ADVANCES IN
VAGAL AFFERENT NEUROBIOLOGY
FRONTIERS IN NEUROSCIENCE

Series Editors
Sidney A. Simon, Ph.D.
Miguel A.L. Nicolelis, M.D., Ph.D.

Published Titles

Apoptosis in Neurobiology
Yusuf A. Hannun, M.D., Professor of Biomedical Research and Chairman/Department of Biochemistry and Molecular Biology, Medical University of South Carolina
Rose-Mary Boustany, M.D., tenured Associate Professor of Pediatrics and Neurobiology, Duke University Medical Center

Methods for Neural Ensemble Recordings
Miguel A.L. Nicolelis, M.D., Ph.D., Professor of Neurobiology and Biomedical Engineering, Duke University Medical Center

Methods of Behavioral Analysis in Neuroscience
Jerry J. Buccafusco, Ph.D., Alzheimer’s Research Center, Professor of Pharmacology and Toxicology, Professor of Psychiatry and Health Behavior, Medical College of Georgia

Neural Prostheses for Restoration of Sensory and Motor Function
John K. Chapin, Ph.D., Professor of Physiology and Pharmacology, State University of New York Health Science Center
Karen A. Moxon, Ph.D., Assistant Professor/School of Biomedical Engineering, Science, and Health Systems, Drexel University

Computational Neuroscience: Realistic Modeling for Experimentalists
Eric DeSchutter, M.D., Ph.D., Professor/Department of Medicine, University of Antwerp

Methods in Pain Research
Lawrence Kruger, Ph.D., Professor of Neurobiology (Emeritus), UCLA School of Medicine and Brain Research Institute

Motor Neurobiology of the Spinal Cord
Timothy C. Cope, Ph.D., Professor of Physiology, Emory University School of Medicine

Nicotinic Receptors in the Nervous System
Edward D. Levin, Ph.D., Associate Professor/Department of Psychiatry and Pharmacology and Molecular Cancer Biology and Department of Psychiatry and Behavioral Sciences, Duke University School of Medicine

Methods in Genomic Neuroscience
Helmin R. Chin, Ph.D., Genetics Research Branch, NIMH, NIH
Steven O. Moldin, Ph.D, Genetics Research Branch, NIMH, NIH

Methods in Chemosensory Research
Sidney A. Simon, Ph.D., Professor of Neurobiology, Biomedical Engineering, and Anesthesiology, Duke University
Miguel A.L. Nicolelis, M.D., Ph.D., Professor of Neurobiology and Biomedical Engineering, Duke University
The Somatosensory System: Deciphering the Brain’s Own Body Image
Randall J. Nelson, Ph.D., Professor of Anatomy and Neurobiology,
University of Tennessee Health Sciences Center

The Superior Colliculus: New Approaches for Studying Sensorimotor Integration
William C. Hall, Ph.D., Department of Neuroscience, Duke University
Adonis Moschovakis, Ph.D., Institute of Applied and Computational Mathematics, Crete

New Concepts in Cerebral Ischemia
Rick C. S. Lin, Ph.D., Professor of Anatomy, University of Mississippi Medical Center

DNA Arrays: Technologies and Experimental Strategies
Elena Grigorenko, Ph.D., Technology Development Group, Millennium Pharmaceuticals

Methods for Alcohol-Related Neuroscience Research
Yuan Liu, Ph.D., National Institute of Neurological Disorders and Stroke, National Institutes of Health
David M. Lovinger, Ph.D., Laboratory of Integrative Neuroscience, NIAAA

In Vivo Optical Imaging of Brain Function
Ron Frostig, Ph.D., Associate Professor/Department of Psychobiology, University of California, Irvine

Primate Audition: Behavior and Neurobiology
Asif A. Ghazanfar, Ph.D., Primate Cognitive Neuroscience Lab, Harvard University

Methods in Drug Abuse Research: Cellular and Circuit Level Analyses
Dr. Barry D. Waterhouse, Ph.D., MCP-Hahnemann University

Functional and Neural Mechanisms of Interval Timing
Warren H. Meck, Ph.D., Professor of Psychology, Duke University

Biomedical Imaging in Experimental Neuroscience
Nick Van Bruggen, Ph.D., Department of Neuroscience Genentech, Inc., South San Francisco
Timothy P. L. Roberts, Ph.D., Associate Professor, University of Toronto

The Primate Visual System
John H. Kaas, Department of Psychology, Vanderbilt University
Christine Collins, Department of Psychology, Vanderbilt University

Neurosteroid Effects in the Central Nervous System
Sheryl S. Smith, Ph.D., Department of Physiology, SUNY Health Science Center

Modern Neurosurgery: Clinical Translation of Neuroscience Advances
Dennis A. Turner, Department of Surgery, Division of Neurosurgery, Duke University Medical Center

Sleep: Circuits and Functions
Pierre-Hervé Luoou, Université Claude Bernard Lyon I, Lyon, France

Methods in Insect Sensory Neuroscience
Thomas A. Christensen, Arizona Research Laboratories, Division of Neurobiology, University of Arizona, Tucson, AZ

Motor Cortex in Voluntary Movements
Alexa Riehle, INCM-CNRS, Marseille, France
Eilon Vatadia, The Hebrew University, Jerusalem, Israel

Advances in Vagal Afferent Neurobiology
Bradley J. Undem, Johns Hopkins Asthma Center, Baltimore, MD
Daniel Weinreich, University of Maryland, Baltimore, MD
ADVANCES IN
VAGAL AFFERENT NEUROBIOLOGY

Edited by
Bradley J. Undem
Johns Hopkins Asthma Center
Baltimore, MD

Daniel Weinreich
University of Maryland
Baltimore, MD
Dedication

This volume is devoted to
the loving memory of Dr. Cinda Helke
Series Preface

The Frontiers in Neuroscience series presents the insights of experts on emerging experimental techniques and theoretical concepts that are or will be at the vanguard of neuroscience. Books in the series cover topics ranging from methods to investigate apoptosis to modern techniques for neural ensemble recordings in behaving animals. The series also covers new and exciting multidisciplinary areas of brain research, such as computational neuroscience and neuroengineering, and describes breakthroughs in fields like insect sensory neuroscience, primate audition, and biomedical engineering. The goal is for this series to be the reference that every neuroscientist uses in order to get acquainted with new methodologies in brain research. These books can be given to graduate students and postdoctoral fellows when they are looking for guidance to start a new line of research.

Each book is edited by an expert and consists of chapters written by the leaders in a particular field. Books are richly illustrated and contain comprehensive bibliographies. Chapters provide substantial background material relevant to the particular subject. Hence, they are not the usual type of method books. They contain detailed “tricks of the trade” and information as to where these methods can be safely applied. In addition, they include information about where to buy equipment and web sites helpful in solving both practical and theoretical problems. Finally, they present detailed discussions of the present knowledge of the field and where it should go.

We hope that, as the volumes become available, the effort put in by us, the publisher, the book editors, and the individual authors will contribute to the further development of brain research. The extent to which we achieve this goal will be determined by the utility of these books.

Sidney A. Simon, Ph.D.
Miguel A.L. Nicolelis, M.D., Ph.D.
Series Editors
Vagus is a Latin word with a root that also gives us the term vague. This is appropriate, as much of what we know about vagal sensory neuroscience remains vague and imprecise. This is partly due to a decided lack of information. The number of scientific studies aimed directly at investigating the primary afferent nerves within the vagi pale in comparison with the somatosensory nerves of the dorsal roots. Given the pivotal role they play in visceral physiology as well as pathophysiology, one could reasonably argue that vagal sensory nerves are relatively understudied by today’s standards. As a surrogate for direct information, it is often tempting to infer knowledge about vagal afferent nerves from the many elegant studies carried out on somatosensory nerves. Beyond some basic fundamental principles, however, this practice may lead to more confusion than clarity. This is because sensory nerve phenotype is directed from embryological “top down” signals as well as from tissue-derived “bottom up” signals. The vagal sensory neurons have their own unique embryological history and the tissues within the visceral organs they innervate provide another set of signals that render the nerves dissimilar in many ways to their somatosensory cousins.

Also contributing to the lack of focus in vagal neuroscience is the fact that investigators in this field are spread across many disciplines. The vagus meanders through the viscera, providing important regulatory influences over the thoracic and abdominal organs. This leads to a situation in which experts in vagal neurobiology are often “organo-centric” presenting their work in organ-specific journals and discussing their findings within their own organ-specific meetings. One of the purposes of this book is to present coherent summaries of vagal afferent neuroscience obtained within the body’s organ systems. By juxtaposing these chapters in a single text, it is anticipated that cross-fertilization of ideas will provide new insights and focus on this important topic.

In Latin, the term vagus also means wandering. In this context, this book can be considered vagal. There have been outstanding resources published on specific aspects of vagal neuroscience, but there are few, if any, publications that provide a broad overview of vagal sensory neurobiology. With this in mind, this volume has been designed with chapters that wander through all aspects of vagal sensory nerve biology. This occurs on a lateral scale with chapters that deal with vagal nerves in the central nervous system (CNS), lungs, esophagus, heart, and gastrointestinal tract. The book also wanders on a vertical scale as it covers critical aspects ranging from vagus nerves in the embryo to vagus nerves in the adult, and from activity in individual neurons to whole animal studies of the reflexes and sensations such activity engenders. It is hoped that by painting vagal afferent biology with the broadest brush possible, this book will be a unique and useful resource for the
student, as well as for the established neuroscientist interested in the visceral nervous system.

Such nonreductionism runs the risk of failing for lack of discriminating detail. This is not the case here. In each chapter, experts cover a particular area of vagal afferent neuroscience. The authors provide an overview of the given topic and, in many cases, details pertaining to specific experiments and techniques. This approach has resulted in an informative reference for the vagal sensory neurobiologist.

Bradley J. Undem
Daniel Weinreich
Contributors

K.D. Alfrey
Biomedical Engineering Program
Purdue School of Engineering and Technology
Indiana University/Purdue University Indianapolis, Indiana

Clare V.H. Baker
Department of Anatomy
University of Cambridge
United Kingdom

Michael J. Beyak
Department of Biomedical Science
University of Sheffield
Sheffield, United Kingdom
and Gastrointestinal Diseases Research Unit
Queen’s University
Kingston, Ontario, Canada

Ann C. Bonham
Department of Pharmacology
School of Medicine
University of California, Davis
Davis, California

Simon J.H. Brookes
Department of Human Physiology and Centre for Neuroscience
Flinders University
Adelaide, South Australia

Brendan J. Canning
Johns Hopkins Asthma and Allergy Center
Baltimore, Maryland

Michael J. Carr
GlaxoSmithKline
King of Prussia, Pennsylvania

Chao-Yin Chen
Department of Pharmacology
School of Medicine
University of California, Davis
Davis, California

Marcello Costa
Department of Human Physiology and Centre for Neuroscience
Flinders University
Adelaide, South Australia

James Deuchars
Department of Physiology
School of Medical Sciences
University of Leeds
Leeds, United Kingdom

David Grundy
Department of Biomedical Science
University of Sheffield
Sheffield, United Kingdom

Musa A. Haxhiu
Department of Physiology and Biophysics
Specialized Neuroscience Research Program
Howard University College of Medicine
Washington, D.C.
Cinda J. Helke  
Department of Pharmacology and Neuroscience Program  
Uniformed Services University of the Health Sciences  
Bethesda, Maryland

Wilfrid Jänig  
Department of Physiology  
Christian-Albrechts-Universität zu Kiel  
Kiel, Germany

Sergey Kasparov  
Department of Physiology  
School of Medical Sciences  
University of Bristol  
Bristol, United Kingdom

Prabha Kc  
Department of Physiology and Biophysics  
Specialized Neuroscience Research Program  
Howard University College of Medicine  
Washington, D.C.

Lu-Yuan Lee  
Department of Physiology  
University of Kentucky Medical Center  
Lexington, Kentucky

B.Y. Li  
Biomedical Engineering Program  
Purdue School of Engineering and Technology  
Indiana University/Purdue University Indianapolis, Indiana

Stuart B. Mazzone  
Department of Neurobiology  
Howard Florey Institute  
University of Melbourne  
Melbourne, Australia

Constance T. Moore  
Department of Physiology and Biophysics  
Specialized Neuroscience Research Program  
Howard University College of Medicine  
Washington, D.C.

Julian F.R. Paton  
Department of Physiology  
School of Medical Sciences  
University of Bristol  
Bristol, United Kingdom

Robert J. Phillips  
Department of Psychological Sciences and Integrative Neuroscience Program  
Purdue University  
West Lafayette, Indiana

Giovanni Piedimonte  
Departments of Pediatrics, Medicine, and Molecular/Cellular Pharmacology  
University of Miami School of Medicine  
Miami, Florida

Terry L. Powley  
Department of Psychological Sciences and Integrative Neuroscience Program  
Purdue University  
West Lafayette, Indiana

Steven C. Schachter  
Department of Neurology  
Beth Israel Deaconess Medical Center  
Harvard Medical School  
Boston, Massachusetts

J.H. Schild  
Biomedical Engineering Program  
Purdue School of Engineering and Technology  
Indiana University/Purdue University Indianapolis, Indiana
Harold D. Schultz
Department of Cellular and Integrative Physiology
University of Nebraska College of Medicine
Omaha, Nebraska

Jyoti N. Sengupta
Division of Gastroenterology and Hepatology
Medical College of Wisconsin
Milwaukee, Wisconsin

Reza Shaker
Professor and Chief
Division of Gastroenterology and Hepatology
Medical College of Wisconsin
Milwaukee, Wisconsin

Bradley J. Undem
Department of Medicine
Johns Hopkins Asthma and Allergy Center
Baltimore, Maryland

Sheng Wang
Department of Physiology
School of Medical Sciences
University of Bristol
Bristol, United Kingdom

Danny Weinreich
Department of Pharmacology and Experimental Therapeutics
University of Maryland
Baltimore, Maryland

Vladimir P. Zagorodnyuk
Department of Human Physiology and Centre for Neuroscience
Flinders University
Adelaide, South Australia
Contents

**PART I  Development and Plasticity**

**Chapter 1**  The Embryology of Vagal Sensory Neurons ........................................ 3  
*Clare V. H. Baker*

**Chapter 2**  Vagal Afferent Neurons: Neurotrophic Factors and Epigenetic Influences ................................................................. 27  
*Cinda J. Helke*

**PART II  Vagal Sensory Ganglion Neurons**

**Chapter 3**  Voltage-Gated Ion Channels in Vagal Afferent Neurons .............. 77  
*J.H. Schild, K.D. Alfrey, and B.Y. Li*

**Chapter 4**  Electrophysiological Studies of Target-Identified Vagal Afferent Cell Bodies ................................................................. 101  
*Danny Weinreich*

**PART III  Vagal Sensory Nerve Terminals**

**Chapter 5**  Advances in Neural Tracing of Vagal Afferent Nerves and Terminals ................................................................. 123  
*Terry L. Powley and Robert J. Phillips*

**Chapter 6**  Mechanotransduction by Vagal Tension Receptors in the Upper Gut ................................................................................. 147  
*Simon J.H. Brookes, Vladimir P. Zagorodnyuk, and Marcello Costa*
Chapter 7  Chemical Transduction in Vagal Afferent Nerve Endings .......... 167
Michael J. Carr

PART IV  Connection in the CNS

Chapter 8  Synaptic Transmission in the Nucleus Tractus Solitarius (NTS) ... 193
Ann C. Bonham and Chao-Yin Chen

Chapter 9  Nitroxergic Modulation in the NTS: Implications for Cardiovascular Function ................................................................. 209
Julian F.R. Paton, James Deuchars, Sheng Wang, and Sergey Kasparov

Chapter 10  Monoaminergic Modulation in the Brainstem: Implication for Airway Function ................................................................. 247
Musa A. Haxhiu, Constance T. Moore, and Prabha Kc

PART V  Organ-Specific Afferent Nerves

Chapter 11  Bronchopulmonary Vagal Afferent Nerves............................ 279
Lu-Yuan Lee and Bradley J. Undem

Chapter 12  Vagal Afferents Innervating the Gastrointestinal Tract .............. 315
Michael J. Beyak and David Grundy

Chapter 13  Cardiac Vagal Afferent Nerves .................................................. 351
Harold D. Schultz

PART VI  Vagal Reflexes and Sensation

Chapter 14  Vagal Afferent Nerve Stimulated Reflexes in the GI Tract .......... 379
Jyoti N. Sengupta and Reza Shaker
Part I

Development and Plasticity
The Embryology of Vagal Sensory Neurons

Clare V. H. Baker

CONTENTS

1.1 Introduction ...................................................................................................... 4
   1.1.1 The Spatial Segregation of Functionally Distinct Cranial Sensory Neurons Reflects Their Different Embryonic Origins .......................... 4
   1.1.2 Cranial Ectodermal Placodes: An Introduction ..................................... 7
   1.1.3 The Neural Crest: An Introduction ....................................................... 7
   1.1.4 Placode and Neural Crest Formation at the Neural Plate Border ...... 8

1.2 Nodose Neurons: Placode Development and Neurogenesis ...................... 8
   1.2.1 Evidence for a Pan-Placodal Primordium at the Neural Plate Border ................................................................. 9
   1.2.2 Formation of Individual Placodes: Involvement of the Pax/Six/Eya/Dach Regulatory Network? .............................................. 9
   1.2.3 Many Individual Placodes, Including the Nodose Placode, Originate from Distinct Multi-Placodal Primordia ...................... 10
   1.2.4 Induction of the Epibranchial Placodes (Geniculate, Petrosal, and Nodose) ........................................................................ 11
   1.2.5 Neurogenesis within the Epibranchial Placodes ................................. 12
      1.2.5.1 Proneural Genes and Notch Signaling in Neurogenesis: A Brief Outline ................................................................. 12
      1.2.5.2 Neurogenins Are Required for Neurogenesis in the Epibranchial Placodes ................................................................. 13
      1.2.5.3 Phox2a and Phox2b Confer Neuronal Subtype Identity and Are Required for Neuronal Survival ........................... 14
   1.2.6 Neural Crest Cells Are Required for the Formation of Central Connections by Epibranchial Neurons .............................................. 15
   1.2.7 Summary of Nodose Neuron Development ...................................... 15

1.3 Jugular Neurons: Neural Crest Development and Sensory Neurogenesis..... 16
   1.3.1 Induction of the Neural Crest ............................................................. 16
   1.3.2 Neural Crest Lineage Diversification: General Principles ................. 17
   1.3.3 Sensory Neurogenesis within the Neural Crest ................................. 19
      1.3.3.1 Wnt Signaling Instructively Promotes Adoption of a Sensory Neuron Fate ................................................................. 19
1.3.3.2 Sensory-Fated and Sensory-Committed Precursors Are Present in the Migrating Neural Crest Population.............20
1.3.3.3 Neurogenin2 Biases Trunk Neural Crest Cells to a Sensory Fate...........................................................................20
1.3.3.4 Neurogenin1 Is Required for the Formation of Jugular Neurons ...........................................................................20
1.3.3.5 Inhibition of Notch Signaling Is Required for Sensory Neuron Differentiation........................................................21

1.3.4 Summary of Jugular Neuron Development.......................................21

References................................................................................................................22

1.1 INTRODUCTION

The sensory neurons of the vagal nerve (cranial nerve X) are collected into two separate ganglia, the nodose and jugular ganglia. The neurons in these ganglia are functionally distinct: visceral sensory neurons are found only in the nodose ganglion, while somatic sensory neurons are found only in the jugular ganglion. These two different types of neuron are formed during embryogenesis from two different embryonic cell populations. The nodose placodes, paired bilateral patches of thickened surface ectoderm by the hindbrain (the most caudal in a series of “epibranchial” placodes), give rise to the visceral sensory neurons of the nodose ganglion. The neural crest, a migratory population of cells that delaminates from the neuroepithelium to migrate throughout the embryo, gives rise to the somatic sensory neurons of the jugular ganglion. In the embryo, these two types of neuron are also morphologically distinct: placode-derived nodose neurons are large in diameter, while neural crest-derived jugular neurons are small.

This chapter provides an introduction to the embryology of nodose and jugular neurons. Relatively little is known about the formation of nodose and jugular neurons specifically, while much more is known about the formation of placode-derived and neural crest-derived sensory neurons in general. Hence, this chapter will necessarily take a fairly general approach, but will emphasize nodose and jugular neurons where possible. Section 1.1 gives a brief general introduction to cranial ectodermal placodes and the neural crest. Section 1.2 describes what is currently known about nodose neuron development, embedded in a general outline of epibranchial placode formation and neurogenesis. Finally, Section 1.3 covers jugular neurons, with a general description of neural crest formation, and sensory neurogenesis within the neural crest.

1.1.1 THE SPATIAL SEGREGATION OF FUNCTIONALLY DISTINCT CRANIAL SENSORY NEURONS REFLECTS THEIR DIFFERENT EMBRYONIC ORIGINS

As described previously, vagal sensory neurons fall into two general functional classes that are spatially segregated in distinct ganglia on the vagal nerve (cranial nerve X). Somatic sensory neurons are found only in the jugular ganglion (Figure 1.1). General visceral and special visceral (gustatory) sensory neurons are found only in the nodose ganglion (Figure 1.1). This segregation reflects the different
embryonic origins of these neurons: the somatic sensory neurons in the jugular ganglion are derived from the neural crest, while the general and special visceral sensory neurons in the nodose ganglion are derived from cranial ectodermal placodes, specifically the nodose placodes (the third and more caudal placodes in the epibranchial series of placodes) (Figure 1.2). The neural crest and cranial placodes are both migratory cell populations that arise from ectoderm at the border between the prospective neural plate (which will eventually form the brain and spinal cord) and the prospective epidermis (skin). Together, neural crest and cranial placodes form the entire peripheral nervous system (reviewed in References 1 and 2).

The same spatial segregation of somatic sensory neurons in proximal ganglia (“root” ganglia, close to the brain), and general visceral and special visceral (gustatory) sensory neurons in distal ganglia (“trunk” ganglia, further from the brain), is also seen for the sensory neurons of cranial nerves VII (facial) and IX (glossopharyngeal) (Figure 1.1). Again, the spatial segregation of functionally distinct neurons into proximal and distal ganglia reflects their different embryonic origins. Just as for the vagal nerve, the proximal ganglia of the facial and glossopharyngeal nerves (root of VII and superior respectively) contain neural crest-derived somatic sensory neurons, while the distal ganglia (geniculate and petrosal respectively) contain placode-derived visceral sensory neurons (Figure 1.1 and Figure 1.2B).
Advances in Vagal Afferent Neurobiology

Most other cranial sensory neurons are placode-derived. The olfactory receptor neurons in the olfactory epithelium are formed by the olfactory placode. The special sensory neurons in the vestibuloacoustic (VIII) and lateral line ganglia (in anamniotes), which provide afferent innervation to the sensory hair cells of the inner ear and lateral line system respectively, are derived from the otic and lateral line placodes respectively (Figure 1.1 and Figure 1.2B). The somatic sensory neurons in the trigeminal ganglion are “mixed” in origin, with neurons derived both from the neural crest and from the two trigeminal placodes (the ophthalmic and maxillomandibular trigeminal placodes). However, neurons of different origin within the trigeminal ganglion are spatially segregated, with small neural crest-derived neurons proximally and large placode-derived neurons distally (Figure 1.1 and Figure 1.2B). (Note that there is no relationship between the different size classes of neuron in the embryonic ganglion and the different cytological classes of neuron in the mature ganglion.3). Both neural crest- and placode-derived neurons in the trigeminal ganglion are somatic sensory neurons, proving an exception to the general rule that placode-derived neurons mainly form special sensory (olfactory, otic, lateral line), special visceral (gustatory) and general visceral sensory neurons.

The precise embryonic origin of the neurons in cranial sensory ganglia (i.e., neural crest, placode, or both) was debated for decades (e.g., References 4 through 9). Ablation of one or the other cell population was first used to attack the question, but this was superseded by cell labeling techniques, in particular the permanent label

FIGURE 1.2 (A) Schematic to show the location of cranial ectodermal placodes in a 3-day chick embryo. Each of the four epibranchial placodes (geniculate, petrosal, and two nodose placodes) is located just caudal to a pharyngeal cleft. The bi-lobed trigeminal ganglion is already forming. gen, geniculate placode; nod, nodose placode; pet, petrosal placode; ves, vesicle. (Adapted from Le Douarin, N.M., Fontaine-Pérus, J., and Couly, G., Trends Neurosci., 9, 175, 1986, and from Abu-Elmagd, M. et al., Dev. Biol., 237, 258, 2001.) (B) Schematic to show the distribution of placode-derived (dark grey) and neural crest-derived (black) sensory neurons in an 8-day chick embryo. Roman numerals indicate the appropriate cranial nerve. drg, dorsal root ganglion; gen, geniculate ganglion; mmV, maxillomandibular lobe of the trigeminal ganglion; opV, ophthalmic lobe of the trigeminal ganglion; pet, petrosal ganglion; and vest-ac, vestibulo-acoustic ganglion. (Adapted from Le Douarin, N.M., Fontaine-Perus, J., and Couly, G., Trends Neurosci., 9, 175, 1986.)
afforded by grafting quail tissue into chick hosts.\textsuperscript{10} The quail-chick chimera method enabled precise and unequivocal labeling of either neural crest or placodes in avian embryos, and was used to demonstrate conclusively the picture of cranial sensory ganglion development outlined above.\textsuperscript{8,11,12}

### 1.1.2 Cranial Ectodermal Placodes: An Introduction

Cranial ectodermal placodes are transitory patches of thickened, columnar epithelium in the embryonic head (Figure 1.2A).\textsuperscript{2,13,14} Although hairs, feathers and teeth also arise from focal ectodermal thickenings called placodes, the term “cranial placodes” here refers only to those placodes that arise from ectoderm at the neural plate border in the head and that are associated with the sensory nervous system. As a group, cranial placodes form a wide variety of derivatives, primarily associated with the paired sense organs, but also including the endocrine cells of the adenohypophysis (anterior pituitary gland). Different cell types formed by cranial placodes include ciliated sensory receptor cells, sensory neurons, endocrine and neuroendocrine cells, and supporting cells, including some glial cells (olfactory ensheathing cells). Placodes are vital for the formation of the paired sense organs. The entire olfactory epithelium, the lens of the eye, the entire inner ear and, in amniotes, the lateral line system, are all derived from different placodes (olfactory, lens, otic and lateral line placodes respectively). Most of the neurons in cranial sensory ganglia are also placode-derived (see Section 1.1.1) (Figure 1.1 and Figure 1.2B). In addition to forming the inner ear, the otic placode forms the afferent innervating neurons for inner ear hair cells collected in the vestibuloacoustic ganglion (cranial nerve VIII). Similarly, in amniotes, the lateral line placodes form not only the mechanosensory and electoreceptive hair cells of the lateral line system, but also their afferent innervating neurons, collected in the lateral line ganglia. Two trigeminal placodes (ophthalmic and maxillomandibular) give rise to somatic sensory neurons in the distal regions of the eponymous lobes of the trigeminal ganglion (V) (Figure 1.2). Finally, and most relevant for this chapter, a series of epibranchial placodes (geniculate, petrosal and nodose) gives rise to all the general visceral and special visceral sensory neurons of the distal sensory ganglia of cranial nerves VII (geniculate), IX (petrosal) and X (nodose) (Figure 1.2). Clearly, cranial ectodermal placodes are essential for the formation of the majority of the peripheral sensory nervous system in the vertebrate head.

### 1.1.3 The Neural Crest: An Introduction

Neural crest cells are formed from ectoderm at the lateral borders of the neural plate, which are lifted up during neurulation to form the neural folds. The neural folds ultimately fuse, leading to formation of the neural tube from what was originally the flat neural plate, and bringing most neural crest cell precursors to the dorsal midline (“crest”) of the neural tube. Some neural crest cells, particularly cranial neural crest cells, may delaminate from the neuroepithelium before neural fold fusion occurs so they are never part of the neural tube proper. Neural crest cells delaminate in a rostrocaudal wave, and migrate along well-defined pathways in both head and
trunk to form an enormous variety of derivatives.¹ These include most peripheral neurons and glia, with the exception of placode-derived cranial sensory neurons and olfactory ensheathing cells. They also form melanocytes, various endocrine cell types (e.g., adrenal chromaffin cells), most craniofacial bones and cartilages, and teeth. They give rise to all the somatic sensory neurons of the proximal sensory ganglia of cranial nerves VII (root), IX (superior) and X (jugular), together with somatic sensory neurons in the proximal region of the trigeminal ganglion (V) (Figure 1.1 and Figure 1.2B). All the glial cells of the cranial sensory ganglia originate from the neural crest, so neural crest cells are required for the formation or maintenance of even the vestibuloacoustic, lateral line, geniculate, petrosal and nodose ganglia, whose neurons are entirely placode-derived.

1.1.4 Placode and Neural Crest Formation at the Neural Plate Border

All fate-mapping studies to date have demonstrated that both neural crest and cranial placodes originate from ectoderm at the border between the prospective neural plate (future central nervous system) and the prospective epidermis (reviewed in References 1 and 2). Current molecular models for the induction of the neural plate are reviewed in Reference 15. Older fate maps (e.g., References 16 and 17) show the neural crest- and placode-forming regions as distinct, segregated strips of ectoderm, with prospective neural crest cells lying just lateral to the neural plate, and placodes lying between the neural crest and the epidermis (see Reference 2). However, more careful recent analyses, involving the labeling either of single cells or small groups of cells at the neural plate border, have shown that this view is greatly over-simplified. Individual cells at the trunk neural plate border can form epidermis, neural crest, and neural tube derivatives,¹⁸ while at the cranial neural plate border, precursors of epidermis, placodes, neural crest, and neural tube are intermingled.¹⁹ Although prospective placodal territory reaches further laterally than prospective neural crest territory, placodal and neural crest precursors are intermingled more medially.¹⁹ Therefore, it is incorrect to think of prospective placode and neural crest territories as being completely segregated from one another at neural plate stages of development.

1.2 Nodose Neurons: Placode Development and Neurogenesis

Nodose neurons are derived from the nodose placode, the most caudal of the epi-branchial series of placodes that forms above the pharyngeal (branchial) clefts (Figure 1.2, see Section 1.2.4). In this section, the formation of placodes in general, and the epi-branchial placodes in particular, are described, followed by a discussion of our current understanding of the control of neurogenesis within the epi-branchial placodes (geniculate, petrosal and nodose). As will be seen, nothing is currently known that distinguishes nodose placode neurogenesis from neurogenesis in the other epi-branchial placodes.
1.2.1 Evidence for a Pan-Placodal Primordium at the Neural Plate Border

Despite the intermingling of precursors of neural tube, neural crest, placodes, and epidermis at the neural plate border (see Section 1.1.4), there is increasing evidence to support the existence of a preplacodal field, or panplacodal primordium, around the anterior neural plate (for a more detailed discussion, see Reference 2). This field is delineated by the expression of several genes, mainly encoding transcription factors implicated in placode development, in a “horseshoe” around the anterior neural plate. These genes include the homeodomain transcription factors Dlx3, Dlx5, Dlx7, Six1 and Six4, the HMG-domain transcription factor Sox3 (also expressed in the neural plate), and the transcription co-factors Eya1 and Eya2 (for original references, see Reference 2). In the chick, these genes are expressed in a series of overlapping domains, rather than being strictly coincident.19

Fate-mapping data show that the preplacodal domain correlates neither with the site of origin of all placodal precursor cells, nor with determination toward a placodal fate. For example, not all otic placode precursors originate from the Six4+ domain, and some cells within the Six4+ domain form neural crest, epidermis or neural tube derivatives, rather than placodal derivatives.19 What, therefore, is the significance of the preplacodal “horseshoe” of gene expression? It is possible that it represents a zone of ectoderm that is competent to respond to specific placode-inducing signals, although as yet there is no functional evidence to support this. There is a large amount of ectodermal cell movement at the neural plate border.19 Precursors of a given placode, such as the zebrafish olfactory placode20 or the chick otic placode,19 arise from a relatively large ectodermal region at the anterior neural plate border that subsequently converges to form the placode proper. This may suggest a model in which cells moving into the preplacodal gene expression domain upregulate preplacodal genes, while cells leaving the domain downregulate these genes. Expression of a sufficient complement of preplacodal genes may render cells competent to respond to signals that induce the formation of specific placodes. However, the fate that is ultimately adopted by a particular cell within the preplacodal domain will be determined by the specific combination of signals it subsequently receives. Hence, despite being competent to contribute to a placode, it may instead form epidermis or neural crest.

1.2.2 Formation of Individual Placodes: Involvement of the Pax/Six/Eya/Dach Regulatory Network?

Members of the Pax family of paired domain, homeodomain transcription factors are expressed in different combinations in virtually all placodes. Pax6 is found in the olfactory and lens placodes, Pax3 in the ophthalmic trigeminal placode, Pax2/5/8 in the otic placode, and Pax2 in the epibranchial placodes (reviewed in Reference 2). Knockout studies in mice have shown that Pax6 is required for proper olfactory and lens placode development, while Pax2 is essential for various features of otic placode development (reviewed in Reference 2). Given the above, it is fascinating that several of the genes expressed in the preplacodal domain are members of the
Six and Eya gene families. Pax, Six, and Eya family members, together with the transcription co-factor Dach, act in an intricate cross-regulatory network in both eye and muscle development (see Reference 21). Dach family members are expressed in part of the preplacodal domain and in various placodes. It is intriguing to speculate that expression of Six, Eya, and perhaps also Dach genes within the preplacodal domain may provide a pan-placodal regulatory module. Different Pax family members are induced in different locations within the preplacodal domain by specific placode-inducing signals from neighboring tissues (for a comprehensive review of known placode-inducing tissues and signals, see Reference 2). The different Pax proteins might then interact with Six, Eya and Dach members to specify the identities of individual placodes. There is currently no real evidence for this model, but it is eminently testable.

1.2.3 Many Individual Placodes, Including the Nodose Placode, Originate from Distinct Multi-Placodal Primordia

The previous section discussed a model in which placodes become “individualized” within the preplacodal domain of ectoderm by receiving different inducing signals from adjacent tissues. However, in many or most species, discrete domains of thickened ectoderm are found that ultimately give rise to two or more placodes (the ectoderm in between the final placodes usually seems to become thin). This is certainly the case for the epibranchial placodes, which form the special visceral (gustatory) and general visceral sensory neurons in the geniculate, petrosal and nodose ganglia. In Xenopus embryos, the lateral line and otic (and possibly epibranchial) placodes seem to originate from a common “dorsolateral placode area” that expresses a common set of molecular markers, including Pax2.26,27

Data from both zebrafish and chick embryos support the origin of the epibranchial placodes from a molecularly and morphologically distinct region of ectoderm that includes the otic placode. In the zebrafish, the winged-helix transcription factor Foxi1, which is necessary for both otic and epibranchial placode formation28,29 is expressed in a “lateral cranial placodal domain” that encompasses the otic and epibranchial placodes.29 In the chick embryo, Pax2 is expressed in a broad region of ectoderm that includes both otic and epibranchial placode precursors.19,30 Furthermore, fate-map data combined with the expression of the pan-placodal marker gene Sox3, which generally correlates with the location of thickened ectoderm, suggest that the geniculate and otic placodes arise from a common domain of Sox3 expression, while the petrosal and the two nodose placodes (see Figure 1.2A) arise from a second, more caudal domain of Sox3 expression.31,32 The ectoderm lying between the epibranchial placodes subsequently thins, while the ectoderm that will ultimately form the placodes remains thick and maintains Sox3 expression.31,32 Taken together, these results suggest that a broad domain of thickened ectoderm near the hindbrain initially shares a set of common molecular markers, including Pax2 and Foxi1, and is subsequently partitioned into multiple individual placodes. This partitioning is likely to involve the maintenance of some molecular markers (such as Sox3) and
the induction of new placode-specific genes, by signals from adjacent tissues. Models of individual placode formation, therefore, must take these data into account.

1.2.4 Induction of the Epibranchial Placodes (Geniculate, Petrosal, and Nodose)

The epibranchial placodes form above the pharyngeal (branchial) clefts (Figure 1.2A). These clefts are formed by the outpocketing of pharyngeal (foregut) endoderm as a series of pouches that fuse with the overlying ectoderm to form slits in the wall of the pharynx. The pharyngeal (branchial) arches represent the tissue between the successive pharyngeal clefts; the number of arches (and clefts) is variable between different vertebrates. In aquatic vertebrates, the pharyngeal clefts form the gill slits and the pharyngeal (gill) arches support gills. The first pharyngeal cleft in tetrapods forms the cavity of the middle ear; the first arch in all jawed vertebrates forms the jaw. Cranial neural crest cells migrate into the pharyngeal arches to fill the subectodermal space around the core of paraxial mesoderm (Figure 1.3); they form all the skeletal elements of the arches, and the connective components of the striated muscles formed by the paraxial mesoderm core.

**FIGURE 1.3** Schematic to show the migration pathways of cranial neural crest cells (black arrows) from the caudal diencephalon, midbrain and hindbrain, into the pharyngeal arches, relative to the location of the epibranchial placodes, in a 2-day-old chick embryo. The source of neural crest cells migrating into each arch is indicated by the white text on the black arrows. Neural crest cells from the postotic hindbrain contribute neurons to the jugular ganglion; gen, geniculate placode; mb, midbrain; nod, nodose placode; olf, olfactory pit; pet, petrosal placode; and r, rhombomere. (Adapted from Helms, J.A. and Schneider, R.A., *Nature*, 423, 326, 2003, and from Trainor, P.A. and Krumlauf, R., *Curr. Opin. Cell Biol.*, 13, 698, 2001.)
The first epibranchial placode (geniculate or facial) arises above the first pharyngeal cleft, and forms special visceral (gustatory) and general visceral sensory neurons in the distal (geniculate) ganglion of the facial nerve (cranial nerve VII) (Figure 1.2). The second epibranchial placode (petrosal or glossopharyngeal) arises above the second pharyngeal cleft, and forms special visceral (gustatory) and general visceral sensory neurons in the distal (petrosal) ganglion of the glossopharyngeal nerve (cranial nerve IX) (Figure 1.2). The third epibranchial placode (nodose or vagal) arises above the third pharyngeal cleft, and forms special visceral (gustatory) and general visceral sensory neurons in the distal (nodose) ganglion of the vagal nerve (cranial nerve X) (Figure 1.2). Additional nodose (vagal) epibranchial placodes arise above more posterior pharyngeal clefts, where these are present, and contribute neurons to the nodose ganglion or ganglia (see References 2 and 32). In the chick, for example, there are two nodose placodes that each contribute neurons to the nodose ganglion (Figure 1.2A). The neural crest, as described previously, gives rise to all the satellite glial cells in these ganglia.8,12

The close association of epibranchial placode formation with pharyngeal cleft and arch formation, in both space and time, raises at least two possibilities for the source of epibranchial placode-inducing signals, or perhaps more accurately, for those signals that maintain, for example, Sox3 and Pax2 expression and upregulate epibranchial placode-specific gene expression (see Section 1.2.3). Firstly, there may be signals from the outpouching pharyngeal endoderm and/or the overlying surface ectoderm. Secondly, there may be signals from the migrating neural crest streams that enter the pharyngeal arches between the forming pharyngeal clefts (Figure 1.3). By mechanically ablating or genetically removing neural crest cells, various studies have shown that neural crest cells are not required for epibranchial placode formation.5,33,34 In contrast, in vitro co-culture experiments in the chick demonstrated that pharyngeal endoderm is sufficient to induce epibranchial (Phox2a-positive) neurons in nonplacode-forming cranial ectoderm5,33,34 (see Section 1.2.5).

1.2.5 Neurogenesis within the Epibranchial Placodes

1.2.5.1 Proneural Genes and Notch Signaling in Neurogenesis: A Brief Outline

Before discussing the genes currently known to be involved in neurogenesis within the epibranchial placodes, a brief introduction to the general classes of genes that control neurogenesis is required. In vertebrates and in Drosophila, where they were first discovered, the expression of proneural basic helix-loop-helix (bHLH) transcription factors bestows neuronal potential and specifies the identity of neural progenitor cells (reviewed in Reference 35). Proneural transcription factors activate the expression of ligands of the Notch receptor, such as Delta, Serrate, and Jagged. Cells with low levels of Notch activity, i.e., cells that are not receiving Notch ligands from surrounding cells or that have inherited intrinsic Notch inhibitors via asymmetric cell division, adopt a “primary” cell fate, e.g., differentiate as neurons (e.g.,
Cells with high levels of Notch activity, that is, whose neighbors are secreting high levels of Notch ligands, downregulate their own expression of Notch ligands (thus signal less to surrounding cells) and adopt a “secondary” cell fate, for example, differentiate as supporting or glial cells.

Two classes of proneural genes operate in the *Drosophila* peripheral nervous system: the *achaete-scute* complex and *atonal* (reviewed in Reference 37). The homologues of the *achaete-scute* complex in vertebrates include *ash1* (*Mash1* in mice, *Cashl* in chick, etc.) plus various species-specific genes (e.g., *Mash2* in mice, *Cash4* in chick). The homologues of the *atonal* class in vertebrates are much more numerous, and can be divided into different subfamilies (reviewed in Reference 35). One of these families comprises the *neurogenins* (*ngns*), which are particularly relevant here, as they are essential for the sensory lineage in both placodes and neural crest cells.

### 1.2.5.2 Neurogenins Are Required for Neurogenesis in the Epibranchial Placodes

Section 1.2.3 mentioned that the winged helix transcription factor Foxi1, which is expressed in a “lateral cranial domain” that includes the prospective otic and epibranchial placodes, is required for both otic and epibranchial placode formation in the zebrafish.28,29 Foxi1 is essential for the expression of neurogenin1 (*ngn1*) in the epibranchial placodes.29 *Ngn1* is also expressed in the neural crest, and after *Ngn1* function is abrogated in zebrafish using antisense morpholinos, all peripheral sensory ganglia are lost.38

In the mouse and chick, two different *Neurogenin* genes (*Ngn1* and *Ngn2*) partition between them the functions apparently encompassed by *ngn1* alone in the zebrafish. In the chick, *Ngn1* alone is expressed in the epibranchial placodes.39 In the mouse, *Ngn2* is expressed at higher levels, and *Ngn1* at lower levels, in the epibranchial placodes and cells delaminating from them, before neuronal differentiation occurs.40 When *Ngn2* is knocked out genetically in mice, cells in the geniculate and petrosal placodes fail to delaminate, migrate or differentiate as neurons.40 At the molecular level, they fail to express *Ngn1* and other bHLH genes in the cascade leading to neuronal differentiation, such as *Math3*, *NeuroD* and *Nsc11*.40

The block in geniculate ganglion formation is only transient, however, as a geniculate ganglion eventually forms; the authors suggest that neural crest cells compensate for the loss of geniculate placode-derived neurons 40 (see Section 1.2.6). The nodose placode develops normally in *Ngn2* mutants, and it is thought that *Ngn1*, which is still present in the nodose placode (though at lower levels than normal) may compensate for the loss of *Ngn2* expression.40,41

In all the epibranchial placodes, *Ngn2* is necessary for the expression of the Notch ligand Delta-like1, suggesting that neurogenesis in the epibranchial placodes involves Notch-Delta signalling40 (see Section 1.2.5.1). Interestingly, expression of the epibranchial neuron marker *Phox2a* (see next section) is unaffected by the loss of *Ngn2*,40 suggesting that *Phox2a* and *Ngn2* expression are regulated independently.
1.2.5.3 Phox2a and Phox2b Confer Neuronal Subtype Identity and Are Required for Neuronal Survival

As described in Section 1.2.4, pharyngeal endoderm can induce Phox2a+ neurons in nonplacodal cranial ectoderm. BMP7, which is produced by pharyngeal endoderm, is able to substitute for pharyngeal endoderm in this assay. Furthermore, a BMP7 inhibitor (follistatin) decreases the neuron-inducing activity of the pharyngeal endoderm in vitro, supporting the hypothesis that pharyngeal endoderm-derived BMP7 is involved in neurogenesis in the epibranchial placodes in vivo.

Phox2a is a paired-like homeodomain transcription factor that is required for the development of the noradrenergic phenotype (reviewed in References 42 and 43). It is expressed by a subset of cells within domains of Ngn2+ expression in the epibranchial placodes (indeed, seemingly only in the cells with the highest levels of Ngn2 expression); it is also expressed in most Ngn2+ cells delaminating from the placodes, and in all epibranchial placode-derived neurons. Both Phox2a and a related transcription factor, Phox2b, directly activate the promoter of the gene encoding the catecholamine synthesis pathway enzyme dopamine β-hydroxylase (DBH) (reviewed in References 42 and 43). Epibranchial placode-derived neurons transiently express both DBH and another enzyme in the catecholamine synthesis pathway, tyrosine hydroxylase (TH) (e.g., References 46 and 47).

In epibranchial placode-derived ganglia, Phox2a lies genetically upstream of Phox2b, which is in turn required for DBH expression. Phox2a is neither required for the delamination or aggregation of epibranchial placode-derived cells, nor for their differentiation as neurons, but it is required for DBH expression, and also expression of the gene encoding the receptor tyrosine kinase cRet. Phox2a is therefore probably required for neuronal survival in response to the Ret ligand, GDNF (glial cell line-derived neurotrophic factor). Indeed, in Phox2a-mutant mice, the nodose and petrosal ganglia are severely atrophied through apoptosis: it is possible that the geniculate ganglion, which develops relatively normally, is rescued by redundancy with Phox2b. Phox2b is expressed later than Phox2a, in cells that have already delaminated from the placodes. Like Phox2a, Phox2b is required for the survival of the epibranchial placode-derived ganglia, which are all severely atrophied in Phox2b-null mice. Since the visceral sensory neurons derived from the epibranchial placodes provide afferent innervation to the heart, lungs and other visceral organs, they are required for medullary autonomic reflexes. Remarkably, Phox2b-null mice (but not Phox2a-null mice) lack all of the neural circuits underlying medullary autonomic reflexes (see References 42, 43, and 49). Phox2b therefore appears to be a true pan-autonomic marker.

In summary, Phox2a and Phox2b are not required for neurogenesis within the epibranchial placodes. However, they may determine the neuronal subtype identity of epibranchial placode-derived neurons, and are required for their survival.
1.2.6 **Neural crest cells are required for the formation of central connections by epibranchial neurons**

As described previously (Section 1.2.4), ablation experiments in chick and mouse have demonstrated that epibranchial placode formation, neurogenesis and gangliogenesis proceed essentially as normal in the absence of neural crest cells.\(^5\),\(^3\),\(^3\) In mice double mutant for \textit{Hoxa1} and \textit{Hoxb1}, loss of rhombomere 4-derived neural crest cells in the second pharyngeal arch has no apparent effect on the formation of either the geniculate or petrosal ganglia (see Figure 1.3).\(^3\) Mechanical ablation of second-arch neural crest cells results in delayed delamination of cells from the geniculate placode, and in the formation of aberrant central projections from the displaced ganglion that eventually forms.\(^5\) Thus, the presence of migrating neural crest streams from the hindbrain (see Figure 1.3) may play a role in guiding the central projections of epibranchial placode-derived neurons.

Neural crest cells may initially contribute neurons to the epibranchial ganglia in the chick,\(^5\) but these presumably die, as they are not seen at later stages.\(^1\) It has been suggested (though not proven) that neural crest cells contribute neurons to the geniculate ganglion in \textit{Ngn2}-null mice, which lack epibranchial placode-derived neurons (see Section 1.2.5.2) but in which the geniculate ganglion eventually forms.\(^4\) Also, when the nodose placode is mechanically ablated, neural crest cells from the same axial level form neurons in the nodose ganglion.\(^5\) However, the heart does not function properly in nodose placode-ablated embryos,\(^5\) suggesting that neural crest-derived neurons in the nodose ganglion are unable to substitute fully for nodose placode-derived neurons.

1.2.7 **Summary of nodose neuron development**

Nodose neurons are derived from the nodose placodes, bilateral patches of thickened surface ectoderm at the level of the postotic hindbrain. These are the most caudal in a series of epibranchial placodes that form above the pharyngeal (branchial) clefts: the geniculate, petrosal and nodose placodes. These form all the neurons in the eponymous sensory ganglia on cranial nerves VII, IX and X, respectively. All placodes originate from ectoderm at the neural plate border. There is increasing evidence to suggest the existence of a preplacodal domain or panplacodal primordium in a horseshoe around the anterior neural plate, from which all placodes arise. This domain is characterized by a specific suite of overlapping transcription factor gene expression domains, including \textit{Sox3}, plus \textit{Six}, \textit{Eya}, and \textit{Dlx} family members. The epibranchial placodes form via the partitioning of a larger region of thickened ectoderm adjacent to the hindbrain that includes the otic placodes, and is characterized by \textit{Sox3}, \textit{Pax2} and \textit{Foxi1} expression. The individual epibranchial placodes form above the pharyngeal clefts, shortly before or concomitant with pharyngeal cleft formation via outpocketing and fusion of pharyngeal endoderm with overlying surface ectoderm. The neural crest cells that migrate into the pharyngeal arches, between the pharyngeal clefts, are not required for neurogenesis within the epibranchial placodes, although they do seem to be necessary for appropriate for-
mation of the central connections of epibranchial-derived neurons. *Foxi1* is necessary for expression of the proneural gene *neurogenin1* in the epibranchial placodes in zebrafish. Neurogenins are essential for epibranchial neurogenesis. In both zebrafish and chick, *Ngn1* seems to be required, while in the mouse, *Ngn2* is required, although in the nodose placode, *Ngn1* seems able to compensate for loss of *Ngn2*. *Phox2a* and *Phox2b* are also expressed (independently of the *Ngns*) in epibranchial neurons; they are required for transient noradrenergic marker expression (and thus neuronal subtype identity) and neuronal survival. BMP7 may be the pharyngeal endoderm-derived signal that induces *Phox2a*+ neurons within the epibranchial placodes.

As may be seen from the above, a general picture of the tissues and molecules involved in epibranchial placode development is beginning to take shape. Very little is known about how (or, indeed, whether) the different epibranchial placodes (geniculate, petrosal and nodose) become distinct from one another. The transcription factor *Hoxb5* is expressed specifically in the nodose placode and nodose neurons, as well as in neural crest cells in the caudalmost pharyngeal arches. This is the only molecule identified to date that shows a differential pattern of expression within the epibranchial placodes, but it does not seem to be necessary for the formation of nodose neurons. Given that the geniculate, petrosal and nodose ganglia contain broadly similar neuronal subtypes (general visceral and gustatory sensory neurons), though in different proportions depending on their peripheral targets, it is possible that few or no differences in their embryology will be discovered. Hence, our understanding of the embryology of nodose neurons is, at least to date, identical to our understanding of the embryology of all epibranchial placode-derived neurons.

### 1.3 Jugular Neurons: Neural Crest Development and Sensory Neurogenesis

Jugular neurons arise from neural crest cells that emigrate from the postotic hindbrain, at the level of the first three somites (see Figure 1.2A and Figure 1.3). In this section, a brief description is given of our current understanding of neural crest induction at the neural plate border, followed by a general discussion of lineage diversification within the neural crest. Finally, recent progress in elucidating the mechanisms underlying neural crest cell adoption of a sensory neuronal fate is described.

#### 1.3.1 Induction of the Neural Crest

The mechanisms and molecules thought to underlie neural crest induction have been exhaustively reviewed in recent years. A brief summary is given here.

Neural crest induction can be divided into three main steps. Step 1 is the formation of the neural plate border region, which seems to be dependent on specific levels of bone morphogenetic protein (BMP) activity (e.g., References 56 and 61–64). The positioning of the neural plate border also seems to be dependent on the activity of Dlx transcription factors during gastrulation. However, neither specific BMP activity levels nor Dlx activity is sufficient to induce neural crest cells alone.
Step 2 of neural crest induction is the posteriorization of the neural plate border and induction of neural crest cell precursors within it. These processes can be experimentally uncoupled (e.g., References 68 and 69), but the Wnt and fibroblast growth factor (FGF) signaling pathways are implicated in both processes.60,69,70 Wnts are both necessary and sufficient to induce neural crest cells from neuralized ectoderm (reviewed in Reference 60), and Wnt family members are expressed both in epidermis and paraxial mesoderm, tissues that have long been implicated in neural crest induction (for a historical review, see Reference 71). FGF signaling is required for the induction of neural crest cells by paraxial mesoderm in *Xenopus*,69 however, so FGF involvement in neural crest induction cannot be ruled out. It is likely that the transcription factors AP2α, Sox9 and Sox10 are crucial downstream targets of BMP and Wnt/FGF signals in the formation of neural crest precursors.72–74 AP2α seems to be upstream of Sox9, which in turn induces the expression of multiple other neural crest precursor markers, including the transcription factors Slug and FoxD3.

Step 3 of neural crest cell induction is the epithelial-mesenchymal transition that transforms a (potential) neural crest precursor within the dorsal neural tube, into a *bona fide* neural crest cell that has moved from the neuroepithelium into the periphery. Therefore, induction of delamination is the final step in neural crest cell induction. FoxD3 and Slug can promote delamination,75,76 but when both genes are induced in ventral neural tube cells by Sox9 overexpression, the Slug+FoxD3+ cells fail to delaminate, suggesting an additional signal in the dorsal neural tube is required.73 Perhaps relevant to the nature of this additional signal, there is strong evidence that BMP activity in the dorsal neural tube is both necessary and sufficient to promote neural crest cell delamination.77–79 A recent review of the molecular mechanisms underlying neural crest cell delamination can be found in Reference 80.

The control of neural crest cell migration is beyond the scope of this chapter, but numerous recent reviews may be consulted on this topic (e.g., References 80 and 81). The remainder of the chapter concentrates on how neural crest cells adopt a sensory neuronal fate, beginning with a general discussion of neural crest lineage diversification.

### 1.3.2 Neural Crest Lineage Diversification: General Principles

Neural crest cells clearly give rise to an enormous variety of derivatives, from cartilage to pigment cells to neurons. Here, we are concerned with the formation specifically of sensory neurons. The sensory neurons of the jugular ganglion are derived from neural crest cells emigrating from the postotic hindbrain, at the level of the first three somites (see Figure 1.2A and Figure 1.3).8,12

Two different developmental questions arise when considering the extraordinary lineage diversification of neural crest cells and how, for example, sensory neurons develop.82 Firstly, neural crest cells emigrating at different axial levels do not all form sensory neurons during normal development. Indeed, neural crest cells from different rostrocaudal levels of the neuraxis give rise to very different subsets of derivatives, a phenomenon called axial fate restriction. For example, cranial neural crest cells do not form sympathetic neurons, while trunk neural crest cells do not
form cartilage. What mechanisms underlie axial fate restriction? Are neural crest cells that emigrate at different axial levels intrinsically different, such that they only have the potential to form the derivatives they are fated to form, or do cells that emigrate at different axial levels encounter different environments, and thus different instructive differentiation cues? Secondly, neural crest cells that emigrate at the same or adjacent axial levels form multiple derivatives. For example, the “vagal” neural crest cells that emigrate from the postotic hindbrain at the level of the first 7 somites, give rise not only to sensory neurons in the superior and jugular ganglia and the most rostral dorsal root ganglia, but also to parasympathetic neurons, sympathetic neurons, enteric neurons, Schwann cells, satellite glial cells, calcitonin-producing cells, the carotid body, and the aorticopulmonary septum of the heart. How is this lineage diversification achieved from cells at the same axial level? Are emigrating neural crest cells totally naïve, responding to whichever differentiation cues they encounter, or are they a heterogeneous collection of predetermined cells?

The results of numerous heterotopic grafting experiments have shown that axial fate restriction does not seem to reflect axial-specific restrictions in potential, rather, it reflects axial-specific differences in the environmental signals encountered by neural crest cells (reviewed in Reference 1). For example, cranial neural crest cells grafted into the trunk will readily form sympathetic neurons. Indeed, recent experiments suggest that the most longstanding apparent exception to the rule, namely that trunk neural crest cells lack the potential to form cartilage, may finally have been removed. Most current evidence, therefore, suggests that neural crest cells are fairly malleable, at least at the population level, and that there are no insurmountable restrictions associated with their rostrocaudal level of origin.

Lineage diversification at the same axial level could in theory be accounted for by two opposing hypotheses: instruction and selection. The first (instruction) proposes that neural crest cells emigrate as a homogeneous population of naïve cells that are instructed to differentiate into particular derivatives depending on where they end up. The second (selection) proposes that emigrating neural crest cells are a heterogeneous collection of determined cells that only form particular derivatives and that are eliminated from inappropriate environments. Both are compatible with the heterotopic grafting experiments used to attack the problem of axial fate restriction. The evidence, as so often in such cases, suggests that the truth lies somewhere in-between. Single cell lineage analysis, both in vivo and in vitro, suggests the existence of multipotent neural crest cells with the capacity to form multiple derivatives in response to instructive signals (reviewed in References 1, 84, and 85). Furthermore, several such instructive signals have been identified in recent years, such as BMPs for sympathetic neurons; neuregulin 1 type II (glial growth factor) for satellite glial cells; neuregulin 1 type III for Schwann cells; and Wnts for both melanocytes and sensory neurons (see Section 1.3.3.1).

However, fate-restricted neural crest cells have also been identified, even in neural crest cell populations that have just left the neural tube, suggesting that the migrating neural crest cell population is indeed heterogeneous (reviewed in References 82 and 92). Neural crest precursors are, of course, exposed to environmental cues while they are within the neural tube: for example, Wnt family members are expressed in the dorsal neural tube, and these can instructively promote melanocyte and sensory...
neural fates\textsuperscript{90,91} (see Section 1.3.3.1). It seems likely that early exposure to instructive cues, plus community effects (neural crest cell-cell interactions) that lead to early fate restrictions (e.g., Reference 93), together underlie the heterogeneity seen in the migrating neural crest cell population. It is important to note, however, that restriction in fate does not necessarily imply restriction in potential: this can only be tested experimentally, by challenging the cell with a different environment, ideally \textit{in vivo}.

### 1.3.3 Sensory Neurogenesis within the Neural Crest

#### 1.3.3.1 Wnt Signaling Instructively Promotes Adoption of a Sensory Neuron Fate

The signaling molecule Wnt1 is expressed in the dorsal midline of the entire developing central nervous system except the telencephalon, throughout the period of neural crest cell emigration (see Reference 94). Neural crest cells do not themselves express \textit{Wnt1} mRNA, but they are derived from \textit{Wnt1}-expressing precursors in the dorsal midline of the neural tube, and the \textit{Wnt1} promoter can be used to drive gene expression in neural crest cells\textsuperscript{94} (also see References 91, 95, and 96). Genetic ablation of β-catenin, an essential component of the canonical Wnt signaling pathway (reviewed in Reference 97), in neural crest precursors and migrating neural crest cells (under the control of the \textit{Wnt1} promoter) results in a complete lack of melanocytes and a severe reduction in sensory neural cells (both neurons and satellite cells) in peripheral sensory ganglia.\textsuperscript{98} Mutant neural crest cells fail to form dorsal root ganglia and instead contribute to sympathetic and enteric ganglia.\textsuperscript{98} These results suggested that Wnt signaling is essential for adoption of both sensory neural and pigment cell fates.

Conversely, expression of constitutively active β-catenin in neural crest precursors and migrating neural crest cells (again under the control of the \textit{Wnt1} promoter) leads to the formation of sensory neurons at the expense of almost all other neural crest derivatives.\textsuperscript{91} Clonally cultured early mammalian neural crest cells respond to Wnt1 primarily by generating small (fewer than 5 cells) clones of sensory neurons (over half formed a single sensory neuron),\textsuperscript{91} while β-catenin-deficient neural crest stem cells do not form sensory neurons in response to Wnt1.\textsuperscript{91} Taken together, these results strongly suggest that Wnt signaling instructively promotes the adoption of a sensory neuron fate by early neural crest cells.

The additional requirement for Wnt signaling for melanocyte formation\textsuperscript{90,98} may reflect Wnt action at different times during development (see Reference 99). It is possible that Wnt signaling promotes a sensory fate in neural crest precursors, but a pigment cell fate in neural crest cells at a later stage of development.\textsuperscript{99} In zebrafish, injection of mRNA encoding a constitutively active β-catenin, into neural crest precursors normally fated to form neurons, leads them to adopt a pigment cell fate.\textsuperscript{90} This seems to contradict the results described previously in mice. The difference seen may reflect a delay in the production and accumulation of constitutively active β-catenin after mRNA injection, such that the cells respond by forming pigment cells instead; however, this hypothesis remains to be tested. Given that both \textit{Wnt1} and \textit{Wnt3a} are expressed in the dorsal neural tube (and indeed that neural crest cells
Advances in Vagal Afferent Neurobiology

are formed from Wnt1-expressing precursor cells), the question also arises as to why only a subset of neural crest cells adopts a sensory neural fate. Community effects (i.e., interactions among neighbor neural crest precursors or neural crest cells) and the presence of other competing signals are likely to be important here, but much work remains to be done to clarify our understanding of these processes.

1.3.3.2 Sensory-Fated and Sensory-Committed Precursors Are Present in the Migrating Neural Crest Population

As described in the preceding section, Wnt signaling instructively promotes a sensory neuronal fate in early neural crest cells. Wnts are expressed in the dorsal neural tube, suggesting that at least some neural crest precursors are likely to be specified toward a sensory fate even prior to delamination. Indeed, clonal analysis of migrating neural crest cells in the chick showed that some clones (which included both neurons and glia) were restricted either to dorsal root ganglia or sympathetic ganglia, suggesting that the fate of some cells and their progeny is restricted to either a sensory or an autonomic fate. Furthermore, it appears that the migrating mammalian trunk neural crest cell population already contains cells that are committed to a sensory fate. Even when cultured in the presence of a potent autonomic neurogenic signal, BMP2, some proliferating rat trunk neural crest cells form sensory neurons. This early commitment to a sensory neuronal fate may, therefore, reflect prior exposure of some neural crest precursors to Wnts in the dorsal neural tube.

1.3.3.3 Neurogenin2 Biases Trunk Neural Crest Cells to a Sensory Fate

In the mouse, the proneural transcription factor Neurogenin2 (Ngn2; see Section 1.2.5.1) is expressed both in cells in the dorsal neural tube, and in a subpopulation of migrating trunk neural crest cells; it is maintained into the early stages of dorsal root ganglion formation. Elegant genetic experiments have shown that the Ngn2+ subpopulation of migrating neural crest cells is not absolutely committed to a sensory fate, but is strongly biased toward it. Ngn2 is not expressed in cranial neural crest cells, however, and all neural crest-derived cranial sensory ganglia are normal in Ngn2-mutant mice.

1.3.3.4 Neurogenin1 Is Required for the Formation of Jugular Neurons

All peripheral sensory ganglia are missing in zebrafish where Ngn1 function has been abrogated using antisense morpholinos (in fish, Ngn1 seems to encompass all functions of amniote Ngn1 and Ngn2). In Ngn1 mutant mice, the superior-jugular complex and the trigeminal ganglion are entirely missing. The bHLH cascade that leads to neurogenesis is abrogated in neural crest cells that populate the nascent superior-jugular and trigeminal ganglia in Ngn1-mutant mice, as neither NeuroD nor Math3 are detected, and no neurons form. Hence, Ngn1 function is essential for the formation of jugular neurons.
1.3.3.5 Inhibition of Notch Signaling Is Required for Sensory Neuron Differentiation

Neuronal differentiation within the dorsal root ganglia depends on the inhibition of signaling through the transmembrane receptor Notch; conversely, glial differentiation depends on Notch activation (see Section 1.2.5.1). In the dorsal root ganglia, dividing cells on the ganglionic periphery preferentially express Notch1, while the Notch ligand, Delta1, is expressed by differentiating neurons in the ganglionic core. Constitutive activation of Notch signaling in quail trunk neural crest cells in vitro inhibits neuronal differentiation and transiently increases cell division, suggesting that Notch activity must be inhibited for neurogenesis to take place. Asymmetric inheritance of the Notch antagonist Numb may be important for determining which cells within the ganglion form neurons and which continue to cycle and/or form satellite glial cells. When Numb is knocked out genetically in mice, sensory neurons fail to form in the dorsal root ganglia, providing additional support for this model. Cranial sensory neurons were not explicitly examined in this study, although apparently neurogenesis in the trigeminal ganglion was normal. Hence, it is unclear how important the asymmetric inheritance of Numb is for sensory neurogenesis in the cranial sensory ganglia.

Although there is little or no information specifically on the importance of Notch inhibition for the formation of neural crest-derived cranial sensory neurons, studies of placodal neurogenesis have shown that Ngn1 (see previous section) is required for expression of the Notch ligand Delta1. Since all cranial neural crest-derived sensory neurons are missing when Ngn1 function is abrogated, and given the importance of Notch inhibition for sensory neurogenesis in the dorsal root ganglia, it seems likely that Notch inhibition is also required for sensory neurogenesis in cranial sensory ganglia.

1.3.4 SUMMARY OF JUGULAR NEURON DEVELOPMENT

Jugular neurons are derived from the neural crest, a population of ectodermal cells that originates from ectoderm at the lateral borders of the neural plate. Neurulation, that is, the rolling up of the neural plate to form the neural tube, brings neural crest precursors to the “crest” of the future brain and spinal cord. Neural crest cells subsequently delaminate and migrate throughout the embryo on stereotypical migration pathways. Neural crest cells that form jugular neurons migrate from the postotic hindbrain, at the level of the first three somites. Neural crest cells are induced in three steps:

1. Formation of the neural plate border
2. Posteriorization of the neural plate border and induction of neural crest precursors within it
3. Epithelial-mesenchymal transition to form migrating neural crest cells

Neural crest cells form an enormous array of different derivatives. Lineage diversification is achieved by a combination of instructive environmental cues and
early fate-restrictions (probably due to neural crest cell-cell interactions, i.e., community effects) that mean the migrating neural crest is a heterogeneous population of multipotent and fate-restricted cells. Wnt signaling instructively promotes adoption of a sensory neuronal fate within the early neural crest. Wnts are expressed in the dorsal neural tube, and at least some early migrating neural crest cells are already restricted to a sensory fate. The proneural basic helix-loop-helix transcription factor Neurogenin1 is required for the formation of neurons in all proximal cranial sensory ganglia, including the jugular ganglion. It is likely that inhibition of the Notch signaling pathway, which is necessary for neurogenesis in dorsal root ganglia, is also required for neurogenesis in cranial sensory ganglia.

REFERENCES

Advances in Vagal Afferent Neurobiology

41. Ma, Q. et al., *neurogenin1* is essential for the determination of neuronal precursors for proximal cranial sensory ganglia, *Neuron*, 20, 469, 1998.


60. Nguyen, V.H. et al., Ventral and lateral regions of the zebrafish gastrula, including the neural crest progenitors, are established by a *bmp2b/brwirl* pathway of genes, *Dev. Biol.*, 199, 93, 1998.


76. del Barrio, M.G. and Nieto, M.A., Overexpression of Snail family members highlights their ability to promote chick neural crest formation, *Development*, 129, 1583, 2002.


88. Hagedorn, L. et al., The Ets domain transcription factor Erm distinguishes rat satellite
89. Leimeroth, R. et al., Membrane-bound neuregulin1 type III actively promotes
90. Dorsky, R.I., Moon, R.T., and Raible, D.W., Control of neural crest cell fate by the
91. Lee, H.Y. et al., Instructive role of Wnt/beta-catenin in sensory fate specification in
92. Dorsky, R.I., Moon, R.T., and Raible, D.W., Environmental signals and cell fate
94. Echelard, Y., Vassileva, G. and McMahon, A.P., Cis-acting regulatory sequences
95. Serbedzija, G.N. and McMahon, A.P., Analysis of neural crest cell migration in
96. Zirlinger, M. et al., Transient expression of the bHLH factor neurogenin-2 marks a
97. Nusse, R., Wnts and Hedgehogs: lipid-modified proteins and similarities in signaling
98. Hari, L. et al., Lineage-specific requirements of beta-catenin in neural crest develop-
100. Fraser, S.E. and Bronner-Fraser, M., Migrating neural crest cells in the trunk of the
102. Ma, Q. et al., NEUROGENIN1 and NEUROGENIN2 control two distinct waves of
103. Wakamatsu, Y., Maynard, T.M., and Weston, J.A., Fate determination of neural crest
cells by NOTCH-mediated lateral inhibition and asymmetrical cell division during
2 Vagal Afferent Neurons: Neurotrophic Factors and Epigenetic Influences

Cinda J. Helke

CONTENTS

2.1 Introduction ....................................................................................................28
2.2 Epigenetic Influences on Development/Survival of Vagal Afferent Neurons ..........................................................29
  2.2.1 Neurotrophins in Development ..........................................................29
    2.2.1.1 NGF and TrkA ....................................................................29
    2.2.1.2 BDNF, NT-4, and TrkB ......................................................31
    2.2.1.3 NT-3 and TrkC ....................................................................34
  2.2.2 Other Neurotrophic Factors/Receptors in Developing Ganglia ........36
    2.2.2.1 Glial Cell Line-Derived Neurotrophic Factor (GDNF) Family ..........................................................36
    2.2.2.2 Ciliary Neurotrophic Factor (CNTF) Family ......................37
  2.2.3 Activity- and Ion Channel-Dependent Plasticity of Vagal Ganglia in Development ..........................................................38
  2.2.4 Trophic Actions of Nonneuronal Cells Associated with Developing Nodose Ganglia ..........................................................39
2.3 Epigenetic Influences on Maintenance of Mature Vagal Afferent Neurons ........................................................................40
  2.3.1 Epigenetic Influences on Neurochemistry of Mature Vagal Afferent Neurons ..........................................................40
    2.3.1.1 Nitric Oxide ........................................................................40
    2.3.1.2 Tyrosine Hydroxylase .........................................................41
    2.3.1.3 Neuropeptides .....................................................................42
    2.3.1.4 c-Jun ....................................................................................45
  2.3.2 Neurotrophins .....................................................................................45
    2.3.2.1 Neurotrophins Associated with Vagal Afferent Neurons ...46
    2.3.2.2 Neurotrophin Receptors Associated with Vagal Afferent Neurons ..........................................................46

27
2.1 INTRODUCTION

Visceral afferent neurons, including vagal and glossopharyngeal afferent neurons, are critical to autonomic and visceral homeostasis. Establishing and maintaining the appropriate functions of these neurons is thus necessary to the integrity of visceral afferent signals to the brain and subsequent influences on autonomic reflexes. Developmental influences on afferent neurons and the ways in which mature visceral afferent neurons respond to injury or neuropathic insults are likely to be important in determining the success or failure of the neurons to maintain visceral afferent reflexes. The functions of these neurons are altered in chronic disease states such as hypertension (chronic overloading of baroreceptors) and congestive heart failure (chronic overloading of cardiac stretch receptors). Injury to visceral afferent nerves can occur from chemical agents (antineoplastic agents), trauma, tumors, and disease (e.g., diabetes mellitus, Guillain-Barre syndrome).

Numerous alterations in the neuronal phenotype, growth, regenerative capacity, and survival are evident during the life span of these neurons, from development through maturity. Knowledge of the influences of trophic factors, injury, and other epigenetic influences on visceral afferent neurons is growing but remains incomplete. Knowledge of the regulatory influences on visceral afferent neurons is essential to understanding the responses of the neurons to deprivation of target-derived factors, and the potential roles of neurotrophins in alleviating specific dysfunctions of these neurons. Moreover, understanding neurotrophic receptor activation, transport, and actions in this important neuronal system will extend our ability to safely and wisely use neurotrophic factors as therapeutic agents in a variety of neuropathic and degenerative disorders affecting the autonomic nervous system.

This chapter provides current information on the neurotrophic and other influences affecting these important visceral afferent neurons.
2.2 EPIGENETIC INFLUENCES ON DEVELOPMENT/SURVIVAL OF VAGAL AFFERENT NEURONS

2.2.1 NEUROTROPHINS IN DEVELOPMENT

The best-studied neurotrophic responses are those of the neurotrophin family of neurotrophic factors. The neurotrophins, a structurally related group of polypeptide neurotrophic factors, include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4). Nodose ganglion neurons express two classes of transmembrane neurotrophin receptors, which display pharmacologically distinct neurotrophin binding sites. Neurotrophin receptors include the low-affinity p75 receptor and the Trk family of receptor tyrosine kinases. NGF is the preferred ligand for TrkA, whereas TrkB is activated by both BDNF and NT-4, and NT-3 is the preferred ligand for TrkC.30,68,122

During development, nodose ganglion neurons are dependent for survival on BDNF, NT-3, and NT-4 but less, if at all, dependent on NGF.32,142,213 Studies of mice with targeted mutations of the neurotrophin genes reveal the dependence of developing nodose ganglion neurons on BDNF, NT-3, and NT-4; however, NGF gene knockout mice and rodents autoimmune to NGF show deficits of nodose ganglion neurons only in narrow developmental periods.213

2.2.1.1 NGF and TrkA

NGF mRNA is not detected in E13 to E18 rat nodose or petrosal ganglia.53 TrkA mRNA is present in the nodose/petrosal ganglia as early as E11 and peaks at E18. As development progresses, the TrkA labeling becomes isolated in the larger cells in the nodose ganglion and becomes less intense.75 Modest labeling of TrkA mRNA in the nodose and petrosal ganglia is noted at postnatal day 1 (Figure 2.1).252

Most studies suggest that NGF has little, if any, effect on survival of developing nodose ganglion neurons. Many studies report that NGF has no trophic effect on cultured nodose ganglion neurons.108,141,142,155,186 Studies using NGF gene knockout mice and rodents autoimmune to NGF also show no deficits of nodose ganglion neurons.108,186,213

In contrast to the numerous studies not showing an NGF dependence of developing nodose ganglion neurons, recent studies suggest that NGF may play a role at specific developmental stages in the survival of ganglion neurons. Katz et al. (see Reference 117) showed that NGF supports a small percentage of placode-derived neurons in explant culture for a narrow embryologic period (E13.5 to E14.5). Mice rendered NGF deficient had fewer neurons at middle to late (E16 and E18) embryologic stages and an increase in dying cells at middle stages (E16) only.63 NGF promotes neuronal survival in cultured E12 and E13, as well as P1 nodose/petrosal ganglia, but has no effect on the embryologic stages in between.63 Moreover, NGF increased the total dendritic growth in newborn nodose ganglion neurons.41

The presence of NGF in neonatal nodose ganglion cultures increases the amount of the neuropeptides calcitonin gene-related peptide (CGRP) and substance P (SP)
FIGURE 2.1 NGF, TrkA, and vagal afferents. (A) Dark-field photomicrographs showing adult nodose (NG), petrosal (PG), and jugular (JG) ganglia hybridized in situ with an 35S-labeled antisense oligonucleotide probe for TrkA mRNA. Bar = 250 µm. (Adapted from Zhuo and Helke, 1996.) (B and C) Double immunofluorescent photomicrographs for TrkA-ir (B) and calbindin D-28k-ir (C) in the rat nodose ganglion. Arrows indicate neurons labeled for both TrkA- and calbindin D-28k-ir; arrowheads indicate TrkA neurons lacking calbindin D-28k-ir. Bar = 50 µm. (Adapted from Ichikawa and Helke, 1999.) (continued)
expressed by the neurons. Nodose ganglion neurons cultured in the presence of NGF have double the amount of SP. The increased amount of CGRP in these neurons is variable, little change to 50% increase, with each experiment compared with those nodose ganglion neurons cultured without NGF present. Newborn nodose ganglion neurons cultured with NGF show an increase in the amount of SP-immunoreactivity (ir) in normally expressing cells and in the total number of cells expressing SP. In more recent studies, the level of TH mRNA in the nodose/petrosal ganglia is not affected by NGF deficiency, but addition of NGF doubles the percentage of tyrosine hydroxylase (TH)-positive neurons at both E14.5 and E16.5.

### 2.2.1.2 BDNF, NT-4, and TrkB

Numerous studies support the role for BDNF and many studies support a role for NT-4 in the development of nodose ganglion neurons. In addition, there appears to be an interactive relationship between these two neurotrophins, and among these agents and other trophic factors during development (Figure 2.2).

BDNF mRNA is found in the E13 to E18 rat nodose ganglion, but not in the petrosal ganglion. BDNF mRNA and protein are first detected in the nodose/petrosal at E16.5 and the levels increase until P0. BDNF is expressed transiently in nodose/petrosal target tissues coincident with sensory innervation and nodose/petrosal neuronal dependence on BDNF in vitro. BDNF is expressed in hindbrain and heart (central and peripheral targets) prior to arrival of the earliest sensory axon.

TrkB mRNA is detected in 1-day-old neonatal rat nodose and petrosal ganglia. TrkB mRNA is first detected in the chick nodose ganglion at E21 and its level continues to rise to E24 (no measurement performed at later stages), while p75 mRNA is first detected at E20 and its level continues to rise to E24. The addition

**FIGURE 2.1 (CONTINUED)** (D) Bar graph showing the percent of enriched dissociated mature nodose/petrosal ganglia neurons immunoreactive for tyrosine hydroxylase (TH), vasoactive intestinal peptide (VIP), and calcitonin gene-related protein (CGRP) after 5 days in cultures treated with NGF at 0, 50, 100 ng/ml or 100 ng/ml in the presence of an NGF neutralizing antibody (+AB). Neurotrophins and neutralizing antibodies were present throughout the 5-day culture period. a = P<0.05 compared with control, b = P<0.05 compared with corresponding 100 ng/ml data. (Adapted from Helke and Verdier-Pinard, 2000.) (E and F) Low power dark-field photomicrographs of the ipsilateral nodose ganglion (E) and nucleus of the solitary tract (NTS) (F) showing the retrogradely transported autoradiographic labeling of cell bodies in the NG and terminals in the NTS (-13.2 caudal to bregma) and cell bodies in the dorsal motor nucleus of the vagus (DMV) after application of 125I-NGF to the cervical vagus nerve. IV = 4th ventricle, arrow indicates ipsilateral solitary tract. Note absence of labeling in the petrosal ganglion. Bar in E = 500 µm; bar in F = 200 µm. (Adapted from Helke et al., 1998.) (G and H) Dark-field photomicrographs showing the in situ hybridization labeling of mRNAs for NGF (G) and TrkA (H) in the proximal stump of the transected cervical vagus nerve 1 day after axotomy. Arrows indicate the site of transection. Bar = 500 µm. (Adapted from Lee et al., 2001a.) (I) Bar graph showing the effect of streptozotocin (STZ)-induced diabetes on NGF content of the intact right cervical vagus nerve at the 8-, 16-, and 24-week time points. a = P<0.05 compared with control, b = P<0.05 compared with diabetic+insulin-treated counterparts. (Adapted from Lee et al., 2001b.)
of BDNF to the cultured medium increases TrkB mRNA only after 48 hours. There is no difference in the amount of TrkB mRNA between BDNF-deficient, BDNF-heterozygous, and wild-type mouse nodose ganglia.

During development, BDNF and NT-4 promote nodose neuronal survival in vitro and in vivo. BDNF increases nodose ganglion

---

**FIGURE 2.2** BDNF, TrkB, and vagal afferents. (A and B) Dark-field photomicrographs (A) and bright-field (B) showing autoradiographic labeling of the nodose ganglion (NG) in neonatal (P1) rats using 35S-labeled antisense oligonucleotide probe for TrkB mRNA. BOcc = basiocipital bone; TyBU = tympanic bulla. Bar = 250 µm. (Adapted from Zhuo and Helke, 1996.) (C and D) Dark-field photomicrographs of the ipsilateral nodose ganglion (C) and nucleus of the solitary tract (NTS) (D) showing the small number retrogradely transported autoradiographic labeling of cell bodies in the NG and absence of terminals in the NTS (~13.2 caudal to bregma) after application of 125I-BDNF to the cervical vagus nerve. Note the cellular labeling in vagal efferent neurons in the dorsal motor nucleus of the vagus (DMV). CC = central canal; AP = area postrema. Bar = 200 µm. (Adapted from Helke et al., 1998.) (E and F) Dark-field photomicrographs showing the in situ hybridization labeling of mRNAs for BDNF (E) and TrkB (F) in the proximal stump of the transected cervical vagus nerve 1 day after axotomy. Arrows indicate the site of transection. Bar = 500 µm. (Adapted from Lee et al., 2001a.)
Neurotrophic Factors

neurite outgrowth in E12 chick nodose ganglion,15 both the neurite length and density of late embryologic stage and newborn rat nodose ganglion explants.160,180 Although early studies reported that NT-4/NT-5 had little effect on nodose ganglion neurons,15,74 later studies report that NT-4 supports cultured developing nodose ganglion neurons86 and induces neurite outgrowth from the cultured adult nodose ganglion.245

Studies in mice rendered neurotrophin-deficient reveal a distinct dependency of developing nodose or petrosal ganglion neurons to BDNF and NT-4.32,51,52,110,144 Deficiencies in BDNF or NT-4 affect the nodose/petrosal neuronal number at different stages in development. A deficiency in BDNF has no effect on the neuronal survival of nodose/petrosal from E11 and E12 mouse embryos; however, addition of exogenous BDNF increases neuronal survival in both mutant and wild-type mice.98 BDNF added to the culture of E3.5 chick nodose ganglion neurons for 72 hours has no effect on neuronal survival compared with control, and these neurons die faster than control after discontinuation of the BDNF treatment.237 Mice deficient for BDNF have increased neuronal loss and increased apoptosis in the nodose/petrosal ganglia at later embryologic stages (E14 to P1).49 From this, it is apparent that BDNF’s role in nodose/petrosal neuronal development comes at later stages. Nodose ganglion neuronal dependence on BDNF is accelerated by a 12-hour pulse of BDNF added to the culture of E3.5 chick nodose ganglion neurons after 48 hours. Once the BDNF is removed at 60 hours, these neurons die faster than controls.237 A 12-hour pulse prior to 48 hours in culture has no effect on BDNF dependence;237 thus, the nodose neurons are only responsive to BDNF at certain embryologic developmental stages. In transgenic mice that overexpress BDNF in epithelial target tissues of sensory neurons, a 38% increase in neurons comprising the placode-derived nodose–petrosal complex occurred.135

Mice deficient in NT-4 lack 55% of vagal sensory neurons, yet are viable.32,144 Mice deficient in NT-4 have increased neuronal loss, more apoptosis, and less proliferation in the nodose/petrosal ganglia at middle embryologic stages (E12 to E14).49 NT-4 deficient mice show severe loss of vagal afferent structures from the duodenum and ileum but not the stomach.64 Knockout of BDNF, NT-4, or both decreases the neonatal nodose/petrosal neuronal survival.49–51,136 NT-3 and BDNF together rescue 90% of these neurons from death.94 Almost all nodose/petrosal neurons are lost in newborn mice lacking functioning TrkB receptors.51

BDNF and NT-4 have nonredundant, but compensatory, actions in the nodose/petrosal ganglion, and each target separates subpopulations of the ganglia.51 Without functioning BDNF or NT-4 there is a loss of about 50% in nodose/petrosal neuronal number, and with neither there is a loss of about 90%.51 A large subpopulation of nodose/petrosal neurons can be supported by NT-4 when BDNF is not available, which suggests that NT-4 can compensate for the lack of BDNF during development.51 The number of surviving neurons is proportional to the number of functioning BDNF alleles. In contrast, a single functioning NT-4 allele can support a large population of neurons.51

An organ-specific loss associated with a neurotrophin deficiency occurs in BDNF mutants wherein arterial chemoreceptors in the carotid body and baroreceptors (a class of mechanoreceptors) in the carotid sinus are lost.18,51 Thus, receptors of two modalities but of the same organ system (arterial beds) are affected by loss of BDNF
during development. *In vivo* chemosensory afferents are selectively supported by treatment with TrkB ligands (BDNF and NT-4), whereas NGF and NT-3 had no effect.\(^{86}\) In the absence of target tissues, a large proportion of carotid body afferents is rescued by implants containing BDNF.\(^{86}\)

### 2.2.1.3 NT-3 and TrkC

NT-3 mRNA is found in the E13 to E18 rat petrosal ganglion, but not in the nodose ganglion.\(^{53}\) TrkC mRNA is detected in the nodose but not petrosal ganglion of the embryologic chick\(^ {246}\) and rat.\(^ {53}\) The nodose and petrosal ganglia from 1-day-old neonates expressed TrkC mRNA (Figure 2.3).\(^ {252}\)

Embryologic nodose neurons are responsive to NT-3.\(^ {105}\) In nodose ganglion, a small population of neurons exclusively responds to NT-3 and a larger population of nodose ganglion neurons that can be supported by NT-3 or by BDNF.\(^ {136}\) The presence of NT-3 supports the survival of 30% of embryologic nodose ganglion neurons, NT-3 and BDNF together rescue 90% of these neurons from death.\(^ {94}\) NT-3 increases nodose ganglion neurite outgrowth in E12 chick nodose ganglion,\(^ {16}\) and both the neurite length and density of late embryologic stage and newborn rat nodose ganglion explants.\(^ {160,180}\)

NT-3 deﬁcient mice have fewer nodose/petrosal neurons than wild-type mice.\(^ {57}\) Mice deﬁcient for NT-3 have increased neuronal loss, more apoptosis, and less proliferation in the nodose/petrosal ganglia at early embryologic stages (up to E14).\(^ {49}\) In newborn single mutant mice, heterozygotes have fewer neurons in the nodose/petrosal ganglia than wild-type and the fewest neurons are in the homozygotes.\(^ {49}\) Newborn mice deﬁcient in BDNF, NT-3, or NT-4 have 30 to 66% fewer neurons in the nodose ganglia compared with wild-type and the effect is additive in triple-deﬁcient mice where there were 96% fewer neurons in the nodose ganglia compared with wild-type.\(^ {145}\) Vagal afferent structures in the esophagus are reduced in adult mice deﬁcient for NT-3 or TrkC.\(^ {191}\)

BDNF acts only via the TrkB receptor during nodose neuronal development, while NT-3 acts via both the TrkB and TrkC receptors during nodose neuronal development. The percent survival in E13 nodose neurons after 36 to 48 hours is less than 10% in the presence of BDNF or NT-3 when TrkB is not functional and
Seventy-five to eighty percent of the E13 nodose neurons survive in the presence of BDNF with functioning TrkB receptors, regardless of TrkC genotype, and 55 to 60% of the nodose neurons survive in the presence of BDNF with the heterozygous (+/−) TrkB genotype, regardless of TrkC genotype.

FIGURE 2.3 NT-3, TrkC, and vagal afferents. (A) Dark-field photomicrographs showing adult nodose ganglion hybridized in situ with an 35S-labeled antisense oligonucleotide probe for TrkC mRNA. Bar = 250 µm. (Adapted from Zhuo and Helke, 1996.) (B) Bar graphs showing the percent of nodose/petrosal ganglia neurons immunoreactive for tyrosine hydroxylase (TH), vasoactive intestinal peptide (VIP), and calcitonin gene-related peptide (CGRP) after 5 days in dissociated enriched neuronal cultures treated with NT-3 at 0, 50, 100 ng/ml or 100 ng/ml in the presence of NT-3 neutralizing antibody (+AB). Neurotrophins and neutralizing antibodies were present from the time of plating, throughout the 5-day culture period. a = P<0.05 compared with control, b = P<0.05 compared with corresponding 100 ng/ml data. (Adapted from Helke and Verdier-Pinard, 2000.) (C and D) Low-power dark-field photomicrographs of the ipsilateral nodose ganglion (C) and nucleus of the solitary tract (NTS) (D) showing the retrogradely transported autoradiographic labeling of cell bodies in the NG and terminals in the NTS (-13.5 caudal to bregma) after application of 125I-NT-3 to the cervical vagus nerve. AP = area postrema, arrow indicates ipsilateral solitary tract. Note absence of labeling in the petrosal ganglion and in the dorsal motor nucleus of the vagus (DMV). Bar in C = 500 µm, bar in D = 200 µm. (Adapted from Helke et al., 1998.) (E and F) Dark-field photomicrographs showing the in situ hybridization labeling of mRNAs for NT-3 (E) and TrkC (F) in the proximal stump of the transected cervical vagus nerve 1 day after axotomy. Arrows indicate the site of transection. Bar = 500 µm. (Adapted from Lee et al., 2001a.)
Fifty to fifty-five percent of the E13 nodose neurons survive in the presence of NT-3 with functioning TrkB receptors, regardless of TrkC genotype. NT-3 promotes levels of survival in neurons with heterozygous TrkB receptors that are similar to the wild-type TrkB numbers as long as there is a functioning TrkC receptor. The percent survival of the neurons decreases by about 20% with heterozygous TrkC receptors and by about 50% with homozygous −/− receptors.40

The presence of BDNF during neuronal development can alter the transmitter phenotype of nodose and/or petrosal ganglion neurons. BDNF attenuates the carotid body glomectomy-induced catecholaminergic cell loss in the petrosal ganglion of newborn rats.86,87 The absence of BDNF produces a loss in dopaminergic petrosal ganglion neurons between E14.5 and P0.50 Mice deficient for BDNF had about half as many TH-ir neurons at P0 than wild-type, whereas mice deficient for NT-3 and NT-4 have similar TH-ir in nodose neurons in all embryological stages.49 Moreover, all TH-ir neurons in the newborn petrosal ganglion are BDNF-ir.18

2.2.2 Other Neurotrophic Factors/Receptors in Developing Ganglia

Other neurotrophic factors, including glial cell line-derived neurotrophic factor (GDNF), ciliary neurotrophic factor (CNTF), and leukemia inhibitory factor (LIF), also have trophic actions on visceral sensory neurons.

2.2.2.1 Glial Cell Line-Derived Neurotrophic Factor (GDNF) Family

GDNF, originally isolated from the rat glial cell line B49, is found in many peripheral tissues, such as heart, lung, liver, kidney, spleen, blood,217 and CNS.214 The GDNF family includes GDNF, persephin, neurturin, and artemin.8 GDNF exerts its actions by binding to the transmembrane tyrosine kinase Ret and GDNF family receptors GFR alpha 1, 2, 3, and 4.

GDNF is found in nodose/petrosal target tissues, including the carotid body, at the same time as initial innervation by the neurons and coincident with neuronal dependence on GDNF.50,181,230 GDNF receptors are found in the nodose ganglion during embryologic development.131 The mRNA levels of the GDNF receptors GFR-1 and Ret are two to three times greater than that of the GDNF receptor GFR-2 at E8 to E12.62

GDNF supports the survival of developing chick nodose sensory neurons,25,230 particularly at early embryologic stages (E8 to E12).62 GDNF also supports rat embryologic nodose and petrosal ganglion neuronal survival in culture.50 Neurturin, although less potent than GDNF, also supports nodose ganglion neuronal survival at early embryologic stages.52

At middle chick embryologic stages (E13 and E16), there is neurite growth in the absence of neurotrophic factors.131 Thus, GDNF is not necessary for nodose ganglion neuronal neurite outgrowth at any embryologic stage, but GDNF can stimulate the outgrowth of fibers in the chick embryonic nodose ganglion.47 Addition of GDNF alone or GDNF plus the GDNF receptor GFR-1 to cultured chick nodose
ganglion neurons increases neurite growth at all stages. Likewise, GDNF increases neurite outgrowth from E18 and P1 rat nodose ganglion explants but not from adult ganglia.

Moore et al. report that newborn (P0) mice deficient for GDNF have fewer neurons in the nodose and petrosal ganglia complex compared with wild-type. However, the absence of GDNF does not affect the nodose neuron number but does reduce the number of petrosal ganglion neurons suggesting that nodose neurons do not require GDNF for survival while petrosal ganglion neurons do require GDNF. Moreover, no nodose ganglion neurons are lost in mice deficient in neurturin, GRF-2, or GFR-3. It has been proposed that BDNF and/or NT-4 support the survival of GDNF-dependent neurons in these mutant mice or that some nodose ganglion neurons become dependent on GDNF during development.

GDNF null mutant mice have reduced numbers of petrosal ganglion neurons. The absence of GDNF produces a loss in dopaminergic petrosal ganglion neurons between E15.5 and E17.5. GDNF is detected in petrosal ganglion targets at E15.5, at the same stage where petrosal neuron loss begins when the cells do not have GDNF, suggesting that it is a target-derived survival factor.

GDNF increases nodose neuronal survival in dissociate cultures as well as total and TH-ir neuron number. GDNF increases the total number of neurons and the number of TH-ir neurons in explant cultures of petrosal ganglia; however, the percentage of TH-ir cells remains constant, indicating that the increase in TH-ir is due to an increase in neuronal number and not an upregulation of TH. BDNF and GDNF together cause an additive increase in the number of TH-ir cells in the petrosal ganglion when they are administered at subsaturating, but not saturating, concentrations. In double knock-out (BDNF−/− and GDNF−/−) mice, 98% of TH-ir and RET-ir neurons are lost, showing that a large subset of dopaminergic petrosal ganglion neurons requires both BDNF and GDNF for survival in vivo.

### 2.2.2.2 Ciliary Neurotrophic Factor (CNTF) Family

CNTF is structurally related to interleukin 6 (IL-6), interleukin 11 (IL-11), leukemia inhibitory factor (LIF), cardiotrophin-like cytokine (CL-1) and oncostatin M (OSM). The high affinity receptor complex, which mediates the biological action of CNTF, contains the ligand-binding subunit (CNTF R) and two signal-transducing proteins, LIF R and gp130. CNTF and LIF receptor components are present in both E16.5 and newborn ganglia. Rat nodose and petrosal sections incubated with iodinated LIF have strong binding at E18 and P0, also indicating the presence of receptors for these factors.

CNTF was initially shown to promote the survival of the chick ciliary ganglion neurons. However, a study by Oppenheim suggested that CNTF had no effect, good or bad, on nodose survival, while a study by Rudge et al. suggested that CNTF increased nodose neuronal survival early in development. CNTF supports the survival of E16.5 but not neonatal rat nodose and petrosal ganglia neurons in dissociate cultures. LIF supports the survival of dissociated embryonic nodose ganglion neurons in culture. LIF appears to support a subset of nodose ganglion neurons that is also responsive to BDNF, NT-3, and NT-4. Throughout development, nodose
ganglion neurons in culture survive better when in the presence of CNTF, LIF, OSM, and CT-1. This effect, however, is not present at birth where only 10% of P0 neurons survive in the presence of LIF and CNTF. The neuronal survival benefits of IL-6 are seen in the later embryologic stages only where up to 45% of neurons survive in culture. Dose response analysis indicates that, of these neurotrophic factors, CNTF is the most potent, followed by OSM, LIF, CT-1, and, lastly, IL-6. The positive effects of CNTF on nodose neuronal survival are abolished when agents that block or inhibit PI3 kinase or inhibit lipid kinase are added to the culture medium. The positive effects of CNTF, LIF, and CT-1 on nodose neuronal survival are abolished when NFκB activity is blocked, suggesting that these factors work via a system that requires NFκB. Normal expression of STAT3 in mouse nodose ganglion neurons produces greater neuronal survival in the presence of CNTF and LIF compared with defective STAT3 expression. Mice deficient for p65 have less nodose neuronal survival in the presence of CNTF, LIF, CT-1, or IL-6 and an increased number of dying cells (measured by the presence of pyknotic nuclei) compared with wild-type. There are no differences in the number of cytokine receptors between p65 deficient and wild-type mice. Whereas p65 deficiency and blocking of NFκB activity prevent the positive effects of the cytokines CNTF, LIF, and CT-1 on neuronal survival, they have no effect on the increased neuronal survival produced by BDNF suggesting that BDNF does not act via a system that requires NFκB.

2.2.3 Activity- and Ion Channel-Dependent Plasticity of Vagal Ganglia in Development

Depolarizing concentrations of potassium increase neuronal survival in petrosal neurons from late embryologic (E19.5) and newborn (P0) cultures, but have no effect on the survival of nodose neurons at these, or any other, stages of development. Intracellular Ca²⁺ appears to play a role in neuronal survival before the chick nodose neurons have begun to express L-type Ca²⁺ channels. Addition of a cytosolic free Ca²⁺ chelator caused complete nodose neuronal death within 36 to 48 hours. Chronic addition of caffeine, which depletes intracellular stores of Ca²⁺, to the culture medium at 12, 24, and 48 hours causes complete neuronal loss by 48 hours after caffeine administration began. Simultaneous addition of KCl to these cultures at 12 hours increases neuronal survival to 10% after 48 hours. Simultaneous addition of KCl to these cultures at 24 hours increased neuronal survival to 30 to 40% after 48 hours. Simultaneous addition of KCl to these cultures at 48 hours prevented the caffeine-induced neuronal death. These data show the significance of the expression of the L-type Ca²⁺ channels and Ca²⁺ on neuronal survival. BDNF can also prevent the neuronal death produced by caffeine if administered after the neurons become dependent on BDNF for survival.

Stage 18 chick nodose neurons express L-type Ca²⁺ channels after 48 hours in culture and began to depend on BDNF for survival at 72 hours. Addition of KCl to the culture medium of stage 18 chick nodose neurons increases neuronal survival even in the absence of neurotrophic factors. Addition of nifedipine and verapamil
Neurotrophic Factors

(L-type Ca\(^{2+}\) channel antagonists) completely blocks the survival-promoting effect of the KCl, while addition of an L-type Ca\(^{2+}\) channel agonist increases the survival-promoting effect of KCl. KCl and BDNF both promote nodose neuronal survival, although the culture receiving KCl had higher percentages of neurons surviving after 120 hours than BDNF. Addition of L-type Ca\(^{2+}\) channel antagonists produced no change in the effect of BDNF, indicating that BDNF does not act via Ca\(^{2+}\) influx through L-type Ca\(^{2+}\) channels.

Activity-related cues can change the neurotransmitter phenotype of a population of neurons of the nodose ganglion. The presence of KCl in culture medium increases the number of TH-ir cells in both the nodose and petrosal ganglia throughout embryologic and neonatal development. KCl has no effect on the expression of SP in either the nodose or petrosal neurons.

In the fetal petrosal ganglion, where only 10 to 20% of the neurons are TH-ir under basal conditions, the TH expression and the dopamine content are increased tenfold by potassium or veratridine-induced depolarization. After 15 days in culture, the number of TH-ir neurons in E16.5 petrosal neuronal culture increases as the amount of time that KCl is administered increases. The timing of KCl administration appears to be important as cultures administered KCl for the final 3 days have more TH-ir neurons than when the KCl is administered for the initial 3 days. Block of L-type Ca\(^{2+}\) channels, but not N-type, significantly inhibits the TH induction by KCl in the petrosal neuron cultures.

Continuous exposure of KCl to newborn nodose/petrosal neurons induces the neurons to release BDNF as measured by the amount of extracellular BDNF levels compared with neurons with no KCl. Certain patterns of electrical stimulation are even more effective at inducing nodose/petrosal neurons to release BDNF.

2.2.4 TROPHIC ACTIONS OF NONNEURONAL CELLS ASSOCIATED WITH DEVELOPING NODOSE GANGLIA

The presence of nonneuronal cells in the nodose ganglion appears to play an important role in the development of neuronal phenotype (dopaminergic vs. cholinergic) especially in the 2 weeks prior to the neurons becoming committed to a sensory phenotype. When neonatal nodose neurons are cultured in the presence of nonneuronal satellite cells, the neurons develop typical nodose neuron phenotypes; however, when the nonneuronal cells are not present, the nodose neurons develop functioning cholinergic nicotinic synapses with each other. In nodose neuronal cultures without ganglionic satellite cells, 60% of the neurons develop synapses with each other and 75% are acetylcholine-sensitive. In nodose neuronal cultures with ganglionic cells, few synapses are formed between the cells and only 15% are acetylcholine-sensitive. Moreover, when neonatal nodose neurons are cultured in the presence of nonneuronal ganglion cells, NGF has no effect on the proportion of Acetylcholine-sensitive neurons or the density of acetylcholine receptors on individual neurons; however, when nonneuronal cells are not present, NGF increases both the proportion of sensitive neurons and the density of the receptors on individual neurons.
2.3 EPIGENETIC INFLUENCES ON MAINTENANCE OF MATURE VAGAL AFFERENT NEURONS

2.3.1 EPIGENETIC INFLUENCES ON NEUROCHEMISTRY OF MATURE VAGAL AFFERENT NEURONS

Multiple neurotransmitters, neuropeptides, calcium binding proteins, and other neuroactive substances are associated with vagal afferent neurons of the nodose ganglion. The neurochemistry of the nodose ganglion was initially studied with the histochemistry and immunoassay, and subsequently with immunohistochemistry, in situ hybridization histochemistry, and RT-PCR. Putative neurotransmitters (glutamate, catecholamines, serotonin, and acetylcholine), and numerous neuropeptides [substance P (SP), neurokinin A (NKA), vasoactive intestinal peptide (VIP), calcitonin gene-related peptide (CGRP), galanin, enkephalin (ENK), somatostatin (SOM), cholecystokinin (CCK), neuropeptide Y (NPY)], calcium binding proteins, and other neuroactive molecules (e.g., nitric oxide) are identified in the neurons of the nodose and petrosal ganglia. Many neurons colocalize two or more neuroactive substances, creating the potential for complex interactions of neurochemical signals in the NTS. Neurons of the nodose ganglion also contain a variety of receptors that respond to transmitters, inflammatory mediators, and neurotrophic factors.

The content and expression of these neurochemicals and receptors are not static. In response to epigenetic influences, such as axonal injury or transection, inhibited axonal transport, disease, alterations of the neuronal expression and content of specific neuroactive agents and proto-oncogenes are triggered in mature vagal afferent neurons. The neurochemical changes in mature visceral afferent neurons include the downregulation of specific neuropeptides and neurotransmitter enzymes and the upregulation of some neuropeptides and protooncogenes.

Alterations in the presence and expression of neurotransmitter enzymes include changes in neuronal nitric oxide synthase (nNOS) and tyrosine hydroxylase (TH).

2.3.1.1 Nitric Oxide

Neuronal nitric oxide synthase (nNOS)-immunoreactivity (ir), nNOS mRNA, and NADPH-diaphorase (a cofactor of NOS) are present in nodose ganglion neurons, and increase following transection of the vagus nerve. A crush lesion of the vagus nerve increases the expression of iNOS-ir in the nodose ganglion at a time course paralleling neuronal regeneration. Induction of nNOS, the enzyme that synthesizes neuronal nitric oxide, is a sensitive marker of injury to sensory neurons. Acute hypoxia also upregulates NADPH-d/nNOS-ir in the nodose ganglion. If the increase in nNOS protein also represents an increase in enzyme activity, and if the cofactors and L-arginine are present in the neuron, the result would be elevated nitric oxide. Nitric oxide is detrimental to the neurons when it forms peroxynitrite with O$_2^\cdot$. In the presence of oxidative stress, e.g., diminished GSH or SOD, this process is increased.
Neurotrophic Factors

Diabetic autonomic neuropathy profoundly alters autonomic reflexes, and is associated with morbidity and poor prognosis. A component of this dysfunction is due to altered visceral afferent neurons of the vagus nerve. The numbers of nNOS-ir neurons are increased in the nodose ganglion of diabetic compared with control rats at the 8- and 16-week time points, but not at the 24-week time point. However, no change is noted in the nNOS mRNA content of the diabetic nodose ganglion at either time point. Because anterograde and retrograde axonal transport are affected in the diabetic vagus nerve, the increase in the number of nNOS-ir cells in the neurons of the nodose ganglion may be attributed to a diabetes-induced inhibition of axonal transport that either causes a build up of protein in the cell body, interrupts neurotrophic support to the neurons, or both.

nNOS is bidirectionally transported in the vagus nerve as shown in studies wherein ligation-induced interruption of axonal transport resulted in an accumulation of nNOS in the nerve segments proximal and distal to the ligation. Nerve transection abolishes NADPH-d labeling distal to the ligature, suggesting that the accumulation of NADPH-d in the nerve is due to altered axonal transport rather than to an acute local production of inducible NOS as a result of injury.

2.3.1.2 Tyrosine Hydroxylase

Mature nodose and petrosal ganglia neurons express tyrosine hydroxylase (TH). In the adult nodose and petrosal ganglia of the rat in vitro, potassium-induced neuronal depolarization doubles TH enzyme activity suggesting an activity-dependent regulation in TH synthesis.

The numbers of TH-ir and mRNA containing neurons decrease shortly after axotomy or blockade of axonal transport. The number of tyrosine hydroxylase mRNA-containing neurons is significantly reduced at 3, 7, and 14 days after axotomy-induced deafferentation compared with intact and sham-operated controls. Labeling density of tyrosine hydroxylase mRNA-containing neurons is significantly reduced at 3 and 7 days. Thus, axotomy-induced deafferentation reduces the number of TH mRNA-containing cells in the nodose ganglion and the amount of mRNA in the remaining positive cells. Since cell area measurements show no significant differences between the population of labeled neurons in the intact and the axotomized groups, the decrease in labeling density cannot be attributed to the dilution of an equal amount of mRNA in an enlarged cell. Cell death is not likely to be the sole cause of this reduction. Sensory neurons do not die within 30 days, only a small cell loss (<20% of neurons) occurs by 60 days after axotomy, and a partial recovery in the number of TH-ir and TH mRNA-containing neurons is seen at 14 days after axotomy. Likewise, disconnection of petrosal ganglion neurons and their targets by transection of the carotid sinus nerve results in a transient decrease in TH catalytic activity and the TH-ir in the petrosal ganglia of young female rats.

Inhibition of axonal transport with application of vinblastine to the cervical vagus nerve, decreases the number of TH mRNA-containing and TH-ir neurons in the nodose ganglion. Likewise, blockade of axonal transport in the carotid sinus nerve decreases TH-ir in visceral sensory neurons of the petrosal ganglion. The pattern of alterations in TH mRNAs and immunoreactivity resulting from the selective
inhibition of axonal transport in the vagus nerve is similar to that seen following the axotomy-induced deafferentation of the nodose ganglion. Therefore, it is likely that the selective inhibition of axonal transport by application of vinblastine to the vagus nerve may have contributed to the actions of the axotomy-induced deafferentation on TH expression in nodose ganglion neurons.

Neither the numbers of TH-ir neurons nor the content of TH mRNA is altered in the nodose ganglion of diabetic rats at the 8- and 16-week time points. However, 24 weeks of diabetes results in a reduction in the numbers of TH-ir neurons in the diabetic nodose ganglia when compared with control, an effect not seen in diabetic rats receiving insulin. The late onset (24-week time point) decrease in TH-ir in diabetic nodose ganglia may reflect either a diabetes-induced nerve injury (milder and later than that noted with axotomy) or the loss of an axonally transported regulatory factor such as NGF. Because NGF increases the numbers of TH-ir nodose ganglion neurons in culture, it follows that the loss of NGF secondary to diabetes-induced reductions in axonal transport may diminish the numbers of TH-ir neurons. A decrease in TH mRNA levels is not seen in the diabetic nodose ganglia, therefore it is possible that the decreased availability of retrogradely transported regulatory factors, e.g., neurotrophins, is either directly or indirectly affecting the post translational processing, and/or stability of the products rather than affecting the production of mRNA.

Studies of the epigenetic factors regulating nodose ganglion visceral afferent neurons benefit from in vitro approaches wherein environmental influences are more clearly defined and controlled. Helke and Verdier-Pinard used cultures of dissociated mature nodose and petrosal ganglia neurons to evaluate the presence of TH in the absence of neurotrophic support or depolarizing influences. At each time point in culture (<1d with no preplating, 1, 5, 7 days after plating), TH-ir nodose/petrosal ganglia neurons are present. TH, downregulated in the nodose ganglion after vagus nerve transection or inhibition of axonal transport, is somewhat surprisingly present in a greater percentage of neurons in culture than in intact nodose/petrosal ganglia (21% at 5d versus 9%). The percentage of TH-ir neurons in the nodose/petrosal ganglia cultures is similar to those obtained when each ganglion is cultured separately.

2.3.1.3 Neuropeptides

The content of neuropeptides and/or their mRNAs in the nodose or petrosal ganglia can be altered by injury, alterations in axonal transport, and disease. Whereas substance P (SP) and calcitonin gene-related peptide (CGRP)-containing visceral afferent petrosal ganglion neurons of the glossopharyngeal nerve are reduced in number by axotomy, comparable changes are not detected in nodose ganglion neurons. However, this difference may reflect the lack of sensitivity resulting from the small number of SP and CGRP neurons in the nodose ganglion compared with the petrosal ganglion because reductions in the numbers of CGRP mRNA-containing neurons are reduced in each ganglion following peripheral deafferentation. A role for neuronal activity in the regulation of SP is suggested by the findings that the synthesis and transport of SP in the nodose ganglion of guinea pigs is inhibited by veratridine,
an effect reversed by tetrodotoxin. Peripheral axotomy also decreases the number of CCKA-receptor mRNA-expressing nodose ganglion neurons and conversely increases the number of CCKB-receptor, CCK mRNA, and preproCCK-like ir neurons.22

The selective inhibition of axonal transport in the vagus nerve increases the number of CGRP mRNA-containing and CGRP-ir neurons in the nodose ganglion, whereas axotomy-induced deafferentation results in a decrease in the CGRP mRNA-containing neurons and no change in the CGRP-ir neurons.80,97 The different results between the vinblastine treatment and the axotomy-induced deafferentation on the CGRP neurons are likely due to the different experimental procedures on the vagus nerve, resulting in a differential response of the CGRP neurons. Both procedures interrupt axonal transport, axotomy additionally injures the neuron and interrupts conduction of depolarizing impulses from the periphery to the cell body. The results of these studies suggest that neurons with distinct neurochemical natures may have differential reaction following the same kind of experimental treatment, and neurons with the same neurochemical nature may have different responses following different treatment.

Whereas blocked axonal transport increases CGRP-ir and mRNA and vagotomy decreases both, the absence of a change in the numbers of CGRP-ir neurons in diabetic rat nodose ganglion could be interpreted as a result of the counteracting diabetes-induced alterations in axonal transport and of nerve injury. Alternatively, the apparent absence of changes in the diabetic ganglion may also reflect a lack of sensitivity of the approach of counting CGRP-ir neurons in detecting changes in the relatively small number of CGRP-ir neurons found in the rat nodose ganglion.

Allergic inflammation of the guinea pig airways induces SP and CGRP production in large-diameter vagal afferent neurons. The number of SP/NKA-containing vagal afferent neurons of the guinea pig nodose ganglion is increased during respiratory viral infection leading to both small diameter nociceptive-like neurons and large diameter nonnociceptive neurons expressing tachykinins. This abnormal pattern of tachykinin expression may contribute to the abnormal physiology associated with respiratory viral infections. Conversely, chronic administration of ACTH or corticosterone decreases the quantity of peripherally transported SP in the afferent vagus nerve.

Relatively few neurons in the normal intact mature nodose ganglion contain vasoactive intestinal peptide (VIP). Following axotomy, VIP-ir and VIP mRNA markedly increase, the number of VIP mRNA-containing neurons increases (from virtually undetectable to readily apparent) significantly at 3, 7, and 14 days, while the labeling density of vasoactive intestinal peptide mRNA-containing neurons also increases at 1, 3, 7, and 14 days. These results suggest that there may be a peripherally derived inhibitory influence on VIP mRNA expression which is removed by axotomy-induced deafferentation. When axonal transport is blocked, the VIP immunoreactivity and mRNA content are also increased. Vinblastine treatment of the vagus nerve increases the numbers of VIP mRNA-containing neurons and VIP-ir neurons in the nodose ganglion at 3, 7, and 14 days. The average labeling density of VIP mRNA-containing neurons also increases following vinblastine treatment.
Because the pattern of alterations in VIP mRNA and immunoreactivity resulting from the selective inhibition of axonal transport in the vagus nerve is similar to that seen following the axotomy-induced deafferentation of the nodose ganglion, it is likely that inhibition of axonal transport may contribute to the effects of axotomy on VIP expression in nodose ganglion neurons.

The function of the increased VIP mRNA and resultant VIP-ir in neurons of the nodose ganglion is of interest. One possibility is that VIP functions as a neurotransmitter/neuromodulator. Consistent with this idea is the finding that VIP increases in central terminal fields in a coordinate fashion with the increase in cell body content. VIP may also play a role in promoting cell survival after neuronal injury. When added to the culture medium of dorsal root ganglion and retinal cells, VIP delays tetrodotoxin-induced and glutamate analogue-induced cell death.

Although VIP-ir and mRNA in the nodose ganglion neurons increase after vagotomy and after axonal transport is blocked, diabetes has no apparent effect on their levels in these neurons. It appears that either the nature or the extent of nerve injury resulting from the diabetic state is insufficient to trigger the pronounced increases in VIP noted in other situations. An alternate explanation may be that diabetes results in a reduced ability of the VIP gene to upregulate expression.

Although a few neurons of the intact nodose and petrosal ganglia express prepro-neuropeptide Y (NPY) mRNA, the synthesis of NPY in the nodose and petrosal ganglia is upregulated in response to transection of the vagus nerve. The numbers of NPY-ir and NPY mRNA-containing neurons in the nodose ganglion are dramatically increased in response to cervical vagus nerve axotomy. Reimer and Kanje show that peripheral axotomy of the sensory neurons in the nodose ganglion increase the number of the C-terminal flanking peptide of neuropeptide Y, galanin, and VIP, whereas central axotomy does not. Zhang et al. show that galanin, NPY, and VIP -ir and mRNA-containing neurons increase in the nodose ganglion after peripheral axotomy.

Helke and Verdier-Pinard (Reference 81 and unpublished data) used cultures of dissociated mature nodose and petrosal ganglia neurons to evaluate the presence of neuropeptide (VIP, CGRP, and NPY)-containing neurons in vitro in the absence of neurotrophic factors. At each time point in culture (<1d with no preplating, 1, 5, and 7 days after plating), VIP, CGRP, and NPY-ir nodose/petrosal ganglia neurons are present. VIP and NPY, agents that are upregulated in nodose ganglion neurons after vagus nerve transection and/or inhibition of axonal transport, are also present in greater percentages of neurons at each time in culture than in tissue sections of ganglia from intact rats. VIP increases from < 10% in situ to >20% of labeled neurons in culture (elevated at all time points in culture, including <1d unenriched cultures). The elevation in numbers of NPY-ir cells is less (12% in situ to 21% at 5 d in culture). The percentage of VIP-ir neurons in the nodose/petrosal ganglia cultures is similar to that obtained when each ganglion is cultured separately. CGRP is present in 21% of the combined nodose and petrosal ganglia cultures (5d), a similar percentage to that seen in tissue sections (20%) from intact ganglia. Given the larger numbers and significantly higher percentage of CGRP-ir neurons in the intact or in the separately cultured petrosal ganglion compared with the nodose
ganglion (34% versus 14% of neurons), it is likely that the majority of CGRP-ir neurons are from the petrosal ganglion.

Pituitary adenylate cyclase-activating polypeptide (PACAP)-ir and mRNA-containing neurons also increase in the cultured nodose ganglion over time in culture.\(^{194}\) PACAP is transported and released at the site of a crush injury to the vagus nerve.\(^{194}\)

The functional significance of alterations in neuropeptide expression in nodose ganglion neurons after vagotomy is not clear. Certain peptides (e.g., galanin, VIP, CCK, and CCK-B receptors) whose expression is increased may have trophic functions during nerve regeneration and may protect against further injury.

### 2.3.1.4 c-Jun

Another neurochemical marker of injury is induction of the transcription factor c-Jun. The constitutive levels of c-Jun in the nodose ganglion are low; however, following vagotomy, there is a rapid (within 10 hours) and long-lasting (up to 100 days) induction of c-Jun mRNA and c-Jun protein, which are closely linked to the intensity of the cell-body reaction.\(^{83,84}\)

The number of nodose ganglion neurons labeled for c-Jun is slightly increased in the diabetic nodose ganglia at the 8-week time point and is reversed with insulin treatment.\(^{192}\) The increase in c-Jun-ir neurons is not found at 16 or 24 weeks of diabetes.

Because of this short-lived response to diabetes, c-Jun may be transiently increased in an initial, nonsustained response to injury, and/or transient early changes in availability of NGF.

Because anterograde and retrograde axonal transport are affected in the diabetic vagus nerve,\(^{127,133,134,229}\) the increase in the number of c-Jun-ir cells in the neurons of the nodose ganglion may be attributed to a diabetes-induced inhibition of axonal transport that either causes a build up of protein in the cell body, interrupts neurotrophic support to the neurons, or both.

Thus, although Regalia et al.\(^{192}\) note that certain of the diabetes-induced changes noted in the content of specific neurochemical agents (nNOS, TH, c-Jun) are similar to those noted after nerve injury due to vagotomy or blocked axonal transport,\(^{80,84,107,240,254}\) the diabetes-induced neurochemical responses of the vagal afferent neurons are not as large in magnitude, as extensive (e.g., no changes in VIP and CGRP), or reflected by changes in mRNA content as those noted after vagotomy or after blocked axonal transport.\(^{80,97,254,255}\)

### 2.3.2 Neurotrophins

The altered expression of neuropeptide and neurotransmitter enzyme mRNAs in mature nodose ganglion neurons by local inhibition of vagus nerve axonal transport suggested that a transported factor (perhaps a target-derived neurotrophic factor) is important for the regulation of neuropeptide and neurotransmitter enzyme mRNAs in visceral sensory neurons of the nodose ganglion.\(^{254}\) Retrogradely transported neurotrophic factors influence the expression of transmitters in somatic sensory dorsal root ganglion (DRG) neurons.\(^{72,140}\) However, because sensory neurons of the
Advances in Vagal Afferent Neurobiology

Advances in Vagal Afferent Neurobiology

neural crest-derived dorsal root ganglion differ from sensory neurons of the placode-derived nodose ganglion embryologically (placodal- versus neural crest-derived), morphologically (absence of RT97 labeled large neurons, and distinct cell size distribution profile, Helke et al., unpublished), neurochemically (TH-ir neurons are primarily in the nodose ganglion119,241), and in trophic factor responsiveness during development,140 it is necessary to evaluate neurotrophic factors, their transport, and receptors in vagal visceral sensory neurons.

In mature peripheral sensory neurons, neurotrophins are important for development and maintenance of morphological integrity, for physiologic and phenotypic differentiation (including transmitter phenotype), and neuronal response to injury.21,37,138,147 Moreover, axonal caliber is maintained by retrogradely transported neurotrophins via regulation of neurofilament synthesis.226 Neurotrophins bind to specific receptors, are retrogradely transported from target tissues to the cell body, and affect synthesis of neurochemicals and structural proteins.10,37,42,124,161

2.3.2.1 Neurotrophins Associated with Vagal Afferent Neurons

Wetmore and Olson243 report that 25% of nodose ganglion neurons contained BDNF mRNA, and BDNF-ir but no NGF or NT-3 mRNA-containing neurons. Other studies show no NGF, BDNF, or NT-3 mRNA-containing neurons in either intact or vagotomized nodose ganglia.134 NGF and NT-3 proteins are detected by ELISA in the intact vagus nerve trunk, where they are likely to represent neurotrophins that are axonally transported from target tissues.133 However, NGF, BDNF, or NT-3 mRNA-containing neurons were detected in non-neuronal cells in the vagus nerve trunk after axotomy.134

Neurotrophins and their mRNAs are present in numerous peripheral tissues (including atrium, stomach, ileum) innervated by the vagus nerve.113,132,160,200,205,225,251 For example, NT-3 is detected in visceral targets of the nodose ganglion including heart, liver, and pancreas.113 Thus, through retrograde axonal transport (see below) of neurotrophins, intact mature vagal afferent neurons are exposed to endogenous neurotrophins in vivo.

2.3.2.2 Neurotrophin Receptors Associated with Vagal Afferent Neurons

Whereas mature visceral sensory neurons do not require exogenous neurotrophins for survival,81,130,139,140 neurotrophin receptors are clearly present on mature vagal afferent neurons and likely play a role in the maintenance of neuronal functions.

Mature nodose ganglion neurons and the vagus nerve express two classes of transmembrane neurotrophin receptors that display pharmacologically distinct neurotrophin binding sites, the low-affinity receptor (p75) and higher-affinity Trk receptors (Figure 2.4).231,243,253

2.3.2.2.1 Nodose Ganglion

As presented earlier, all three Trks are present in nodose ganglion neurons in the early postnatal period; in adulthood, expression of TrkB is reduced, while that of
Neurotrophic Factors

TrkC and TrkA remains. In adult rats, the presence of mRNAs to the tyrosine kinase (Trk) receptors for neurotrophins was studied in visceral afferent neurons of the nodose and petrosal ganglia using in situ hybridization histochemistry (with oligonucleotide probes and with oligoprobes), immunocytochemistry and RT-PCR. Neurons containing TrkA mRNA are found in the intact adult nodose and petrosal ganglia. At least 10% of nodose ganglion neurons and 38% of petrosal ganglion neurons contain TrkA mRNA. TrkC mRNA is found in approximately 9% of nodose, and 11% of petrosal ganglion neurons of adult rats. Slightly more robust labeling for both TrkA and TrkC is noted with riboprobes than with oligonucleotide probes. The cell size profiles of the TrkA and TrkC mRNA-containing nodose ganglion neurons were similar with average neuronal diameters of 35 ± 3 µm for each Trk. The presence of TrkA and TrkC mRNAs is verified in the nodose ganglion using RT-PCR. Lamb and Beilefeldt found a majority of rat nodose ganglion neurons express TrkA and TrkC receptor-ir after 1 day in culture, whereas Molliver et al. found a small minority of nodose ganglion neurons were TrkA positive.

Ichikawa and Helke defined the presence of the neurotrophin receptor, TrkA, in neurochemically identified vagal and glossopharyngeal sensory neurons of the adult rat. TrkA is colocalized with CGRP, and with the calcium-binding proteins, parvalbumin, or calbindin D-28k, in mature neurons of the rat nodose and petrosal ganglia. Although nearly one half of the TrkA-ir neurons in the nodose ganglion contain calbindin D-28k-ir, few or no TrkA-ir neurons in the petrosal ganglion are also labeled for either calcium binding protein. Conversely, whereas only a few of the numerous TrkA-ir neurons in the nodose ganglion contain CGRP-ir, about one half of the TrkA-ir neurons in the petrosal ganglion contained CGRP-ir. In contrast, no TrkA-ir neurons in these ganglia colocalize TH-ir. These data show

FIGURE 2.4 Schematic of a mature vagal afferent neuron in the nodose ganglion, the presence of TrkA, TrkC, and p75 neurotrophin receptors on neuronal perikarya, the ability of the cervical vagus nerve to retrogradely transport specific 125I-labeled neurotrophins to the cell body and transganglionically to the CNS terminals in the nucleus of the solitary tract (NTS), the retrograde transport of endogenous NGF and NT-3 in cervical vagus nerve, and the ability of NGF and NT-3 to affect neurotransmitter/neuropeptide presence in the ganglia. (Based on data from Zhuo and Helke, 1996; Helke et al., 1998; Helke and Verdier-Pinard, 2000; Lee et al., 2001b.)
distinct colocalizations of TrkA with specific neurochemicals in vagal and glossopharyngeal sensory neurons, and suggest that nerve growth factor, the neurotrophin ligand for TrkA, plays a role in functions of specific neurochemically-defined subpopulations of mature vagal and glossopharyngeal sensory neurons. This study also shows the coexistence of TrkA with CGRP, parvalbumin, and calbindin D-28k, but not with TH, in neurons of the nodose, petrosal, and jugular ganglia.

Whereas TrkB mRNA is detected in 1-day-old neonatal nodose and petrosal ganglia, few or no TrkB mRNA-containing neurons were detected in the adult nodose and petrosal ganglia. These data, obtained using two different approaches to in situ hybridization histochemistry (oligonucleotide probes and riboprobes), fit well with the absence of the retrograde transport of ²⁵I-BDNF by vagal afferent neurons and absence of effects of exogenous BDNF on neuropeptides in nodose/petrosal ganglion cultures. However, other studies using different probes report that a majority of intact nodose ganglion neurons contain TrkB. Michael and Priestley found that the majority of small visceral sensory neurons of the nodose ganglion express VR1 mRNA in conjunction with TrkB but not with TrkA. Moreover, mature nodose ganglion neurons in cultures or whole mounts show numerous TrkB containing neurons.

The p75 receptor is also present in the nodose ganglion. A majority of the neurons of the nodose and petrosal ganglia contains p75NTR mRNA or has p75NTR - ir. The presence of p75 mRNA in the ganglion is verified with RT-PCR.

2.3.2.2 Vagus Nerve Trunk
In contrast to the neurons of the nodose ganglion, in situ hybridization histochemistry of the normal intact cervical vagus nerve trunk does not reveal mRNAs for Trk receptors. p75 mRNA is not found in non-neuronal elements of the vagus nerve either before or after injury. However, using RT-PCR mRNAs of TrkA, TrkC and p75 are detected in the vagus nerve of normal adult rats. The detection of TrkA and TrkC mRNAs in the nerve trunk using RT-PCR but not when using in situ hybridization histochemistry suggests a low level of activity that may not be histochemically localized in intact tissue but which can be detected and localized to non-neuronal elements after nerve injury (see the following).

2.3.2.3 Axonal Transport of Neurotrophins by Vagal Afferent Neurons
Neurotrophins initiate their effects, in part, by binding to high-affinity receptors, followed by uptake and retrograde transport to the cell body. The ability of neurons to respond correlates with transport. Likewise, retrograde transport may be predictive of neuronal types selectively responsive to NGF, BDNF, NT-3, or NT-4. Neurons can transport neurotrophins by both Trk and p75-dependent mechanisms.

Mature vagal afferent neurons retrogradely transport I-NT-3, I-NGF, and I-NT-4 to perikarya in the ipsilateral nodose ganglion and transganglionically to the NTS. More recently, the retrograde axonal transport of endogenous NGF and NT-3 was demonstrated. The receptor mechanisms of axonal transport of
neurotrophins by nodose ganglion neurons are consistent with the expression of Trk and p75 receptors in these neurons. Moreover, vagal neurons have unique profiles of neurotrophin transport in comparison with somatic sensory and motor neurons.

125I-NGF is retrogradely transported from the cervical vagus nerve to neuronal cell bodies in the ipsilateral but not the contralateral nodose ganglion. The size of 125I-NGF neuronal profiles is 499 ± 28 µm². The presence of excess unlabeled NGF diminishes the transport of 125I-NGF to the nodose ganglion by >80%, whereas other unlabeled neurotrophins do not significantly alter the transport of 125I-NGF. Autoradiographic grains are distributed in the NTS and the solitary tract after application of 125I-NGF to the cervical vagus nerve, although considerably fewer are noted than with 125I-NT-3. Retrograde axonal transport of endogenous NGF is also demonstrated in the cervical vagus nerve using a double-nerve ligation model and ELISA to measure accumulated NGF. Little, if any, anterograde transport is noted using this approach. It is likely that the contributions of afferent versus efferent vagal fibers to the transport of endogenous NGF are similar to those noted with the exogenous iodinated NGF and thus reflect largely afferent vagal nerve transport.

125I-NT-3 is retrogradely transported from the cervical vagus nerve to neuronal perikarya in the ipsilateral nodose ganglion. The average cell size of 125I-NT-3 neuronal profiles is 459 ± 38 µm². The transport of 125I-NT-3 to the nodose ganglion is reduced by 85% in the presence of unlabeled NT-3 and by 30% in the presence of unlabeled NGF. Unlabeled BDNF and NT-4 had no significant effect on the transport of 125I-NT-3 to the nodose ganglion. Autoradiographic grains are also present in the NTS and the solitary tract after application of 125I-NT-3 to the cervical vagus nerve. The densest rostrocaudal labeling of the NTS is found at the level of the area postrema. Retrograde but not anterograde axonal transport of endogenous NT-3 is also demonstrated in the cervical vagus nerve using a double-nerve ligation model and ELISA to measure accumulated NT-3. It is likely that the contributions of afferent versus efferent vagal fibers to the transport of endogenous NT-3 are similar to that noted with the exogenous iodinated NT-3 and thus reflect both afferent and efferent vagal nerve transport.

The content of 125I-BDNF in the ipsilateral nodose ganglion after application to the cervical vagus nerve is not different from the content of 125I-cytochrome C and is significantly less than the content of each of the other iodinated neurotrophins after similar application. Autoradiography shows the presence of few labeled neurons scattered throughout the ipsilateral ganglion. No autoradiographic grains are accumulated in any NTS subnuclei subsequent to the application of 125I-BDNF to the cervical vagus nerve. In contrast, 125I-BDNF is retrogradely transported to CNS nuclei by efferent vagal axons.

125I-NT-4 is retrogradely transported from the cervical vagus nerve to neuronal perikarya in the ipsilateral nodose ganglion. The presence of excess unlabeled NT-4 diminishes the transport of 125I-NT-4 to the nodose ganglion by 86%. Interestingly, the presence of 22- to 25-fold excess of each of the other three unlabeled neurotrophins (NGF, BDNF, NT-3) also significantly alters the transport of 125I-NT-4.

The studies using iodinated neurotrophins demonstrate the receptor-mediated retrograde transport of neurotrophins from axons of the afferent vagus nerve to
Advances in Vagal Afferent Neurobiology

perikarya in the nodose ganglion, a profile of efficacy of NT-4>NT-3>NGF>>BDNF, and the transganglionic transport to the central terminal fields of vagal afferent neurons in the NTS.

Based on evidence that NGF is the preferred ligand for TrkA, whereas TrkB is activated by both BDNF and NT-4, and NT-3 is the preferred ligand for TrkC and is a secondary ligand for TrkA,\textsuperscript{30,68,122} the retrograde transport data are consistent with the demonstrated presence of TrkA and TrkC (but few TrkB) mRNA-containing neurons. Thus, the competition profile for \textsuperscript{125}I-NT-3 transport (inhibition of transport by excess NT-3 and NGF) is consistent with the involvement of TrkC and to a lesser extent TrkA (Curtis and DiStefano, unpublished data). The competition profile for \textsuperscript{125}I-NGF transport (significant inhibition of transport by excess NGF) is consistent with the involvement of TrkA. The absence of \textsuperscript{125}I-BDNF transport is consistent with the minimal presence of TrkB in the adult nodose ganglion.\textsuperscript{76,252} The finding that each of the excess unlabeled neurotrophins competed for the transport of \textsuperscript{125}I-NT-4 to the nodose ganglion suggests a lack of involvement of specific Trk receptors in the transport of \textsuperscript{125}I-NT-4 and is consistent with the minimal presence of TrkB mRNA. However, p75 may be involved in the transport of \textsuperscript{125}I-NT-4. p75 binds all neurotrophins with comparable affinity\textsuperscript{74,215} and is retrogradely transported.\textsuperscript{109,121} The transport of NT-4 and BDNF (but not NGF) to the DRG is dependent on p75,\textsuperscript{36} and p75 plays a role in regulating biological responsiveness to NT-4 but not to BDNF or NT-3.\textsuperscript{203}

Thus, the presence of TrkA and TrkC and the retrograde transport of NGF and NT-3 suggested that mature nodose ganglion neurons are able to respond to NGF and NT-3, and perhaps NT-4, but they are unlikely to respond to BDNF.

2.3.2.4 Neurotrophins and Neurochemical Expression in Vagal Afferent Neurons

An established action of neurotrophins (NGF, BDNF, NT-3, and NT-4) is the maintenance of normal neurotransmitter and neuropeptide phenotype expression in mature neurons.\textsuperscript{137,138,143,228}

After NGF injections into the tracheal wall, about 10% of the large diameter nodose neurofilament positive neurons projecting fibers to the trachea become SP-positive, suggesting that NGF not only increases SP expression in airway neurons, but changes the neuronal phenotype such that large, capsaicin-insensitive nodose neurons provide a component of the tachykinergic innervation.\textsuperscript{99}

Helke and Verdier-Pinard\textsuperscript{81} evaluated neurotrophin influences on the presence of neuropeptides and neurotransmitter enzymes in mature visceral sensory neurons. Exogenous NGF (10 to 100 ng/ml) increases the TH-ir and decreases VIP-ir neurons in the nodose/petrosal ganglia cultures over a 5-day period.\textsuperscript{81} Given that \textit{in vivo} NGF is a retrogradely transported target-derived neurotrophin, and that loss of contact with target (either through vagotomy or inhibition of axonal transport in the cervical vagus nerve) results in a decrease in TH and an increase in VIP neurons,\textsuperscript{80,254,255} these data are consistent with a role for endogenous NGF \textit{in vivo} to maintain normal neurotransmitter phenotype in nodose/petrosal ganglia neurons. These data, coupled
with the presence of TrkA mRNA and of the retrograde transport of NGF by vagal afferent neurons, indicate an important role for NGF in the functions (including maintenance of normal transmitter phenotype) of these visceral afferent neurons. The mechanism through which NGF alters the numbers of TH and VIP neurons is not known. One possibility is that NGF is acting directly through TrkA receptors on specific cultured nodose and petrosal ganglion neurons. Although we have preliminary evidence for the presence of TrkA mRNA in cultured nodose/petrosal ganglia neurons (Zhuo, Verdier-Pinard, and Helke, unpublished data), we do not know if the TrkA mRNA containing neurons are those in which the content of TH or VIP is altered. Moreover, TH-positive neurons of intact nodose and petrosal ganglia that co-expressed TrkA are not found. NGF is also a ligand for p75 and nearly all nodose ganglion neurons contain p75. Thus, the roles of neuronal TrkA, and p75, remain to be defined in this system. Likewise, the possibility that a non-neuronal cell type (e.g., fibroblasts) remaining in these neuronally enriched cultures responds to the addition of NGF with the secretion of a factor that secondarily alters neuronal phenotype requires additional studies.

Whereas NT-3 (up to 150 ng/ml) does not affect peptide expression in newborn DRG, NT-3 does affect the numbers of VIP-ir neurons in the mature nodose/petrosal ganglia cultures. NT-3 increases the number of VIP-ir neurons in the nodose/petrosal ganglia cultures and does not alter the numbers of TH- or CGRP-ir neurons. The addition of an NT-3 neutralizing antibody attenuates the effects of NT-3 on VIP-ir neurons. Because the increase in the numbers of VIP-ir neurons is the opposite effect noted in in vivo studies with removal of access to the target tissue, perhaps locally derived (not target derived) NT-3 is involved in the elevated VIP seen in vivo after injury or inhibition of axonal transport. The induction of NT-3 mRNA is noted in non-neuronal cells of the vagus nerve trunk immediately proximal and distal to a nerve lesion within 1 day after injury. In vivo this non-neuronally derived NT-3 may have access to the injured neuron and, coupled with the loss of NGF, be involved in the elevation of neuronal VIP.

Although not required for survival, when adult murine nodose ganglion neurons are placed in culture, BDNF and NT-4 but not NGF or GDNF demonstrated the ability to stimulate axonal outgrowth. NT-3 showed weak stimulation of outgrowth. Likewise, neurite outgrowth from adult rat nodose ganglion placed in explant cultures is increased by BDNF but not by NGF, NT-3 or GDNF.

2.3.3 OTHER NEUROTROPHIC FACTORS

Messenger RNAs for c-ret and GFR alpha 1, signaling receptors of GDNF family ligands, are found in 30 to 40% of nodose ganglion neurons, whereas GFR alpha 2 and GFR alpha 3 are not detected nodose ganglion neurons. The latter finding suggests that mature nodose ganglion neurons do not respond to neurturin and artemin but have the capacity to respond to GDNF. Addition of GDNF to adult rat nodose ganglion neurons in culture rapidly increases cytosolic calcium due to release from intracellular stores.
2.4 EFFECT OF VAGUS NERVE DAMAGE AND DISEASE ON NEUROTROPHINS ASSOCIATED WITH VAGAL AFFERENT NEURONS

2.4.1 NEUROTROPHIN AND NEUROTROPHIN RECEPTOR MRNAS AFTER VAGUS NERVE INJURY

Neurotrophins and neurotrophin receptors play an important role in survival and growth of injured peripheral nerves. The injury-mediated neurotrophic response in autonomic nerves has been investigated by studying changes in mRNA expression of neurotrophins and their receptors in the transected vagus nerve and nodose ganglion. The presence and distribution of neurotrophin and neurotrophin receptor mRNAs in the nodose ganglion and in the cervical vagus nerve trunk after nerve injury were assessed at various time points (17 hours to 45 days). In situ hybridization histochemistry was used to detect mRNAs for the neurotrophins, NGF, BDNF, NT-3, and the neurotrophin receptors, TrkA, TrkB, TrkC, and p75NTR in the vagus nerve at multiple time points after axotomy and ELISA to detect NGF and NT-3 proteins at one time point after axotomy.

In nodose ganglion neurons at 17 hours after cervical vagotomy, there are no readily apparent differences in the numbers, distribution, or the labeling intensity of neurons containing TrkA, TrkB, or TrkC mRNA or p75-ir. However, at more extended times after vagotomy, alterations in the expression of Trk receptors are noted in nodose ganglion neurons. By 48 hours after nerve injury, the numbers and labeling densities of TrkA and TrkC mRNA-containing neurons are reduced. By 3 days, the receptor mRNA levels are nearly absent and remained markedly depressed for more than 28 days after the axotomy. Neuronal expression of the neurotrophins was also examined in intact and axotomized neurons to evaluate the potential of autocrine effects of neurotrophins in the injured nerve. No NGF, BDNF, or NT-3 mRNA-containing neurons are detected in intact or vagotomized nodose ganglia.

Because vagal sensory neurons of the nodose ganglion can retrogradely transport 125I-NGF and 125I-NT-3 and endogenous NGF and NT-3, it is likely that the transection-induced interruption of axonal transport of target-derived factors is involved in the decreased expression of TrkA, TrkC, and p75NTR in the nodose ganglia after nerve injury. However, it is also possible that a more generalized perikaryal reaction to the axonal injury resulted in the downregulation of the Trk mRNAs.

In contrast to the neurons of the nodose ganglion, the normal intact cervical vagus nerve trunk does not contain mRNAs for Trk receptors or neurotrophins. However, cervical vagotomy results in the expression of mRNAs for each neurotrophin, and for TrkA, TrkB, and TrkC receptors in non-neuronal cells at both the proximal and distal segments of the transected nerve. The induction of each neurotrophin and Trk receptor mRNA is apparent within 1 day after the axotomy and is sustained at least 7 days. The increased neurotrophin and Trk receptor mRNAs in the proximal segments is limited to a short distance from the site of the transection, whereas in the distal segment the expression of these mRNAs is also noted at a greater distance from the transection. Moreover, NGF protein is increased in the distal end, and NT-3 protein is increased in both the distal and proximal ends of the
transected nerve 3 days after axotomy. The expression of neurotrophins and Trk receptors in the non-neuronal cells at or near the site of transection may be an attempt to compensate for the loss of target-derived trophic support and/or the injury-induced down-regulation of neuronal neurotrophin receptors.

The expression of mRNAs for neurotrophins and Trk receptors in the non-neuronal cells of the transected vagus nerve return to the normal control level 45 days after axotomy and coincide with regeneration of the nerve (as verified by retrograde transport of FluoroGold from the wall of the stomach to the ganglion). The return of the mRNA levels to the low pre-axotomy levels with reinnervation suggests that the restoration of target innervation either reduces the signals responsible for, or restores signals suppressive to, the induction of the neurotrophin and Trk mRNAs in the vagus nerve. In addition, the induction of individual neurotrophin mRNAs in the vagus nerve coincided with the increase of its preferred Trk receptor after axotomy.134 This spatial and temporal co-localization of neurotrophin and Trk mRNA expression supports the idea that neurotrophins promote or guide axonal regeneration via their Trk receptor. Because the proximal end of a transected nerve no longer has access to target-derived trophic support, the injured vagus nerve may depend on local production of neurotrophin and their receptors resulting from the upregulation of their mRNA, for axonal survival and regrowth.

The induction of neurotrophin and Trk receptor mRNAs in the transected vagus nerve (but not in neuronal cell bodies) in proximity to the site of nerve transection and relation of the mRNA hybridization signals to underlying cellular elements suggests an injury-induced upregulation of mRNA expression in non-neuronal elements of the vagus nerve. Non-neuronal cells including Schwann cells and macrophages can produce neurotrophin and Trk mRNAs after nerve injury.66,67,90,91,250 Schwann cells and macrophages are individually or cooperatively involved in Wallerian degeneration and regeneration.71,183,187 Macrophages secrete a variety of proteins or factors that initiate and enhance proliferation of Schwann cells.187 Schwann cells produce various cell adhesion molecules and neurotrophic factors that promote the outgrowth of regenerating axons5,172,211,218,219 (see reviews by Ide104 and Weinstein242).

p75 mRNA is not found in non-neuronal elements of the vagus nerve either before or after injury.134 p75NTR can play a role as a positive regulator of Trk-mediated neurotrophin activity12,159 and/or generate an apoptotic signal.9,58,65 However, because cervical vagotomy fails to induce expression of p75NTR mRNA in the injured vagus nerve, expression of p75NTR mRNA at the site of the injury does not appear to be involved in the process of vagus nerve response to injury.

2.4.2 DIABETES AND NEUROTROPHINS ASSOCIATED WITH VAGAL AFFERENT NEURONS

2.4.2.1 Neurotrophins and Neurotrophin Receptors in Diabetes

The loss of neurotrophic support has several consequences to mature sensory neurons. Alterations in neurotransmitters and neuropeptides are key manifestations of
deficient neurotrophic support in injury or neuronal disease such as diabetic neuropathy.\textsuperscript{227} Moreover, the neuropeptide changes in somatic sensory neurons and sciatic nerves of diabetic rats can be normalized by strict glycemic control, treatment with an aldose reductase inhibitor, or with exogenous NGF.\textsuperscript{204,207,227,228} Deficient neurotrophic support may also be a factor in the defective neuronal repair and regeneration known to occur in diabetic neuropathy in humans and in animal models.\textsuperscript{17,146,227} The established role for neurotrophins in nerve regeneration, and the finding that NGF assists sensory nerve regeneration in STZ diabetic rats\textsuperscript{244} support this idea. Thus, even in the presence of other causative mechanisms (e.g., immunological, increased aldose reductase pathway activity, microvascular abnormalities) for the diabetic neuropathy, correction of an alteration in function or availability of the appropriate neurotrophic factors may enhance maintenance of normal transmitter phenotype, neuronal survival, and regeneration.\textsuperscript{3,222,227}

Autonomic nerve dysfunction is a serious and common complication in diabetes mellitus\textsuperscript{54,55,92,256} and contributes to the high risk of cardiovascular mortality and morbidity.\textsuperscript{54,185,257} Cardiovascular reflex tests show the presence of autonomic neuropathy in 17 to 40\% of diabetic patients, and 85\% of diabetic patients with any peripheral neuropathy show evidence of autonomic neuropathy.\textsuperscript{178,233} Diabetic cardiovascular autonomic neuropathy is associated with severe postural hypotension, exercise intolerance, impaired cardiac function, increased incidence of myocardial infarction and ischemia, and a poor prognosis.\textsuperscript{178,234,256} Increased mortality is noted among diabetic patients with symptomatic autonomic neuropathy or with abnormal cardiovascular reflex tests.\textsuperscript{157,233,256}

Whereas visceral afferent neurons, including those of the vagus nerve, are critical to autonomic reflexes including the baroreceptor reflex, our understanding of the involvement of specific reflex components in diabetes is incomplete. However, visceral afferent fibers appear to be implicated in the symptomatology of diabetic autonomic neuropathy, and their involvement considered part of the neuropathy.\textsuperscript{4,92,189,224,256} For example, postural hypotension occurs because of an altered baroreceptor reflex and it has been suggested that damage to the afferent component of this reflex contributes to this deficit.\textsuperscript{4} Low et al.\textsuperscript{148} proposed that diabetes-induced postural hypotension results from degeneration of afferent baroreceptor fibers and of sympathetic neurons innervating the vasculature and the heart. Moreover, baroreceptor afferent nerve involvement in diabetic autonomic neuropathy is inferred from the postural hypotension and the altered circulatory reflexes in patients with intact efferent vasomotor pathways.\textsuperscript{4,208} In addition, a component of abnormal baroreceptor afferent neurons could explain why parasympathetic responses in diabetic patients may be subnormal during increases of arterial baroreceptor input\textsuperscript{48} but normal during increases of trigeminal nerve input provoked by face immersion.\textsuperscript{14} Important to this premise is the recent demonstration of an impairment of the afferent limb of the baroreceptor reflex in experimental diabetes.\textsuperscript{69,70}

In addition to the functional deficits mentioned above, morphologic, metabolic and neurochemical changes are found in diabetic vagal and/or baroreceptor afferent nerves. Marked lesions, demyelination and loss of myelinated axons in the vagus nerve, occur with symptomatic diabetic autonomic neuropathy.\textsuperscript{46,73,125} Autonomic neuropathy affecting the vagus nerve is characterized by progressive axonal atrophy
Neurotrophic Factors

of myelinated and unmyelinated fibers, which is preceded and accompanied by vagal dysfunction substantiated by impaired heart rate variability. Diabetic gastroparesis has been associated with a marked reduction in the density of unmyelinated vagal axons. Thus, it appears that both myelinated and unmyelinated vagal fibers and visceral afferent fibers are affected in diabetes. Diabetes-induced metabolic, oxidative, and neurochemical changes in the vagus nerve and nodose ganglion have been demonstrated. The vagus nerves from STZ-induced diabetic rats had elevated glucose, fructose, and sorbitol, and decreased reduced glutathione. Diabtes-induced alterations in the neurochemical content of visceral afferent neurons are consistent with nerve injury (see above and Reference 192).

2.4.2.1.1 Neurotrophin Content of the Vagus Nerve

The vagus nerves have elevated NGF protein content at early stages of STZ-induced diabetes (Figure 2.5). Cervical vagus nerves of streptozotocin (STZ)-induced diabetic rats were studied at 8, 16, and 24 weeks after the induction of diabetes. Elevations in vagus nerve hexose (glucose and fructose) and polyol levels (sorbitol), and their normalization with insulin treatment, verify that the STZ treatment results in hyperglycemia-induced metabolic abnormalities in the nerve. Intact right vagus nerves were used to determine whether STZ-induced diabetes altered the nerve content of endogenous NGF and NT-3. The vagus nerves of diabetic rats have elevated NGF at 8 weeks (50%) and at 16 weeks (35%), but not at the 24-week time point, when compared with the control or the diabetic+insulin groups. The intact cervical vagus nerve of the diabetic rats at each of the three time points (8, 16, and 24 weeks) show no significant changes in NT-3 compared with the control or the diabetic+insulin groups. The expression of NGF mRNA and NT-3 mRNA in the cervical vagus nerve, measured by RT-PCR, shows no significant differences in the relative levels of vagal nerve NGF or NT-3 mRNA from diabetic rats compared with the control or the diabetic+insulin rats at the 8- or 16-week time points. The amounts of neurotrophin receptor (p75, TrkA, TrkC) mRNAs in the vagus nerve and vagal afferent nodose ganglion are not reduced in diabetic rats.

Whether the elevated NGF at the early stages of diabetes is a regenerative response to an analogous hyperglycemia-induced injury to the nerve is not known. If it is, the loss of the elevated NGF at the later stages of diabetes may signal a failure of regenerative support of the neurons by locally produced neurotrophins. The elevation in vagal NGF at the early stages of STZ-induced diabetes is likely due to local production of the neurotrophin as other potential sources are not increased, e.g., there is no increase in transported NGF nor is there an additional increase in NGF (above that seen in the nerve) in the nodose ganglia content of NGF (data not shown). Most likely the elevated NGF originated in non-neuronal cells such as Schwann cells and macrophages, as these non-neuronal cells have the ability to produce NGF after nerve injury. The increase in NGF protein in the absence of elevations in vagus nerve NGF mRNA in diabetic rats may be due to an alteration in a post-transcriptional event. Regulation of NGF production can occur at a post-transcriptional level, e.g., increases in NGF protein can result from increases in NGF mRNA stability and/or an increased translational efficiency of NGF protein. It is unclear whether increased levels of NGF present in the vagus nerve
FIGURE 2.5 Bar graphs (grey bars = controls, black bars = diabetic, cross hatch bars = diabetic+insulin replacement) showing the effect of streptozotocin (STZ)-induced diabetes at the 8-, 16-, and 24-week time points on the retrograde transport of NGF and NT-3 in cervical vagus nerve segments 24 hours after placement of constricting double ligatures. The retrogradely transported NGF and NT-3 were calculated from the content in the distal segment minus the content in the intermediate segment of the double-ligated vagus nerve. $a = P<0.05$ compared with control, and $b = P< 0.05$ compared to diabetic+insulin-treated counterparts. (Adapted from Lee et al., 2001b.)
trunk during the early stages of diabetes results in an increased availability of the vagal afferent and/or efferent neuronal perikarya to NGF.

2.4.2.1.2 Neurotrophin Content in Peripheral Targets of the Vagus Nerve

The effects of diabetes on the contents of NGF and NT-3 protein and mRNAs are studied in tissues innervated by the vagus nerve (Reference 132 and Cai, Lee, and Helke, unpublished data). Using RT-PCR, NGF and NT-3 mRNAs are detected in multiple targets tissues of the vagus nerve, e.g., atrium, stomach and duodenum. At the 16-week time point, the expression of NT-3 mRNA in the right atrium, stomach and duodenum is not different among the control, diabetic or diabetic+insulin groups. At the 8-week time point, a small increase (22%) in the NT-3 mRNA in the diabetic right atrium compared with controls is noted but the increase is not reversed by insulin treatment. The expression of NT-3 mRNA in left ventricle, stomach and duodenum of diabetic rats at the 8-week time points is not different from control or from diabetic+insulin groups. Assessment of the neurotrophin protein content (using ELISA) in the stomach at the 16-week time point does not show differences in the content of NGF or NT-3 among control, diabetic, or diabetic+insulin groups.

2.4.2.1.3 Neurotrophin Receptors in the Vagus Nerve and Nodose Ganglion

To assess possible changes in the expression of the neurotrophin receptors (TrkA, TrkB, p75) in the diabetic vagus nerve, we used RT-PCR to measure the mRNAs for TrkA, full-length TrkC, and p75 in vagus nerves and nodose ganglia (Reference 132 and Cai et al. unpublished data). The mRNAs of TrkA, TrkC, and p75 are clearly detected in the nodose ganglia and the vagus nerve of normal adult rats by RT-PCR. After 8 weeks of STZ-induced diabetes, there are no differences in the mRNA levels of TrkA, full-length TrkC, and p75 in the nodose ganglia or the vagus nerves among control, diabetic, and diabetic+insulin groups. Likewise, at the 16-week time point, no reductions in the mRNA levels of TrkA, TrkC, or p75 are found in the nodose ganglia or the vagus nerves from diabetic rats. A modest increase (29%) in the expression of TrkA mRNA in vagus nerve, but not in the nodose ganglion, of diabetic rats is not reversed by insulin treatment.13

2.4.2.2 Axonal Transport of Neurotrophins in Diabetes

Using a double-ligation model to assess the transport of endogenous neurotrophins, the retrograde transport of both NGF and NT-3 is found to be significantly reduced in the cervical vagus nerve at later stages of streptozotocin (STZ)-induced diabetes (16 and 24 weeks).133 Anterograde transport of NGF or NT-3 is not apparent in the vagus nerve of diabetic or control rats. The reduction in retrograde transport of endogenous NGF and NT-3 in the vagus nerve of diabetic rats could be due to a decline in the transport in the afferent and/or the efferent vagal nerve fibers. However, given that the vagal transport of both agents is altered in diabetic rats (this study), that the vagus nerve is more than 70% afferent,160,188 and that iodinated NGF is retrogradely transported by the afferent but not the efferent vagus nerve,76 it is likely...
that the primary decrement of NGF and NT-3 transport is in the afferent component of the vagus nerve.

The finding that the retrograde transport of both NGF and NT-3 is affected suggests that diabetes may affect the transport of each agent by mechanisms that are shared by both. No changes in the NGF and NT-3 protein or mRNA levels in the stomach or atrium, two vagally innervated organs, are noted after 16 or 24 weeks of diabetes. Moreover, the amounts of neurotrophin receptor (p75, TrkA, TrkC) mRNAs in the vagus nerve and vagal afferent nodose ganglion are not reduced in diabetic rats. These data suggest that neither diminished access to target-derived neurotrophins nor the loss of relevant neurotrophin receptors accounts for the diabetes-induced alteration in the retrograde axonal transport of neurotrophins. However, the diabetes-induced reduction in retrogradely transported NGF and NT-3 by vagal nerve fibers at the later stages of diabetes suggests a deficit in the neurotrophin-dependent trophic support to vagal afferent and efferent neurons.

The possibility of more general diabetes-induced changes in the ability of the neuron to maintain machinery involved in retrograde transport is suggested by data showing that the anterograde transport of proteins (choline acetyltransferase, muscarinic and opioid receptors) is reduced in the vagus nerve of diabetic rats. To assess whether diabetes causes a defect in axonal transport that may not be specific to neurotrophin transport, we studied the ability of a neuronal tracer (FluoroGold) to be retrogradely transported by vagal neurons of control and diabetic rats. After 24 weeks of diabetes, FluoroGold is retrogradely transported from the stomach to more than 50% fewer afferent and efferent vagal neurons in the STZ-diabetic compared with control rats. The diabetes-induced deficit in retrograde axonal transport of FluoroGold is likely to reflect alterations in basic axonal transport mechanisms in both the afferent and efferent vagus nerve that contribute to the previously observed reductions in neurotrophin transport.

The interaction between neurotrophins and Trk receptors can activate the phosphatidylinositol-3 kinase (PI3 kinase)/Akt (protein kinase B) signal pathway which mediates neuron survival, differentiation, axon growth, protects neurons from apoptosis, and promotes nerve regeneration. The PI3 kinase/Akt signal pathway located in the distal axon of neurons has a unique role in the retrograde transport of NGF and brain-derived neurotrophic factor (BDNF) in sympathetic, sensory neurons and motoneurons. Inhibition of PI3 kinase in the distal axons of neurons attenuates the retrograde transport of NGF and also induces neuron apoptosis. Furthermore, retrogradely transported neurotrophins play a critical role in the activation of downstream effectors of PI3 kinase/Akt in neuronal perikarya. Inhibition of the PI3 kinase/Akt signal pathway in distal axons attenuated the retrograde transport of NGF to the neuronal cell body, which reduced the activation of the Akt in the neuronal cell body. Thus, it is plausible that impaired retrograde transport of neurotrophins, deficits in nerve regeneration, and neuronal apoptosis in diabetes could be due to impairment in the PI3 kinase/Akt pathway in the neuronal perikarya and/or axons.

To assess the potential involvement of an impaired PI3 kinase/Akt signal pathway in the diabetes-induced reduction in retrograde axonal transport of neurotrophins in the vagus nerve, Cai and Helke characterize diabetes-induced changes in the PI3
kinase/Akt signal pathway in the vagus nerve and vagal afferent neurons. Control 
and streptozotocin (STZ)-induced diabetic rats with a duration of 16 weeks, kinase 
assays, western blotting, and immunocytochemistry show that diabetes results in 
alterations in activity and protein expression of the PI3 kinase/Akt signal pathway 
in the vagus nerve and vagal afferent neurons. Diabetes causes a significant decrease 
in enzymatic activity of PI3 kinase and Akt (52% and 36% of control, respectively) 
in the vagus nerve. The reduced enzymatic activity is not associated with decreased 
protein expression of the p85 subunit of PI3 kinase, Akt and phosphorylation of Akt 
(ser473). In contrast, there is a significant increase in the phosphorylation of p70s6 
kinase (thr421/ser424), along with a normal protein expression of p70s6 kinase in 
the vagus nerve of diabetic rats. However, diabetes induces an overall decrease in 
immunoreactivity of the p85 subunit of PI3 kinase, phospho-Akt (ser473) and phos-
pho-p70s6/p85s6 kinase (thr421/ser424) in vagal afferent neurons. Thus, Cai and 
Helke26 demonstrate that STZ-induced diabetes resulted in impairment in the PI3 
kinase/Akt signal pathway in the vagal afferent neurons and the vagus nerve after 
16 weeks of diabetes. The findings provide evidence to support previous observations 
that reduced retrograde transport of NGF and NT-3 in the vagus nerve of diabetic 
rats is not due to deficient neurotrophin production and neurotrophin receptors. The 
impaired PI3 kinase/Akt signal pathway could be, at least in part, responsible for 
the reduced retrograde transport of NGF and NT-3 in the vagus nerve.

ACKNOWLEDGMENTS

C.J.H. was supported by NIH grant R01-DK55143 and USU protocol R075IU. Jen 
Regalia provided extensive technical and editorial assistance in the preparation of 
this chapter.

REFERENCES

1. Agostoni, E., Chinnock, J.E., Burgh Daluy, M.D., and Murray, J.G. Functional and 
histological studies of the vagus nerve and its branches to the heart, lungs, abdominal 
2. Alonzi, T., Middleton, G., Wyatt, S., Buchman, V., Betz, U.A., Muller, W., Musiani, 
P., Poli, V., and Davies, A.M. Role of STAT3 and PI 3-kinase/Akt in mediating the 
3. Apfel, S.C., and Kessler, J.A. Neurotrophic factors in the therapy of peripheral 
4. Appenzeller, O. Peripheral autonomic neuropathies, in Disorders of the Autonomic 
Nervous System, Robertson, D. and Biaggioni, I., Eds., Harwood Academic Publish-
5. Araki, T. and Milbrandt, J. Ninjurin, a novel adhesion molecule, is induced by nerve 
ganglia and the potentialities of their component cells studied in quail-chick chimeras, 


Neurotrophic Factors


141. Lindsay R.M. and Rohrer, H. Placodal sensory neurons in culture: Nodose ganglion neurons are unresponsive to NGF, lack NGF receptors but are supported by a liver-derived neurotrophic factor, *Dev Biol*, 112: 30, 1985.


Neurotrophic Factors


Part II

Vagal Sensory Ganglion Neurons
3 Voltage-Gated Ion Channels in Vagal Afferent Neurons

J.H. Schild, K.D. Alfrey, and B.Y. Li

CONTENTS

3.1 Introduction ....................................................................................................77
3.2 Voltage-Gated Sodium Ion Channels ............................................................79
  3.2.1 TTX-Sensitive Na, .............................................................................79
  3.2.2 TTX-Resistant Na, .............................................................................80
3.3 Voltage-Gated Calcium Ion Channels ...........................................................82
3.4 Voltage-Gated Potassium Ion Channels ........................................................84
3.5 Dynamic Properties of Ionic Currents in Vagal Afferent Neurons...............87
  3.5.1 Na+ and Ca²⁺ Current Dynamics........................................................89
  3.5.1.1 Functional Role of ITTXS and ITTXR ........................................91
  3.5.2 K⁺ Current Dynamics.........................................................................92
  3.5.3 Continued Study of the Ionic Currents Underlying Membrane
    Excitability .........................................................................................94
3.6 Voltage Dependent Calcium-Activated Potassium Ion Channels ..........94
3.7 Hyperpolarization Activated Cyclic Nucleotide-Gated Cation Channels .....94
3.8 Conclusion......................................................................................................95
References................................................................................................................95
Appendix: Total Transmembrane Current as a Function of Membrane
  Potential.................................................................................................................98

3.1 INTRODUCTION

Numerous cellular and subcellular mechanisms contribute to the overall excitability of vagal afferent neurons. Those that alter the electrical state of charge across the plasma membrane can be broadly classified as either being functionally dependent upon transmembrane voltage or not. This chapter focuses upon the superfamily of voltage-gated ion channels expressed in the somata of vagal afferent neurons. Of particular interest here are the functional properties of the whole-cell currents that play a major role in setting the resting membrane potential, threshold for action
potential discharge, and voltage trajectory over the upstroke, downstroke, and initial afterhyperpolarization of the somatic action potential waveform.

Voltage-gated ion channels (VGC) are complex protein assemblies that function as specialized transmembrane conduits for ions flowing along a concentration gradient. Electrophysiological analyses of single VGC have demonstrated that these most often operate as unitary conductances, rapidly switching between a closed and open state in a voltage- and time-dependent manner. Underlying this bimodal transition in channel conductance are multiple molecular configurations of the protein complex most often described as nonconducting substates. The time a VGC spends occupying particular substates and the manner in which the transition or “gating” between these substates occurs can be influenced by a great many factors. However, only when the VGC occupies a final open state will ions flow along the concentration gradient. This redistribution of ionic charge imparts a change in transmembrane potential, which, in turn, further influences the gating properties of the VGC. The net effect of a large population of these VGC is often described in terms of a single, whole-cell Na+, K+, or Ca2+ ion current or, in a few cases, as a mixed cation current such as with the family of hyperpolarization activated, cyclic nucleotide-gated cation channels (HCN). The whole-cell current may or may not be comprised of multiple current components arising from a superfamily of molecularly distinct VGC termed Na,X.Y, K,X.Y, and Ca,X,Y, in which X and Y are integer classifiers for separate channel subtypes. (See the IUPHAR ion channel compendium at http://www.iuphar-db.org/iuphar-ic/ for an excellent summary) Another family of transmembrane proteins that can rapidly influence membrane excitability is ionic transporters such as the NaK and Ca2+ ATPases, Na+-Ca2+ exchangers, and other mechanisms associated with ionic homeostasis. The net effect of a large population of these transporters is also most often described in terms of a single, whole-cell current. However, over the span of physiological membrane potentials associated with mammalian sensory neuron discharge (i.e., −90 to +60 mV), the amplitudes of these transmembrane currents are primarily dependent upon ionic concentration gradients across the cell membrane and are several orders of magnitude smaller than the peak, whole-cell currents arising from VGC over the course of action potential discharge. Similar considerations exist for other classes of whole-cell currents arising from transient receptor potential (TRP), cyclic nucleotide-gated (CNG), and mixed classes of inward rectifier (KIRX,Y and K2PX,Y) cation channels. Many of these are markedly influenced by transmembrane and subcellular signaling pathways and are most often associated with long-term modulation of neuronal excitability, especially when considering subthreshold membrane potentials.

Vagal afferent neurons are quiescent until depolarizing events drive membrane potential toward the threshold for action potential discharge. Once achieved, the neuron sequences through a stereotypical trajectory of membrane voltage that is delineated by a rapidly depolarizing upstroke, a slower repolarizing downstroke, and an afterhyperpolarization in membrane potential that is generally several times longer than the duration of the action potential waveform. This review will focus on those voltage-dependent Na+, Ca2+, or K+ ionic currents that dominate the total transmembrane current over the time course immediately leading up to and for a few hundred milliseconds following a somatic action potential waveform. As there is no evidence
for any differences in the voltage- and time-dependent properties of ion channels expressed in myelinated or unmyelinated vagal afferents the data will be presented without regard to fiber type.

3.2 VOLTAGE-GATED SODIUM ION CHANNELS

To date, only four isoforms of the voltage-gated sodium ion channel family (Nav1.6, Nav1.7, Nav1.8, and Nav1.9) have been found in the peripheral nervous system. Of these, the tetrodotoxin (TTX) sensitive Nav1.7 and the TTX resistive Nav1.8 are the most likely candidates expressed in vagal afferent neurons. The Nav1.7 whole-cell Na⁺ current can be blocked by low nanomolar concentrations of TTX (TTX-sensitive, TTXS), while Nav1.8 whole-cell current is unaffected by high micromolar concentrations of TTX (TTX-resistant, TTXR). Cellular observations suggest these two classes of Na⁺ are expressed at high densities (>100 channels/µm²) in the nodose ganglia and are well suited for supplying the regenerative current flux necessary for the neural encoding of sustained physiological stimuli as repetitive patterns of action potential discharge. Voltage and current clamp studies have also demonstrated that Na⁺ have the capacity to contribute much more to the dynamic neural encoding properties of vagal sensory afferents than simply helping to establish the number of action potentials produced per unit of physiological stimulation.

3.2.1 TTX-SENSITIVE NAV

Under voltage clamp conditions suitable for isolation of the whole-cell Na⁺ current, rat nodose neurons exhibit an inward current transient that is reversibly blocked by submicromolar concentrations of TTX (I_{TTXS}). Under conditions of voltage clamp the I_{TTXS} has dynamic features that are typical of the large and fast TTXS inward Na⁺ currents observed in other sensory neuron preparations. Numerical analysis of the current records reveals activation and inactivation kinetics that are typical of the voltage- and time-dependent properties of this current. Rapid activation begins as membrane potentials approach approximately -50 mV, with the amplitude of the current being highly dependent upon the rate of membrane depolarization relative to the magnitude of the inactivation time constants at that potential. Above approximately -10 mV the current follows an ohmic reduction in peak magnitude suggesting full availability of TTXS Na⁺ beyond this potential. The transmembrane voltage required for activation of one-half of the available population of TTXS Na⁺ (V_{1/2}) is in the range of approximately -40 to -30 mV, while the V_{1/2} range for inactivation covers approximately -75 to -65 mV. At these respective half-activation potentials an analysis of chord conductance using a Boltzmann function reveals e-slope factors in the range of approximately 7 for activation and 6 for inactivation. The voltage-dependent profile of steady-state activation and inactivation of the available population of TTXS Na⁺ reveals a small Na⁺ activation window (note arrow, Figure 3.2). The time-dependent profile of the whole-cell current shows time constants of activation (τₘ) and inactivation (τᵢₙ) that are markedly voltage dependent and well below 10 msec for membrane potentials typical of somatic action potentials. Furthermore, under conditions of voltage clamp,
two pulse protocols have demonstrated that $I_{\text{TTXS}}$ recovers from inactivation rather slowly, requiring more than 100 msec to return within 70 to 80% of control magnitudes, as is the case in dorsal root ganglion neurons.\textsuperscript{7,18}

\subsection{3.2.2 TTX-Resistant $I_{\text{Na}}$

In the presence of micromolar concentrations of TTX approximately 70 to 80\% of adult vagal afferent neurons exhibit a large inward whole-cell Na$^+$ current ($I_{\text{TTXR}}$)

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure3_1.png}
\caption{Two components of the whole cell Na$^+$ current in rat nodose neurons. The upper voltage clamp traces represent that component of the whole cell Na$^+$ current ($I_{\text{Na}}$) that is sensitive to submicromolar concentrations of tetrodotoxin (n.b. subtracted traces). The $I_{\text{TTXS}}$ begins activation as membrane potentials approach $-40$ mV. Rapid inactivation follows a peak response, which generally occurs within a few milliseconds of the voltage clamp step. The lower voltage clamp traces represent that component of the $I_{\text{Na}}$, which remains in the presence of 10 $\mu$M tetrodotoxin. Here, membrane potentials must approach $-20$ mV to activate $I_{\text{TTXR}}$. While activation rates are comparable to those of $I_{\text{TTXS}}$, the $I_{\text{TTXR}}$ rate of inactivation is markedly slower. Recording protocol consisted of 40 msec voltage steps from a holding potential of $-90$ mV, 5 mV increments up to 35 mV with an interstep interval of 3 sec. Solutions consisted of $[\text{Na}^+]_i = 7$ mM and $[\text{Na}^+]_o = 50$ mM. See Schild and Kunze\textsuperscript{7} for complete details concerning the electrophysiological methods. The vertical scale bar is 1 nA for the TTXS and 0.5 nA for the TTXR Na$^+$ current records, respectively. The horizontal scale bar is 10 msec for both current recordings.}
transient that is reversibly blocked by metals such as zinc, cadmium and cobalt. The whole-cell $I_{\text{TTXR}}$ has dynamic features that are typical of the slower inactivating TTXR Na$^+$ currents observed in other sensory neuron preparations (Figure 3.3). Numerical analysis of the current records reveals activation and inactivation kinetics that are typical of the voltage- and time-dependent properties of this current (Figure 3.2). Rapid activation does not begin until membrane potentials approach approximately $-30$ mV. Above approximately $10$ mV the current follows an ohmic reduction in peak magnitude suggesting full availability of TTXR Na$^+$ beyond this potential. The $V_{1/2}$ of activation for the TTXR Na$^+$ is in the range of approximately $-15$ to $-5$ mV, while the $V_{1/2}$ for inactivation ranges from approximately $-35$ to $-25$ mV. At this respective $V_{1/2}$ an analysis of chord conductance using a Boltzmann function reveals $S_{1/2}$ factors in the range of $-5$ for activation and $5$ for inactivation. These modest slope factors coupled with the rather depolarized steady-state activation and inactivation profiles for $I_{\text{TTXR}}$ result in a surprisingly large 40 mV Na$^+$ current activation window that is centered on a membrane potential of approximately $-20$ mV (note arrow, Figure 3.2). The voltage-dependent profile and magnitude of the $\tau_m$ and $\tau_h$ for $I_{\text{TTXR}}$ are comparable to those of $I_{\text{TTXS}}$. However, because the peak values are 30 to 40 mV more depolarized than those for $I_{\text{TTXS}}$, over membrane potentials typical of somatic action potentials the activation rates for $I_{\text{TTXR}}$ are moderately slower, but the inactivation rates are four to six times slower than those for $I_{\text{TTXS}}$ (Figure 3.2). Interestingly, results of two pulse protocols demonstrate that 

**FIGURE 3.2** Voltage- and time-dependent channel gating for $I_{\text{TTXS}}$ and $I_{\text{TTXR}}$. (Left) Hodgkin and Huxley (1952) ion channel gating variables can be derived from the voltage- and time-dependent activation and inactivation characteristics of $I_{\text{TTXS}}$ and $I_{\text{TTXR}}$. Steady-state profiles of the (activation, inactivation) gating variables for $I_{\text{TTXS}}$ ($m_{\text{TTXS}}, h_{\text{TTXS}}$) and $I_{\text{TTXR}}$ ($m_{\text{TTXR}}, h_{\text{TTXR}}$) can reveal the region of the action potential waveform over which each inward current begins to contribute to membrane depolarization. Of particular importance are the “window currents” that result from an overlap in the voltage-dependent profiles of the activation and inactivation gating variables (i.e., the shaded arrows, see text). (Right) An examination of the voltage-dependent time constants ($\tau$) for these gating variables provides insight into how quickly individual gating variables can respond to changes in membrane potential. (Center) C-type action potential waveform produced by a computational model utilizing the voltage-dependent gating variables and time constants presented in Figure 3.2, Figure 3.4, and Figure 3.6, a Hodgkin–Huxley model of the BK-type $I_{kCa}$ in Figure 3.7 and other currents as described in Schild et al.2
Advances in Vagal Afferent Neurobiology

ITXR recovers from complete inactivation quite rapidly, requiring less than 10 msec to return within 90% of control magnitudes, as is the case in dorsal root ganglion neurons.\(^7,18\)

3.3 VOLTAGE-GATED CALCIUM ION CHANNELS

Voltage-gated calcium ion channels (Ca\(^+_2\)) are heterogeneous in molecular structure and endogenous regulation. Evidence for most isoforms of the Ca\(^+_2\) family has been found in the peripheral nervous system, in which multiple members often function...

FIGURE 3.3 The N- and T-type whole-cell Ca\(^+_2\) currents in rat nodose neurons. The upper voltage clamp traces present the whole cell Ca\(^+_2\) current (I\(_{\text{Ca, total}}\)), which presents distinct current components with either low-voltage or high-voltage activation thresholds. As membrane potentials approach -70 mV, a transient inward Ca\(^+_2\) current (I\(_{\text{Ca,t}}\)) is observed with characteristics typical of members of the Ca\(^+_2\) family. On account of its small amplitude and rapid inactivation kinetics, this I\(_{\text{Ca,t}}\) is obscured as membrane potential approaches the -30 mV threshold for the much larger magnitude high-voltage activated Ca\(^+_2\) current components in I\(_{\text{Ca, total}}\). In the presence of 1 \(\mu\text{M}\) \(\omega\)-conotoxin, a selective blocker of the Ca\(_{\text{v},2.2}\) Ca\(^+_2\) channel, the I\(_{\text{Ca, total}}\) is reduced by more than 70%. Subtraction of this remnant from I\(_{\text{Ca, total}}\) reveals this N-type whole cell Ca\(^+_2\) current (I\(_{\text{Ca,n}}\)). Recording protocol consisted of 400 msec voltage steps from a holding potential of -100 mV, 10 mV increments up to 40 mV with an interstep interval of 3 sec. The vertical and horizontal scale bars are 1 nA and 200 msec, respectively, for both recordings and 0.5 nA and 100 msec, respectively, for the inset. Solutions consisted of \([\text{Ca}^{2+}]_i = 100 \text{nM (BAPTA-K}^+\text{)}\) and \([\text{Ca}^{2+}]_o = 2 \text{mM. See Mendelowitz and Kunze}^{21}\) for complete details concerning the electrophysiological methods.

I\(_{\text{ITXR}}\) recovers from complete inactivation quite rapidly, requiring less than 10 msec to return within 90% of control magnitudes, as is the case in dorsal root ganglion neurons.\(^7,18\)
within a single cell type. The selective action of high-specificity calcium channel antagonists has led to the classification of five functionally distinct Ca\textsubscript{v} subtypes, which are further identified as high-voltage activated (HVA) or low-voltage activated (LVA) on account of channel-gating thresholds in the range of −30 to −20 mV or −60 to −50 mV, respectively\textsuperscript{20-22} The HVA Ca\textsubscript{v} exhibit selective IC\textsubscript{50} values in the range of 100’s nM to low µM for nifedipine (L-type, presumably Ca\textsubscript{v}1.2), 10’s nM to 100’s nM for omega-conotoxin GVIA (N-type, presumably Ca\textsubscript{v}2.2), subnanomolar to 10’s nM for omega-agatoxin IVA (P-type, presumably Ca\textsubscript{v}2.1), low 100’s nM for omega-agatoxin IVA or low µM for omega-conotoxin MVIIC (Q-type, also presumably Ca\textsubscript{v}2.1). Those Ca\textsubscript{v} resistant (R-type, presumably Ca\textsubscript{v}2.3) to all these antagonists may represent a single subtype or perhaps multiple, as yet unidentified Ca\textsubscript{v} subtypes. The LVA Ca\textsubscript{v} exhibit selective IC\textsubscript{50} values in the low 10’s nM for kurtoxin (T-type, the Ca\textsubscript{v}3 family) with strong evidence for Ca\textsubscript{v}3.2 in nodose neurons\textsuperscript{23}.

Although the relative contribution of each Ca\textsubscript{v} subtype to the I\textsubscript{Ca} is quite variable across the general population of vagal afferent neurons, the majority of the whole-cell Ca\textsuperscript{2+} current (I\textsubscript{Ca}) at the cell body arises from N-type Ca\textsubscript{v}.\textsuperscript{21,24} Our recordings from rat nodose neurons show that I\textsubscript{Ca} peaks at approximately −10 mV within a few milliseconds of the voltage clamp step (Figure 3.3). The I\textsubscript{Ca} decays along a multi-exponential time course for several hundred milliseconds before settling at a magnitude that is about one half the peak current magnitude for any particular clamp step beyond approximately −40 mV. From a holding voltage of −100 mV, 70 to 80% of the I\textsubscript{Ca} is carried by a HVA N-type Ca\textsubscript{v} that is completely blocked by 1 µM omega-conotoxin (I\textsubscript{Ca,n}, Figure 3.3). This HVA Ca\textsuperscript{2+} current is responsible for the majority of the steady-state inward Ca\textsuperscript{2+} current in nodose neurons, giving rise to incomplete inactivation characteristics for I\textsubscript{Ca,n} (Figure 3.4). The total Ca\textsuperscript{2+} current that remains in the presence of ω-CTX exhibits comparable activation characteristics, but is far less inactivating and more sustained than the N-type current, common features of all the remaining four HVA Ca\textsubscript{v}. Occasionally, a small LVA transient calcium current (<100-200 pA in 2 mM [Ca\textsuperscript{2+}]) is observed with activation and inactivation characteristics typical of a T-type Ca\textsubscript{v} (Figure 3.3, inset). This current is completely inactivated at steady-state conditioning voltages more depolarized than −50 mV (Figure 3.3 and Figure 3.4).

Numerous aspects of neurobiological development and neuropathic injury responses are known to influence the relative expression of channel subtypes from the Ca\textsubscript{v}1 and Ca\textsubscript{v}2 families of HVA calcium channels.\textsuperscript{24} Such dynamic distributions may be indicative of specialized functions for particular ion channel subtypes along the afferent fiber pathway (peripheral, somatic and central regions.) For example, the N-type Ca\textsubscript{v} present in the cell body of nodose neurons are known to regulate synaptic transmission of vagal afferent information in the NTS, but a functional role is less apparent at the peripheral terminal ending.\textsuperscript{25} The functional role of the Ca\textsubscript{v}3 family of transient currents is less well understood. However, as the activation window for I\textsubscript{Ca,t} resides within the range of −70 to −60 mV there exists a small, but sustained inward Ca\textsuperscript{2+} current from these channels that may play an important role in establishing the resting membrane potential (Figure 3.4). Upon action potential discharge, a Ca\textsubscript{v}3.2 current component may contribute over the time course of late.
membrane repolarization and early afterhyperpolarization, which, in turn, may influence cell excitability of nodose neurons.³³

### 3.4 VOLTAGE-GATED POTASSIUM ION CHANNELS

The considerable biological homology present across the superfamily of voltage-gated potassium channel (Kv) proteins has made it possible to demonstrate that only modest differences in molecular sequences, or perhaps membrane lipid dynamics, separate a disparate range of voltage- and time-dependent gating properties.²⁶,²⁷ To date, the superfamily of Kv gives rise to the most functionally diverse and extensively studied class of transmembrane ion channel currents.²⁸ There exists a broad landscape of voltage- and time-dependent whole-cell K⁺ currents that respond to the depolarizing action of the inward Na⁺ and Ca²⁺ currents. The net effect of all these Kv channel subtypes is a total transmembrane outward K⁺ current (IK,total) that makes a critically important contribution in defining the membrane excitability characteristics of vagal afferent neurons. Evidence for many isoforms of the Kv family has been found in the central and, to a lesser extent, the peripheral nervous systems. The action of Kv antagonists has led to the broad classification of two functional forms of whole-cell K⁺ currents, those that are noninactivating and those that rapidly inactivate.

Under conditions of voltage clamp and with K⁺ as the major membrane permeant ion, the IK,total in rat nodose neurons exhibits threshold currents starting near −50 mV followed by a near ohmic current recruitment until presentation of a rather modest
transient outward peak at the highest clamp potentials (Figure 3.5). From this peak, the $I_{K,\text{total}}$ decays along a multieponential time course toward a sustained activation that requires several seconds to achieve. Pharmacological dissection of the $I_{K,\text{total}}$ into specific VGC subtypes is limited by the selectivity of $K^+$ channel antagonists, most of which exhibit an overlapping concentration-dependent block of multiple $K^+$ current subtypes. The channel antagonists with the greatest specificity are those derived using recombinant techniques or from purified animal or plant neurotoxins such as the family of dendrotoxin peptides, which are highly selective for the $K_v1.1$, $K_v1.2$ and $K_v1.6$ subtypes with IC$_{50}$’s on the order of 1 to 10 nM. At a concentration of 10 nM this whole-cell $K^+$ current ($I_{\text{DTX}}$), obtained by subtracting the current in the presence of $\alpha$-dendrotoxin from that in its absence, exhibits rapid activation characteristics beyond a threshold of approximately -40mV and effectively no inactivation over the time course of 400 msec clamp steps. The voltage-dependent recruitment of $I_{\text{DTX}}$ exhibits an e-fold slope ($s = 7.5$) and half activation ($V_{1/2} = -10mV$) that are comparable to the traditional delayed rectifier present in these cells (see below) but with an activation rate that is 5 to 10 times faster and, as a result, $I_{\text{DTX}}$ contributes to setting discharge threshold from rest potentials (Figure 3.6).

In the presence of 10 nM $\alpha$-dendrotoxin there is a component of the remaining $I_{K,\text{total}}$ that is sensitive to the $K^+$ channel antagonist 4-aminopyridine (4AP), that is presumed to be comprised of multiple, independent current components arising from members of the $K_v1$, $K_v2$, $K_v3$, and $K_v4$ families of $K^+$ VGC (Figure 3.5). Rapid activation (<10 msec) at low membrane potentials (<-50 mV) is a characteristic property of this whole-cell 4AP sensitive current ($I_{\text{4AP}}$), as is the complex inactivation profile that often follows a multieponential time course of decay toward a sustained (or very slowly inactivating) outward current component. It is this multieponential time course of decay that has led to the breakdown of $I_{\text{4AP}}$ into transient ($I_A$) and delayed ($I_D$) 4AP sensitive current components. In some preparations it has been possible to discriminate these two current components based upon modest differences in sensitivity to 4AP and thresholds for voltage activation but further study is required for definitive classification of the VGC subtypes. The $I_A$ is likely comprised of $K_v1.4$ and members of the $K_v3$ and $K_v4$ families of VGC. The origins of the $I_D$ component remain unclear although some members of the $K_v1$ and $K_v2$ families do exhibit the requisite insensitivity to $\alpha$-dendrotoxin and an inactivation time constant large enough (> 3 sec) to account for the voltage- and time-dependent profile of this current.

In the presence of 10 nM $\alpha$-dendrotoxin and 5 mM 4AP there is a component of the remaining $I_{K,\text{total}}$ that is blocked by 15 mM of the ubiquitous $K_v$ channel antagonist tetraethylammonium (TEA). This whole-cell current has voltage- and time-dependent features typical of a classical delayed rectifier current, $I_K$. Although at 15 mM TEA it is likely that at least some of this current, along with a significant component of the $I_{K,\text{total}}$ that remains in the presence of these three antagonists, is comprised of a calcium-activated $K^+$ current ($K_{Ca}$, see below). Threshold for voltage activation of $I_K$ occurs at relatively depolarized membrane potentials greater than -30 mV ($V_{1/2} = -5 mV$) with an activation time constant greater than 30 msec and an extremely gradual e-fold slope of greater than 15 (Figure 3.6).
FIGURE 3.5 The $\alpha$-DTX, 4-AP and TEA sensitive K$^+$ currents in rat nodose neurons. The upper voltage clamp traces present the whole cell K$^+$ current ($I_{K,\text{total}}$), which is comprised of multiple subtypes of K$^+$ currents that can only be separated through judicious application of selective channel antagonists. Alpha-dendrotoxin ($\alpha$-DTX) has a high specificity for select members of the K$_v$1 family. A 10 nM concentration of $\alpha$-DTX reveals a K$_v$1 current ($I_{\text{DTX}}$) that is $\sim$25% of $I_{K,\text{total}}$. At low millimolar concentrations 4-aminopyridine (4-AP) is moderately selective for members of the K$_v$1 family that exhibit both transient ($I_A$) and sustained ($I_D$) K$^+$ current components. A 5 mM concentration of 4-AP reveals a K$^+$ current ($I_{4\text{AP}}$) with peak and sustained responses that are $\sim$25% and $\sim$10% of $I_{K,\text{total}}$. Tetraethylammonium (TEA) blocks a broad spectrum of K$_v$ channels in a concentration-dependent manner. A 15 mM concentration of TEA reveals a composite of K$_v$ currents with delayed rectifier ($I_K$) characteristics that is $\sim$25% of $I_{K,\text{total}}$. Recording protocol consisted of 400 msec voltage steps from a holding potential of $-80$ mV, 10 mV increments up to 40 mV with an interstep interval of 3 sec. [K$^+$]$_i$ = 140 mM, [K$^+$]$_o$ = 5.4 mM, [Ca$^{2+}$]$_i$ = 10 nM (BAPTA-K$^+$) and [Ca$^{2+}$]$_o$ = nominally, 1-10 µM. Agonist application was sequential and additive, i.e., 10 nM $\alpha$-DTX, followed by 10 nM $\alpha$-DTX and 5 mM 4-AP, followed by 10 nM $\alpha$-DTX, 5 mM 4-AP and 15 mM TEA, in producing the subtracted traces. Vertical and horizontal scale bars are 2 nA and 100 msec, respectively, for all traces. See Schild et al.$^2$ and Glazebrook et al.$^{29}$ for complete details concerning the electrophysiological methods.
Voltage-gated potassium channels that also exhibit voltage-dependent gating properties ($K_{vCa}$) are most often classified according to measures of single-channel conductance and pharmacological sensitivities. These include a large (>200 pS) and an intermediate (10 to 60 pS) $K_{vCa}$ conductance that are blocked by low-nanomolar concentrations of charybdotoxin and, therefore, presumably members of the $K_{vCa1}$ and $K_{vCa3}$ families, respectively, and a small $K_{vCa}$ conductance (<10 pS) that is blocked by low-nanomolar concentrations of apamin and presumably a member of the $K_{vCa2}$ family.31–34

Under conditions of voltage clamp, with potassium as the major membrane permeant ion and $[Ca^{2+}]_i$ buffered to 10 nM using BAPTA-K, the extracellular application of 10 nM charybdotoxin selectively blocks a whole-cell $I_{KCa}$ in nodose neurons that is approximately one fourth of the peak magnitude of $I_{K, total}$. The voltage- and time-dependent activation characteristics are typical for this BK-type $K_{vCa}$. Beyond a ~10 mV threshold, the $I_{KCa(BK)}$ achieves a sustained magnitude within 10 to 15 msec of the clamp step that scales in an ohmic manner with increasing potential (Figure 3.7).

3.5 DYNAMIC PROPERTIES OF IONIC CURRENTS IN VAGAL AFFERENT NEURONS

The voltage clamp technique has become the de facto standard for quantification of the voltage- and time-dependent properties of whole-cell ionic currents. When combined with molecular and pharmacological methodologies, it is possible to dissect out the individual contributions of particular VGC subtypes to the whole cell...
Advances in Vagal Afferent Neurobiology

Such data can provide insight concerning the impact of a particular ionic current upon membrane excitability, albeit under very limited and very contrived conditions. A more comprehensive assessment of ion channel function can be obtained through the application of pharmacological blockers of proven specificity and selectivity under conditions of current clamp, or through the use of antisense technologies in conjunction with more integrative experimental paradigms. However, an interpretation of the functional consequences of the selective elimination or even over expression of a particular VGC subtype upon neural discharge cannot follow the simple logic of superposition. The complex interplay of the transmembrane currents arising from the collective of VGC is inextricably bound to the magnitude and time course of

FIGURE 3.7 The charybdotoxin sensitive $K_{Ca}$ current in rat nodose neurons. The upper voltage clamp traces present the whole cell $K^+$ current ($I_{K,\text{total}}$) of which a component is sensitive to the influx of $Ca^{2+}$ ($K_{Ca}$). At nanomolar concentrations, charybdotoxin (ChTX) is a selective blocker of the large conductance $K_{Ca}\,3.1$ channel in neurons. A 10 nM concentration of ChTX reveals an $I_{K,\text{Ca(BK)}}$ that is ~25% of $I_{K,\text{total}}$ at a $[Ca^{2+}]_i$ of 10 nM. Recording protocol consisted of 400 msec voltage steps from a holding potential of $-80$ mV, 10 mV increments up to 40 mV with an interstep interval of 3 sec. $[K^+]_i = 140$ mM, $[K^+]_o = 5.4$ mM, $[Ca^{2+}]_i = 10$ nM (BAPTA-K$^+$) and $[Ca^{2+}]_o$ = nominally, 1-10 µM. Vertical and horizontal scale bars are 2 nA and 100 msec, respectively, for all traces.
membrane potential. Therefore, it is not surprising that the changes in neuronal discharge properties observed in such deletion studies are often more subtle and less revealing than what might have been predicted based solely upon whole cell current magnitudes.\textsuperscript{23,29} Computational models derived from a foundation of voltage and current clamp data from a single cell type can serve as a useful platform for studying the nonlinear ionic current dynamics underlying membrane excitability. However, even the most complex model of membrane excitability is, at best, only an approximation to the actual system or data under investigation. Although it generally presents but one abstract snapshot of highly integrated and nonlinear biophysical mechanisms the effort can reveal possibilities regarding ion channel function that are beyond the reach of existing experimental paradigms. For the computational simulations presented below the individual membrane currents were modeled using Hodgkin and Huxley (1952) formalisms that were parameterized through numerical fits to the voltage clamp data presented in this chapter (Figure 3.1 through Figure 3.6) with whole cell conductances scaled according to the relative contribution each individual current makes to the whole cell current (e.g., $I_{K,total}$, Figure 3.5).\textsuperscript{2} The corresponding somatic action potential waveform is that of a C-type neuron as all the known ionic channel currents in rat nodose neurons are represented. Unlike the A-type, which do not exhibit $I_{TTXR}$ and have an outward $K^+$ current that is dominated by the transient $I_A$.\textsuperscript{3}

### 3.5.1 \(Na^+\) and \(Ca^{2+}\) Current Dynamics

Over the course of a single action potential waveform $I_{TTXS}$, $I_{TTXR}$, and $I_{Ca,n}$ comprise the majority of the total inward ionic current. Of these, $I_{TTXS}$ is the first to respond to a depolarizing event from resting membrane potential with a rapid inward current that reaches a maximum near 0 mV but quickly subsides as the overshoot approaches the reversal potential for $Na^+$ ions and the inactivation processes begin to dominate channel gating (Figure 3.8). Both $I_{TTXR}$ and $I_{Ca,n}$ exhibit voltage-activation profiles that are considerably more depolarized and activation time constants that are slower than those for $I_{TTXS}$ (Figure 3.2 and Figure 3.4). These factors substantially limit the recruitment of these currents over the rapid membrane depolarization leading up to the peak of the action potential waveform (Figure 3.8). Over the course of the action potential upstroke there is sufficient time and membrane depolarization to recruit a substantial $I_{K,total}$ (see below), which eventually results in a reversal in the trajectory of the action potential waveform. As repolarization proceeds, the membrane potential moves away from the reversal potential for both $Na^+$ and $Ca^{2+}$ ions and back through the activation windows for $I_{TTXR}$ and $I_{Ca,n}$ (Figure 3.2 and Figure 3.4). The time required to traverse from the action potential peak back toward threshold potentials for these currents is well below the magnitude of their activation time constants. This factor, combined with the slow rates of inactivation for these currents, results in a substantial $I_{TTXR}$ and $I_{Ca,n}$ over the downstroke of the action potential waveform (Figure 3.8). The net magnitude of the inward $Na^+$ and $Ca^{2+}$ currents are substantially less than the $I_{K,total}$ (see below) and, therefore, produce only a transient loss of total
outward current but it is of sufficient magnitude to slow the progression of membrane voltage over the downstroke of the action potential waveform (Figure 3.8). This suggests that the counteractive influences of $I_{\text{TTXR}}$ and $I_{\text{Ca,n}}$ upon the $I_{\text{K,total}}$ plays an important role in setting the duration of the action potential waveform, which further impacts the recruitment of the $K_V$ VGC and, ultimately, neuronal excitability.
### 3.5.1.1 Functional Role of $I_{\text{TTXS}}$ and $I_{\text{TTXR}}$

A-type nodose neurons, i.e., those with narrow ($\leq 1$ msec) somatic action potential waveforms and deep but short-lasting afterhyperpolarization transients, are known to give rise to myelinated axons.\(^{36,37}\) The total inward Na\(^+\) current in A-type neurons is comprised exclusively of $I_{\text{TTXS}}$. The characteristically low $V_{1/2}$ values, steep slope factors, small activation window and brief time constants coupled with a rather sluggish recovery from inactivation define the functional dynamics of $I_{\text{TTXS}}$ over the range of membrane potentials typically exhibited by somatic action potentials. The $I_{\text{TTXS}}$ is well suited for the low activation threshold and high discharge frequencies typically exhibited by myelinated vagal afferent fibers. In contrast, the rather depolarized activation profile of the $I_{\text{TTXR}}$ in vagal afferents is not well suited for initiation of action potential discharge and is, therefore, always co-expressed with $I_{\text{TTXS}}$. Cells expressing both $I_{\text{TTXS}}$ and $I_{\text{TTXR}}$ are often classified as C-type, exhibiting broad (2 msec or more) somatic action potential waveforms with a hump or delay in repolarization phase and a shallow afterhyperpolarization transient that requires nearly 100 msec to return to baseline. These C-type nodose neurons are known to give rise to unmyelinated axons.\(^{36,37}\) The physiological rationale behind the functional expression of two distinctly different Na\(^+\) currents in a single nodose neuron remains unresolved. However, a closer examination of the dynamic profiles of $I_{\text{TTXS}}$ and $I_{\text{TTXR}}$ under conditions of both voltage and current clamp along with mathematical simulations offers some intriguing possibilities (Figures 3.1, 3.2, and 3.8).

Starting from a resting membrane potential of -60 mV, approximately 60% of the total population of TTXS Na\(_{\text{v}}\) in nodose neurons is available for action potential generation (Figure 3.2). Over the time course of repetitive discharge, the rather slow recovery from inactivation steadily reduces the peak magnitude of the regenerative $I_{\text{TTXS}}$, which, in turn, plays an important role in setting the maximum discharge frequency of vagal afferent neurons. In contrast, nearly 100% of the population of TTXR Na\(_{\text{v}}\) is available for action potential discharge from rest potentials on account of the depolarized activation and inactivation profiles for these VGC. Furthermore, the rapid recovery from inactivation exhibited by TTXR Na\(_{\text{v}}\) ensures that the peak magnitude of the regenerative $I_{\text{TTXR}}$ remains essentially unchanged at discharge frequencies approaching 100 Hz. Therefore, neurons with an $I_{\text{TTXR}}$ that comprises the majority of the total inward Na\(^+\) current may be slowly adapting, assuming sufficient $I_{\text{TTXS}}$ remains to drive membrane potential toward the activation threshold for TTXR Na\(_{\text{v}}\). It would appear that the relative availability of the rapidly inactivating TTXS Na\(_{\text{v}}\) and the slowly inactivating TTXR Na\(_{\text{v}}\) is an important factor in defining the excitability, action potential waveshape, and capacity for repetitive discharge of vagal afferent neurons. There are multiple mechanisms that can influence this ratio of channel availability. Perhaps the simplest would be increasing the depth of the afterhyperpolarization in order to accelerate the recovery of the inactivated TTXS Na\(_{\text{v}}\). Alternatively, neuropeptides, inflammatory mediators, and other chemical signaling agents are known to affect the voltage-dependent properties of TTXR Na\(_{\text{v}}\), raising the possibility that the relative expression and availability of TTXS and TTXR Na\(_{\text{v}}\) may play an important role in the physiological response characteristics of vagal...
afferents to (as an example) neuropathic injury or conditions of sustained inflammation.8,14,38–40

3.5.2 K⁺ CURRENT DYNAMICS

Over the course of a single action potential waveform $I_A$, $I_D$, $I_{DTX}$, $I_K$, and $I_{K,Ca}$ comprise the majority of the total outward ionic current. Each of these individual, pharmacologically identified whole-cell currents is comprised of multiple subtypes of K⁺ VGC, which are only beginning to be identified and characterized in vagal afferent neurons. Not surprisingly, $I_A$ and $I_D$ are the first to respond to a depolarizing event on account of the hyperpolarized activation profile and fast activation time constants for these currents (Figure 3.6 and Figure 3.9). As membrane potential enters into a phase of rapid depolarization there is a marked recruitment of $I_{DTX}$ and $I_{K,Ca}$, which are the primary outward K⁺ currents responsible for terminating the action potential upstroke and initiating a reversal in the trajectory of the membrane potential. This effect is most apparent in the phase plot where beyond approximately 0 mV $I_{DTX}$ dominates the outward current, being joined and eventually surpassed by $I_{K,Ca}$ near the peak of the action potential waveform (note shaded arrowheads, Figure 3.9). As repolarization proceeds from peak back toward 0 mV $I_A$, $I_D$, and $I_K$ are recruited to a greater extent, eventually peaking near the midpoint of the action potential waveform. These three currents are slow to join $I_{DTX}$ and $I_{K,Ca}$ but for different reasons. Both $I_A$ and $I_D$ are inactivating currents with gating profiles that are far more negative than those of the other K⁺ currents (Figure 3.6). The inactivation time constant for $I_A$ is on the order of 10 to 15 msec over the more depolarized phases of the action potential waveform. This factor, coupled with a whole-cell conductance that is comparable to or just slightly smaller than for $I_{DTX}$ and $I_{K,Ca}$ (see below), markedly restricts the peak whole-cell current from these transient VGC. This is in contrast to $I_K$ and $I_D$, which are both considerably larger than $I_A$ and present gradual current profiles that are quite similar in magnitude and time course (note inset, Figure 3.9). Interestingly, $I_D$ has a whole-cell conductance that is nearly equal to that of $I_A$ but only about one-third that of $I_K$ (n.b. at the end of the $I_{4AP}$ traces, Figure 3.5) $I_A$ has nearly completely inactivated and the current that remains is $I_D$ and this has a magnitude only one third that of $I_K$ across all clamp voltages). The larger current magnitudes also come about because of the sustained nature of these currents, $I_K$ is noninactivating and $I_D$ has an inactivation time constant of 7500 msec. By far the largest contributor to the total outward transmembrane current over the course of an action potential is $I_{K,Ca}$ (Figure 3.9).² This is best explained by first re-examining the voltage clamp response of this charybdotoxin-sensitive current (Figure 3.7). In voltage clamp, the [Ca²⁺]i has been buffered to 10 nM, but more importantly the [Ca²⁺]o is nominally calcium free with an unbuffered concentration on the order of 1 to 10 µM. While far below the influx of Ca²⁺ ions that give rise to the $I_{Ca,n}$ over the time course of a somatic action potential (Figure 3.8), this low micromolar concentration is sufficient to support the recruitment of a BK-type $I_{K,Ca}$ that is approximately one fourth of the $I_{K,total}$. Under conditions of normal [Ca²⁺]o at 2 mM the [Ca²⁺]i transient will be well above concentrations that have been shown
to markedly increase single channel open probability. Model predictions suggest that such a $[Ca^{2+}]_i$ would produce a voltage clamped $I_{K, Ca(BK)}$ several times larger than that presented in Figure 3.7, which is consistent with the simulations of the somatic action potential waveform (Figure 3.9).
3.5.3 Continued Study of the Ionic Currents Underlying Membrane Excitability

Knowledge of the molecular identity of the superfamily of VGC underlying the major transmembrane currents in vagal afferent neurons lags well behind that for somatic and central nervous system neurons. This, however, presents exciting opportunities for future scientific investigation of these afferents. When coupled with a definitive identification of sensory modality (e.g., through the use of fluorescent lipophilic tracers) such in vitro studies would advance the physiological relevance of using the cell body as a model system for investigating the molecular foundations of the ion channel currents participating in the neurosensory transduction and spike encoding processes. Furthermore, there remain several identified subclasses of channel proteins in vagal afferents in which a functional interpretation of the impact of the corresponding whole-cell current upon membrane excitability is lacking. Two such subtypes are the KCa and hyperpolarization activated, cyclic nucleotide-gated cation (HCN) channels.

3.6 Voltage Dependent Calcium-Activated Potassium Ion Channels

To date, the combined effects of [Ca$^{2+}$] magnitude and transmembrane potential upon the availability of KvCa(BK) in vagal afferent neurons has not been well characterized. This is also true for the small fraction (<5%) of the IK,total that is comprised of SK-type apamin sensitive and intermediate KvCa current components. Both electrophysiological and computational studies have demonstrated that IKCa(BK) can have a significant impact upon action potential waveshape and repetitive discharge in vagal afferent neurons (Figure 3.9). Further elucidation of the role of KCa dynamics in vagal afferent discharge will require a more intensive investigation of the subcellular mechanisms that can effect the transient change in [Ca$^{2+}$], associated with membrane depolarization. A more comprehensive investigation of the gating properties of KvCa channels along with molecular identification of KCa channel subtypes in vagal afferent neurons is needed. Likewise, existing mathematical models of the Ca$^{2+}$-dependent gating mechanisms of KCa channels must be advanced to include regional [Ca$^{2+}$] dynamics that are influenced by both transmembrane and intracellular sources of Ca$^{2+}$ ions.

3.7 Hyperpolarization Activated Cyclic Nucleotide-Gated Cation Channels

The channels underlying the HCN current (IH) comprise four distinct subtypes that contribute to the oscillatory characteristics of neurons in the central nervous system, but differ with regard to cAMP sensitivity and activation dynamics. Considerably less is known regarding the functional roles for HCN channels expressed in the normally quiescent cell bodies of vagal afferents, but as sustained suprathreshold stimulation can elicit repetitive discharge, a similar functional role is presumed.
Immunohistochemical studies have shown HCN2 and HCN4 immunoreactivity across all neurons in the rat nodose ganglia, but only 20% of these cells presented with immunoreactivity for HC1. Interestingly, HCN1 expression was present in nearly all neurons that carried positive for markers for myelination and was expressed at the mechanosensitive terminals of myelinated (A-type) but not unmyelinated (C-type) sensory fibers. In contrast, the HCN2 and HCN4 subtypes were found in the receptor terminals of both A- and C-type afferents.\textsuperscript{35,46}

Glazebrook et al. (2002) demonstrated that under conditions of voltage clamp the capacitance normalized peak magnitude of the whole-cell mixed cation \( I_\text{H} \) in A-type nodose neurons was nearly ten times greater than that in C-type neurons.\textsuperscript{29} In both cell types, \( I_\text{H} \) activated at potentials negative to \(-50\) mV and developed slowly with time. At \(-120\) mV \( I_\text{H} \) activated approximately twice as fast in A-type as compared with C-type neurons. Under current clamp conditions, \( I_\text{H} \) exhibits a time-dependent rectification in response to hyperpolarizing current injections from resting membrane potentials. Further experimental and computational study are required to resolve the differential role \( I_\text{H} \) may have across the population of A- and C-type vagal afferents such as active regulation of the resting membrane potential and limiting the substantial \( I_{\text{KCa}} \)-mediated hyperpolarization that can occur following periods of elevated membrane discharge.\textsuperscript{44}

### 3.8 CONCLUSION

To this point we have focused on a select group of \( \text{Na}^+ \), \( \text{Ca}^{2+} \) and \( \text{K}^+ \) VGC whole-cell currents that make up the vast majority of the total transmembrane current over the course of a somatic action potential waveform. The magnitude of the whole-cell currents arising from these VGC is a direct reflection of the somatic expression density (i.e., channels/\( \mu \)m\(^2\)) and the nonlinear dynamic properties of channel gating (Figure 3.8 and Figure 3.9). Many questions remain concerning the molecular identity and functional distribution of these VGC subtypes along the vagal afferent pathway. Ion channel expression at the peripheral terminal ending is a critically important component of the sensory transduction process, while expression at synaptic terminations most certainly impacts the central integration of neurosensory information. Further \textit{in vitro} study of the dynamic properties of particular subtypes of VGC can advance the understand of the role these transmembrane proteins play in neuronal excitability. Interpretation of these data in terms of issues relevant to health and disease of organ systems along the vagal afferent pathway will require more comprehensive molecular, electrophysiological and computational methodologies than can likely be provided by isolated cellular recordings alone.

### REFERENCES


APPENDIX: TOTAL TRANSMEMBRANE CURRENT AS A FUNCTION OF MEMBRANE POTENTIAL

The “dynamics” of ionic current refers to how the current changes over the course of time and transmembrane voltage. An important objective of the mathematical theory of dynamical systems is to characterize and quantify system behavior by identifying critical interrelationships between system subcomponents.

The net transmembrane current functions electrically to alter the total charge of the whole cell capacitance which is most often observed as a change in membrane potential. As a result, the net ionic current flow can be calculated according to the product of whole-cell capacitance and the negative time derivative of membrane voltage (i.e., \(-C_m dV/dt\)). When presented as a function of membrane voltage, the ensuing phase plot forms a closed loop that makes possible detailed quantification of subtle changes in discharge threshold and waveshape over the more rapid phases of the action potential trajectory such as the upstroke and downstroke (Figure A.1). During the action potential upstroke, the net inward transmembrane current manifests as a downward trajectory in the phase plot, which rapidly returned to zero as the waveform peaked, i.e., total inward and total outward transmembrane currents were equal. The upward trajectory represents a net outward transmembrane current during the action potential downstroke, which was markedly distorted in neurons that exhibited delayed repolarization, i.e., a prominent “hump” over the time course of the action potential downstroke. On account of the temporal sensitivity of this and other dynamical systems analysis techniques, subtle changes in the action potential waveform as a result of experimental manipulation of whole cell transmembrane currents can be readily assessed.
FIGURE A.1
4 Electrophysiological Studies of Target-Identified Vagal Afferent Cell Bodies

Danny Weinreich

CONTENTS

4.1 Introduction ..................................................................................................102
4.2 Anatomy of Vagal Afferent Cell Bodies......................................................103
4.3 Dissociation of Vagal Afferent Cell Bodies from Adult Vagal Ganglia .... 104
4.4 Selection of Dye for Retrograde Labeling Vagal Ganglion Neurons ....... 105
4.5 Preparations Useful for Studying Vagal Afferent Cell Bodies................. 107
4.6 Attributes, Limitations, and Concerns when Studying Isolated Target-Identified Vagal Sensory Cell Bodies...............................................107
   4.6.1 Attributes ..........................................................................................107
   4.6.2 Limitations and Concerns ................................................................110
4.7 Physiological Studies of Target-Identified Vagal Afferent Cell Bodies.....111
   4.7.1 Studies of Baroreceptors.................................................................111
   4.7.2 Airway Vagal Afferent C-Fibers Comprise Two Distinct Phenotypes .................................................................112
   4.7.3 Electrophysiological Properties and Chemical Responsiveness of Parasympathetic and Sympathetic Primary Sensory Neurons Innervating the Airways .................................................................113
   4.7.4 Bradykinin Excites Airway Sensory Neurons by Promoting a Calcium-Dependent Chloride Current .................................................................113
4.8 Pathophysiological Studies of Target-Identified Vagal Afferent Cell Bodies.................................................................114
   4.8.1 Gastric Inflammation ......................................................................114
   4.8.2 Airway Inflammation .....................................................................115
4.9 Conclusions and Future Directions .............................................................115
Acknowledgments..................................................................................................116
References..............................................................................................................116
4.1 INTRODUCTION

The observation that cell bodies (somata) of primary sensory neurons are endowed with receptors similar to those that exist on their central or peripheral nerve terminals has been known for more than 30 years. Nishi et al.\textsuperscript{50} demonstrated that activation of GABA receptors on somatosensory (dorsal root ganglion, DRG) neurons produced a membrane depolarization that is mediated by the efflux of chloride ions. From this observation, they proposed that the mechanism underlying GABA-evoked depolarization of primary afferent nerve terminals might also be due to an increase in chloride permeability. The correspondence between the chemosensitivity of vagal primary afferent nerve terminals and their cell bodies for a panel of excitatory/sensitizing substances and inflammatory mediators (serotonin, histamine, bradykinin, Substance P, and prostaglandins) was initially and elegantly delineated in a series of publications by Higashi and co-workers.\textsuperscript{24} These results were derived from intracellular recordings of primary vagal afferent cell bodies in intact vagal ganglia, \textit{in vitro}. Working with intact ganglia it was possible to measure conduction velocities of the impaled neurons and thus to correlate somal chemosensitivity with a neuronal phenotype, conduction velocity. Type A neurons have axons with conduction velocities 6 to 12 m/sec and somal action potentials that are completely blocked by tetrodotoxin (TTX, 0.1 to 0.5 FM). Type C neurons that have axons with conduction velocities 0.3 to 1.4 m/sec and somal action potentials resistant to TTX (1 to 5 µM). These two classes of neurons are also distinguished by their sensitivity to inflammatory mediators. Type C neurons are depolarized by serotonin, bradykinin or histamine, while type A neurons are relatively insensitive to these mediators. One drawback of this preparation is that intact vagal ganglion contain many different cell types besides primary afferent somata. Satellite cells, endothelial cells, and immune cells (macrophages and mast cells) are present in primary sensory ganglia\textsuperscript{42} and may respond to inflammatory mediators and autacoids by releasing signal molecules that, in turn, alter the excitability of primary afferents.\textsuperscript{23} Thus, when studying the actions of autacoids on neurons in intact vagal ganglia, interpretation of their site-of-action can be compromised by the heterogeneity of the tissue.

In 1983, two key papers were published that set the stage for using isolated primary afferent cell bodies as a tractable model for physiological and pharmacological studies of the primary afferent nerve terminal. Baccalini and Hogan\textsuperscript{2} reported that dissociated primary afferent somata (DRG neurons) maintained in culture expressed chemosensitive properties similar to those expressed by their peripheral nerve terminals and that these properties persisted in the absence of other cell types. In the same year, Belmonte and Gallego\textsuperscript{3} reported that primary afferent somata with axons having different peripheral receptors had distinct electrophysiological properties. Cell bodies of chemoreceptor neurons had action potentials with prominent humps on their falling phase and a prolonged spike after-hyperpolarization, while the action potentials recorded in the cell bodies of baroreceptor neurons had action potentials with little or no deflections on their falling phase and they had much shorter duration spike after hyperpolarizations. Together, these two key papers revealed that somatic plasma membranes of primary afferent neurons possess distinct
sets of electrophysiological and chemosensitive properties that could reflect pharmacologic and biophysical properties of their nerve terminal membranes.

A third development that facilitated physiological studies of re-identifiable primary vagal afferent cell bodies was the introduction of fluorescent retrograde labeling techniques for the functional study of visceral-specific vagal afferent neurons. Mandelowitz and Kunze (1992)* were among the first investigators to combine axonal tracing methods, vital fluorescent dyes, with patch-clamp recording to examine electrophysiological and pharmacological properties of isolated, target-identified, primary afferent neurons. They measured whole-cell calcium currents from isolated aortic baroreceptor cell bodies that were identified by the fluorescent tracer, 4-(4-dihexadecylaminostyryl)-N-methylpyridinium iodine (DiA), previously applied to the uncut aortic nerve. A detailed methodological examination of the use of fluorescent dyes to study the electrophysiological properties and chemosensitivity of acutely isolated retrogradely labeled viscera-specific vagal afferent somata (nodose ganglion neurons) appeared the following year.13

The development of cell isolation techniques for adult primary sensory cell bodies and the application of retrograde fluorescent tracers to identify target-specific cell bodies have yielded a powerful technique to study numerous physiological, pharmacological and biophysical properties of vagal afferent nerve cells. This chapter deals with the attributes, limitations, and concerns about combining retrograde tracing techniques and sensory cell body isolation methods with intracellular recording methods for studying target-identified primary vagal afferents. It also considers several examples of how studies of target-identified vagal afferent cell bodies have furthered our understanding of the physiology and pathophysiology of the vagus nerve.

4.2 ANATOMY OF VAGAL AFFERENT CELL BODIES

A vagal primary afferent neuron is a pseudo-unipolar cell consisting of a spheroidal cell body (somata), an initial tract of axon (stem process) that extends from the cell body to a site of bifurcation (a distance ranging from a few µm to 100s of µm), and peripherally and centrally projecting processes.42 The cell bodies of vagal primary afferents are housed in two separate vagal ganglia. Cell bodies in the superior vagal ganglion or jugular ganglion (JG), like neurons from spinal ganglia, are derived from the neural crest (see Chapter 1). Axons of JG neurons innervate the pharynx and larynx,18 lower airways,36,54 skin of the external acoustic meatus, and dura of the posterior cranial fossa.4 The inferior vagal ganglion or nodose ganglion (NG) contains cell bodies that are derived from the placodal ectoderm (see Chapter 1), and innervate almost all viscera, including airways, heart, gastrointestinal tract, liver, thymus, uterus, neuro-epithelial bodies, and vagal paraganglia.4,46

* Though Mandelowitz and Kunze’s 1992 paper appears to be the first report of electrophysiological studies of target-identified primary afferent cell bodies, a series of preliminary observations from Chuck deGroat’s laboratory appeared in 1989 and 1990, detailing the application of fluorescent retrograde tracers with patch-clamp recording to study isolated, target-identified dorsal root ganglion neurons; a full account of these observations did not appear until 1994 (Yoshimura et al., 1994).
There are approximately three times as many NG neurons as JG neurons (~25,000 vs 8,500 for vagal ganglia of the cat. Based upon measurements of cross-sectional area, cell diameters, or membrane capacitance, the distribution of cell body sizes appears to be unimodally distributed for vagal ganglion neurons (VGNs). In rat, for example, NG neurons range in size from 56 µm² to 1631 µm² (mean, 595 µm²), while JG cell body sizes varied between 80 µm² and 1288 µm² (mean, 391 µm²). Corresponding values for rat VGN size based upon membrane capacitance are: 20 pF to 60 pF (mean, 31 pF) for NG neurons, and 22 pF to 59 pF (mean, 32 pF) for JG neurons. Approximate mean cell body size based upon diameters measured from acutely isolated NG neurons for mouse, rat, guinea pig, rabbit, and ferret are: 33 µm, 35 µm, 39 µm, 61 µm, and 54 µm, respectively.

Like most sensory ganglia, detectable synaptic profiles have not been described in the adult JG or NG (Reference 42), although NG somata can chemically communicate with one another. The nature of the mediator(s) supporting this form of neuronal communication remains to be identified.

4.3 DISSOCIATION OF VAGAL AFFERENT CELL BODIES FROM ADULT VAGAL GANGLIA

Ikeda and coworkers initially developed an enzymatic dispersion procedure to isolate single nodose ganglion neurons from adult rats to study voltage-sensitive sodium and calcium currents with patch-clamp recording techniques. Their procedure used a combination of trypsin (type III, 1 mg/ml), collagenase (type 1A, 1mg/ml), and DNAase (type III, 0.1 mg/ml). General protocols for isolating adult primary sensory cell bodies have recently been published by Spigelman et al. We have developed a relatively uncomplicated procedure for isolating nodose and jugular ganglion neurons from adult ganglia from various species (mouse, rat, guinea pig, rabbit, and ferret) using collagenase and dispase. This procedure consistently provides high yields of viable vagal cell bodies (in the case of rabbit > 90 % recovery of nodose neurons).

After removing vagal ganglia, they are transferred to a dissecting chamber containing ice cold (4°C) calcium-free, magnesium-free Hank’s Balanced Salt Solution (HBSS). Using a dissecting microscope, the adhering connective tissue, blood vessels, and other debris are removed from the ganglia with the aid of fine-sharpened watchmaker forceps and iris scissors. When suitably trimmed, it should be possible to visualize individual cell bodies with the dissecting microscope. What is visualized is a sack of cells surrounded by a thin transparent sheath. The reason for carefully trimming the ganglia is to reduce exposure time and concentration of digestive enzymes. In the case of larger nodose ganglia, those from guinea pig, rabbit, and ferret, nicks are made into the ganglia with iris scissors or the ganglia are cut into three to six segments.

The ganglia, or ganglion fragments, are transferred to a sterile 15 ml conical tube containing 5 ml cold, filter sterile (0.22 µm millex-Gs, Millipore) HBSS with 1 mg/ml collagenase (Type 1A, Sigma, C-9891) and 1 mg/ml dispase (grade II, Boehringer, Mannheim, A-7292). The tube containing the ganglia is either incubated
at 37°C for 2 hours, or placed in a 4°C refrigerator for 4 hours, or overnight. For the 37°C incubate, at 45 min, and 90 min the tissue is gently triturated with a fire-polished Pasteur pipette five times, taking care to avoid bubbles. At two hours the ganglia are triturated with a small-bore fire-polished Pasteur pipette. The orifice of the pipette should be just larger than the ganglia or ganglion segments. Only a few (3 to 8) triturations should be necessary to completely dissociate the neurons; the fewer the number of triturations, the less cell damage occurs. After trituration the neurons are centrifuged (700g, 45 sec) and the pelleted cells are resuspended in L15 media (GiBCO BRL containing heat-inactivated 10% fetal bovine serum; JRH Biosciences). The neurons are washed two more times by centrifugation and then 150 µl aliquots of cells are transferred onto circular 15- or 25-mm glass cover slips coated with poly-D-lysine (0.1 mg/ml, Sigma) lying on the floor of 35 mm petri dishes. The cell bodies adhering to the cover slips are maintained for two hours after plating at 37°C and then they are used for recording or an additional 2 ml of culture medium is added to the petri dishes and the cells are transferred to a room-temperature incubator for storage. For the cold-incubated ganglia, at 4 hours, or longer, 4.5 ml of enzyme solution is removed and replaced with 4 ml of prewarmed enzyme solution and the tissues are incubated for 5 to 10 min at 37°C. After trituration (1 to 3 times) with a large-bore, fire-polished Pasteur pipette, the cells should dissociate completely. Then the cells are processed as described above for the 2-hour incubation. Though we have not performed a systematic investigation on the electrophysiological properties of the cell bodies prepared by these two methods, there do not appear to be any obvious differences in the passive or active membrane properties or to the responses to a panel of autacoids between the two procedures. The advantage of the cold incubation procedure is that tissue can be prepared in the evening then used the following morning. Isolated VGNs maintained at room temperature are used for recording for up to 48 hours.

We have not used antibiotics in our protocols because, with careful sterile technique, we have rarely encounter bacterial contamination. Over the years, we have found that the largest variable with the dissociation procedure is the activity of the collagenase. Adjustment between different lots can be made based upon specific activity and time of incubation. We have noted that once a suitable lot is optimized, then a year’s supply is purchased and stored at –80°C. Manufacturers will often provide gratis small samples of different lots in order to choose an appropriate lot. A month’s supply of enzyme solution can be prepared and divided aliquots stored at –20°C, facilitating the time necessary to prepare cells.

### 4.4 SELECTION OF DYE FOR RETROGRADE LABELING VAGAL GANGLION NEURONS

Honig and Hume popularized the use of long-chain carbocyanine fluorescent dyes for neuronal labeling and pathway tracing. These dyes are ideal for retrogradely labeling neurons in intact tissues; they were essentially nontoxic, and there was no significant spread of dye from labeled cells to other cells. Subsequently, it was determined that there was no loss of label following acute dissociation of neurons
Advances in Vagal Afferent Neurobiology

from primary sensory ganglia previously retrogradely labeled with fluorescent dyes. A complete list of available fluorescent dyes and their attributes for retrograde labeling can be found in Handbook of Fluorescent Probes and Research Products.

There are a large number of fluorescent tracers available for retrograde tracing; choosing the most suitable dye depends upon the end organ being labeled. Currently, the most commonly used dyes for retrograde labeling VGNs are long-chain lipophilic carbocyanine dyes (DiI, DiO, DiD, and DiR). In-depth information about the chemistry and use of these dyes can be found in chapter 14 of A Handbook of Fluorescent Probes and Research Products. Because these dyes have a wide range of wavelength emission peaks, ranging from ~500 to 800 nm, it is possible to combine an appropriate retrograde dye with another fluorescent indicator. For example, simultaneous whole-cell patch-clamp and Fura-2 microfluorimetric recordings of Ca²⁺ currents and intracellular Ca²⁺ concentration can be accomplished from target-identified VGNs retrogradely labeled with DiD. In addition, because the carbocyanine dyes have absorption and fluorescence emission maxima separated by ~65 nm, several lipophilic carbocyanine dyes can be used to identify VGNs innervating two or more visceral organs or different areas within the same organ. Two newer dyes, Fast DiI and Fast DiO, have diunsaturated linolely (C₁₈:2) tails instead of saturated octadecyl tails (C₁₈). These chemical modifications are touted to substantially improve the rate of dye migration over that observed with DiI or DiO; however, little data is currently available about the migration rates of these dyes with respect to retrogradely labeling VGNs.

Numerous methods exist for applying fluorescent tracers to tissues. Two of the most common are direct application of dye crystals to intact axons, or injection of the tracer into organs or the vagus nerve. When injected, lipophilic carbocyanine dyes are dissolved in either dimethylsulfoxide, (up to 10%), dimethylformamide (0.5 to 10%), or ethanol or methanol (2 to 5%). Usually multiple injections of (3–20 µl/site) are used. When labeling VGNs innervating lungs and airway much larger volumes of dye are used, 2 × 200 µl of 0.2 to 0.4 mg/ml DiI in 1% ethanol, in saline is instilled into the lumen of the airway.

The time required to label VGNs with a retrograde tracer varies with target distance, nature of the dye, species, and age of the animal. In the adult rat (>150 gm), labeled nodose ganglion neurons innervating baroreceptors, stomach, or airways with DiI dye was detected after 7 to 10 days.

One of the contentious problems generic to using retrogradely labeled somata for physiological studies of voltage- and ligand-gated channels is the argument that the properties of ion channels and chemoreceptors being studied may be altered by the labeling procedure. This problem is particularly applicable with the use of carbocyanine dyes because for injection they must be dissolved in methanol, DMSO, or other solvents that can cause localized inflammation or direct damage to the very cells that are being labeled. It is difficult to circumvent this argument directly. Several strategies are employed to minimize this argument. Waiting extended time periods (one to two weeks) after VGNs are retrogradely labeled before making physiological measurements should minimize injury- or inflammation-induced changes in the VGNs evoked by tracer application. In addition, it is possible to determine that the
electrical membrane properties (action potential waveform, chemosensitivity, resting conductance, etc.) of labeled VGNs are similar to those recorded from adjacent nonlabeled VGNs residing on the same coverslip.

4.5 PREPARATIONS USEFUL FOR STUDYING VAGAL AFFERENT CELL BODIES

There are a variety of experimental preparations that can be used when studying target-identified VGNs. These range from intact animals to acutely isolated somata. Though in principle there should be no experimental limitation in recording intracellularly from target-identified primary vagal afferent somata in intact animals, such recordings have not yet been reported. This is unfortunate because much could be learned from such preparations (e.g., testing the efficacy and magnitude of chemical communication between target-identified VGN somata labeled from different visceral).52

Recordings of target-identified VGNs in intact vagal ganglia either in vivo or in vitro have the advantage of correlating somatic electrical and chemical properties with action potential discharge patterns and conduction velocities in peripheral and central vagal axons. It has recently become feasible to apply whole-cell patch clamp recording techniques to adult intact nodose ganglia.41 With this preparation it will be possible to classify somal voltage-and ligand gated ion channel types with action potential conduction velocities with target-identified vagal afferents.

Patch clamp recording has several advantages over “sharp” micropipettes: less shunting of the resting conductance yielding approximately an order-of-magnitude lower resting conductances; control of the internal ionic milieu for accurate determination of reversal potential values, or to isolate specific classes of ionic currents; the ability to introduce large molecular weight or nonionic substances (enzymes, antibodies, caged reagents, etc.) into somal compartments, and the capability to extract the intracellular milieu for studies of genes and gene products. The most common preparation utilizing target-identified VGNs is the acutely isolated VGN. Here investigators can combine the attributes of target-identified vagal somata with the methodological advantages afforded by using isolated primary afferent cell bodies; in particular, application of patch-clamp recording.

4.6 ATTRIBUTES, LIMITATIONS, AND CONCERNS WHEN STUDYING ISOLATED TARGET-IDENTIFIED VAGAL SENSORY CELL BODIES

4.6.1 ATTRIBUTES

Ikeda et al.30 pioneered the use of primary vagal afferent cell bodies isolated from adult vagal ganglia for electrophysiological studies. They developed methods to enzymatically disperse nodose ganglion neurons with surface membranes sufficiently free of debris that it was possible to form giga-ohm seals, thereby allowing the application of patch-clamp techniques to VGNs. The same attributes associated
Advances in Vagal Afferent Neurobiology

with patch-clamp recording from identified VGNs described earlier for studies of VGNs in the intact ganglia also hold for isolated VGNs with the additional experimental advantage that it is far easier to perform patch-clamp recordings with isolated VGNs.

Isolated VGNs possess many properties that make them very attractive for studying the electrical properties and chemosensitivity of vagal afferents. Anatomically, dissociated VGNs are isolated from satellite cells that normally surround each VGN in the intact ganglia. This situation is not only indispensable for patch-clamp recording and allowing rapid exchange of extracellular milieu, it is critical in for determining that drug- or pathology-induced changes in neuronal excitability are occurring directly from the neuron under study. It is known, for example, that satellite cells surrounding primary afferent cell bodies can release substances that alter the excitability of the primary afferent neuron. Bradykinin, for example, can exert a direct nociceptive effect on isolated NG neurons by blocking spike frequency adaptation and by depolarizing the membrane potential by activating a chloride conductance. It can also indirectly affect the excitability of primary afferent neurons by evoking an inward current that requires contact between the primary afferent soma and nonneuronal satellite cells.

During the isolation procedure, the soma is sheared away from the stem process yielding nearly round neurons, a geometry that minimizes space-clamp errors. Isolated VGNs can be derived from adult animals, thereby obviating the reliance on the use of neurons derived from neonatal animals. Importantly, many voltage-gated currents as well as ionotropic and metabotropic receptors observed in the neurons from intact ganglia are not significantly altered by the isolation procedure. In an in-depth study, Leal-Cardoso et al. compared the passive and active electrical membrane properties and the chemosensitivity of intact and acutely dissociated adult rabbit NG neurons (see Table 4.1, Table 4.2, and Table 4.3). Their results revealed

<p>| TABLE 4.1 | Comparison of Passive Membrane Properties of Acutely Isolated Rabbit Nodose Neurons with Neurons from Intact Nodose Ganglion In Vitro |</p>
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Intact Ganglion</th>
<th>Acutely Dissociated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting Potential (mV)</td>
<td>−58 ± 0.6 (29)</td>
<td>−59 ± 0.6 (108)</td>
</tr>
<tr>
<td>Input Resistance (MΩ)</td>
<td>81 ± 4.4 (29)</td>
<td>80 ± 5.3 (104)</td>
</tr>
<tr>
<td>Time Constant (msec)</td>
<td>8.5 ± 0.8 (50)</td>
<td>5.7 ± 0.5 (47)</td>
</tr>
<tr>
<td>Membrane Resistance (kΩ/cm²)</td>
<td>9.1</td>
<td>9.3</td>
</tr>
<tr>
<td>Membrane Capacitance (µF/cm²)</td>
<td>0.92</td>
<td>0.78</td>
</tr>
<tr>
<td>Cell Diameter (µm)</td>
<td>60 ± 1.2 (35)</td>
<td>61 ± 1.6 (90)</td>
</tr>
</tbody>
</table>

Source: Data from Leal-Cardoso et al. Values are means ±SEM. Numbers in parentheses are n values. All parameters were recorded at room temperature (21° to 23°C) with sharp micropipettes filled with 2 M KCl and 1 M K acetate. Recordings from isolated neurons were made between 2 to 4 hours after plating cells on glass coverslips. Recordings from intact nodose ganglion neurons were made between 1 to 2 hours after the nodose ganglion was in vitro.
essentially no differences between the membrane properties recorded from acutely isolated NG neurons and nodose neurons in intact vagal ganglia. Both ionotropic and metabotropic receptor-mediated ion channel function were unaffected by the isolation procedures. Noteworthy, the labile and metabolically dependent ($Q_{10} = 3–4$) slow spike afterhyperpolarization that is observed in ~ 40% of the rabbit NG neurons

### TABLE 4.2
Comparison of Action Potential Properties of Acutely Isolated Rabbit Nodose Neurons with Neurons from Intact Nodose Ganglion In Vitro

<table>
<thead>
<tr>
<th>Action Potential Parameter</th>
<th>Intact Ganglion</th>
<th>Acutely Dissociated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amplitude (mV)</td>
<td>91 ± 0.7 (230)</td>
<td>79 ± 1.2 (105)</td>
</tr>
<tr>
<td>Duration (msec)</td>
<td>3.5 ± 0.1 (23)</td>
<td>5.2 ± 0.1 (92)</td>
</tr>
<tr>
<td>Overshoot (mV)</td>
<td>26 ± 0.9 (23)</td>
<td>21 ± 1.0 (105)</td>
</tr>
<tr>
<td>Max. Rate of Rise (V/sec)</td>
<td>120 ± 9.4 (22)</td>
<td>52 ± 4.1 (80)</td>
</tr>
<tr>
<td>Spike Threshold (mV)</td>
<td>−41 ± 0.4 (23)</td>
<td>−38 ± 0.9 (98)</td>
</tr>
<tr>
<td>AHP$_{fast}$ Duration (msec)</td>
<td>89 ± 14.1 (16)</td>
<td>64 ± 3.8 (86)</td>
</tr>
<tr>
<td>Amplitude (mV)</td>
<td>13 ± 4.0 (20)</td>
<td>10 ± 0.5 (86)</td>
</tr>
<tr>
<td>AHP$_{slow}$ Duration (sec)</td>
<td>19.2 ± 0.8 (12)</td>
<td>13.1 ± 1.6 (23)</td>
</tr>
<tr>
<td>Amplitude (mV)</td>
<td>7.5 ± 0.3 (12)</td>
<td>9.5 ± 1.2 (23)</td>
</tr>
</tbody>
</table>

*Source:* Data from Leal-Cardoso et al. 40 Values are means ± SEM. Numbers in parentheses are n values. All parameters were recorded at room temperature (21° to 23°C) with “sharp” micropipettes filled with 2 M KCl and 1 M K acetate. Recordings from isolated neurons were made between 2 to 4 hours after plating cells on glass coverslips. Recordings from intact nodose ganglion neurons were made between 1 to 2 hours after the nodose ganglion was in vitro. AHP$_{fast}$ and AHP$_{slow}$ are fast and slow spike afterhyperpolarizations, respectively. AHP$_{slow}$ elicited by four action potentials at 10 Hz.

### TABLE 4.3
Chemosensitivity of Acutely Isolated Rabbit Nodose Neurons to Various Autacoids

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Serotonin</th>
<th>Bradykinin</th>
<th>Acetylcholine</th>
<th>Histamine</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of Neurons Tested</td>
<td>28</td>
<td>21</td>
<td>23</td>
<td>23</td>
</tr>
<tr>
<td>Resting Potential (mV)</td>
<td>−58 ± 0.6</td>
<td>−59 ± 0.5</td>
<td>−59 ± 0.5</td>
<td>−58 ± 0.7</td>
</tr>
<tr>
<td>Input Resistance (Mohm)</td>
<td>67 ± 5.0</td>
<td>73 ± 6.6</td>
<td>71 ± 5.3</td>
<td>72 ± 5.2</td>
</tr>
<tr>
<td>Response (mV)</td>
<td>22 ± 2.1</td>
<td>3 ± 0.7</td>
<td>5 ± 0.8</td>
<td>4 ± 0.7</td>
</tr>
<tr>
<td>Percent Responding</td>
<td>79 (80)$^a$</td>
<td>43 (36)</td>
<td>43 (33)</td>
<td>30 (24)</td>
</tr>
</tbody>
</table>

*Source:* Data from Leal-Cardoso et al. 40 Values are means ± SEM. All parameters were recorded at room temperature (21-23°C) with sharp micropipettes filled with 2 M KCl and 1 M K acetate. Recordings from isolated neurons were made between 2 to 4 hours after plating cells on glass coverslips.

$^a$ Values in parentheses are from data of Higashi et al. 25 obtained from intact ganglia.
in intact nodose ganglia\textsuperscript{21} was not significantly altered in its magnitude, duration, or distribution in acutely dissociated NG neurons.

In many, but not all, respects acutely isolated somata of VGNs are convenient models for the less accessible, small, and morphologically complex peripheral and central nerve terminals. Voltage-gated ion channels (see Chapter 3), chemoreceptors, and physical transducers that exist in vagal afferent nerve terminals are also present and functional in the membranes of VGNs. Representative examples of somal receptors include: mechanoreceptors,\textsuperscript{59} osmoreceptors,\textsuperscript{15,17} TRV1 receptors,\textsuperscript{5,43} Ca\textsuperscript{2+} sensing receptor/ion complex,\textsuperscript{61} bradykinin receptors,\textsuperscript{35,65} neurokinin receptors,\textsuperscript{32,66} histamine H\textsubscript{1} receptors,\textsuperscript{31,62} CCK-8 receptors,\textsuperscript{19} serotonin receptors,\textsuperscript{12,25} eicosanoid receptors,\textsuperscript{56,62} and purinergic receptors.\textsuperscript{27,33,34}

When considering the presence of functional autacoids and transmitter receptors in the membranes of VGNs, it is important to recognize that many agonists do not produce measurable changes, or only minimal changes, in excitability when recordings are carried out at room temperature (21\textdegree{} to 23\textdegree{}C) but do show prominent effects when recordings are made at physiological temperatures (33\textdegree{} to 37\textdegree{}C). Representative examples include: angiotensin,\textsuperscript{67} bradykinin,\textsuperscript{53} dopamine,\textsuperscript{39} and Substance P.\textsuperscript{64}

### 4.6.2 LIMITATIONS AND CONCERNS

There are a number of notable limitations and concerns when using acutely isolated vagal sensory cell bodies in general and target-identified VGNs in specific. By the very nature of isolating vagal afferent somata, these neurons have become axotomized. Axotomy can elicit a profound spectrum of morphological, physiological, and biochemical alterations in the nerve cell body, a process designated the retrograde response or the axon reaction. For example, vagotomy can dramatically decrease the excitability of NGNs, increasing action potential threshold by more than 200\%, and reducing action potential discharge by up to 80\% in response to a suprathreshold depolarizing stimuli.\textsuperscript{38} These changes require >24 hours to develop. To minimize the effects of vagotomy, isolated VGNs are studied within a few hours after dissociation. The effects of vagotomy can also be minimized by maintaining neurons at room temperature. This has the effect of extending the useful life of the isolated neurons and reducing growth of neurites that can markedly affect the control of membrane voltages. VGNs can be maintained at room temperature (22\textdegree{}C) for several days, a procedure initially developed for isolated sympathetic neurons.\textsuperscript{44}

A particularly confounding problem associated with the use of isolated VGNs is the potential redistribution of receptors upon cell isolation. There is a growing number of reports showing that some chemoreceptors, mechano- and thermo-transducers that reside in nerve terminals are not always present in membranes of cell bodies in the intact sensory ganglion, yet they become expressed and functional when the somata are isolated and held in culture. Cell bodies of primary afferent neurons do not ordinarily respond to mechanical stimulation, yet mechanosensitive currents can be readily recorded from these after a few days in culture.\textsuperscript{45,59} Similarly, the excitatory actions of cold temperature are not observed in primary sensory somata.
(trigeminal ganglion cells) but are evident at the level of the nerve terminal. The discrepancy between the presence of chemoreceptors in isolated primary afferent somata but absent in cell bodies situated in sensory ganglia has also been noted. This problem was first revealed in the classic paper of Baccaglini and Hogan in which they reported that the number of capsaicin-sensitive neurons and the fraction of neurons staining for Substance P were much larger for primary afferent neurons (dorsal root ganglion cells) in culture than was observed in intact ganglia. More recently Stebbing et al. reported that < 6% of somata in intact dorsal root ganglia express functional purinoreceptors while ~ 90% of acutely dissociated somata responded to ATP with an inward current. These observations point out that caution should be exercised when studying receptor-mediated currents or voltages in isolated cell bodies and extrapolating this information to nerve terminals without information that the same receptors exist at the nerve terminals of the vagal afferents.

It is important to recognize that potential deleterious effects can occur when using fluorescent dyes to identify neurons for physiological studies. Protracted fluorescent excitation of labeled neurons can lead to profound changes in electrophysiological parameters, depending on the nature of the tracer, its concentration, excitation wavelength, and duration of epifluorescent illumination (see Table II and Figure 5 in Christian et al., and also Figure 3 in Yoshimura et al.). For example, when using dextran-tetramethylrhodamine, Fast Blue, or Fluorogold dyes, obvious signs of action potential broadening and depolarization of the membrane potential can occur with 30 to 40 sec of illumination. By contrast, when neurons are illuminated for a minimal amount of time to identify cell bodies with a fluorescent tracer (1 to 3 sec), neurons exhibited stable passive and active membrane properties similar to those recorded in nonlabeled neurons. The long-chain carbocyanine lipophilic neuronal tracer like DiA and other analogs appear to produce less deleterious membrane effects. To minimize the possibility of fluorescent tracers altering electrophysiological parameters, it would be prudent to restrict the duration of epifluorescent illumination to a minimal time necessary to identified labeled neurons. This process can be greatly facilitated by the use of an electronic shutter positioned between the epifluorescent illuminator and the microscope in conjunction with an inexpensive CCD camera.

4.7 PHYSIOLOGICAL STUDIES OF TARGET-IDENTIFIED VAGAL AFFERENT CELL BODIES

4.7.1 STUDIES OF BARORECEPTORS

The nerve terminals of baroreceptor neurons respond to increases in arterial blood pressure with a barrage of action potential activity triggering CNS-mediated reflexes vital to maintaining cardiovascular regulation. Nodose ganglion contain the cell bodies of aortic baroreceptors that contain transcripts for DEG/ENaC subunits, putative mechanosensitive ion channels. By labeling baroreceptor endings in the aortic arch with DiI it is possible to isolate target-identified nodose ganglion
baroreceptor cell bodies for physiological and pharmacological studies with patch-clamp techniques. Labeled baroreceptor cell bodies are significantly more sensitive to hypoosmotic stretch than nonlabeled nodose cell bodies. Hypoosmotic stretch produces an inward current with an increased membrane conductance and a reversal potential value consistent with the activation of a nonselective cation channel. These electrophysiological observations suggest that these mechanosensitive ion channels may be involved in mechanoelectric transduction in baroreceptor nerve terminals.

Baroreceptor neurons possess a variety of low- and high-threshold calcium channels. Low-voltage, T-type calcium channels likely contribute to activation of baroreceptors and support repetitive action potential activity in these neurons. High-voltage calcium channels are known to mediate many intracellular calcium-dependent processes in NGNs; in particular, they trigger activation of calcium-dependent calcium-release pools of intracellular calcium. High-voltage calcium channels in target-identified nodose baroreceptor neurons are modulated by µ-opioid receptor activation perhaps contributing to the opioid-mediated attenuation of baroreflex activity.

### 4.7.2 Airway Vagal Afferent C-Fibers Comprise Two Distinct Phenotypes

Using target-identified VGNs, Undem and his colleagues applied immunocytochemistry, extracellular, and intracellular recording techniques to reveal that vagal C-fiber innervation of the intrapulmonary system is different from the extrapulmonary airways and that these two C-fiber populations represent distinct phenotypes. Retrograde tracing from the airways with fast blue showed that C-fibers innervating the pulmonary system are derived from vagal cell bodies situated in NG and JG. The nerve terminals of vagal afferents in the lungs can be activated by capsaicin and bradykinin but only the nodose C-fibers nerve terminals responded with action potential discharges following application of ATP or the P2X selective receptor agonist α,β-methylene ATP. Patch-clamp recordings from retrogradely labeled airway cell bodies revealed capsaicin responsive C-type cell bodies existed in both JG and NG. Lung-specific C-fiber nodose cell bodies expressed functional P2X receptors whereas lung-specific jugular C-fiber somata were categorically unresponsive to purinergic agonists. The nodose and jugular C-fibers projecting to the bronchopulmonary system are not only different with respect to their chemosensitiveness but they also differ in their neuropeptide content. Most Substance P containing vagal C-fiber somata innervating the airway reside in the JG. These data indicate that two classes of vagal C-fibers can be distinguished based upon their distribution within the lungs, their neuropeptide content, and their responsiveness to nociceptive substances. They also indicate that the chemosensitive properties of sensory airway nerve endings depend on the ganglionic origin of the supplying nerve fiber. These results illustrate how target-identified VGNs can be used to answer fundamental questions about the function of vagal sensory neurons.
Electrophysiological Properties and Chemical Responsiveness of Parasympathetic and Sympathetic Primary Sensory Neurons Innervating the Airways

Visceral organs, including airway, are dually innervated by sympathetic and parasympathetic neurons. Parasympathetic primary sensory neurons (vagal afferents) run through vagus nerves and have their cell bodies situated in NG and JG. The cell bodies of sympathetic airway sensory neurons (somatic afferents) reside in upper thoracic (T1-4) DRG. By contrast to the wealth of information about the physiology of parasympathetic airway sensory afferents, there is only limited amount of information about the function and cellular characteristics of sympathetic airway afferents. Oh et al.\textsuperscript{51} utilized airway-identified sensory cell bodies isolated from NG, JG, and DRG to compare the electrophysiological properties and chemosensitivity of sympathetic and parasympathetic airway afferent neurons.

Parasympathetic and sympathetic airway sensory neurons were comprised of both A- and C-type neurons as determined by neuronal size, action potential waveforms, and capsaicin sensitivity. Both parasympathetic and sympathetic afferent cell bodies fired tonically following a depolarizing current step regardless of ganglia and neuronal type. Thus, all airway primary sensory neurons could be classified as nonaccommodating or tonic neurons. Exogenously applied serotonin (5-HT, 10 µM) increased the excitability of airway sensory neurons from all three ganglia. However, the cellular mechanism underlying the actions of 5-HT differed depending upon the location of the primary afferents. In the majority of NG of neurons (78%) 5-HT evoked an inward current (~2.0 nA) that was associated with an increased membrane conductance. By contrast, 5-HT did not elicit measurable membrane currents in most of the airway sensory JG (72%) or DRG (68%) neurons. In these cells, 5-HT lowered the membrane potential for spike initiation (~4 mV) and reduced the current required to reach spike threshold.

ATP induced long-lasting (>10 sec) inward currents in 100% of airway-identified NG cell body tested. By contrast, ATP evoked relatively short duration responses (<1 sec) in 27% and 32% of airway JG and DRG cell bodies, respectively. These data show that airway primary afferent neurons existing in different sensory ganglia possess distinct cellular mechanisms underlying autacoid-induced changes in membrane excitability. These differences may reflect the existence of distinct receptors or second messenger pathways. Such differences may provide a basis to develop therapeutics that may be selective for different subpopulations of airway sensory neurons.

Bradykinin Excites Airway Sensory Neurons by Promoting a Calcium-Dependent Chloride Current

While studying the excitability changes produced by inflammatory mediators on isolated airway-identified sensory neurons, Oh and Weinreich\textsuperscript{53} characterized a novel cellular mechanism underlying the excitatory action of bradykinin (BK), a nonapeptide inflammatory mediator. BK can alter the function of distinct populations of ion channels to exert increases in neuronal excitability. It can block a slowly activating
potassium current (M-current),\textsuperscript{26} obtund a calcium-dependent potassium current (I\textsubscript{AHP} current),\textsuperscript{63,65} evoke an inward sodium current,\textsuperscript{8} and activate nonselective cation channels via modification of TRPV1 receptors.\textsuperscript{11} In airway-identified VGNs, BK causes a membrane depolarization, evokes action potential activity, and induces an inward ionic current (I\textsubscript{BK}). Measurements of reversal potential values in conjunction with changes in extracellular and intracellular ion composition revealed that chloride ions were the major charge carriers for I\textsubscript{BK}. Reducing the concentration of extracellular calcium had no effect on I\textsubscript{BK} but buffering intracellular calcium with BAPTA or bath application of niflumic acid, a calcium-activated chloride channel blocker, inhibited I\textsubscript{BK}. These results imply that the BK-evoked a chloride current that was dependent upon a rise in intracellular calcium concentration. These observations demonstrated, for the first time, that BK can excite primary afferent neurons by modifying an anion channel.

4.8 PATHOPHYSIOLOGICAL STUDIES OF TARGET-IDENTIFIED VAGAL AFFERENT CELL BODIES

Electrophysiological techniques have been widely used to examine how injury (physical, chemical, or immunological) produces changes in primary afferent neurons. Many injury-induced changes occur at or near the site of injury, often in the peripheral nerve terminals. The morphological complexity, small size, and inaccessibility of nerve endings does not support the use of intracellular or patch-clamp recording techniques for direct cellular studies of injury-induced changes in these neurons. However, many injury-induced changes to primary afferent neurons are also reflected by alterations in electrophysiological properties of their cell bodies. It is often observed that voltage-sensitive currents are changed in somal membranes of neurons whose axons projects to a tissue that is inflamed. For example, chronic bladder inflammation sensitizes mechanosensitive afferents and increases the excitability of isolated bladder-identified afferent cell body, in part by suppressing an A-type, voltage-sensitive, potassium current.\textsuperscript{68} The success of these experiments critically depended upon the ability to re-identify primary afferent cell bodies (DRG neurons) innervating the bladder. In this section, several examples are provided illustrating how studies of target-identified vagal afferent cell bodies have been used to gain insights into cellular basis of injury-provoked changes in excitation or sensitization.

4.8.1 GASTRIC INFLAMMATION

Gebhart and associates\textsuperscript{6,16} used target-identified vagal cell bodies to study the mechanisms associated with excitability changes in primary afferents triggered by gastric inflammation. Using a rat model of gastric hyperalgesia, produced by multiple injections of acetic acid into the stomach wall, these investigators examined voltage-sensitive currents in isolated NG and DRG gastric-identified cell bodies. Patch-clamp recordings of retrogradely dye-labeled cells revealed that gastric inflammation altered both sodium and potassium currents. Sodium currents recovered significantly more rapidly from inactivation in neurons obtained from animals in the ulcer group compared with controls due, in part, to an enhanced contribution of the TTX-resistant
sodium current to the peak sodium current. The recovery kinetics of the TTX-sensitive sodium current was faster in the ulcer group. Gastric inflammation also reduced the density of A-type potassium current, shifted its steady-state inactivation to a more hyperpolarized membrane potential, and accelerated the inactivation kinetics. These changes in ionic currents are likely to contribute to the enhanced excitability of gastric afferents observed during gastric inflammation and perhaps during dyspeptic symptoms. An interesting observation from this work was that inflammation increased TTX-resistant current in both vagal and spinal afferents. Changes in TTX-resistant currents are often associated with the development and maintenance of pain, hyperalgesia, and allodynia, sensations not typically associated with parasympathetic afferents.

4.8.2 Airway Inflammation

Application of retrograde tracers to the airways of guinea pigs revealed that the cell bodies of the primary afferents innervating the airway are housed in two separate ganglia, the JG and the NG.54 Though many of the cell bodies in both these ganglia contain tachykinins, such as Substance P and Neurokinin A, under normal conditions all the vagal tachykininergic fibers innervating the guinea pig airways are derived nearly exclusively from cell bodies in the JG.54 Viral infection10 or allergen-induced infection20,49 of the airways causes an induction of tachykinin expression in NG cell bodies that innervate the airways. Interestingly, this phenotypic switch in the expression of tachykinins in NG neurons occurred in large, fast-conducting low-threshold mechanically sensitive Aδ-type neurons.49 Electrophysiological studies of these airway-labeled Aδ-type somata revealed that they were insensitive to nociceptive stimuli such as capsaicin and bradykinin. Thus, it is possible that during airway inflammation non-noxious activation of low-threshold mechanosensory nerve terminals can cause tachykinin release that can contribute to exaggerated reflexes that accompany inflammatory diseases.

4.9 Conclusions and Future Directions

It has been just over a decade since the first report was published describing the use of acutely isolated, target-identified, primary vagal afferent cell bodies for electrophysiological studies. It is now clear that this preparation is an invaluable model system for answering many fundamental physiological, pharmacological, and pathological questions about the vagus nerve. The ability to re-identify subpopulations of vagal sensory somata based upon the organs they innervate allows detailed cellular and molecular measurements to be made before and subsequent to brief and chronic manipulations of an end organ.

Most neuronal populations are heterogeneous with respect to the expression of plasma membrane proteins; primary vagal afferent neurons are no exception. To convert specific visceral modalities into electrical signals and pass these signals to the CNS, vagal sensory neurons have evolved a specific set of receptor proteins that are specialized to detect and transduce specific sensory stimuli. These vagal receptors include: mechanoreceptors (rapidly and slow adapting) chemoreceptors (alkali-and
Advances in Vagal Afferent Neurobiology

acid sensitive receptors; amino acid receptors, glucoreceptors, autacoid receptors, etc.); thermoreceptors (cold and heat); osmoreceptors and nociceptors. Some of these transduction proteins are being identified and characterized. For example, the serpentine protein transient receptor potential V1 (TRPV1) is part of a receptor complex that responds to noxious stimuli, protons, heat, and endogenous ligands (fatty acids and eicosanoid derivatives); DEG/ENaC family of proteins (DEG-1, MEC-4, MEC-10, UNC-105, and UNC-8) may serve as mechanosensory channel proteins. In the future, it should be possible to visually identify subpopulations of vagal afferent neurons based upon vital markers that can recognize these specialized receptor proteins or their distinct accessory proteins. Thus, functional subsets of vagal afferents innervating a given organ may be studied and manipulated for experimental and therapeutic purposes.

ACKNOWLEDGMENTS

The author is grateful to Mr. Tony Gover and Ms. Thais Moreira for helpful comments on this chapter. Our research program on the vagus nerve has been enriched by a collaboration of almost 20 years with Dr. Brad Undem, a colleague and friend. Financial support for our research program and for preparation of the manuscript was provided by Grant NS-22069 from the NINDS of the National Institutes of Health.

REFERENCES


Part III

Vagal Sensory Nerve Terminals
Advances in Neural Tracing of Vagal Afferent Nerves and Terminals

Terry L. Powley and Robert J. Phillips

CONTENTS

5.1 Overview: Neural Tracers Provide Assignable Phenotypes .................................................. 124
5.2 Tracers and Tracer Protocols for Vagal Afferents .............................................................. 125
  5.2.1 Wheatgerm Agglutinin-Horseradish Peroxidase (WGA-HRP) ........................................ 125
  5.2.1.1 WGA-HRP Protocol ......................................................................................... 126
  5.2.1.2 Notes on WGA-HRP Protocols ....................................................................... 128
  5.2.2 Carboxyamine Dyes .................................................................................................. 128
  5.2.2.1 DiI Protocol ....................................................................................................... 129
  5.2.2.2 Notes on DiI Protocols ....................................................................................... 129
  5.2.3 Dextran Amines ........................................................................................................ 130
  5.2.3.1 Dextran, Tetramethylrhodamine (TMR or “Fluoro-Ruby”) Protocol .................. 130
  5.2.3.2 Notes on TMR Protocols .................................................................................. 131
  5.2.4 Biotin Conjugated Dextran Amines ............................................................................ 131
  5.2.4.1 Dextran, Tetramethylrhodamine, and Biotin (TMR-B or 10K “mini-ruby” and 3K “micro-ruby”) Protocol ................................................................. 131
  5.2.4.2 Notes on TMR-B Protocols ............................................................................... 134
5.3 Controls and Validations ..................................................................................................... 134
5.4 Selecting Tracers for Particular Applications ...................................................................... 138
  5.4.1 Issues of Field of View, Magnification, and Population Surveys ................................. 139
  5.4.2 Resolution or Definition of Endings ......................................................................... 140
  5.4.3 Compatibility with Immunohistochemistry and Counterstaining ............................. 140
  5.4.4 Double or Multiple Tracer Injections .................................................................... 141
  5.4.5 Permanence of the Labeling ...................................................................................... 141
  5.4.6 Transport Time ........................................................................................................ 142
5.5 Summary ............................................................................................................................. 142
Acknowledgments ....................................................................................................................... 142
References .................................................................................................................................. 143
5.1 OVERVIEW: NEURAL TRACERS PROVIDE ASSIGNABLE PHENOTYPES

Experiments on vagal afferents are often hampered by a lack of information about the precise distributions, as well as the specialized terminal architectures, of the peripheral processes of the neurons. As discussed elsewhere, full analysis of an afferent system and its transduction mechanisms is impractical under such conditions. When the precise locations of the peripheral terminals are unknown, when the structural characteristics of the terminals are uninvestigated, and when any juxtapositions of the neurites with accessory cells or tissues are unknown, physiological investigations are significantly curtailed. This principle is clear in the abstract: Where would visual or auditory neuroscience be if such information about the retina and the cochlea were missing? Nonetheless, experimental approaches to the vagus frequently proceed under such handicaps.

Traditionally, there were few, if any, practical means of recognizing and analyzing the peripheral processes of vagal afferents. The viscera that the vagus innervates are commonly innervated by other visceral afferents, by extrinsic — including vagal — efferents, and by intrinsic neural networks. The complexity and heterogeneity of such innervation patterns means that vagal afferents cannot be unequivocally recognized unless they can be discriminated by some particular feature(s) from other neurites in the target tissues. Though vagal neurons display a variety of consistent chemical phenotypes (see other chapters in this volume), no neurochemical marker is specific for only vagal afferents and is inclusively characteristic of all vagal afferents. Thus, investigators traditionally have not been able to readily identify the terminals of all vagal afferents in a target organ or tissue and distinguish them from other circuitry by any unique neurochemical signature.

Recent advances and applications of neural tracing techniques, however, have made it practical to label selectively vagal afferents so that they can be unequivocally recognized in any target tissue. The tracers delineate neuronal processes with sufficient fidelity, in many cases, that they also provide significant structural information about these different vagal afferent projections. These tracers are exogenous molecules that are internalized and transported by neurons. In effect, neural tracers can be used to assign a specifiable and unique phenotype to vagal neurons. Once labeled, the peripheral processes of the neurons can be readily identified in situ and then mapped, inventoried, and analyzed structurally.

The last two decades have seen the development of a number of tracers and the proliferation of processing protocols for these labels (for reviews see, for example, References 18, 36, and 42). This armamentarium has become so extensive, it can be unclear which compound might be most promising for a particular application. Choosing processing protocols that are especially well suited to vagal afferents, as well as to a given tracer compound, also becomes an issue. In the present survey, we describe four different tracer strategies that we have found to be particularly suitable and powerful for labeling the peripheral processes and terminals of nodose neurons. Though this set is by no means complete, it offers a place to start if one does not want to screen through the entire list of candidate tracers. The survey also discusses some of the distinctive strengths and limitations of the four tracers, thus
underscoring issues an investigator might want to consider in choosing among the four for a given application or for evaluating additional tracer candidates. This overview also considers some of the control observations that should be used to validate a tracer protocol in a particular situation.

5.2 TRACERS AND TRACER PROTOCOLS FOR VAGAL AFFERENTS

We have found the four tracers described here to be exceptionally useful for analyses of vagal afferents in the gastrointestinal tract. They have also been used individually or in different combinations by different labs to describe vagal afferent projections to the heart, lungs, liver, and abdominal paraganglia as well. These different compounds meet a set of critical requirements for successful in vivo labeling of the peripheral neurites and terminals of the vagus. For example, the compounds are all readily incorporated by vagal neurons, they all (though not all of their solvents — cf. the carbocyanine dyes) appear to be tissue nonreactive, and they all are transported over at least the substantial distances from the nodose to the distal gastrointestinal tract of the rat (~15 cm).

For illustration and to provide some starting points for those interested in using the tracers, we also describe protocols for the four tracers that we have found effective in work on the rat gastrointestinal tract. The parameters reported would need, of course, to be adjusted for other species and other organ systems. In the case of the dextran amines, we also include examples of variations on the protocol in which counterstaining and immunohistochemistry are added to the basic procedures.

We should repeat, however, that each of the four tracers can be employed with a very large number of different protocols that might capitalize on the tracer's strengths, while optimizing them for other applications. There are far too many protocol variants to discuss in a short review, but investigators wishing to adapt one of the tracers we review (or others, for that matter) would want to consider utilizing some of the myriad different protocols that can be used with the labels. A partial list of such variants would include the use of alternate chromagens in processing, the different intensification and amplification methods that have been developed, photostabilization or photoconversion strategies to stabilize the fluorescent labels, and immunohistochemistry with antibodies directed against the less stable tracers.

5.2.1 WHEATGERM AGGLUTININ-HORSERADISH PEROXIDASE (WGA-HRP)

The complex of wheatgerm agglutinin and horseradish peroxidase has been used extensively for neural tracing and applied to the vagus. WGA is a lectin that appears to be universally bound to membrane glycoproteins on vagal afferents (as well as many other cell types). When the lectin is conjugated to the enzyme HRP, neurons actively bind, incorporate, and then transport this complex throughout their neurites. Subsequent processing with a chromogen (TMB, DAB, etc.) developed by the HRP then labels the neuron. The glycoprotein binding sites are found particularly on somata, as opposed to neurites, thus nodose injections label vagal
afferents without concomitantly labeling en passage the efferent axons coursing through the nodose ganglion. Because the WGA conjugate capitalizes on this active binding mechanism to facilitate incorporation into cells, WGA-HRP injection provides much stronger labeling than administration of free or unconjugated HRP.

Our research group has used a WGA-HRP strategy extensively to inclusively label vagal afferents to an organ in order to appreciate the complete innervation pattern of vagal afferents. Figure 5.1, for example, is a low-power view of a whole mount of the proximal duodenum of the rat that illustrates the pattern of afferent vagal axons in the smooth muscle wall of the intestine. The course of the fibers in the myenteric plexus connectives can be clearly traced. Figure 5.2 is a somewhat higher-power view that illustrates vagal afferent projection patterns in the wall of the stomach. At this magnification, one can see individual bundles of axons in connectives and even the vagal terminal apparatuses in smooth muscle (i.e., intraganglionic laminar endings and intramuscular arrays). Using such material, it has been possible to inventory and map the different types of vagal afferents to GI tract smooth muscle.

5.2.1.1 WGA-HRP Protocol

- The nodose ganglion is exposed and injected with WGA-HRP (3 µl; 4%; Vector Laboratories, Burlingame, CA)
- At 72 hrs post injection, the animal is overdosed with a lethal injection of sodium pentobarbital and injected in the left ventricle of the heart with 0.25 ml Heparin (1,000 units/ml; Elkins-Sinn, Inc., Cherry Hill, NJ) to prevent coagulation and 0.25 ml propranolol (Ayerst Laboratories, Inc., Philadelphia, PA) to produce vasodilation
- The animal is perfused transcardially with 200 ml 0.9% saline at 40°C followed by 500 ml 3% paraformaldehyde and 0.4% glutaraldehyde in 0.1 M sodium phosphate buffer saline (PBS), pH 7.4, at 4°C
- Whole mounts of the circular and longitudinal smooth muscle, containing the myenteric plexus, are prepared by removing the mucosal and submucosal layers
- Tissue is then processed with 3,3,5,5-tetramethylbenzidine (TMB; Sigma, St. Louis, MO) according to the protocol of Mesulam
  - Buffer at pH 3.3: Add 200 ml of 1.0 M sodium acetate to 200 ml distilled water; add 190 ml of 1.0 M HCL; make up the volume to 1 L with distilled water. Titrate with concentrated acetic acid or sodium hydroxide in order to bring the final pH to 3.3
  - Solution A: Mix 92.5 ml of distilled water, 5 ml of pH 3.3 buffer and 50 mg of sodium nitroferricyanide (Sigma)
  - Solution B: Add 5 mg of TMB to 2.5 ml absolute ethanol, which has been heated to 37°C to 40°C to dissolve the TMB
  - Mix solutions A and B and incubate the tissue for 20 min
  - Add 2.0 ml 0.3% H₂O₂ per 50 ml incubation solution and let react for 15 min
  - 6 × 5 min rinses in a post-reaction buffer consisting of 50 ml of the sodium acetate buffer, pH 3.3, in 950 ml distilled water
Mount the tissue on gelatin coated slides and air dry overnight

2 × 30 min in xylene and coverslip using DPX (Aldrich, Milwaukee, WI)

FIGURE 5.1 A photomontage of a duodenal whole mount from a rat that had received a left nodose injection of wheatgerm agglutinin-horseradish peroxidase (WGA-HRP). The intestinal segment was opened with a longitudinal cut along the mesenteric attachment, and large bundles of axons can be seen entering from the mesenteric attachment (left and right sides of the whole mount) and traveling both parallel to the mesenteric border and radially toward the antimesentery (center of the montage). The completeness of the label and the ability to view the label at low power magnification makes WGA-HRP a useful tracer for making both qualitative and quantitative descriptions of the vagal afferent innervation of the GI tract smooth muscle. Scale bar = 2 mm.
5.2.1.2 Notes on WGA-HRP Protocols

Such injections tend to yield only afferent labeling and to label effectively all the afferents. Larger injections or larger pipettes (we use pulled glass pipettes with IDs of 25 µm and ODs of \(\sim 35−40 \mu m\)) that produce more mechanical disruption of the ganglion, however, tend to produce some efferent labeling \textit{en passage} as well as the afferent labeling\(^{17}\) and should generally be adopted cautiously.

To map the vagal afferent terminals in gastrointestinal smooth muscle, we typically employ a survival time of 72 hours in the rat or 24 hours in the mouse. By adjusting the timing slightly for transport, it is possible to maximize the labeling for a particular caliber of axon or a particular site within the GI tract, but transport times are similar enough and WGA-HRP accumulated in the terminals is stable enough that the survival times mentioned are typically satisfactory.

Much of the success of the WGA-HRP technique seems to be in the preparation of the target organ tissues. In the case of the vagal afferent innervation of the GI smooth muscle, we have found it particularly effective to work with whole mounts, to separate the tissue into layers to improve visibility and focus, and to mount the tissue so that it can be viewed from a perspective perpendicular to the plane(s) in which the afferent terminals lie.

5.2.2 Carbocyanine Dyes

“DiI” (1,1-diocadecyl-3,3,3,3-tetramethylindocarbocyanine) and the other compounds (e.g., “DiA” and “DiO”) of the carbocyanine tracer family have been used extensively to label neuronal neurites or projections in fixed tissues.\(^{16,37}\) Since the strong lipophilia and hydrophobia of the carbocyanines cause them to intercalate in

\[\text{FIGURE 5.2} \quad \text{A higher power view than that shown in Figure 5.1 of WGA-HRP labeled vagal afferents in the smooth muscle wall of the rat stomach. At this magnification, one can see, in addition to bundles, individual axons in the connectives and both types of vagal afferent terminals located in the smooth muscle wall [i.e., intraganglionic laminar endings (IGLEs) and intramuscular arrays (IMAs)]; see the legend for Figure 5.3 for a description of both endings. Scale bar = 320 µm.}\]
membrane and then to diffuse passively within the membrane, long incubations (e.g., weeks) of fixed tissue can eventually label neurons in their entirety (at least over a few millimeters). The technique has also been widely used as a vital dye for labeling developing neurons and cells in culture and then following their histories.

Our research group and others have, however, also used the carbocyanine dyes as *in vivo* anterograde and retrograde tracer to label over long distances (centimeters — e.g., nodose to the intestines). When a solution of DiI or other carbocyanine dye is injected into neuronal tissue, the dye dissolves in the membrane at the injection site. It appears that this labeled membrane is then slowly incorporated and recycled through endocytotic processes and intracellular vesicular membrane trafficking. Eventually, through such membrane exchange, the entire cellular membranes tend to accumulate the dye.

Utilizing this strategy, it is possible to label strongly a large number of neurons. Because endocytosis tends to occur at cell bodies (and axon terminals), nodose ganglion injections of DiI will selectively label vagal afferents and not label vagal efferents *en passage* (any label diffusing into the axonal membrane of vagal efferents traversing the nodose can spread locally slowly along that membrane by the passive diffusion process, but does not reach distal tissues over the distances and transport times employed).

### 5.2.2.1 DiI Protocol

- The nodose ganglion is exposed and injected with DiI (1 µl; 4% 1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine in methanol; Molecular Probes, Eugene, OR)
- At 3 to 4 weeks post injection, the animal is overdosed with a lethal injection of sodium pentobarbital
- The animal is perfused transcardially with 200 ml 0.9% saline at 40°C followed by 500 ml 10% formalin in 0.1 M PBS, pH 7.4, at 4°C
- Whole mounts of the tissue specimen are prepared
- Tissue is dehydrated through a graded series (70, 90, 100, and 100%) of glycerins
- The tissue is mounted and coverslipped using 100% glycerin and n-propyl gallate (5%; Sigma) to prevent fading

### 5.2.2.2 Notes on DiI Protocols

Though the DiI protocol is extremely simple, the solubility of the dye in solvents routinely used in histology makes the processing somewhat demanding. For example, all the carbocyanine dyes are so soluble in detergents that even trace amounts of the agents on glassware or slides will dissolve the dye from the tissue. Ethanol and most other alcohols will also degrade the label. Xylenes will also solubilize the label.

Another factor that can complicate work with the carbocyanines is that the compounds tend to continue to diffuse in a tissue specimen once it is fixed. DiI was originally used for its ability to diffuse in such fixed tissue, and if a specimen is not sufficiently well fixed (and particularly if the cell membranes have been damaged
and/or juxtaposed by mechanical stresses during processing), the dye will continue to migrate within the tissue. Whereas the dextran amines, in contrast, can be obtained with lysine fixable residues that anchor the label within the fixed tissue, the carbocyanine dyes are not always well stabilized in tissue samples.

5.2.3 DEXTRAN AMINES

Dextran amines conjugated with fluorochromes (Fluoro-ruby; Fluoro-emerald, Micro-ruby, Mini-ruby and other variants — see the Molecular Probes catalog) have also been extensively used as cell markers. They are thought to be incorporated by intact neurons through an active endocytotic process. Since the uptake of the dextran amines, like that of the lectin WGA-HRP, does involve endocytosis, which occurs at somata and terminals, injections into the nodose typically do not label the efferent axons of passage. Once internalized, the dextran complex is distributed throughout the neuron in both retrograde and anterograde directions. As typically used in neuronal tracing, the dextran amine contains lysine residues that are cross-linked to cellular proteins by standard fixation protocols, thus leaving a stable fluorescent marker in fixed tissues. These lysine-fixable dextran amines remain stable through a variety of subsequent processing and counterstaining strategies. Further facilitating neuronal tracing, the dextran amines can be obtained in a variety of different molecular weights (commonly 3K and 10K), which influence the rate at which they are incorporated and transported by living cells as well as their relative storage stabilities, with a range of different fluorophores conjugated with them.

5.2.3.1 Dextran, Tetramethylrhodamine (TMR or “Fluoro-Ruby”) Protocol

- The nodose ganglion is exposed and injected with TMR (3 µl; 7 to 15%; in distilled water; Molecular Probes)
- At 12 to 15 d post injection, the animal is overdosed with a lethal injection of sodium pentobarbital
- The animal is perfused transcardially with 200 ml 0.9% saline at 40°C followed by 500 ml 4% paraformaldehyde in 0.1 M PBS, pH 7.4, at 4°C
- Whole mounts of the tissue specimen are prepared
- Mount the tissue on gelatin coated slides and air dry overnight
- Run the tissue through an ascending series of alcohol (70, 90, 100, and 100%) for 2 min each followed by 2 × 5 min in xylene
- Coverslip using Cytoseal XYL (Richard-Allan Scientific, Kalamazoo, MI)

5.2.3.1.1 TMR with the Putative Pan Neuronal Marker Fluoro-Gold

- 5 d post TMR injection, the animal receives an i.p. injection of 1 mg/ml saline of Fluoro-Gold (Fluorochrome, Inc., Englewood, CO) to label all of the enteric neurons in the gut
5.2.3.1.2 TMR and Immunocytochemistry

- After perfusion the tissue is placed for 1 to 12 h in a blocking buffer (2% bovine serum albumin, 0.5% Triton X-100, 10% normal goat serum in 0.1 M PBS)
- 24 h in the primary antibody of choice (diluted with 2% bovine serum albumin, 0.3% Triton X-100, 2% normal goat serum in 0.1 M PBS)
- 3 × 10 min rinses in the same diluent as the primary 2 h in goat anti-rabbit FITC or goat anti-mouse FITC (1:100; Jackson ImmunoResearch, Laboratories, Inc., West Grove, PA) diluted with the same diluent
- 4 × 5 min PBS rinses
- Mount the tissue on slides and coverslip using VectaShield mounting medium (Vector Laboratories)

5.2.3.2 Notes on TMR Protocols

The fluorescent dextran amines yield excellent, high-definition labeling of vagal afferent axons and terminals (see, for example, Figure 5.3). Though, compared with some other fluorochromes, they are relatively stable when coverslipped with a mounting medium containing an antifade agent, the dextran amines will fade with protracted illumination. The tracer is also subject to the usual limitations of fluorescent markers: There is some degradation of the tracer fluorescence with long term storage, and autofluorescence of the tissue specimen tends to increase with storage. Other strengths and weaknesses of the fluorescent dextran amines are mentioned below in Section 5.4.2

5.2.4 Biotin Conjugated Dextran Amines

The dextran amines are also available with biotin conjugated to the appropriate complexes in order that investigators can capitalize simultaneously on the incorporation efficiency of the dextrans and the exceptional affinity of avidin and biotin for each other to yield a strong and permanent label. They too are widely used in neural tracing applications.9,18,31,35,41,42 The neuronal binding, incorporation, and transport characteristics of biotinylated dextran amines appear to be identical to those of the corresponding dextran amine without the biotin conjugate. Once the material has been transported to the target sites, however, the post-perfusion processing protocol involves the formation of the avidin-biotin complex and subsequent processing with a chromogen that will yield a light-stable permanent marker.

5.2.4.1 Dextran, Tetramethylrhodamine, and Biotin (TMR-B or 10K “mini-ruby” and 3K “micro-ruby”) Protocol

- The nodose ganglion is exposed and injected with TMR-B (3 µl; 7 to 15%; in distilled water; Molecular Probes, Eugene, OR)
- At 12 to 15 days post injection, the animal is overdosed with a lethal injection of sodium pentobarbital
The rat is perfused transcardially with 200 ml 0.01 M PBS at 40°C followed by 500 ml 4% paraformaldehyde in 0.1 M PBS, pH 7.4, at 4°C. Whole mounts of the tissue specimen are prepared with 3 × 5 min PBS rinses, 15 min endogenous peroxidase block (methanol:3% H₂O₂; 4:1), 3 × 5 min PBS rinses, Overnight in PBS with 0.5% Triton X-100 (PBST), 6 × 5 min PBS rinses, 60 min in ABC (prepared as per the directions provided with the VECTASTAIN ELITE ABC kit; Vector Laboratories).

**FIGURE 5.3** Labeling of the vagal afferent terminals in the smooth muscle wall of the rat gastrointestinal tract using either 1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine (DiI; panel A) or dextran-tetramethylrhodamine (TMR; panels B and C). Both tracers allow for high-power viewing of the structure of individual endings with either a fluorescent or confocal microscope; however, both are limited by the constraints of fluorescent microscopy (e.g., fading). A, B: The structures of IGLEs consist of vagal axons entering a myenteric ganglion and terminating as highly arborized laminar endings upon neurons (unlabeled) within the ganglion. C: IMAs originate from a parent axon (see upper right-hand corner) that typically branches several times before terminating within a smooth muscle layer. Upon entering the sheets of muscle, these individual terminals run for several millimeters, creating a distinct pattern of parallel elements. An out-of-focus element of an IMA also appears in the background of panel B. Scale bar = 100 µm for A, 100 µm for B, and 200 µm for C.
• 6 × 5 min PBS rinses
• 10 min DAB reaction
• 6 × 5 min distilled water rinses
• Mounted on slides and air dried overnight
• 4 × 2 min in ascending series of alcohols
• 2 × 8 min xylene
• Coverslip using Cytoseal

5.2.4.1.1 TMR-B with Cuprolinic Blue Counterstaining of Neurons

• Prior to the overnight soak in PBST, 3 × 5 min rinses in distilled water
• 4 h in a humidified slide warmer (38°C) with 0.5% Cuprolinic Blue (quinolinic phthalocyanine; Polysciences, Inc., Warrington, PA) in 0.05 M sodium acetate buffer containing 1.0 M MgCl₂, pH 4.9. Protocol adapted from Holst and Powley 14
• 3 × 5 min distilled water rinses
• 1 to 2 min differentiation in 0.05 M sodium acetate buffer containing 1.0 M MgCl₂, pH 4.9
• 3 × 5 min distilled water rinses
• 3 × 5 min PBS rinses

5.2.4.1.2 TMR-B with NADPHd Histochemistry or Counterstaining of Neurons

• Prior to the endogenous peroxidase block, 3 × 5 min rinses in 0.1 M Tris-HCl; pH 7.9
• 30 to 60 min soak in 0.1 M Tris-HCl (pH 7.6) containing 1.0 mg/ml -NADPH (Sigma), 0.33 mg/ml nitroblue tetrazolium (Sigma), and 0.5% Triton X-100 at 37°C. Protocol adapted from Scherer-Singer et al. 33
• 3 × 5 min 0.1 M Tris-HCl (pH 7.9) rinses

Note: Use VectaMount (Vector Laboratories) instead of Cytoseal to coverslip TMR-B/NADPHd labeled tissue

5.2.4.1.3 TMR-B and Immunocytochemistry

• Following the overnight soak in PBST, 1 h in a blocking buffer (2% bovine serum albumin, 0.5% Triton X-100, 10% normal goat serum in 0.1 M PBS)
• 24 h in the primary of choice (diluted with 2% bovine serum albumin, 0.3% Triton X-100, 2% normal goat serum in 0.1 M PBS)
• 3 × 10 min PBS rinses
• 60 min in ABC solution
• 6 × 5 min PBS rinses
10 min DAB reaction
3 × 5 min distilled water rinses
3 × 5 min rinses in the primary diluent
60 min in the appropriate species specific biotinylated secondary raised in goat (Vector Laboratories or Jackson ImmunoResearch, Laboratories, Inc.) diluted with the same diluent used for the primary
6 × 5 min PBS rinses
60 min in ABC solution
6 × 5 min PBS rinses
5 min reaction in either Vector NovaRED or Vector VIP substrate kits (Vector Laboratories)
3 × 5 min distilled water rinses
Mounted on slides and air dried overnight
4 × 2 min in ascending series of alcohols
2 × 6 min in xylene
Coverslip using VectaMount

5.2.4.2 Notes on TMR-B Protocols

Biotinylated dextran amines generally provide excellent, high-definition labels for vagal afferents. One feature that can limit the use of this family of tracers is the requirement that the ABC processing or avidin binding step requires that the tissue specimen be sufficiently thin and permeable to allow ready penetration. (By comparison, the fluorescent dextran amines and carbocyanine dyes have no such constraints: the signal or fluorophore is part of labeling molecule and thus no penetration issue arises.) Combining TMR-B processing with immunohistochemical protocols similarly requires good tissue penetration by the antibodies in order to achieve staining of additional tissue elements. As an illustration, we have found that it is frequently necessary to separate the circular and longitudinal muscle layers of the GI smooth muscle wall in order to get effective antibody penetration in our immunohistochemical protocols. Since the organs the vagus innervates are large and complex, vagal axons regularly project over circuitous paths (e.g., Figure 5.1 and Figure 5.2), vagal afferent terminals are structurally and spatially complex (e.g., Figure 5.3, Figure 5.4, and Figure 5.5), and success in analyzing the geometry of such endings can often hinge on the use of large blocks of tissue or whole mounts in particular orientations, strategies for achieving adequate penetration are often key to the successful use of the biotin-conjugated dextrans. Other strengths and limitations of this family of tracers are considered below in Section 5.4.

5.3 CONTROLS AND VALIDATIONS

Applications of neural tracers can lead to a variety of false positive and false negative outcomes (see Fox and Powley\(^\text{10}\), for a detailed discussion). Many of
FIGURE 5.4 (A color version of this figure follows page 236.) Biotin-conjugated dextran-tetramethylrhodamine (TMR-B) is compatible with neuronal stains, allowing for permanent visualization of vagal terminals and myenteric neurons. (A) An IGLE (golden brown; TMR-B stained with a DAB reaction) is seen in close approximation to several neurons (light blue; stained with the putative pan-neuronal stain cuprolinic blue) within a myenteric ganglion. (B) TMR-B (golden brown) is also compatible with nicotinamide adenine dinucleotide phosphate diaphorase (NADPHd; dark blue) staining. In the myenteric plexus, neurons that produce nitric oxide synthase can be demonstrated histochemically in aldehyde-fixed tissue using a histochemical reaction for NADPHd. Note the disparate relationship of the IGLEs to the NADPHd-positive and NADPHd-negative (unstained) neurons. Mouse stomach whole mounts; scale bar = 25 µm.
The durability of the TMR-B molecule makes it an excellent choice for lengthy staining protocols, such as immunocytochemistry, that typically require exposure of the tissue to harsh detergents such as Triton X-100. (A) An IGLE (dark brown) visualized using TMR-B stained with a DAB reaction can be seen in close association with several myenteric neurons (stained red using Vector NovaRED) positive for the calcium-binding protein calbindin, which was labeled using a polyclonal antibody to calbindin. (B) TMR-B can also be used in conjunction with both a histochemical reaction and an immunocytochemical reaction. An IGLE is labeled brown using the tracer TMR-B stained with DAB, while two different nonoverlapping phenotypes of myenteric neurons are labeled different colors. A nitric oxide synthase producing neuron is labeled dark blue using an NADPHd reaction, while a calbindin-positive neuron is labeled red using NovaRED. Mouse stomach whole mounts; scale bar = 25 µm.
these complications are minimized in the case of vagal afferent labeling experiments by the facts that:

- Vagal afferents to more distal organs, such as the stomach and intestines, are relatively compactly organized in the nodose ganglion or, for the more cranial organs, in some instances in the jugular ganglion.
- The nodose is well encapsulated and thus possesses a natural limiting barrier that contains even large injections.
- The target tissues are not adjacent to the ganglion where leakage by diffusion could become an issue.

The anatomical organization of the vagus does not, however, mitigate all control issues in tracing analyses. From the perspective of investigations of the vagal afferents, the most problematic feature stems from the fact that the axons of vagal efferents course through (in some species, somewhat superficially to one side of) the nodose. There is always the prospect that injections of tracers will also label en passage the efferent axons. Even when experimental circumstances strongly favor selective labeling of afferents (e.g., tracer compounds such as lectins or dextrans that are selectively bound by somata rather than axons; minimal mechanical damage to the ganglion, small injections, etc.), there remains the prospect that efferent axons can be labeled and that any putative afferent ending in the periphery is actually a motor process.

The procedure that constitutes the “gold standard” for addressing this prospect is a preparation in which the efferents have been eliminated from the vagal trunk and the nodose is then injected. Complete and selective chemical lesions of all vagal efferents in the periphery are presently impractical, so surgical strategies are probably the most feasible means of achieving such controls. Furthermore, since the somata of vagal efferents are distributed centrally in two long and thin columnar bilateral cell groupings (i.e., the dorsal motor nucleus of the vagus and the nucleus ambiguus) and extensive lesions of the brainstem are not readily tolerated, CNS lesions of all efferents are not practical. One strategy for eliminating efferents is to perform an extracranial supranodose vagotomy, to wait for the peripheral efferent axons to undergo dissolution, and then to inject the nodose with tracer. Such an experiment is relatively simple in terms of surgical requirements, but it runs very substantial risks of damaging the nodose. The nodose is situated so close to the jugular foramen (at least in smaller mammals) that a transection above the ganglion must almost invariably damage the rostral pole of the ganglion either by direct mechanical damage or by disruptions of the local circulation.

The alternative to an extracranial supranodose vagotomy to eliminate efferent fibers is an intracranial transection of the motor roots of the vagus, just as they exit the medulla and span towards the jugular foramen. This strategy has proved practical from both a ventral and a dorsal approach. Conveniently too, from the perspective of tracer experiments, there are simple adaptations of the tracers such as HRP and FluoroGold that make it possible to verify the completeness of such selective efferent vagotomies.

The complementary manipulation that makes the recognition of afferents unambiguous is to label the efferents coursing in the vagus in animals that have had the
afferents transected. Experimentally this is most readily achieved by injecting the motor neurons in the brainstem after the vagal afferent rootlets have been cut between the medulla and the jugular foramen. Identifying the motor profiles in the tissues of interest and formally comparing these efferent profiles with the afferent endings makes it practical to subsequently recognize any efferents inadvertently labeled after nodose injections in animals that do not have selective rhizotomies.

Such surgical manipulations and verification strategies are time-consuming, and would be especially tedious if they were routinely and continuously needed. In our experience with the vagal innervation of the heart, as well as the GI tract, though, afferents and efferents differ categorically in their target and accessories tissues and in their morphologies. Once afferents and efferents have been distinguished and characterized with the subtractive surgical analysis to validate the discriminations, it is possible to classify and identify vagal afferents and to distinguish them from any efferent profiles that may occasionally be labeled incidentally.

The issue of efferent profiles raises another point pertaining to a potential false positive analysis. If one regularly checks the dorsal motor nucleus of the vagus and the nucleus ambiguous in the brainstem after tracer injections in the nodose ganglion, it is common to see a small number (or with particularly large injections, a proportionately larger number) of neurons retrogradely labeled with the tracer. These retrogradely labeled somata typically appear without any corresponding labeling of efferent profiles in the periphery. It appears that any efferent axons interrupted or transected by the pipette when the nodose was impaled will effectively take up the tracer and transport it retrogradely. Just as in a classic cut-nerve-soak study, though, when the axons have been transected there is no mechanism for the tracer to be transported into the peripheral efferent terminals, hence the absence of efferent labeling in the periphery.

On the other hand, a second process, though it is more hypothetical, may also occur in some preparations. It is possible that some of the retrogradely labeled motor neurons one can observe possess transiently damaged but not destroyed peripheral axons. In these hypothetical cases, subsequent anterograde transport of the tracer might label efferent terminals in the periphery. Indeed, this mechanism seems likely to be responsible for the motor profiles we have observed occasionally in peripheral target organs after nodose injections. It might also be the case that different combinations of protocol manipulations (e.g., larger pipette tips, more mechanical damage, more chemical injuries from nonoptimal solvents, different survival periods tailored for the longer time course of retrograde then anterograde transport, etc.) might substantially increase the amount of adventitial efferent labeling one obtained. At any rate, the rhizotomy control experiments outlined earlier unequivocally eliminate such a source of erroneous labeling.

5.4 SELECTING TRACERS FOR PARTICULAR APPLICATIONS

With the extensive battery of tracers available, choosing the most useful compound for a particular experimental design can be a challenge. As outlined earlier, we have
found the four tracer protocols described in this chapter to be particularly successful in inventories and characterizations of vagal afferents. Each of the tracer strategies has distinct advantages and disadvantages when compared with the others. Their complementarities mean that they serve collectively as a useful arsenal of techniques that can be substituted for one another, depending upon the details of the experimental application. These complementarities, discussed below, also underscore some of the issues that one might consider when selecting and adapting additional tracer protocols.

5.4.1 Issues of Field of View, Magnification, and Population Surveys

The proportion of the entire vagal afferent population that the different tracers label is one such issue. WGA-HRP will label virtually the entire population of nodose neurons. The enzyme is highly soluble in aqueous solutions, it is delivered in distilled water or a buffer or solvent with an osmolarity similar to that of extracellular fluids, and the solution appears to readily diffuse throughout the ganglion, delivering the enzyme to all nodose neurons. Furthermore, vagal afferent neurons all apparently express binding sites for the lectin. The net effect is that WGA-HRP serves as a particularly powerful marker when one is seeking to label the entire afferent population. When processed with a chromogen such as TMB that amplifies the signal, the compound can be observed in whole mounts at even low power (e.g., Figure 5.1), and we think it is the method of choice for this purpose.

In contrast, the carbocyanine dyes will label extensively, but they are not ideal in applications requiring labeling of the entire population. Being lipophilic and hydrophobic, the carbocyanine dyes must be dissolved in methanol or other solvents that tend to damage cells. Furthermore, when the injected solution contacts extracellular fluids, the carbocyanine dyes tend to rapidly precipitate out of solution and produce a depot of dye at the injection site, rather than diffusing through the ganglion. For some applications where tracer spread is a problem this is a decided advantage, but for labeling a large population of neurons, it is a disadvantage. To distribute the carbocyanine material throughout the ganglion, multiple injections may be required, but then the risk of tissue damage and potential destruction of some afferents is greatly increased.

The dextran amines, with or without biotin conjugated, are soluble in aqueous media and can be easily injected throughout the ganglion (gauged by visible monitoring of the colored solution), and they do not produce any observable necrosis in the ganglion. Nonetheless, the dextran amines only label a small percentage of all neurons. This selectivity is unrelated to particular classes of neurons. Though the mechanism of the selectivity has not been established, the injected dextrans effectively label only those neurons located in close proximity to the injection sites. Typically, one finds a number of afferents strongly labeled (those immediately proximal to the pipette tip), a larger number that are relatively lightly labeled (those less proximal, but still close to the tip), and a still larger population of essentially unlabeled neurons (those a significant distance from the injection site, perhaps where the effective concentration of the dextran falls below a threshold level). Multiple
injections increase the number of labeled neurons, but it is impractical to label the entire nodose population this way.

5.4.2 Resolution or Definition of Endings

A second issue that might influence any tracer selection relates to whether the intended application will involve relatively low-power surveys or higher-power, higher-definition analyses of individual endings. WGA-HRP is particularly effective for evaluating large fields or whole mounts, in part because, as mentioned earlier, the enzyme can label the entire population of vagal afferents. Additionally, WGA-HRP lends itself to wide-field inventories, in part because the signal can readily be amplified with TMB processing to generate a darkfield-compatible label that accentuates the endings. Neither the carbocyanine dyes nor the fluorescent dextran amines are as useful for low-power surveys. In these latter cases, the effective signal is limited both because fluorescent labels tend to label only a percentage of the neurons and because fluorescent emission signals are not sufficiently strong or stable to yield a robust signal through low-power optics of relatively low numerical aperture. Of the two limitations that plague the fluorescent labels, the first also applies to biotinylated dextran amines, though the second does not limit the permanent labeling strategies for dextrans.

At the other end of the magnification range, different considerations determine the relative strengths of the different tracers. The dextran amines produce high-definition smooth and continuous labeling of neurites that compares favorably with the benchmarks of neural tracing such as PHA-l processing. Where long-term stability and nonfading signals are not essential, the fluorescent dextran amines can be readily photographed (cf. Figure 5.3) or used for confocal microscopy. Where stability and fading are issues, permanent labeling with the biotinylated dextrans is particularly practical (cf. Figure 5.4 and Figure 5.5). Carbocyanine labeling of vagal afferents is frequently more grainy and less continuous or smooth than the dextrans (compare for example, Figure 5.3A and Figure 5.3B), but, at its best, carbocyanine labeling provides almost the same quality of fine-process definition. In contrast, WGA-HRP labeling provides a much less satisfactory image for high-definition analyses of individual endings. HRP labeling is typically grainy, and with TMB processing, the crystalline reaction product tends to aggregate on the ending without continuously and smoothly labeling only the axoplasm or membrane of the neurite.

5.4.3 Compatibility with Immunohistochemistry and Counterstaining

A third issue on which the four tracing techniques have different strengths is in their relative compatibility with immunohistochemistry and other techniques for double labeling. The dextran amines are in general the most compatible with such additional processing. Indeed, they produce exceptional results. The fixation protocols and tissue preparations for the dextrans are readily compatible with both immunohistochemistry (e.g., Figure 5.4 and Figure 5.5) and counterstaining methods (e.g.,
Figure 5.4). Furthermore, the dextran amines can be used for fluorescence or permanent-label immunohistochemistry and counterstaining.

In contrast, both WGA-HRP and the carbocyanine dyes are somewhat less satisfactory for routine double and triple staining protocols. WGA-HRP seldom can match the definition and quality of neurite filling seen readily with the dextrans (or with the carbocyanine labels). In addition, when TMB is used as the chromagen for WGA-HRP processing, the crystals are less stable and more subject to fading and to being dissolved when exposed to some of the reagents employed for immunohistochemistry and some counterstaining. The carbocyanine dyes are notoriously soluble in detergents and agents used to enhance penetration for immunohistochemistry. In addition, as commonly used, the carbocyanine dyes are limited to fluorescence and subject to the fading and oxidation that limit some applications. To date, with only a few limited exceptions, the successes in routinely combining the carbocyanine labels with immunohistochemistry have been modest (e.g., see discussion in Berthoud4).

**5.4.4 DOUBLE OR MULTIPLE TRACER INJECTIONS**

In a variety of experiments, tracer protocols that simultaneously distinguish two (or more) different subpopulations can be very powerful. For example, the separate peripheral innervation fields, as well as the target regions of overlap of the left and right nodose ganglion projections, can be distinguished with injections of different tracers into the two sides. Similarly, double tracer experiments provide practical means of comparing the projections of vagal afferents and efferents in the same tissue specimen, or the distinct projection fields of the rostral and caudal pole of the nodose ganglion, or the patterns of inputs of vagal versus dorsal root ganglion afferents. The carbocyanine dyes and dextran amines are readily adaptable for such experiments insofar as both families of tracers are available with several different fluorochromes conjugated to the basic complex. WGA-HRP and biotinylated dextran are somewhat less versatile in this regard, although protocols have been devised for combining other conjugates of HRP (e.g., WGA-HRP vs. cholera-toxin-HRP or free HRP) and different dextrans (e.g., a simple dextran amine vs. biotin-conjugated dextran amine). Though the fluorescent markers appear to be somewhat more practical in this type of double labeling experiment, it should be mentioned that the different variants within a marker family do not all appear to label neurons equally strongly. For example, the rhodamine-related forms of the fluorochromes (DiI and Fluoro-ruby, respectively) generally produce stronger labeling (or detectability) than the FITC variants, thus producing a need to counterbalance the different labels across the different injection sites.

**5.4.5 PERMANENCE OF THE LABELING**

Permanence of the different labels is another factor that has to be considered in selecting a tracer for a particular application. Of the four candidates under discussion, permanently labeled biotinylated dextrans appear to be the most stable for long-term storage. They are not affected by light, and they do not obviously deteriorate in
long-term storage (when they are well fixed prior to processing). WGA-HRP also does not fade when illuminated, and it is relatively stable, though even under ideal conditions (stored refrigerated in the dark), it does deteriorate slowly over several months. The quality of the initial fixation is important in ensuring the relatively long-term stability of the TMB reaction product. Both the carbocyanine family of dyes and the fluorescent dextran amines fade with sustained illumination. Furthermore, both of the types of fluorescent labels do tend to lose intensity in long-term storage, to diffuse into surround connectives and tissue if the fixation is sub-optimal, and to slowly become masked by the autofluorescence of the surrounding tissues that evolves over weeks or months of storage.

**5.4.6 TRANSPORT TIME**

Transport (and incorporation) times vary widely and constitute another factor that, given the constraints of a particular experiment, might clearly dictate the use of one or another of the four tracers we have discussed. WGA-HRP is rapidly incorporated and transported to the peripheral terminals of vagal afferents. For the rat, we typically allow 72 hours between the injection of WGA-HRP into the nodose and the perfusion step; for the mouse, we typically use 24 hours. The dextran amines (both free and biotin-conjugated) are considerably slower. Typical survival periods for optimal dextran amine labeling of vagal afferents in the GI tract of the rat would be 14 days and of the mouse would be 7 days. This interval varies somewhat depending upon which compartment of the organ system is being investigated and which type of afferent terminal is of more interest. Optimal labeling with the carbocyanine tracers is still much slower. For analyses of the GI tract innervation, we typically wait three to four weeks between injection and perfusion.

**5.5 SUMMARY**

The development, over the last two decades, of powerful neural tracers and sensitive processing protocols for these labels has made it practical to investigate the distributions and finer structural details of vagal afferents. Such information is needed if the understanding of vagal sensory processes is going to progress toward parity with that of other sensory systems. Particularly effective tracers for the nodose neurons include WGA-HRP, carbocyanine dyes, dextran amines, and biotinylated dextran amines. These families of tracers have different and complementary strengths and weaknesses as well as specific control considerations, and such factors should shape decisions or choices of labeling strategies for different experimental applications.

**ACKNOWLEDGMENTS**

Support was provided by NIH DK27627. Technical assistance was provided by Elizabeth Baronowsky.
REFERENCES


6 Mechanotransduction by Vagal Tension Receptors in the Upper Gut

Simon J.H. Brookes, Vladimir P. Zagorodnyuk, and Marcello Costa

CONTENTS

6.1 Introduction ..................................................................................................147
6.2 Identification of the Morphological Structure of Vagal Tension Receptors ........................................................................................................149
6.3 Mechanotransduction ...................................................................................151
6.4 Mechanical Activation of Muscle Mechanoceptors ....................................152
   6.4.1 Activation of Mechanoreceptors in Intact Organs ..........................155
   6.4.2 Receptive Fields of Mechanoreceptors............................................156
6.5 Evidence that Transduction Is Not Chemically Mediated .......................157
6.6 Modulation of Vagal Afferent Nerve Endings by Endogenous Chemicals ........................................................................................................159
6.7 Molecular Basis of Transduction in IGLEs...............................................160
Acknowledgments..................................................................................................161
References..............................................................................................................162

6.1 INTRODUCTION

Vagal afferents to the gut form at least three different morphological types of endings: mucosal endings, intraganglionic laminar endings in myenteric ganglia, and intramuscular arrays in the muscularis externa. Electrophysiologically, two functional types of vagal afferents have been identified in most preparations from the upper gut: mucosal afferents, which respond to mucosal stroking and various chemical stimuli, and muscle afferents, which appear to function largely as in-series tension receptors. Few would dispute that mucosal endings probably correspond to the units that are activated by mucosal stroking. Intraganglionic laminar endings have been shown to correspond to the transduction sites of tension receptors. The role of intramuscular arrays is currently unclear; it has been argued that they may be length receptors, but physiological evidence for a specialized population of length receptors is currently lacking. Intraganglionic laminar endings respond on
a millisecond timescale to distortion and their ability to transduce mechanical stimuli is not blocked in calcium-free solution. These observations suggest that they probably transduce mechanical stimuli directly, rather than being activated indirectly by mediators released from other cells. The mechanosensitive ion channels responsible for transduction by IGLEs remain to be identified. A variety of substances released from various cells in the gut wall, including ATP and glutamate, may modulate their integrative properties, as can a number of exogenous neurochemicals, opening possibilities for pharmacological manipulation of mechanoreceptor activity in gastrointestinal disorders.

Vagal afferent nerve fibers innervating the upper gastrointestinal tract have been divided into two basic functional types; mucosal receptors, which respond to chemical or mechanical stimulation of the mucosa but not to stretch of the gut wall, and muscle receptors, which respond to stretch but are not activated by mucosal stimuli. In the ferret esophagus, a third type of vagal afferent has been described with intermediate properties, the so-called tension-mucosal (TM) receptors. Muscle receptors, which we will interchangeably refer to as vagal mechanoreceptors, have been extensively studied by many groups in a large number of preparations. They are functionally important as they underlie the sensations of esophageal and gastric distension and are the afferent limb of reflexes such as receptive relaxation and gastric accommodation. The first single unit recordings were made using the fiber-teasing technique developed by Adrian (1933), which was shown to be capable of recording the smallest diameter axons. In 1955, Iggo demonstrated that vagal mechanoreceptors in the goat stomach behaved as if they were in-series tension receptors. They increased their firing to both stretch and contractile activity in the muscular wall of the stomach, indicating that their firing did not simply reflect unidirectional length changes of their receptive fields. Since then, a number of studies have confirmed this basic finding: vagal mechanoreceptors appear to function largely as tension receptors in the ferret stomach, sheep stomach, rat, mouse, dog, and guinea pig esophagus and stomach.

The observation that vagal mechanoreceptors behave as if they were in-series tension receptors is, at least anatomically, surprising. The concept of in-series tension receptors arose from studies of Golgi tendon organs. These are encapsulated structures located at the junction between skeletal muscle fibers and the tendon proper and thus are truly in-series with the muscle. Contraction of the muscle stretches the Golgi tendon organ and this leads to firing of group Ib somatic afferents. It has been hypothesized that linear stretch of the receptor by muscle contraction straightens out collagen fibrils within the tendon organ. These then squeeze and distort mechanosensitive branches of the afferent fiber, which intertwine between them. In the gut wall, there are, of course, no tendons and the muscle layers form a continuous ring. Afferent fibers cannot, therefore, be aligned solely in-series with the muscle; they must always be aligned in parallel to it. This then raises questions as to how an in-parallel arrangement could give rise to mechanoreceptors being sensitive to tension rather than length. For this, one needs to consider the morphology of the nerve endings, their transduction sites and their relationship with the surrounding tissue.
In the skin and other organs of the body, identifying the morphology of physiologically characterized afferent fibers was relatively straightforward. Hunt in 1961 identified Pacinian corpuscles as the endings of specialized, rapidly adapting, vibration-sensitive mechanoreceptors. This was made possible by locating the receptive fields of an afferent unit with the appropriate physiological activity, marking it in the tissue, and identifying the associated neuronal structures in either stained or unstained tissue. This is possible when receptors have a relatively low density in the tissue and when the density of all types of motor and sensory innervation is similarly low. In most viscera, the latter condition is not met; there is extensive innervation, both efferent and afferent, by autonomic neurones (sympathetic, parasympathetic, and intrinsic) as well as extrinsic sensory neurones. This makes it difficult to identify which particular structures are likely to correspond to a marked receptive field when all nerve fibers are stained in the tissue.

A modified dye-filling technique was reported in 1999, which allowed selective filling, via anterograde transport, of extrinsic axons innervating a piece of tissue from a single fine-nerve trunk. This meant that extrinsic nerve fibers from a single nerve trunk could be labeled without labeling either axons of intrinsic origin, or extrinsic axons that reach the tissue via other nerve trunks. Using this technique, it became possible to correlate dye-filled structures with mechanically sensitive sites in the guinea-pig esophagus and upper stomach. This technique has subsequently revealed comparable specialized flattened endings in the guinea pig rectum.

Specialized vagal afferent endings, now known as Intraganglionic Laminar Endings (IGLEs), were first described by Lawrentjew (1929) using silver staining. Rodrigo and colleagues demonstrated that these endings were of vagal origin as they disappeared following subnodose vagotomy. IGLEs are located within the myenteric ganglia of the esophagus and stomach, but are found in decreasing densities throughout the small and large intestine. A second class of vagal afferent endings were described more recently following anterograde labeling from the nodose ganglion of the rat. These endings had quite different morphological features and were called intramuscular arrays (IMAs). These have a much more restricted distribution, being concentrated in the upper stomach and the lower esophageal sphincter and pylorus. There are also vagal afferent nerve endings in the inner layers of the gut including the mucosa. However, mechanoreceptors activated by stretch of the gut wall do not generally respond to mucosal stroking and removal of the mucosa does not interfere with vagal fiber responses to stretch. This means that either IGLEs or IMAs are likely to be the endings of vagal mechanoreceptors.

6.2 IDENTIFICATION OF THE MORPHOLOGICAL STRUCTURE OF VAGAL TENSION RECEPTORS

The existence of three morphological types of vagal afferent endings in the upper gut (mucosal, IGLEs, IMAs), but only two major electrophysiologically characterized classes is problematic. Identifying the functions of IGLEs and IMAs became feasible when techniques to combine extracellular recording and dye filling were
developed. This required making recordings from fine nerve trunks close to the target tissue, rather than at remote sites far up the vagus nerve. Such recordings made in isolated preparations of guinea pig esophagus\textsuperscript{12} and stomach\textsuperscript{13} showed mechanoreceptors with very similar characteristics to those recorded \textit{in vivo}. Typically, mechanoreceptors showed slow rates of spontaneous firing, had low thresholds to stretch, a wide dynamic range, and adapted slowly during maintained distension. In addition, units typically showed increased firing rates during spontaneous and evoked muscle contractions. This was the case even when the preparation was maintained at a constant length, indicating that the units were behaving similarly to the “in-series tension receptors” described by Iggo and subsequent investigators.

The small, \textit{in vitro} preparations differed in two important aspects from the \textit{in vivo} preparations that had previously been used so widely. First, it was possible to remove the mucosa and submucosa from \textit{in vitro} preparations and thus reduce contamination from other classes of mucosal afferents. Secondly, the preparations could be studied at higher resolution, attaching transducers close to the receptive fields to closely monitor changes in both length and tension at the transduction sites, and probe systematically to identify mechanosensitive sites. Using light von Frey hairs, it was possible to show that nearly all of the mechanosensitive afferents had one or more small sites at which they could be powerfully activated by radial compression. These so-called “hotspots” were marked on the tissue by applying carbon particles on the tip of the von Frey hair, followed by photography. A clear pattern emerged from these studies. Each tension sensitive afferent had from 1 to 6 hotspots, each of which was surrounded by an area of much lower sensitivity. Dye fills from the recorded nerve trunk consistently revealed intraganglionic laminar endings close to, or directly under, each hotspot. While IMAs and viscerofugal nerve cell bodies were also labeled in the anterograde dye fills, these showed no significant association with hotspots. These studies led to the conclusion that vagal afferent mechanoreceptors, at least the slowly adapting, wide dynamic range tension receptors, have transduction sites that correspond to IGLEs and that the parent axons are not themselves mechanosensitive.\textsuperscript{12,13}

The role of IMAs was not determined in the study of Zagorodnyuk and colleagues even though they were anterogradely filled from many of the nerve trunks from which recordings were made. No evidence was seen that IMAs were sensitive to probing with a von Frey hair. Mapping receptive fields failed to reveal the extensive structures extending in parallel to either longitudinal or circular smooth muscle layers, as would be expected for IMAs. The possibility that IMAs are transduction sites for length or stretch receptors has been strongly argued in several publications.\textsuperscript{28,29} This possibility cannot be refuted on the present evidence. The studies of Zagorodnyuk et al. were not carried out to search systematically for a role for IMAs. There may have been selection bias in the units chosen for study; many of the units subsequently shown to have IGLEs had relatively large amplitude action potentials. It is possible that, while IMAs were present in the preparations studied, they may have represented a very small proportion of the total filled fibers and hence been missed in recordings. In support of this, we have seen several IMAs, extending over many square millimeters, arising from a single parent axon, suggesting that the total number of vagal axons giving rise to IMAs may be relatively small relative to
IGLE-bearing tension receptors. Thirdly, it is possible that IMAs are not activated by focal distortion — their adequate stimulus may stretch along their entire length. Lastly, the mucosa and submucosa was always removed from the preparations used in this study. This meant that a restricted range of stretches had to be used to avoid irreversibly damaging the preparation.

It is possible that IMAs are activated only with large-amplitude distensions. Bearing in mind these limitations to the study, no evidence was seen for stretch activated afferents that lacked hotspots. Of 46 units in the stomach that could be activated by stretch, 6 units appeared to lack focal hotspots, of which four of these were rapidly adapting. The other two slowly adapting units were indistinguishable from IGLE-bearing units and probably had inaccessible transduction sites. If IMAs are transduction sites of vagal mechanoreceptors, it would seem that under the conditions used here, they acted as rapidly adapting units and are unlikely to function as length receptors. Nevertheless, the role of IMAs will only be determined by systematically attempting to record from their parent axons and applying a range of mechanical and chemical stimuli until their adequate stimulus is identified.

6.3 MECHANOTRANSDUCTION

The question then arises as to how IGLEs, which are located in parallel to the muscular elements of the muscularis externa, can transduce tension, when elsewhere in the body this is mediated by in-series structures. Two very different mechanisms appear to underlie mechanotransduction by afferent neurones in different systems. The first, which we will refer to as chemical transduction, involves chemicals, released from physically distorted non-neuronal cells, that increase the excitability of afferent nerve endings, beyond the threshold for action potential generation. For example, in slowly adapting mechanoreceptor corpuscles, Merkel cells make ultrastructurally identifiable synapse-like contacts onto afferent nerve fibres. It is believed that release of a transmitter substance, possibly glutamate, may activate the nerve fiber, with the Merkel cell being the actual site of transduction. Likewise, glutamate acting on AMPA receptors probably mediates transmission from mechanotransducing inner hair cells of the cochlea to spiral ganglion neurones. Chemical transduction can be considered to occur when the released substance drives the afferent nerve fiber’s entire response to the mechanical stimulus. This should be distinguished from substances released from damaged, inflamed or distorted tissue that only modify the firing rate of sensory nerve endings by changing their excitability. Thus prostaglandins, histamine, bradykinin, serotonin, ATP, proteases, and protons can all act on receptors or ion channels on afferent nerve endings to modulate their responses to mechanical stimuli. The difference between chemical transduction and modulation of excitability is one of degree. Suffice it to say that to qualify as a chemical transduction mechanism, the release of chemical should be both necessary and sufficient to account for the stimulus/response relationship of the afferent fiber.

The second mechanism, which we will refer to as direct mechanotransduction, is characterized by having all of the essential molecular elements located in the afferent nerve ending, without extracellular mediators being involved. This mechanism does not rely on other cell types except as mechanical anchor points for the
transduction complex. Direct mechanotransduction is believed to be mediated by specific mechanically gated ion channels, which open when the nerve ending is distorted. The resulting ion fluxes produce a generator potential, which then gives rise to a train of action potentials. This is believed to be the case for many types of cutaneous mechanoreceptors to which putative mechanosensitive ion channels are transported. Modulatory mechanisms can affect direct mechanotransduction at multiple points, by altering the function of mechano-gated ion channels, by altering the excitability of the nerve endings via other ion channels, by changing the mechanical activity of surrounding tissues (e.g., smooth muscle), or in the longer term by re-modeling the mechanical coupling to adjacent structures.

For vagal intraganglionic laminar endings the mechanisms of transduction have not yet been positively identified. Nevertheless, evidence is accumulating that direct mechanotransduction is likely to be involved, but the molecular nature of the mechanotransduction complex is yet to be discovered.

6.4 MECHANICAL ACTIVATION OF MUSCLE MECHANORECEPTORS

Vagal tension receptors make intraganglionic laminar endings (IGLEs) located in the myenteric ganglia, within the external muscle layers of the gut wall. They appear to be activated by any stimulus that distorts the IGLE. Thus, stretch in either longitudinal or circumferential axes of the gut wall powerfully activates these endings (Figure 6.1). This occurs even if the gut wall is an intact tube, if the mucosa and submucosa are removed, or if the preparation is prepared as a flat sheet. Significantly, IGLEs are also exquisitely sensitive to compression by a von Frey hair applied perpendicular to the preparation. The lightest von Frey hair that can penetrate the surface tension of the bathing solution (which in our hands exerts a force of 0.08 mN over an area of approximately 0.03 mm²) can evoke brisk firing of a vagal afferent when positioned precisely on an IGLE. However, IGLEs do not only respond to externally imposed stretches — they are also powerfully activated by contractions of the wall musculature under isometric conditions (Figure 6.2). This gave rise to the description that they behave as in-series tension receptors although, as discussed earlier, this description cannot be anatomically accurate.

The responses of primary afferent neurones to mechanical stimuli are substantially affected by the physical environment surrounding the transduction site. This was elegantly demonstrated in Pacinian corpuscles, in which intact corpuscles give rapidly adapting responses to both the onset and offset of a mechanical deformation. In contrast, removal of part or all of the corpuscle (i.e., non-neuronal material) changes the response to a more slowly adapting response that extends further throughout the stimulus. A recent ultrastructural study of Meissner corpuscles provides a convincing explanation of how the mechanical coupling between the terminal axons and surrounding tissue, mediated via collagen fibrils, and enveloping Schwann cells, could explain the rapidly adapting responses to cutaneous indentation. It is likely that the details of the mechanical environment surrounding IGLEs may also strongly influence their firing patterns.
FIGURE 6.1 An L-shaped preparation of guinea pig esophagus is used to examine the effects of both longitudinal and circumferential stretch on two vagal mechanoreceptors. (A) Longitudinal stretch (1 mm for 10 s, left hand traces) evokes brisk firing of both units during the stretch, followed by a silent period after removal of the stimulus. Similarly, circumferential stretch (1 mm, 10 s) also evokes firing of both units (distinguishable by their height in the raw trace), again followed by a silent period. (B) The receptive fields of both units are located in the stretched area. (C) Superimposed action potentials show the different waveforms that distinguish the two units.

FIGURE 6.2 A single electrical stimulus applied to the guinea pig esophagus, maintained at constant length, evokes a rapid twitch in the striated muscle (lower trace). At least two tension-sensitive units are activated during the twitch (upper trace), demonstrating that they behave as if they were in-series tension receptors. The stimulus artifact is marked with an asterisk.
Two questions arise. First, what are the features of the mechanical microenvironment that give rise to this gross pattern of responsiveness? Secondly, what are the molecular mechanisms that convert forces or movements of tissue into electrical signals?

To answer the first question it is necessary to consider the fine structure of IGLEs. They arise from a parent axon via branches, which then typically subdivide and form flattened plate-like structures. Analysis with light microscopy reveals that these laminar endings are most dense close to the surface of the myenteric ganglia; there are few within the neuropil. A single IGLE often supplies laminar endings on both the mucosal surface of the ganglion sheath, and on the serosal surface, typically with endings also wrapping around one edge of the ganglion. In a large ganglion, several IGLEs may be present, arising from the same or different parent axons. Ultrastructurally, IGLEs appear to make junctional contacts with some enteric neurones, with small clear vesicles distributed in a fashion that suggests that these could be sites of transmitter release. However, studies of fos expression following afferent activation suggests that this is unlikely to be a major role for these endings. In addition, the flattened structures of IGLEs are anchored to the ganglion and overlying muscle layer and have a close association with glial processes, which have been hypothesized to allow them to transduce shearing forces associated with change in tension and length.

It would appear then that the location of IGLEs would be suitable to detect distortion of myenteric ganglia, caused by both passive stretch and by active contraction of the muscle layers. How this occurs requires further consideration. IGLEs, the muscle surrounding them and the ganglia are all fluid-filled structures and incompressible, therefore, it is unlikely that the volume of any of the structures changes when force is applied. Given the great sensitivity of IGLEs to radial compression of the ganglia (i.e., perpendicular to the plane of the muscle) it is tempting to speculate that this could be the major type of distortion that physiologically activates the endings during both stretch and contraction. This idea extends the analogy with Golgi tendon receptors, in which straightening of collagen fibrils by external force leads to compression of the branching nerve fibers coursing through the capsule. This idea could, we believe, explain how passive stretch of the tissue activates IGLEs.

During either circumferential or longitudinal stretch of a rectangular flat sheet of gut tissue, the gross surface area of the tissue increases, but as volume must be conserved, the thickness reduces. This is obvious as “pinching” in the middle of a flat sheet preparation. In the pinched region, there must be considerable distortion of the myenteric ganglia, including radial compression, which might be expected to activate IGLEs. In tubular preparations of gut, distension by a balloon inflated in the lumen (or infusion of fluid, or arrival of a semi-solid bolus) would lead to an equivalent reduction in wall thickness and radial compression of ganglia. The question then arises as to how isometric contractions, during which the dimensions of the tissue grossly remain constant, could also activate IGLEs. Typically, under isometric conditions, the overall dimensions of the tissue change very little during contraction, but there is still considerable movement on fine scale, readily visible under a dissecting microscope and recordable with a video camera. As individual
muscle cells contract, they inevitably shorten and increase in diameter as well as becoming more rigid. Compensatory stretching of the passive extracellular matrix presumably takes place, thus allowing the tissue to maintain constant length. The change in muscle cell shape and rigidity may be sufficient to apply radial compression to myenteric ganglia, and thereby activate the IGLEs. In a tubular preparation, the effect of circular muscle contractions may be further enhanced by the radius of curvature, which would tend to evoke a force with a radial vector acting against the resistance of the gut contents.

6.4.1 Activation of Mechanoreceptors in Intact Organs

This crude model, in which IGLEs are activated by radial distortion, can also account for the effects of muscle relaxants that are known to reduce the firing of mechanoreceptors to constant amplitude stretches, since their effect would be to reduce the rigidity of individual muscle cells, and hence their tendency to exert radial compressive forces on ganglia.

The stomach functions as two different organs in terms of motility: the proximal stomach acts as a low pressure reservoir, whereas the antrum acts as a pump, mill, and aliquoting device. The reflex control of the two regions reflect these different functions: Cannon first described receptive relaxation, in which distension of the esophagus leads to relaxation of the upper stomach, in preparations for the arrival of contents. Once food arrives in the stomach a vago-vagal reflex ("gastric accommodation"), activated by gastric distension, leads to further relaxation of the upper stomach and simultaneous excitation of the antral pump The extrinsic accommodatory reflex is supplemented by an intrinsic reflex that can be recorded in the isolated stomach Importantly, these two functions are also mirrored by the myogenic activity of the stomach, in which slow-wave mediated rhythmic activity starts weakly in the upper stomach and gains both force and velocity as it propagates toward the pylorus. These reflex pathways have important consequences for the activation of gastric mechanoreceptors. Vagal mechanoreceptors in the two functional regions of the stomach typically have different patterns of firing, either firing rhythmically, in time with gastric peristaltic contractions or independently of them. This may well be due to the different local mechanical environment surrounding IGLEs in the different regions. It is interesting that while both types exist in the antrum, there appear to be more nonrhythmic units in the upper stomach.

During normal gastric filling, most of the contents tend to accumulate in the low tone-region of the fundus and corpus, with about 20% entering the antrum. Under these conditions, food entering the stomach should cause stretch of the fundus wall with a resulting reduction in wall thickness and tonic radial compression of IGLEs in this region. In the antrum, the waves of propagating contraction activate IGLEs in a rhythmic fashion. Thus the mechanical environment surrounding IGLEs could explain how a single type of receptor may signal to the central nervous system, both the contractile state of the lower stomach and the state of distension of the upper stomach. Thus IGLEs in different parts of the stomach may be able to signal both hunger pangs and sensations of fullness after a large meal, without proposing the existence of a separate class of length receptor.
6.4.2 RECEPTIVE FIELDS OF MECHANORECEPTORS

The functional significance of having multiple IGLEs arising from a single parent axon is worth considering. It is clear that each IGLE is capable of being mechanically activated by a von Frey hair\textsuperscript{12,13} and that it can interact with companion IGLEs, such that the fastest-firing IGLE determines the firing frequency of the whole unit. This was demonstrated by examining the firing of tension receptors during the adapted phase of a maintained distension. Under these conditions, afferents with multiple IGLEs fire at a highly constant rate, as long as spontaneous muscle activity is not present. Simple modeling revealed that such constant firing rate could not be reproduced by two IGLEs firing independently.\textsuperscript{12} Rather, action potentials initiated from one IGLE appears to invade the others arising from the same parent axon and reset their excitability. Support for this idea was provided by the observation that activating a single IGLE strongly with a stiff von Frey hair reduced the response of the whole unit to a stretch stimulus applied moments later.\textsuperscript{12,13} Clearly, the IGLEs that had not been activated by the von Frey hair were unable to respond normally to the subsequent stretch stimulus. This mechanism may involve 4-aminopyridine-sensitive, voltage operated potassium channels, or calcium-activated potassium channels being opened following arrival of antidromic action potentials in the IGLEs.\textsuperscript{51}

It seems likely that the result of multiple IGLEs spread out over a small area is that the firing of the unit reflects the maximal wall tension anywhere within this overall receptive field.

Smooth muscle contraction in the stomach is determined by an interaction between myogenic and neurogenic mechanisms, which give rise to migrating Ca\textsuperscript{2+} waves associated with shortening.\textsuperscript{52} In the stomach it has been shown that myogenic pacemaker potentials arise from Interstitial Cells of Cajal (ICCs) sandwiched between the longitudinal and circular muscle layers.\textsuperscript{53} While the longitudinal and circular muscle layers are innervated by separate populations of motor neurones, and can thus be activated independently,\textsuperscript{54–56} the common pacemaker drive will tend to mean that contractions of one layer are in concert with those of the other. Thus IGLEs are likely to be squeezed from both sides by rhythmic contractions. When neuronal input differs between the longitudinal and circular muscle layers, shear forces may be generated across the myenteric ganglia. It is possible that IGLEs are sensitive to such forces,\textsuperscript{29} although this has not been directly tested to date.

Multiple receptive fields have also been described for mucosal afferents, activated by light mucosal probing.\textsuperscript{57} In the stomach, receptive fields from the same axons could be separated by up to 35mm. Thus some mucosal afferents are likely to sample simultaneously luminal stimuli in two or more widely separated regions of the stomach. Interactions between the different transduction sites were not studied in this investigation, but it would seem likely that the most active site drives the firing frequency of the parent axon, which could apply here too.

In many recordings from vagal muscle mechanoreceptors, spontaneous firing has been observed, even when the gut is in a “no-load” state (i.e., neither stretched nor compressed). It is clear that within the tissue of the gut wall, there may be considerable residual stresses, thus the mucosa of the esophagus is normally compressed, whereas the outer muscle layers are normally under slight tension.\textsuperscript{58} This
is readily demonstrated by the tendency of tubular segments of tissue to flatten, or even curl inside out, when they are opened up into flat sheets. Spontaneous tone of the muscle may also contribute to resting tension around the IGLEs and thus add to their “resting” excitability. Blockers of channels or receptors coupled to contraction (e.g., L-type calcium channel antagonists, muscarinic receptors) often reduce spontaneous firing. Likewise, drugs that actively relax the smooth muscle, such as sodium nitroprusside or other nitric oxide donors, vasoactive intestinal polypeptide, or beta adrenergic agonists can also reduce spontaneous firing rates. These effects can be considered as an indirect form of modulation of mechanoreceptor responses, since they are mediated via changes in smooth muscle contractility or tone.

6.5 EVIDENCE THAT TRANSDUCTION IS NOT CHEMICALLY MEDIATED

A number of experimental approaches have been used to distinguish whether IGLEs are activated directly, via mechano-gated ion channels or whether they could be activated by chemicals released from another cell type that functions at the true mechanoreceptor. The first test of this was to determine how quickly IGLEs respond to mechanical deformation. This was achieved in one study by placing a piezo-operated probe directly above an IGLE and measuring the latency to the first action potential. The probe was then replaced with a focal electrical stimulating electrode and a near-threshold electrical stimulus was given to determine the conduction delay. Typically, this evoked a burst of antidromic action potentials amongst which the particular single unit could not be discriminated, however, a minimum conduction delay could be determined (Figure 6.3). When the conduction delay was subtracted from the latency to the mechanical probe, a transduction delay of less than 5 ms was consistently observed. In several cases, the latency was considerably less than this. This indicates that transduction occurs over a millisecond time scale. However, mechanically activated ATP release from cells occurs over a time scale of seconds, even when this is mediated by “burst release.”

In the bladder, where ATP mediated mechanotransduction has been implicated in determining afferent excitability, there is also no evidence for rapid ATP release on a millisecond timescale. It has been suggested ATP release from epithelial cells also occurs in the gut, where ATP released from epithelia could increase the excitability of visceral afferents. However, this appears again to occur with a slow timescale. However, it is likely that ATP plays an important role in modulating primary afferent sensitivity in a variety of pathological circumstances. A recent paper has elegantly demonstrated an alternative method of chemical transduction that may occur in epithelia. This suggests that there may be ongoing tonic release of a compound into the lateral extracellular space. Mechanical deformation of the epithelium reduces the volume of the lateral extracellular space and thus raised the concentration of, in this case, an EGF-like ligand, sufficiently to evoke a cellular response. Again, this mechanism occurs over a time course of seconds rather than milliseconds and cannot explain the rapid transduction by IGLEs.
ATP and other transmitters can be released very rapidly, during fast synaptic transmission on a timescale well within the 5 ms latency that we identified. Rapid release is believed to be mediated by exocytotic release, which is a calcium-sensitive mechanism. It was of interest then to examine the effects of lowered [Ca$^{2+}$] and raised [Mg$^{2+}$], which blocks such exocytotic release. This did not block mechanotransduction by IGLE-bearing vagal mechanoreceptors. Rather, it increased both basal firing rate and stretch-activated responses, possibly by reducing resting activation of calcium-dependent potassium channels, or by increasing membrane excitability through charge-mediated effects. The voltage-sensitive calcium channel blocker, Cd$^{2+}$, also failed to block IGLE activation by mechanical stimuli. This makes it unlikely that exocytotic release of a chemical mediator, ATP, glutamate, or any other transmitter from a second cell type, is involved in the rapid response of vagal muscle receptors to mechanoreceptors stimuli.

**FIGURE 6.3** Calculating the minimum transduction delay for vagal mechanoreceptors. (A) A piezo-electric probe, with a response time of $<$20 µs was positioned directly above an IGLE of a vagal mechanoreceptor in the guinea pig esophagus. The unit showed slow, spontaneous firing. Immediately after the probe was advanced (stimulus artifact at asterisk) an action potential occurs with a latency of 7.85 ms. An increased rate of firing is also seen for the duration of the 200 ms stimulus (bar), followed by a silent period before spontaneous firing starts again. (B) The latency of the mechanically evoked response is shown on a faster timebase. (C) A focal electrical stimulus, applied at the same site, evoked a compound action potential, which includes the recorded unit, with a delay of 3.3 ms. This indicates that the delay due to transduction was less than 4.55 ms.
6.6 MODULATION OF VAGAL AFFERENT NERVE ENDINGS BY ENDOGENOUS CHEMICALS

Although ATP is unlikely to be responsible for mechanotransduction by IGLEs, it has potent effects on many primary afferent neurones. It has been shown immunohistochemically that P2X2 purinoceptors are present on IGLEs and ATP or its stable analogue, alpha beta methylene ATP strongly increase spontaneous firing of most vagal tension-sensitive afferents. There appear to be striking differences between species. In the mouse, fewer than 50% of vagal mechanoreceptors were activated by ATP and in the ferret, none were excited by it. However, following acid-induced inflammation, many vagal mechanoreceptors in the ferret became sensitive to ATP, suggesting that this could be an important mechanism for inflammation-induced hypersensitivity.

Mouse vagal mechanosensitive afferents also responded to other chemicals such as bile and 5-HT, although typically after latencies of more than 10s, suggesting that other intermediate steps may have been involved. It has recently been reported that both colonic afferents and vagal mechanoreceptors are sensitive to ionotropic glutamate agonists and that channel blockers of both NMDA and non-NMDA channels (memantine and CNQX respectively) can decrease the excitability of endings. However it should be pointed out that mechanotransduction was not blocked even at the highest concentrations of these drugs, which may affect muscle contractility. This suggests that glutamate is neither necessary nor sufficient for mechanotransduction. Rather it appears to be a powerful modulator of mechanosensitive endings in the gut. Again, there are notable differences between species. In the guinea pig, glutamate and agonists did not affect the excitability of vagal mechanoreceptors.

The effects of glutamate are interesting since much of the presynaptic apparatus required for glutamate release appears to be present in IGLEs. It is well established that vagal afferent neurones utilize glutamate as a primary transmitter at their central endings. Several studies have recently shown the presence of vesicular glutamate transporters and elements of the SNARE complex that are involved in fast synaptic transmission, in IGLEs. It is possible then that IGLEs release glutamate, which then acts in an autocrine fashion on ionotropic glutamate receptors on the same endings to increase their excitability. This does not appear to be the case in the guinea pig esophagus, where IGLEs appear to lack both ionotropic and metabotropic glutamate receptors but it could occur in other species or regions.

A number of other neurotransmitters and hormones have been reported to modulate firing of vagal mechanoreceptors. These include cholecystokinin, GABA, met-enkephalin, anandamide, and the various neuropeptides. In at least some cases, part or all of the effect of the agonist is mediated indirectly, via changes in muscle activity. In other cases, the effects are probably direct and may reflect the presence of receptors that are functionally important on the central terminals of vagal afferents also being expressed on peripheral endings.
6.7 MOLECULAR BASIS OF TRANSDUCTION IN IGLES

It seems likely that mechanotransduction is directly mediated in IGLEs. The molecular basis of how mechanical deformation of nerve endings is converted into a cellular response has been the subject of intense interest for a number of years. Much of this has been driven by studies in invertebrates in which behavioral screens have been used to identify mutants with defects in mechanotransduction. Leading the way in this endeavor was the nematode *Caenorhabditis elegans*, or rather the investigators who study it. Studies have revealed twelve genes that are essential for normal mechanosensory function by six touch cells in body of C elegans. Mec 4 and Mec 10 appear to be channel proteins, probably part of a heteromeric complex (Mec 2, a stomatin like protein) appears to be involved in intracellular tethering and is located in many mammalian sensory neurons. Mec 6, a homologue of mammalian paraoxonases, appears to be an essential part of the channel complex. Other mec genes appear to be largely involved in intracellular tethering to the cytoskeleton or extracellularly to the surrounding matrix. Mammalian mechanoreceptors probably also need equivalent tethering proteins. Thus, the tip links of mammalian hair cells are essential for transduction and cadherin 23 has recently been shown to be one of the components.

The ion channel subunits Mec4 and Mec10 are part of a class of ion channels called the degenerins, named after the effects of gain-of-function mutations that lead to selective death of the cells that express them. These channels share sequence homology with vertebrate epithelial sodium channels (ENaCs), a group of ion channels expressed in many cell types of the body. The Deg/ENaC family of ion channels are characterized by two membrane-spanning domains with a large extracellular loop and short intracellular n and c termini. Acid-sensing ion channels or ASICs are a subclass of the Deg/ENaC family, which are expressed in many mammalian sensory neurones and which can be activated by reductions in pH. There is evidence that ASICs may be involved in mechanotransduction too. Thus ASIC2 knockout mice show reductions in sensitivity of low-threshold, rapidly adapting cutaneous mechanoreceptors, although another study did not replicate this finding. ASIC3 knockout mice showed increased sensitivity to light touch but decreased sensitivity to noxious pinch, suggesting that these channels may form a number of types of heteromeric complexes in different mechanosensory or mechano-nociceptive neurones. It should be pointed out, however, that there is some doubt about the exact role of ASICs in the mechanotransduction complex. It has been reported in cultured dorsal root ganglion cells, from ASIC2 and ASIC3 single and dual knockout mice, that the specific, mechanically activated currents, are unaffected. This suggests that ASICs may, in fact, contribute to the integrative properties of afferent nerve endings rather than to mechanotransduction per se. It has recently been suggested that ASIC1 may be involved in mechanotransduction by vagal mechanoreceptors in the mouse esophagus, as in transgenic animals lacking this subunit, mechanosensitivity is enhanced and benzamil sensitivity is altered. Visceral afferents in ASIC2 knockout mice appear to be normal, whereas in ASIC3 knockout mice, tension receptors may be less responsive.
Another subfamily of the Deg/ENaCs, the mammalian ENaCs, has also been implicated for a role in mechanically sensitive primary afferent neurones in mammals, although direct functional evidence of its role is lacking. To date, transgenic mice lacking functional genes for these channels have not been reported. Both beta and gamma ENaC subunits have been localized immunohistochemically in the cell bodies of vagal afferent fibers that project to the aortic arch and gamma ENaC has been shown to be present in the terminal fibers in the aortic arch, in the carotid sinus\(^9\) and in both Merkel cell complexes and Meissners corpuscles in the skin.\(^9\) The role of ENaC subunits in vagal mechanoreceptor function is yet to be determined. Most ASICs and ENaCs are readily blocked by amiloride and its analogues. It is interesting that amiloride is not an effective blocker of IGLEs.\(^3\) Benzamil, its more potent analogue is only effective at very high concentrations (100µM). These observations suggest either that ASICs and ENaCs are not the prime mechanosensitive ion channels in IGLEs, or that they form atypical, possibly heteromeric channel complexes with distinctive pharmacology.

A final set of candidates for mechanosensory channels belongs to a quite different class of ion channels, the Trp channels. Named after the original “transient receptor potential” ion channel located in light sensitive cells in Drosophila, this family of ion channels now has 29 mammalian members, divided into 6 subfamilies.\(^9\) A number of members of the vanilloid receptor subfamily have been shown to be temperature-sensitive and one, TrpV4, can also be osmotically activated and can substitute for osm9 in touch-sensitive cells of *Caenorhabditis elegans*. A number of members of the TrpV family, including TrpV4 have been detected as mRNA transcripts in single nodose ganglion neurones projecting to the stomach.\(^9\) In Drosophila, TRP family members *nompc*\(^9\) and *Nanchung*\(^9\) have been implicated in mechanotransduction in chordotonal organs and Johnston’s organ respectively.

From this brief discussion, it is clear that a number of candidate ion channel subunits have been identified that are likely to be involved in mechanotransduction in mammalian and nonmammalian sensory neurones. One remaining challenge is to identify which are important in visceral mechanoreceptors from the vagus nerve. Furthermore, we need to understand how the presence of particular channels can be related to the sensory neurobiology of different functional regions of the gastrointestinal tract. This will require an account of how mechanosensitive ion channels are physically coupled to the surrounding structures. The mechanotransduction complexes represent potential targets for novel drugs that could selectively interfere with vagal afferent activity in a number of disease states.

**ACKNOWLEDGMENTS**

This work was supported by DK 56986 from the National Institutes of Health of the USA. SJHB is a Senior Research Fellow of the National Health and Medical Research Council of Australia.
REFERENCES

7 Chemical Transduction in Vagal Afferent Nerve Endings

Michael J. Carr

CONTENTS

7.1 Introduction ..................................................................................................168
7.2 Stages of Chemotransduction ......................................................................168
  7.2.1 Transduction .........................................................................................168
  7.2.2 Activation .........................................................................................168
  7.2.3 Amplification .....................................................................................169
  7.2.4 Modulation .......................................................................................171
7.3 Chemotransducers ........................................................................................171
  7.3.1 Ionotropic Receptors ........................................................................171
    7.3.1.1 Transient Receptor Potential (TRP) Family of Channels ............171
    7.3.1.2 ASICs (Acid Sensitive Ion Channels) ........................................174
    7.3.1.3 5-HT3 Receptor .......................................................................174
    7.3.1.4 Ionotropic Purine (P2X) Receptors ........................................175
    7.3.1.5 Nicotinic Acetylcholine (nACh) Receptor ............................176
  7.3.2 Metabotropic Receptors ...................................................................176
    7.3.2.1 Bradykinin B$_2$ Receptor ......................................................177
    7.3.2.2 Cholecystokinin (CCK) Receptors ...........................................177
    7.3.2.3 Neurokinin (NK) Receptors ...................................................178
    7.3.2.4 Histamine H$_1$ Receptors .....................................................178
    7.3.2.5 Eicosanoid Receptors ............................................................180
    7.3.2.6 ORL-1 (Nociceptin) Receptor .................................................180
    7.3.2.7 Opioid Receptors ....................................................................181
    7.3.2.8 GABA$_B$ Receptors ...............................................................181
    7.3.2.9 Cannabinoid (CB) Receptors ..................................................181
7.4 Summary ......................................................................................................182
References ..............................................................................................................182
7.1 INTRODUCTION

Chemical transduction in afferent nerve terminals is the process by which afferent neurons detect encounters of their receptive endings with chemical molecules and encode this encounter into electrical signals. The resulting changes may activate the nerve ending, i.e., evoke action potentials that are carried toward the central terminal of the neuron, or may cause subthreshold changes in the terminal that increase or decrease the sensitivity of the ending to other stimuli. Chemicals that act on vagal afferent terminals may be external in origin or they may be produced endogenously and play a role in normal physiology or pathophysiology.

In vagal afferent neurons as in other afferent neurons, detection and transduction are thought to take place at specialized sites within the peripheral terminal. In some cases, elaborate perireceptor structures comprised of highly specialized detector cells, such as those found in neuroepithelial bodies, are thought to be primary chemotransducers. When activated, these specialized accessory structures release chemical transmitters that then act on the vagal afferent neurons that innervate them. Although these accessory structures are of unquestionable importance, the current discussion is limited to chemotransduction that takes place within the nerve. After a description of the stages of chemical transduction in vagal afferent neurons, a few examples of chemotransducers involved in activation and modulation of vagal afferent nerve terminals are discussed. Vagal afferent neurons innervate a variety of distinct tissues and organs and it is, therefore, no surprise that not all vagal afferent neurons respond to the same chemicals. The properties of tissue- and organ-specific vagal afferent neurons are expertly reviewed in other chapters of this volume.

7.2 STAGES OF CHEMOTRANSDUCTION

7.2.1 TRANSDUCTION

In order for the peripheral terminals of a vagal afferent neuron to detect the presence of a particular chemical, it must possess a transducer for that chemical. The detection of a particular chemical may lead to action potential discharge, referred to here as “activation.” Many chemicals that activate vagal afferent nerve endings are thought to do so via ionotropic receptors whose gating can be initiated by direct interaction of the chemical with a ligand-binding site on the channel. In addition, vagal afferent neurons express a variety of metabotropic receptors, such as members of the G-protein coupled receptor superfamily, that once occupied by agonist initiate a cascade of events with the nerve terminal leading to decreased or increased sensitivity of the nerve terminal or, in some cases, initiate action potential generation. Several examples of ionotropic and metabotropic transducers are discussed in the following sections.

7.2.2 ACTIVATION

The peripheral terminal of vagal afferent neurons are typically embedded in tissue and it is, therefore, difficult to directly experiment on them. Because of this, there is little known about how the peripheral terminals of vagal afferent nerve endings
encode encounters with chemical stimuli into frequency-coded action potentials. However, based on a burgeoning body of evidence from other sensory systems, certain fundamental concepts have emerged that are likely to be shared with vagal afferent neurons.

The encounter of the afferent nerve ending with a stimulus is transduced, presumably at specialized sites in the endings (sensory receptors) into a membrane depolarization. The evoked membrane depolarization is a nonpropagated potential, similar to a synaptic potential. It is referred to as a receptor potential or generator potential. If the generator potential is of sufficient magnitude, the initiation of an action potential occurs. The conduction of these neural signals along axons involves classical voltage-gate currents. In some afferent fibers, generator potential formation and action potential formation may occur at anatomically distinct sites. The anatomical arrangement of the generator initiation site and the spike initiation site in the peripheral terminals of vagal afferent neurons is largely unknown.

There is a close correlation between the amplitude of the generator potential and the frequency of action potential discharge. The generator potential is graded in amplitude in relation to the stimulus, and activation of the nerve ending involves transformation of information from the continuously varying mode of a sensory stimuli into the neural mode of all-or-nothing action potentials. The intervals between action potentials vary in relation to the underlying depolarizing level of the generator potential and its rate of change. In this way, the discharge of action potentials encodes the parameters of the encounter with the stimulus. An increase in the intensity of the stimulus produces an increase in the frequency of action potentials (Figure 7.1). This relationship has been studied in detail for the crayfish stretch receptor and the photoreceptor in the horseshoe crab. In both of these systems, the frequency of discharge is proportional to the log of the stimulus intensity. The log function takes place at the transducer stage, i.e., the generator potential amplitude is a function of the log stimulus intensity while the frequency of action potential discharge is linearly related to the generator potential amplitude.

### Amplification

In some instances, the generator potential evoked by chemical stimuli may be very weak and may require amplification in order to provide the required change in membrane potential to reach threshold for action potential generation. Knowledge of generator potential amplification in vagal afferent terminal is lacking, however, recent descriptions of voltage-gated sodium channels with unique properties consistent with a role in amplification of generator potentials have been described in dorsal root ganglion derived afferent neurons.

Voltage-gated sodium channels are classically considered to carry the fast, transiently active current responsible for the propagation of action potentials. More recently, it has become evident that some neuronal voltage-gated sodium currents function within a voltage range that is sub-threshold for action potential generation. This ability to carry current at potentials around resting membrane potential has led to the hypothesis that voltage-gated sodium channels act not only in the fast all-or-none mode responsible for propagation but that they may also act to amplify...
Advances in Vagal Afferent Neurobiology

generator potentials. The best understood example of a channel that may act in this way is Na\textsubscript{v}1.7 (also known as PN1, NaS, or hNE-Na). This channel does not appear to be expressed in the central nervous system and its expression in the peripheral nervous system is restricted predominately to sensory neurons.\textsuperscript{6} The response of Na\textsubscript{v}1.7 to changes in membrane potential differs from that of classical neuronal sodium channels. The classical, fast, voltage-gated sodium current that carries the action potential requires a sudden, relatively large depolarization in order to be activated, the channels that carry these fast currents do not appear to be activated by slow changes in membrane potential. Thus, they may not open in response to slow ramp-like depolarization close to resting membrane potential such as those provided by the generator potential. In contrast, when expressed in HEK239 cells, Na\textsubscript{v}1.7 opens in response to slow ramp-like changes in membrane potential designed to mimic generator potentials.\textsuperscript{7} This distinctive property suggests that Na\textsubscript{v}1.7 channels in sensory neurons respond to small depolarizing inputs and act to amplify them. Consistent with this role, the Na\textsubscript{v}1.7 channel is localized to the distal end of spinal sensory neurons in culture.\textsuperscript{6} There have been no reports directly assessing the role of the currents carried by Na\textsubscript{v}1.7 or channels with similar characteristics in vagal afferent nerve endings. However, if these or similar channels are located at the peripheral terminals of nerve endings in vagal afferents, they would be suitably located to act in the transduction process.

FIGURE 7.1 Schematic representation of the steps in intensity coding in a sensory receptor. The upper diagrams show the relationship between an applied stimulus of ×1 and ×10 intensity to a generator potential and the relationship of the generator potential to the frequency of action potential discharge. Transduction involves the transformation of information in the graded generator potential to the all-or-nothing mode of action potential discharge. The lower diagram illustrates that the frequency of action potential discharge is proportional to the \log_{10} of the stimulus intensity. The \log_{10} function takes place at the transducer stage; i.e., the generator potential is a function of the \log_{10} stimulus intensity, while the frequency of action potential discharge is linearly related to the generator potential amplitude.
7.2.4 Modulation

The sensitivity of some subsets of afferents is not fixed but rather may be subject to modulation following activation of various chemotransduction pathways within the nerve terminals. For example, electrophysiological studies of vagal primary afferent neurons have revealed an effect of a variety of inflammatory mediators on ion currents that would be consistent with increasing afferent excitability. Activation of various metabotropic receptors expressed at the peripheral terminal of vagal afferents may decrease the excitability of these endings. Such modulation can result in changes in the threshold for action potential generation, an increase or decrease in the number of spikes evoked and an increase or decrease in the frequency of action potential discharge.

The ionic mechanisms by which various chemicals modulate the excitability of vagal afferent nerve endings are likely to be complex. The currents most likely to be influenced during these processes are carried by Na\(^+\), K\(^+\) and potentially Cl\(^-\) ions. Examples of transducers that play a role in the modulation of the excitability of vagal afferent nerve endings are discussed in the following sections. A detailed account of the roles of specific channels controlling the excitability of vagal afferent nerve endings is beyond the scope of the current discussion and is expertly reviewed in other chapters in this volume.

7.3 Chemotransducers

A variety of chemicals interact with various transducers leading to action potential discharge in vagal afferent neurons or modulation of their responsiveness. The following discussion focuses on a few examples that, while still not completely understood, provide examples of ionotropic and metabotropic chemotransducers thought to be important in the activation and modulation of vagal afferent nerve ending responsiveness.

7.3.1 Ionotropic Receptors

Many of the molecular structures that act as transducers in primary afferent neurons are thought to be ion channels. Each ion channel is specifically responsive to some stimulus, a chemical or a physical perturbation. A channel's response to a stimulus is an opening or closing of the pore, regulating passage of a restricted group of ions. Examples of ligand-gated ion channels in vagal sensory neurons include members of the Transient Receptor Potential (TRP) family of channels, the 5-hydroxytryptamine-3 (5-HT\(_3\)) receptor, the P2X receptor, the nicotinic acetylcholine (nACh) receptor and channels of the Acid Sensitive Ion Channel (ASIC) family. Upon agonist binding to these receptors, their ion pore opens, allowing an influx of cations resulting in membrane depolarization and activation of the nerve ending.

7.3.1.1 Transient Receptor Potential (TRP) Family of Channels

Channels belonging to the TRP family are putative six-transmembrane proteins (Figure 7.2) that assemble as tetramers to form cation-permeable channels. In
general members of the family are nonselective with $P_{Ca}/P_{Na} \leq 10$, exceptions include the monovalent-selective TRPM4, and the $Ca^{2+}$-selective TRPV5 ($P_{Ca}/P_{Na} > 100$). Opening of TRP channels results in depolarization of cells and increases in intracellular $Ca^{2+}$ and/or $Na^+$. The known sensory functions of members of the TRP family are diverse and various members are expressed in neurons as well as a variety of non-neuronal cells. TRP channels do not have sharp voltage sensitivity, but they can be activated by intracellular second messengers, endogenous ligands such as protons, pheromones and amino acids, and exogenous ligands such as capsaicin and menthol, and environmental stimuli such as osmolarity, warmth, and cold. TRPV1 (previously know as VR1, the first cloned vanilloid receptor), is a ligand-gated cation channel that is activated by the vanilloids capsaicin and resiniferatoxin. TRPV1 mRNA is found predominately in nociceptive-like primary afferent neurons whose cell bodies reside in dorsal root, trigeminal, or nodose ganglia. An interesting feature of TRPV1 is that it is gated not only by ligands but also by heat. At normal physiological pH the TRPV1 pore is opened at temperatures above approximately 42°C. At low pH (6.3) however, the threshold for channel opening is reduced to approximately 30°C. So it appears that low pH sensitizes TRPV1, so it
may be activated at normal physiological temperatures. It has been proposed that
inflammatory pain may be at least partially due to this sensitization, as the pH in
inflamed tissues is known to be lower than that of uninflamed tissue. Such a role is
supported by studies in mice lacking TRPV1. The sensitivity of TRPV1 to heat
may also be modulated by intracellular second messenger such as phosphatidylinosi-
tol-4, 5-bisphosphate (PIP$_2$), protein kinase C, and 5, 12, and 15 lipoxygenase
products. These findings have lead to the hypothesis that TRPV1 can function in
neuronal metabotropic receptor-mediated sensitization and activation of afferent
nerve endings.

Recent studies suggest differences in the responsiveness of neural crest (jugular
ganglia)-derived and placode (nodose ganglia)-derived vagal afferent neurons to the
TRPV1 agonists capsaicin and olvanil. It was found that capsaicin evoked action
potential discharge in all intrapulmonary vagal C-fibers irrespective of their gangli-
onic origin. Olvanil also effectively evoke action potential discharge in all nodose
derived C-fibers in the lung with an intensity similar to that of the response evoked
by capsaicin. In contrast, only two of twelve jugular derived C-fibers were activated
by olvanil. Using whole-cell patch-clamp recording of retrogradely labeled vagal
neuron cell bodies, it was found that the vast majority of capsaicin-sensitive neurons
from both nodose and jugular ganglia responded to olvanil. These findings suggest
that jugular and nodose derived bronchopulmonary vagal C-fibers express a capsaicin
receptor that can be activated by either capsaicin or olvanil. However, the peripheral
nerve terminals of jugular-derived fibers in the airways are sensitive to capsaicin but
insensitive to olvanil. The reason for this is unknown and is difficult to reconcile
with the established efficacy of these compounds at human, rat, and guinea pig
cloned TRPV1. Andersson and co-workers suggested that the lack of effect of olvanil
may be due to the absence of a transport system necessary for olvanil, but not
capsaicin to enter afferent neuron terminals to access the TRPV1 intracellular agonist
binding site. Alternatively, the different kinetics of olvanil and capsaicin-evoked
ion currents in sensory neurons may be responsible.

TRPV4 (also known as OTRPC4 and VR-OAC) is expressed in a variety of cell
and tissue types including whole nodose ganglia and single nodose cell bodies that
had been retrogradely labeled from the upper gut. In addition to being gated by
physical stimuli such as innocuous (27°C) heat and cell swelling, TRPV4 can be
activated by ligands and sensitized by inflammatory mediators. Hypotonicity was
demonstrated to increase TRPV4-mediated current in nociceptive primary afferent
neurons and this response was sensitized by prostaglandin E$_2$. Phorbol derivatives
such as 4α-phorbol 12,13-didecanoate also activated TRPV4-mediated currents inde-
dependently from activation of protein kinase C, in a manner consistent with direct
agonist gating of the channel. The endocannabinoid anandamide and its metabolite
arachidonic acid also activated TRPV4, but in an indirect manner involving the
cytochrome P450 epoxyenose-dependent formation of epoxyeicosatrienoic acids.

TRMP8 (also known as Trp-p8 and CMR1) was shown by RT-PCR to be
expressed in whole-nodose ganglia. This member of the TRP family is activated
by cold (8°C to 28°C) and by cooling compounds like menthol and icilin. Although
the exact role of TRMP8 in the vagal afferent system is yet to be established, it may
be the transducer responsible for the activation of airway cold “receptors” by
menthol\textsuperscript{33} the activation of which may subsequently reduce the respiratory discomfort associated with loaded breathing\textsuperscript{34} or inhibit cough.\textsuperscript{35}

7.3.1.2 ASICs (Acid Sensitive Ion Channels)

The acid-sensitivity of vagal afferent neurons innervating cardiac tissue and the gastrointestinal and respiratory tracts has been of particular interest.\textsuperscript{36–38} At least two families of proton-gated ion channels are expressed by afferent neurons, the TRP family in particular TRPV1 (see above) and the ASIC family. ASICs belong to the family of voltage-insensitive, amiloride sensitive epithelial sodium channel/degenerin cation channels.\textsuperscript{39}

ASICs are formed by subunits with two membrane-spanning domains (Figure 7.2) that form homo- or hetero-multimeric channels, which differ in their sensitivity to protons and pharmacological blockers.\textsuperscript{39} Members of this family also differ in their activation time course. For example ASIC1a and ASIC1b respond to an external pH of around 6.9 and give rise to a rapidly inactivating current.\textsuperscript{40} In contrast, ASIC2b/ASIC3 heteromers respond to a drop in external pH by generating a biphasic current consisting of a fast inactivating and a sustained current.\textsuperscript{41} ASIC channels are highly expressed in nociceptors and some other sensory neurons including vagal afferents.\textsuperscript{41}

ASICs open when extracellular pH drops and the currents they carry are enhanced by lactate, making them specialized for detecting lactic acidosis. The mechanism of proton-induced gating has been investigated in ASIC3.\textsuperscript{42} Extracellular protons open ASIC3 by speeding release of Ca\textsuperscript{2+} from a high-affinity binding site on the extracellular side of the pore. The bound Ca\textsuperscript{2+} blocks permeation and the channel conducts when multiple H\textsuperscript{+} ions relieve this block. Activation through Ca\textsuperscript{2+} may explain the sensitivity to lactate, which binds Ca\textsuperscript{2+} and thus decreases the extracellular free Ca\textsuperscript{2+} concentration.\textsuperscript{43,44}

7.3.1.3 5-HT\textsubscript{3} Receptor

Intestinal serotonin (5-hydroxytryptamine, 5-HT) acts as a paracrine substance to mediate vagal signal transmission evoked by luminal factors.\textsuperscript{44} In addition, 5-HT and the 5-HT receptor agonist phenyl biguanidine are among the most commonly used chemicals in the study of the activation of pulmonary and bronchial vagal afferent A\textsubscript{δ} and C-fibers.\textsuperscript{45} The activation of vagal afferent neurones by these agonists may occur via the 5-HT\textsubscript{3} receptor, a ligand-gated ion channel that is permeable to sodium and potassium ions.\textsuperscript{8,46–48}

The biophysical and pharmacological properties of 5-HT-evoked currents in rabbit nodose ganglion neurones have been investigated in whole-cell and outside-out membrane patch recording.\textsuperscript{46} In approximately half of the neurones, application of 5-HT elicited an inward current that reversed at approximately −2 mV consistent with activation of a mixed cation current. This was confirmed by ion substitutions that established that the current was mainly mediated by a mixed Na\textsuperscript{+}, K\textsuperscript{+} cation conductance with little or no contribution from Cl\textsuperscript{−} ions. On isolated outside-out membrane patches 5-HT-induced single channel currents exhibited modest inward
rectification. Whole-cell inward currents were concentration-dependently antago-
nized by the selective 5-HT\textsubscript{3} receptor antagonists tropisetron, ondansetron, and bemesetron.\textsuperscript{46} These findings are consistent with a role for a mixed cation current carried by the 5HT\textsubscript{3} channel mediating the excitatory effect of 5-HT on vagal afferent terminals.

7.3.1.4 Ionotropic Purine (P2X) Receptors

Adenosine triphosphate (ATP) activates a subset of vagal afferent nerve fibers. Purines can activate both ionotropic and metabotropic receptors. The ionotropic receptors belong to the P2X family. The seven cloned subunits (P2X\textsubscript{1-7}) are predicted to have two transmembrane spanning domains (Figure 7.2) and can assemble to form homoeric and heteromeric receptors.\textsuperscript{49}

ATP-evoked currents in sensory neurons are non-selective for the cations Na\textsuperscript{2+}, Ca\textsuperscript{2+} and K\textsuperscript{+} and show strong inward rectification.\textsuperscript{49} Afferent neurons express mRNAs for multiple P2X subunits and functional studies on neuron cell bodies from dorsal root ganglia and nodose ganglia have revealed differences in the relative contribution of P2X receptor subunits to ATP evoked currents in these two populations. In contrast to dorsal root ganglion neurons, that respond to ATP with transient, persistent or biphasic inward current\textsuperscript{49-52} rat nodose neurons responded to ATP and its analogue -methylene ATP (\(\alpha\)-meATP) with sustained responses. However, in these neurons, biphasic inhibition curves were obtained with 2',3'-O-trinitrophenyl-ATP (TNP-ATP), an antagonist which is selective for P2X\textsubscript{1}, P2X\textsubscript{3} and P2X\textsubscript{2/3} receptors. Thus, more than one form of multimeric P2X receptor channels are functionally expressed on the cell bodies of individual nodose ganglion neurones.\textsuperscript{53}

The lack of subunit selective agonists/antagonists has so far prevented the direct pharmacological characterization of this mixed population of receptors. P2X mediated responses have however been examined in P2X3 deficient mice.\textsuperscript{54} It was found that that all P2X3-/dorsal root ganglion neurons lacked rapidly desensitizing responses to ATP, and both dorsal root ganglion and nodose neurons from P2X3-/mice no longer responded to \(\alpha\),\(\beta\)-methylene ATP. In contrast, ATP-evoked a persistent inward current in 12% of dorsal root ganglion neurons and 84% of nodose neurons from P2X3-/ mice. Therefore, the \(\alpha\),\(\beta\)-methylene ATP-insensitive persistent responses in nodose neurons was likely to be mediated by P2X2 homomers, which contributed to about 60% of currents evoked by ATP in the wild type.\textsuperscript{54}

Experiments using an \textit{ex-vivo}, vagally innervated lung preparation have shown differences in the sensitivity of two distinct populations of vagal afferent neurons (jugular and nodose derived neurons) to P2X receptor agonists.\textsuperscript{55} The nerve terminals within the lungs of both nodose and jugular C-fibers responded with action potential discharge to capsaicin and bradykinin application, but only the nodose C-fiber population responded with action potential discharge to the P2X selective receptor agonist \(\alpha\),\(\beta\)-methylene-ATP. Whole cell patch clamp recording of capsaicin-sensitive nodose and jugular ganglion neurons retrogradely labeled from the lung tissue revealed that, like the nerve terminals, lung-specific nodose C-fiber neurons express functional P2X receptors, whereas lung specific jugular C-fibers did not.\textsuperscript{55}
Advances in Vagal Afferent Neurobiology

7.3.1.5 Nicotinic Acetylcholine (nACh) Receptor

The nACh receptor is a pentametric assembly of subunits that traverse the cell membrane four times (Figure 7.2).\(^{56}\) Using an RNase protection assay it was established that nodose ganglion neurons express at least six nACh receptor transcripts: \(\alpha_3, \alpha_5, \alpha_7, \beta_2, \beta_3\) and \(\beta_4\). mRNA for \(\beta_2\) is most abundant, the levels of \(\alpha_5, \beta_3\) were at the limit of detection. In contrast, in sympathetic neurons \(\alpha_3, \alpha_7\) and \(\beta_4\) were the most abundant, and \(\beta_3\) was below the limit of detection.\(^{57}\) The expression of nACh receptors in nodose afferent neurons is consistent with the observation that acetylcholine can evoke action potential discharge in baroreceptor afferents.\(^{58}\) With respect to vagal afferents that innervate the respiratory tract, concentrations of nicotine required to activate the nerve endings can be achieved by inhalation of smoke from high-nicotine containing cigarettes.\(^{59}\) Also consistent with the hypothesis of nicotinic receptors on airway afferent nerves are results from psychometric studies in humans\(^{28}\) that showed that blockade of nicotinic receptors in the airways inhibits the sensation of irritation caused by cigarette smoke in normally nonsmoking volunteers.

As discussed by Cooper, nACh receptors have several properties that make them ideally suited to act as transducers in afferent nerve terminals.\(^{60}\) First, they have a relatively large single channel conductance. Second, they allow ions to flow into the cell at negative membrane potentials, but do not allow ions to flow out of the cell at positive membrane potentials (inward rectification). Combined, these characteristics suggest that relatively few nACh channels need to be opened in order to produce a depolarization large enough to reach threshold for action potential initiation and that the strong inward rectification prevents ion flow through nACh receptors from short-circuiting the action potential.

The mechanism underlying the strong inward rectification has been investigated. It does not appear to be a property of the channels themselves, as current carried by them in isolated patches show little or no rectification.\(^{61,62}\) These findings suggest that the rectification is the result of a block of the channel by intracellular molecules. In this respect, it has been shown that neuronal nACh receptors are blocked by spermine, a polyaminepolyamine found in high concentration inside cells, in a voltage dependent manner,\(^{63}\) a finding that could account for the rectification of macroscopic acetylcholine evoked currents.\(^{60}\)

7.3.2 Metabotropic Receptors

A variety of metabotropic receptors are expressed by vagal primary afferent neurons. When occupied by agonist, some of these receptors activate second messenger pathways within the nerve terminal that lead to action potential discharge. Activation of metabotropic receptors may not lead directly to action potential discharge, but may increase or decrease the responsiveness of the nerve terminal to stimuli that do directly evoke action potential discharge. Metabotropic receptors expressed by vagal afferent neurons include receptors for bradykinin, cholecystokinin neurokinins, histamine, eicosanoids, nociceptin, opiates, GABA, and cannabinoids.
7.3.2.1 Bradykinin B$_2$ Receptor

The nonapeptide bradykinin is generated during inflammation from high- and low-weight molecular weight kininogen precursors present in plasma and tissue. Bradykinin is allogenic and leads to action potential discharge in a subset of nociceptive neurons whose cell bodies reside in dorsal root ganglia. Bradykinin also evokes action potential discharge in a subset of vagal afferent neurons. This effect of bradykinin is blocked by the B$_2$ receptor-selective antagonist, HOE140 and is selective for C- and A$_\delta$-nociceptive-like fibers.

The ionic mechanism underlying B$_2$ receptor mediated activation of airway afferent nerve fibers is unknown. Bradykinin B$_2$ receptors have been localized autoradiographically in human nodose ganglion cell bodies. Bradykinin B$_2$ receptor stimulation depolarizes the membrane potential of nodose ganglion neuron cell bodies and inhibits a calcium-dependent potassium current responsible for an after-spike hyperpolarization. Both of these effects are mediated by bradykinin B$_2$ receptors, although the latter effect on the after-spike-hyperpolarization appears to be secondary to prostacyclin (PGI$_2$) production by the neuron.

Stimulation of G-protein coupled receptors such as the bradykinin B$_2$ receptor may also lead to TRPV1 activation indirectly by undefined signaling pathways that may include lipid second messengers and/or release from PIP$_2$ mediated inhibition. Consistent with a role of TRPV1 in B$_2$ receptor-mediated activation of vagal afferent nerve endings, the number of bradykinin-induced action potentials evoked in airway vagal C-fibers was significantly inhibited by the TRPV1 antagonists capsazepine or ruthenium red, and by inhibitors of lipooxygenase enzymes. A role for TRPV1 has also been investigated in isolated airway preparations from TRPV1 knock out mice. In the TRPV1+/+ mice, B$_2$ receptor-mediated activation of vagal afferents by bradykinin was restricted to the capsaicin-sensitive C-fibers. Although bradykinin was effective in evoking B$_2$ receptor-mediated action potential discharge in TRPV1−/− C-fibers, the response was significantly less persistent than in TRPV1+/+ C-fibers. These findings suggest that TRPV1, may be a point of convergence of secondary intracellular mediators activated by agonist occupation of metabotropic receptors in afferent vagal nerve terminals.

7.3.2.2 Cholecystokinin (CCK) Receptors

Cholecystokinin is an important satiety factor, acting via the vagus nerve to influence central feeding centers. Using in-situ hybridization, expression of the CCK$_A$ and CCK$_B$ receptors in the nodose ganglion of rats has been investigated; approximately 30 percent of the neuron profiles contained CCK$_A$ mRNA while only approximately 10 percent contained CCK$_B$ mRNA. Consistent with the dominant expression of CCK$_A$ receptors CCK caused concentration-dependent depolarizations when superfused over the nodose ganglion and both the CCK$_A$ antagonists PD140548 and SR 27897B caused significant rightward shifts in the concentration response curve to CCK.

The precise sequence of events leading to action potential discharge following activation of CCK receptors in vagal afferent nerve terminals is unknown. The
cholecystokinin octapeptide (CCK-8) evoked in the majority of rat nodose ganglion cells, a rapid depolarization associated with a fall of membrane resistance and in a few cells a slow depolarization accompanied by an increase of membrane resistance.\textsuperscript{77} The rapid depolarizations were increased and decreased by membrane hyperpolarization and depolarization and the extrapolated reversal potential was about -10 mV. The response was depressed in a sodium-free solution and by d-tubocurarine but not by a chloride-deficient solution. It was concluded that CCK-8 depolarized the nodose ganglion cells by increasing cation conductances and in a few cells it also produced a slow excitation, the mechanism of which remains to be established.\textsuperscript{77}

### 7.3.2.3 Neurokinin (NK) Receptors

Neurokinins such as substance P are released from a subset of vagal afferent neurons. In addition, these neuropeptides can evoke NK\textsubscript{1} and NK\textsubscript{2} receptor-mediated responses in vagal afferent neurons.\textsuperscript{78,79} Depending on the species, NK\textsubscript{1} receptor agonists have been found to depolarize\textsuperscript{80} or hyperpolarize\textsuperscript{81} the membrane potential of nodose ganglion neuron cell bodies. The hyperpolarization of ferret nodose ganglion neurons is secondary to activation of a calcium-gated potassium current.\textsuperscript{81} In addition, NK\textsubscript{1} is functionally coupled to a slowly developing, noninactivating inward current referred to as a hyperpolarization-activated cation current.\textsuperscript{78} Thus NK\textsubscript{1} receptor activation in ferret vagal ganglia leads not only to a hyperpolarization of the membrane potential, but may also enhance synergistically this inhibition effect by decreasing the magnitude of the hyperpolarization activated cation current.

NK\textsubscript{2} receptor agonists depolarize guinea pig nodose ganglion cell bodies secondary to an increase in a nonselective cation current.\textsuperscript{82} Interestingly, this effect is “unmasked” by 5-HT\textsuperscript{82} and inflammatory mediators released during allergen-induced activation of mast cells\textsuperscript{83} The identity of the channel that carries this current or the second messengers involved in its activation are unknown.

The hypothesis that NK\textsubscript{2} receptors can signal TRP channels was tested using whole-cell patch-clamp recordings of HEK293 cells stably transfected with the human TRP3 channels and transiently transfected with a functional NK\textsubscript{2} receptors.\textsuperscript{84} Substance P induced an inward cation current in the cells expressing both TRP3 and NK\textsubscript{2}. These findings indicate that NK\textsubscript{2} can be functionally coupled to TRP channels in HEK293 cells and suggest that substance P-induced cation currents in vagal primary sensory neurons might be mediated by TRP channels.\textsuperscript{84}

### 7.3.2.4 Histamine H\textsubscript{1} Receptors

Histamine plays an important role in allergic responses. A direct action of histamine on vagal afferent neurons is consistent with H\textsubscript{1} receptor-induced membrane depolarization of a subpopulation of neuron cell bodies from a variety of species. However, unlike ligand-gated ion channel agonists and metabotropic bradykinin B\textsubscript{2} receptor mediated responses, histamine-induced membrane depolarization was typically associated with a decrease in ion conductance.\textsuperscript{8,85} This is consistent with H\textsubscript{1} receptor-induced inhibition of a resting potassium current in nodose ganglion neuronal cell bodies (Figure 7.3).
Histamine appears to directly activate vagal afferent fibers innervating the rat jejunum\textsuperscript{86} and epicardial tissue in guinea pigs,\textsuperscript{87} but did not evoke action potential discharge in vagal afferent neurons. Histamine-induced membrane depolarization is typically associated with a decrease in ion conductance, consistent with H\textsubscript{1} receptor-induced inhibition of a resting potassium current (IK\textsubscript{rest}) in nodose ganglion neuron cell bodies. In addition, H\textsubscript{1} receptors and receptors for a variety of other inflammatory mediators, such as bradykinin, prostacyclin, PGE\textsubscript{2}, PGD\textsubscript{2}, 5-HT, or leukotriene C\textsubscript{4} are coupled to effector systems that inhibit a slowly developing, long-lasting afterspike hyperpolarization (IAHP\textsubscript{slow}). The IAHP\textsubscript{slow} is initiated by the opening of N-type voltage-gated Ca\textsuperscript{2+} (N-Type Ca\textsuperscript{2+}) channels during action potentials, followed by the release of Ca\textsuperscript{2+} from intracellular Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+}-release (CICR) stores, which, in turn, stimulates the opening of calcium-activated potassium channels (SK\textsuperscript{+}). The hyperpolarization caused by K\textsuperscript{+} efflux regulates neuronal excitability and the frequency and pattern of neuronal discharge.

Histamine appears to directly activate vagal afferent fibers innervating the rat jejunum\textsuperscript{86} and epicardial tissue in guinea pigs,\textsuperscript{87} but did not evoke action potential discharge in vagal afferent fibers innervating guinea pig isolated airway preparations.\textsuperscript{86,88} Histamine (and other inflammatory mediators) may not need to evoke action potential discharge in order to influence the function of vagal afferent neurons. For example, activation of H\textsubscript{1} receptors in vagal ganglion cell bodies from some species inhibits voltage-gated calcium currents and the calcium-activated potassium currents that subserve membrane hyperpolarizations that occur after action potentials.\textsuperscript{85,89} One of these is a slowly developing, long-lasting afterspike hyperpolarization (AHP\textsubscript{slow}).\textsuperscript{8,90} In vagal afferent nerves, the channels subserving the AHP\textsubscript{slow} appear similar to the SK1-type KCa.\textsuperscript{90} The AHP\textsubscript{slow} is initiated by the opening of voltage-gated Ca\textsuperscript{2+} channels during action potentials, followed by the release of Ca\textsuperscript{2+} from intracellular Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+}-release stores, which, in turn, stimulates the
Advances in Vagal Afferent Neurobiology

opening of KCa channels (Figure 7.3). The hyperpolarization caused by K+ efflux regulates neuronal excitability and the frequency and pattern of neuronal discharge. Both the amplitude and duration of AHP_{slow} in guinea-pig nodose cell bodies are significantly reduced following activation of H_{1} receptors and metabotropic receptors for other inflammatory mediators such as bradykinin, prostacyclin, PGE_{2}, PGD_{2} 5-HT or leukotriene C_{4}. The inhibition of AHP_{slow} in nodose cell bodies is accompanied by an increase in the frequency at which neurons could successfully elicit repetitive action potentials suggestive of an important role of this current in controlling vagal afferent neuron excitability.

7.3.2.5 Eicosanoid Receptors

Prostaglandins have long been recognized to activate or increase the excitability of afferent nerves. Electrophysiological studies on afferent ganglion neuron cell bodies have demonstrated excitatory affects of several prostaglandins including inhibition of calcium-activated potassium currents involved in the afterspike hyperpolarization, potentiation of hyperpolarization-activated cation currents and tetrodotoxin-resistant, voltage-gated sodium currents. If these effects occur at the nerve terminals, they would likely lead to an increase in the peak frequency of action potential discharge and decreased threshold. Consistent with this, in vivo studies revealed that low concentrations of PGE_{2} did not cause action potential discharge in vagal afferent nerves innervating rat airways, but effectively sensitized pulmonary C-fiber afferents to capsaicin or mechanical stimulation during lung inflation.

There has been little investigation into the potential role of cysteinyl leukotrienes (cys-LTs) on vagal afferent nerve activity. Cys-LTs inhibited the afterspike hyperpolarization in vagal sensory ganglion neuron cell bodies, and caused membrane depolarization of neuron cell bodies isolated from vagal afferent ganglia. The membrane depolarization was due to an inhibition of a resting potassium current and was blocked by the Cys-LT_{1} receptor antagonist, zafirlukast. Other lipoxygenase products, as discussed previously, may interact directly with members of the TRP channel family as discussed previously.

7.3.2.6 ORL-1 (Nociceptin) Receptor

Nociceptin is an opioid-like heptadecapeptide that is an endogenous ligand for the opioid receptor-like (OR-1) receptor. Among other tissues, including dorsal root ganglia, ORL-1 is expressed in vagal afferent ganglia. The observation that nociceptin inhibited cough and citric-acid-induced action potential discharge in jugular-derived vagal afferent nerve terminals innervating guinea pig isolated trachea is consistent with an inhibitory role of ORL-1 receptors expressed at the peripheral terminals of vagal afferent neurons.

The ion currents responsible for ORL-1-induced modulation of vagal afferent neuron excitability are unknown. Nociceptin has been reported to activate a K+ conductance in dorsal raphe neurons and in locus coeruleus neurons and suppressed high-voltage-activated Ca^{2+} channel conductances. In dorsal root ganglion neurons, nociceptin suppressed a low-voltage-activated, transient calcium current,
an effect associated with an inhibition of bursts of action potentials.\textsuperscript{102} Whole-cell patch-clamp experiments failed to provide evidence for G-protein involvement in nociceptin-induced suppression of this current.\textsuperscript{102} It is unknown whether a similar G-protein-independent mechanism is responsible for ORL-1 receptor-mediated inhibition of action potential discharge in vagal afferent nerve terminals.

### 7.3.2.7 Opioid Receptors

In addition to their established effects in the central nervous system, opiates may modulate the function of peripheral sensory neurons. Consistent with this µ-opioid receptors are present in vagal afferent neurons\textsuperscript{103} and mu-opioid agonists inhibited voltage-gated calcium currents in vagal afferent neurons.\textsuperscript{104,105} In guinea pig nodose ganglion neuron cell bodies, the opioid agonist, Tyr-D-Ala-Gly-MePhe-Gly-ol enkephalin had no effect on resting membrane current or on a hyperpolarization-activated cation current, but inhibited the ability of inflammatory mediators to enhance this current.\textsuperscript{106} Thus, opioids may inhibit the increased excitability of afferent nerves induced by mediators such as PGE\textsubscript{2}.\textsuperscript{107}

### 7.3.2.8 GABA\textsubscript{B} Receptors

The GABA\textsubscript{B} receptor is a member of the heptahelical transmembrane G protein-coupled receptor family. GABA\textsubscript{B} receptor agonists such as baclofen have been shown to reduce mechanosensitivity of some, but not all, vagal afferent fibers innervating isolated gastroesophageal tissue.\textsuperscript{108–110} The ionic mechanism underlying this effect is not clear, but electrophysiological studies suggest K\textsuperscript{+} and/or Ca\textsuperscript{2+} channels may be involved. In nodose cell bodies from guinea pigs, baclofen evoked a hyperpolarization in a subset of neurons\textsuperscript{108} consistent with the opening of a K\textsuperscript{+} conductance. In whole-cell recordings of nodose neurons from rats, baclofen inhibited Ca\textsuperscript{2+} currents carried by N-type, but not L-type channels.\textsuperscript{104}

### 7.3.2.9 Cannabinoid (CB) Receptors

Recent studies suggest that the effect of endogenous CBs on appetite may be at least partially mediated by vagal afferent neurons.\textsuperscript{111} In addition, CBs have been shown to inhibit vagal afferent nerve-mediated airway reflexes such as citric-acid-induced cough in guinea pigs.\textsuperscript{112} Two subtypes of CB receptor have been characterized, CB\textsubscript{1} and CB\textsubscript{2}. The latter appears to be expressed mainly by immune cells, whereas CB\textsubscript{1} is expressed by many central and peripheral neurons.\textsuperscript{113} RT-PCR indicated CB\textsubscript{1} and CB\textsubscript{2} expression in rat nodose ganglia.\textsuperscript{114}

The ion currents coupled to CB receptors in afferent neurons are largely unknown. In dorsal root ganglion neurons the CB receptor agonist (+)-WIN55212 inhibited voltage-activated Ca\textsuperscript{2+} currents.\textsuperscript{115} The effects of CB agonists on 5-HT\textsubscript{3} receptor-mediated currents were investigated in rat nodose ganglion neurons.\textsuperscript{116} Anandamide, Win 55212-2, and CP55940 inhibited the 5-HT-induced current in a concentration dependent manner. This inhibition was slowly developing, noncompetitive, not dependent on membrane potential, and not affected by adenosine 3',5'-cyclic monophosphate analogues. These observations suggest that the 5-HT\textsubscript{3}
receptor ion-channel is a site acted upon by CB agonists in the nervous system, and the action of CB agonists on 5-HT₃ receptors may be a possible mechanism for some of the behavioral effects of CBs, such as antiemesis and analgesia.¹¹⁶

7.4 SUMMARY

The peripheral terminals of vagal afferent neurons possess multiple metabotropic and ionotropic receptors that serve as transducers for chemical stimuli. Activation of these transducers results in the encoding of encounters with various chemicals. This interaction may evoke action potential discharge, however, a growing body of evidence indicates that various chemicals may also interact at various stages of activation and thus modulate the threshold and frequency of action potential discharge. The interaction between ionic pathways involved in activation and modulation is not completely understood and is based largely on inferential studies of neuronal cell bodies isolated from vagal afferent ganglia. A more accurate picture of chemical transduction in the peripheral terminals of vagal afferent neurons awaits the development of techniques that can directly access these microscopic structures.

REFERENCES


Part IV

Connection in the CNS
8 Synaptic Transmission in the Nucleus Tractus Solitarius (NTS)

Ann C. Bonham and Chao-Yin Chen

CONTENTS

8.1 Overview ...................................................................................................... 193
8.2 Experimental Approaches ............................................................................ 194
8.3 Core Components of Synaptic Transmission .............................................. 194
  8.3.1 The Input Signal............................................................................... 195
  8.3.2 The Role of the Second-Order Neuron ........................................... 195
  8.3.3 Glutamate Is the Cornerstone of Synaptic Transmission............... 195
  8.3.4 Presynaptic Depression .................................................................... 197
  8.3.5 Physiological Relevance of Synaptic Depression in the NTS ........ 198
  8.3.6 Postsynaptic Mechanisms for Modulating Synaptic Transmission ........................................ 199
    8.3.7 Intrinsic Properties.................................................................... 199
  8.4 Synaptic Plasticity...................................................................................... 200
  8.5 Summary ................................................................................................... 201
  8.6 Where to Next? ..................................................................................... 203
Acknowledgments.................................................................................................. 204
References.............................................................................................................. 204

8.1 OVERVIEW

The nucleus tractus solitarius (NTS) is the first CNS site for synaptic contact of the primary vagal afferent neurons. These synapses are the first site where the primary sensory input can be changed, and the signal conditioning here determines the NTS output to all downstream synapses in a wide array of autonomic reflex pathways. The second-order NTS neurons do not simply relay the input signal, but rather bringing to bear their own intrinsic and synaptic properties, the neurons integrate the sensory input with converging inputs from local networks, higher brain regions and circulating mediators, to ultimately orchestrate a coherent pattern of CNS responses for maintaining homeostasis.
8.2 EXPERIMENTAL APPROACHES

Ideally, experimental approaches to characterize synaptic transmission would cover all elements of the reflexes — the sensory endings, vagal afferent neurons, second-order neurons, local neural network in the NTS, higher brain regions that might modulate the reflex, output neurons, and target organs. At least for the present, this has not been uniformly possible for studying neurotransmission in the NTS. However, the use of the brainstem slice, which contains the primary neural network of the NTS, that is, the vagal afferent neurons coursing in the tractus solitarius (TS), the second-order neurons, and the local interneurons, has allowed for a more exact analysis of synaptic events. Whole-cell voltage-clamping at these synapses allows isolation of presynaptic and postsynaptic currents, without the confounding influences of changes in the postsynaptic membrane potential. There are challenges, however. Although the NTS is organized somewhat viscerotopically, neurons receiving distinct vagal afferent inputs and subserving different autonomic functions are intermingled throughout the nucleus. Thus, functionally and anatomically identifying the second-order neuron is critical for studying specific synapses. The use of lipophilic fluorescent tracers, such as, 1,1′-dioctadecyl 3,3,3′,3′ tetramethylindocarbo-cyanine perchlorate (DiI), which can be transported anterogradely to label terminal vagal boutons on the second-order NTS neurons without altering the basic biophysical properties of the neurons, has allowed for the functional and anatomical identification of the second-order neurons. By also taking advantage of a number of electrophysiological criteria for monosynaptic activation of the second-order NTS neurons, it is now possible to examine the synaptic response of a single neuron to input from a vagal afferent neuron under a variety of different conditions. With these approaches, it may be possible to determine the extent to which the signal processing varies depending on the particular synaptic partners and to determine the range of signal conditioning required at the NTS synapses to assure an appropriate output targeted to the correct distal pathways.

8.3 CORE COMPONENTS OF SYNAPTIC TRANSMISSION

The core components of the synaptic transmission are the vagal afferent neuron, which provides the input signal, the principal neurotransmitter, and the second-order NTS neuron, which processes the input signal. In general, these components may be altered by two fundamental mechanisms — presynaptic events, which can change the nature of the input signal by modifying neurotransmitter release, and postsynaptic events, which can add to or subtract from the primary activation of the second-order neuron. These mechanisms may be set in play by input from higher brain regions, from convergent inputs from other visceral or somatic inputs, and possibly by humoral agents, since regions of the NTS have an incomplete blood brain barrier, rendering the neurons accessible to bloodborne substances.
8.3.1 The Input Signal

At the most basic level, the input signal from the vagal neurons has been characterized based on the organ of origin, specialization of the sensory endings (i.e., mechanosensitive vs. chemosensitive), and whether the neurons are myelinated or unmyelinated as determined by conduction velocity. An additional layer of complexity of the input signal stems from differences in phenotype within the same type of vagal afferent neuron. This was first recognized by the Coleridges, who suggested that the vagal afferent C-fiber neurons in the lung parenchyma were distinct from those along the luminal surface of the airways. More recent studies have expanded on differential phenotypes based on ganglionic location, ionic composition, and neuropeptide content of the cell bodies of the afferent fibers and expression of receptors in their peripheral terminal endings. It seems likely that the input signals monitored by the second-order neurons will vary with the differences in the phenotypes of the vagal synaptic partners. Thus, based on the advances in our understanding of the vagal afferent neurons, presented in previous chapters, it seems axiomatic that the input signals from the presynaptic partner may be considerably more complex than once was appreciated and that the synaptic transmission may reflect these complexities.

8.3.2 The Role of the Second-Order Neuron

Presented with wide-ranging patterns and frequencies of sensory input from the vagal neurons, the role of the second-order NTS neuron is to:

1. Sort and process the input in such a way as to maintain the integrity of input relevant to each reflex (that is, for example, to avoid mixing up incoming cardiac and gastrointestinal vagal signals), while at the same time, allowing for the possibility that multiple inputs (i.e., regarding cardiovascular and respiratory status) might need to be coincidentally processed by the same neuron.
2. Integrate the information spatially and temporally with other inputs from local neural networks or from higher brain regions, including from the limbic forebrain circuits.
3. Ultimately, coordinate outputs to appropriate distal synapses. The intrinsic and synaptic properties of the second order neurons, which likely vary from neuron to neuron, will contribute to how the neuron carries out these signal-conditioning activities.

8.3.3 Glutamate Is the Cornerstone of Synaptic Transmission

The principle neurotransmitter is generally considered to be glutamate, based on classic evidence including ablation of the vagal afferent cell bodies in the nodose ganglion reducing the high-affinity uptake of L-glutamate in the NTS, injections of glutamate agonists in the NTS mimicking the action of glutamate release and glutamate ionotropic receptor antagonists significantly attenuating or abolishing
transmission between the primary vagal afferent neurons and second-order NTS neurons.\textsuperscript{18,19}

Glutamate activation of the ionotropic AMPA receptor which mediates the fast component of glutamate signaling is critical for the neurotransmission.\textsuperscript{18,20} However, this excitatory effect of AMPA can be further shaped by glutamate activation of its other receptors. One important feature of the glutamate signaling is that some second-order NTS neurons also possess functional NMDA receptors, the ionotropic glutamate receptor subtype which mediates a slower-developing, longer-lasting component of glutamate signaling.\textsuperscript{6,21,22} The physiological significance of the NMDA receptors depends to a large extent on the nature of the input signals.\textsuperscript{23} For example, because of the Mg\textsuperscript{2+} block of the NMDA receptor channel at resting membrane potentials, the NMDA receptors may only significantly contribute to synaptic transmission when the cell is depolarized and the Mg\textsuperscript{2+} block of the channel is relieved,\textsuperscript{24} such as occurs during high-frequency sensory input from vagal afferent neurons or under conditions in which excitatory inputs from other sources are integrated.\textsuperscript{25} Once glutamate binds to the NMDA receptors, the prolonged depolarization due to NMDA receptor currents lengthens the time during which otherwise ineffective inputs can be integrated with the primary vagal afferent input. In addition, the NMDA receptors allow the neurons to transduce sensory input, which has no apparent pattern, into patterns. For example, NMDA induces bursting in second-order NTS neurons,\textsuperscript{26,27} and converts irregular firing patterns into regular ones.\textsuperscript{28} The suggestion has been that the bursting pattern may optimize the signal for transmission to subsequent neurons. Finally, the presence of NMDA receptors, through Ca\textsuperscript{2+} activation of second-messenger cascades, provides a neural substrate for long-term modification of synaptic efficacy, that is, long-term potentiation\textsuperscript{29} and windup.\textsuperscript{30,31}

Layered over the synaptic transmission mediated by activation of the postsynaptic ionotropic glutamate receptors is the contribution of the G protein-coupled metabotropic glutamate receptors, which allow glutamate to further modulate its own signaling by both presynaptic and postsynaptic mechanisms. Presynaptic metabotropic glutamate receptors have been examined by RT-PCR and electrophysiological techniques. The gene expression for all eight metabotropic receptor subtypes belonging to the Group I (subtypes 1,5), II (subtypes 2,3) and III (subtypes 4,6,7,8) receptors has been demonstrated in the vagal afferent neuron cell bodies in the nodose and jugular ganglia, an essential step if the receptors are to be moved along their central axons and inserted at the terminals in the NTS.\textsuperscript{32} Whole-cell patch-clamping studies have demonstrated that the Group II and III, but not the Group I, metabotropic glutamate receptors decrease glutamate release in a frequency-dependent manner. At high, yet physiological relevant frequencies of vagal afferent input to the second-order neurons, the glutamate released from the central terminal is sufficient to spill back along the terminal to activate presynaptic Group II and III metabotropic glutamate receptors to put a brake on further glutamate release, while at low frequencies of vagal afferent activity, the signals pass through the synapse unaltered.\textsuperscript{32} Extrapolating from studies of neurons in the nodose ganglia, Hay and
colleagues suggested that one of the mechanisms for the Group III metabotropic glutamate receptors to decrease glutamate release may be through a frequency-dependent modulation of exocytosis. The presynaptic metabotropic glutamate receptors afford glutamate the capacity to depress its own release in a frequency-dependent manner, but as will be discussed later, presynaptic depression of glutamate release is a fundamental feature at the NTS synapses and can be triggered by several neuromodulators.

There are other sources of glutamate impinging at these first central synapses, beyond the glutamate released from activation of the vagal afferent neuron: 1) stimulation of glutamatergic interneurons in the local NTS network (perhaps by descending projections from higher brain regions), also provides monosynaptic excitatory inputs to the second-order neurons; 2) spontaneous glutamatergic synaptic inputs (spontaneous miniature excitatory postsynaptic currents (mEPSCs) provide a tonic low level of synaptic excitability absent stimulated inputs. These multiple modes for releasing glutamate allow for modulation of various aspects of the signal processing regardless of the state of the vagal afferent neuron.

### 8.3.4 Presynaptic Depression

In general, the presynaptic depression of glutamate release from the vagal afferent terminal is one of the most prominent mechanisms in shaping synaptic transmission in the NTS, having been demonstrated both in vitro with whole-cell patch clamping and intracellular recording\(^4,34-37\) 38-41 and in vivo with intracellular and extracellular recording.\(^42-46\) Experimental approaches have used classic electrophysiological methods to demonstrate synaptic depression. These include measuring changes in the amplitude of two consecutively evoked excitatory postsynaptic currents (eEPSCs) or potentials (eEPSPs); this is referred to as paired pulse depression when the amplitude of the second of the two eEPSCs or eEPSPs is smaller than the first or as frequency-dependent depression when the amplitude of the eEPSCs or eEPSPs decreases with trains of stimuli.\(^4,37\) Other approaches record decreases in the frequency of spontaneous synaptic currents,\(^2,40\) or simultaneous decays in the amplitudes of AMPA and NMDA receptor-mediated components of the eEPSCs.\(^36\) The synaptic depression fundamentally reflects a decreased probability of glutamate release,\(^47\) which may relate to changes in vesicle mobilization and depletion \(^48\)and presynaptic calcium current inactivation.\(^49\) These mechanisms are likely engaged by the number of neurotransmitters or neuromodulators shown to contribute to synaptic depression at the first NTS synapses.

Recent reviews have provided complete analyses of the many neuroactive agents and their potential roles in presynaptic and postsynaptic modulation of synaptic transmission.\(^50-52\) This chapter will describe the contributions of a few neuromodulators, which have been implicated by both anatomical and electrophysiological studies. The most ubiquitous of which is the inhibitory amino acid neurotransmitter, \(\lambda\)-aminobutyric acid (GABA).\(^53\) GABA is extensively distributed throughout the local network of interneurons strategically located near vagal afferent terminals to mediate synaptic depression. The inhibitory amino acid has been shown to modestly decrease glutamate release from vagal afferent terminals by binding to presynaptic
G-protein linked GABA$\textsubscript{B}$ receptors located on vagal afferent terminals.\textsuperscript{54,55} The widespread distribution of GABA terminals throughout the NTS suggests that GABA may also inhibit glutamate release at higher order neurons. Other neuromodulators demonstrated to presynaptically depress glutamate release from vagal afferent terminals include dopamine acting at D2 receptors,\textsuperscript{39} and adenosine acting at A1 receptors.\textsuperscript{40} We have recently shown an unexpected result that substance P, in addition to its well-known excitatory postsynaptic effect on NTS neurons, can decrease glutamate release from the presynaptic terminals by activating presynaptic NK1 receptors.\textsuperscript{2}

Such redundant mechanisms by the various neuromodulators to regulate glutamate release confer both flexibility and dependability for regulating synaptic traffic. The challenge is to determine under what conditions which mechanisms are most important in regulating glutamate release. It’s tempting to speculate that inputs from distinct higher brain centers or somatic afferent neurons may utilize different neurotransmitter pathways to regulate presynaptic glutamate release from the vagal terminals. There are relatively few studies on presynaptic facilitation of glutamate release in the NTS; however, ATP has been shown to increase glutamate release via activation of presynaptic purinergic (P2X) receptors based on increased frequencies of glutamatergic sEPSCs recorded in second-order neurons.\textsuperscript{40}

### 8.3.5 Physiological Relevance of Synaptic Depression in the NTS

The extensive observations of synaptic depression in the NTS, both in the whole animal and in the slice suggest that the phenomenon may be physiologically important in modulating synaptic transmission of many autonomic signals conveyed to the NTS.\textsuperscript{4,34-46} At a most basic level, synaptic depression, by serving as a low-pass filter, may improve signal transmission by allowing incoming signals with a wide dynamic range (which are not easily modulated) to be converted to signals with a smaller range (which are more easily modulated). In addition to optimizing signal transmission through filtering, the depression might carefully limit unnecessary glutamate release, which, in excess can be neurotoxic.\textsuperscript{56} Finally, the consequences may extend to the reflex output. By simultaneous recording NTS neuronal activity and lumbar sympathetic nerve activity, we showed that frequency-dependent depression of sensory input from primary aortic baroreceptor afferent fibers to second-order NTS neurons limited the magnitude of the reflex output, that is the decrease in sympathetic nerve activity. Based on curve-fitting analysis, the data predicted that prevention of synaptic depression of as little as 10% in the NTS would allow for a 19% greater reflex output, that is a 56% increase in the reflexly evoked decrease in sympathetic nerve activity.\textsuperscript{45} Thus, in autonomic regulation of blood pressure, synaptic depression at the NTS synapses during high-frequency afferent activity may serve to limit excessive fluctuations in the baroreceptor reflex-mediated changes in arterial blood pressure by limiting high-frequency signal transmission in the NTS.
8.3.6 Postsynaptic Mechanisms for Modulating Synaptic Transmission

Many of the same neurotransmitters or neuromodulators which presynaptically regulate glutamate release also exhibit postsynaptic effects that can either add to or subtract from the neuronal excitability. Here again, the most prominent inhibitory neurotransmitter is GABA. Electrophysiological data obtained in the slice suggest that second-order NTS neurons receive monosynaptic GABA inhibitory input in the form of evoked inhibitory postsynaptic currents (eIPSCs) from stimulating local GABA interneurons in the NTS and from spontaneous synaptic currents (mIPSCs). The eIPSCs and mIPSCs are mediated by activation of postsynaptic GABA\(\alpha\) receptors on the second-order neurons, thus providing a mechanism for GABA to act directly on the postsynaptic neuron to blunt neuronal excitability to synaptic input. Electrophysiological data obtained in vivo indicate that monosynaptic EPSPs evoked by stimulation of the vagal afferent neurons can sometimes be followed by polysynaptic inhibitory postsynaptic potentials (IPSPs), suggesting a network whereby activation of vagal afferent neurons may activate GABA interneurons over polysynaptic pathways to feedback onto the second order neurons.\(^{44,57}\) The notion is reinforced by the finding that blocking the initial glutamatergic transmission with the AMPA-receptor antagonist, 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(f)quinoxaline (NBQX), abolishes the GABA-mediated IPSPs. The physiological relevance of GABA modulation of the excitatory input is further buttressed by data showing that blockade of GABA\(\alpha\) receptors with bicuculline can convert an irregular firing pattern of the NTS neurons to a regular pattern.\(^{28}\)

In contrast to the uniform inhibitory effect of GABA, acting at presynaptic or postsynaptic sites, other neuromodulators that suppress glutamate from the presynaptic terminal can excite the second-order neurons via postsynaptic receptors. Evidence obtained from NTS injections of metabotropic glutamate receptor agonists suggests that activation of Group I metabotropic glutamate receptors on NTS neurons is excitatory;\(^{58,59}\) preliminary studies suggest that the Group I agonists induce a slowly developing depolarization and inward current.\(^{60}\) However, the receptor subtype may or may not be the same. For example, substance P activates postsynaptic NK1 receptors to depolarize the neurons;\(^{61-63}\) while activating presynaptic NK1 receptors on vagal afferent terminals to decrease glutamate release second-order neurons.\(^{2}\) While activation of presynaptic Group II metabotropic glutamate receptors suppresses glutamate release, at least in some postsynaptic neurons, preliminary studies suggest that activation of postsynaptic Group II metabotropic glutamate receptors on a subgroup of second-order NTS neurons is excitatory.\(^{60}\) A picture is emerging in which location of the neuromodulator receptor in the synapse and receptor subtype may provide offsetting mechanisms to modulate synaptic transmission, the balance of which may depend on the level and source of afferent traffic.

8.3.7 Intrinsic Properties

The intrinsic properties of the NTS neurons will also determine the excitability and the responsiveness to neuromodulators, and hence the NTS output. A number of ion
Advances in Vagal Afferent Neurobiology

channels have been characterized in NTS neurons, including the hyperpolarization-activated current I(h) current $^{64}$; transient outward K$^+$ current (I_{TOC})$^{65-71}$, the delayed outward rectifier (I_K)$^{68,71}$ and the small conductance, apamin sensitive Ca$^{2+}$-dependent K$^+$ current (slow I_{AHP}), which have also been implicated in baroreceptor signaling.$^{71,72}$

The relative densities and time- and voltage-dependent kinetics of certain ion channels may help to further distinguish NTS neurons. Distinct electrical phenotypes of NTS neurons have been designated based on different patterns of spiking in response to depolarizations and the extent of the delay in spiking onset following hyperpolarizations.$^{69,70,73}$ The phenotypes have been associated with qualitative differences in the density and kinetics of various currents. Recently, Bailey and colleagues linked the differential expression of I_{TOC} to differential processing of sensory signals conveyed by myelinated vs. unmyelinated vagal afferent neurons.$^{69}$ These studies attempting to further distinguish second-order neurons based on current densities or kinetics provide a new and exciting framework for further characterizing the responses of the second-order neurons to vagal afferent inputs based on their neuronal behavior and differential expression or kinetics of specific ion channels.

8.4 SYNAPTIC PLASTICITY

Aside from acute regulation of synaptic transmission, data are beginning to suggest that changes in the state of the organism may induce structural or functional changes at these first NTS synapses that might have long-term consequences. We have previously found in a primate model of allergic asthma, that extended, repeated exposure to an allergen resulted in an increased excitability of NTS neurons that met electrophysiological criteria for being second-order neurons.$^{74}$ The neurons exhibited a more depolarized resting membrane potential and an increased spiking response to intracellular injections of depolarizing currents. While the precise mechanisms were not determined, the changes could have been mediated by the changes in nature of the vagal afferent neuron input: previous studies of vagal afferent neuronal activity found that exposure to allergen and inflammatory mediators, resulted in an increase in the mRNA encoding substance P,$^{75}$ de novo substance P expression in A $\delta$ vagal afferent cell bodies$^{76}$ and increases in neuronal excitability, including depolarization of the membrane potential and blockade of an anomalous rectifier.$^{77}$ The phenotypic change in the cell bodies of vagal lung neurons raises the possibility that a change in the frequency, magnitude or pattern of the vagal afferent input could have triggered the postsynaptic changes in the postsynaptic neuron. The neuroplasticity of NTS neurons was also observed following episodic exposure to ozone, through complex changes in the synaptic and intrinsic excitability. Exposure of the primate to episodes of ozone resulted in a more depolarized membrane potential, increased membrane resistance, and increased neuronal spiking responses to depolarizing current injections, but at the same time decreased responsiveness to synaptic input from vagal afferent neuron stimulation.

The results, indicating a diminished responsiveness of the postsynaptic neuron to sensory transmission, despite overall increases in excitability, implicate several intriguing mechanisms.$^{78}$ Given that many candidate neurochemicals have been localized in vagal afferent neurons,$^{79-81}$ the findings could be explained by the
possibility that under certain conditions, modulators or transmitters other than glutamate may be important in synaptic transmission. The changes in the postsynaptic neurons could be mediated independently of the sensory neural pathways through changes in local synaptic inputs from other brain regions, including the adjacent area postrema, the most caudal of the circumventricular organs.\textsuperscript{14,15,82} By virtue of its lack of a blood brain barrier and its prominent axonal projections to the NTS, the area postrema provides an anatomical pathway whereby mediators released into the circulation can affect NTS neurons. Finally, regions of the NTS, also appear to lack a complete blood brain barrier and feature local complexes of fenestrated capillaries and perivascular spaces that afford the NTS neurons direct exposure to blood-borne mediators.\textsuperscript{10}

There is a precedent of long-lasting modifications of synaptic transmission during prolonged changes in the activity of synaptic partners in another sensory network that may be informative for considering synaptic plasticity in the NTS. Woolf and Doubell\textsuperscript{83,84} characterized neural mechanisms in the spinal cord underlying chronic neuropathic pain as a prolonged increase in the intrinsic postsynaptic excitability of spinal neurons that might be triggered in part by the combination of:

1. An induced novel input from peripheral A\textsubscript{δ} afferent fibers
2. An exaggerated input from the peripheral nociceptive C fibers
3. Local changes in inhibitory and excitatory synaptic inputs
4. Phenotypic changes in the postsynaptic neurons themselves

Other studies also demonstrate the capacity for plasticity in NTS neurons. Mei et al. showed that second-order baroreceptor neurons, which are only modestly sensitive to inhibitory influences by GABA\textsubscript{B} inputs in the normotensive state, become exquisitely sensitive after four weeks of a model of renal hypertension.\textsuperscript{85} The changes were observed only in second-order neurons, suggesting that the signal processing at this particular synapse was altered by exaggerated responsiveness to GABA\textsubscript{B} receptor activation. The data suggesting functional plasticity are further buttressed by findings that NTS neurons can undergo fine structural plasticity during prolonged excitatory inputs from baroreceptor afferent neurons, such as might occur during hypertension.\textsuperscript{86} Taken together, results from various laboratories suggest that long-term changes in synaptic transmission in the NTS may be important in helping to explain changes in neural control of autonomic function.

### 8.5 SUMMARY

Studies over the past two decades have changed our thinking about the NTS as simply a relay nucleus that relays the input signal from the peripheral sensors to an output signal on a one-to-one basis, to something much more complex and interesting. As illustrated in Figure 8.1, the cornerstone of synaptic transmission — glutamate activation of AMPA receptors — is subject to considerable transformation by presynaptic and postsynaptic events. The second-order NTS neurons, bringing to bear their own intrinsic and synaptic properties, integrate the sensory input with converging inputs from local networks, higher brain regions, and circulating
Cornerstone of synaptic transmission: Glutamate at AMPA-R

Mechanisms for shaping synaptic transmission

A. Presynaptic depression of TS-eEPSCs
B. Functional NMDA-R

C. Spontaneous and stimulated synaptic inputs via local interneurons
D. Intrinsic properties

E. Circulating mediators and hormones

F. Plasticity

FIGURE 8.1 (Top panel) The cornerstone of synaptic transmission at the first central NTS synapses in glutamate activation of AMPA receptors (AMPA-R). (Bottom panel) The output signal of the second-order neuron resulting from the fast-acting AMPA receptor activation can be modulated by a number of mechanisms operating at these synapses, presynaptic events, postsynaptic events, and the intrinsic properties of the neurons determined by ion channels. (A) Presynaptic depression of glutamate release places frequency-dependent limits on the output signal and is mediated by activation of a number of presynaptic receptors, including the presynaptic metabotropic glutamate receptors (mGluR) and receptors for other neuromodulators (Pre-R). (B) The presence of functional NMDA receptors (NMDA-R) on the second-order neurons provides for additional signal conditioning capacity by prolonging the excitatory response. (continued)
mediators to ultimately orchestrate a coherent pattern of CNS responses for maintaining homeostasis. Moreover, the system has the capacity to undergo plasticity, allowing these synapses to provide long-term regulation that can change the nature of autonomic reflex outputs.

8.6 WHERE TO NEXT?

Still, there is more to learn and there are some challenges. First, the increasing recognition of the complexities of phenotypes of vagal afferent neurons — even within a given subtype, to the extent that these different phenotypes somehow convey a different pattern or frequency of input and perhaps using heretofore unrecognized neurotransmitters beyond the traditionally recognized glutamate — suggests an entirely new complexity of synaptic processing at the second order neurons. Second, while a preponderance of evidence suggests that glutamate is the primary excitatory neurotransmitter, anatomical evidence suggests that other candidate agents are contained in the vagal afferent fibers and that, under certain conditions, they may transmit sensory traffic to the second-order neurons. Third, while more attention is being paid to the intrinsic ion channels in the NTS neurons, a challenge is to use approaches to overcome the inherent difficulties of classifying the contribution and function of the various K channels because of the complexities of the channel subunits — i.e., the molecular diversity, heteromultimeric assembly, and lack of truly selective blockers. Fourth, an ideal experimental approach would be to examine synaptic transmission with all the elements of the reflex intact. Recent advances by others in performing whole-cell patch-clamping in the conscious animal could provide us with new ability to simultaneously examine the nature of synaptic transmission at these synapses in the whole awake organism. Finally, what about the next synapse in the NTS neural network? While it is possible to anatomically identify the second-order neuron in terms of afferent input and perhaps even phenotype, based on spiking behavior, after that, the manner in which the subsequent neurons are connected remains a mystery.

The ever-expanding new approaches to study synaptic transmission — site-specific mutagenesis, systems analysis of the neural network, and molecular imaging will likely provide entirely new and more sophisticated reviews of synaptic transmission in the NTS.

**FIGURE 8.1 (CONTINUED)** Numerous other postsynaptic receptors for neuromodulators are indicated by (Post-R). As shown by the traces at voltages from –80 to +40 mV, the classic AMPA-R current occurs at the peak of the tractus solitarius (TS)-evoked EPSC and is followed by the delayed-onset NMDA-R mediated current, detectable at 20 ms after the peak. The IV plots indicate the magnitude and voltage-dependence of the AMPA-R and NMDA-R mediated components of the eEPSCs. (C) Local interneurons provide spontaneous excitatory (sEPSCs) and inhibitory synaptic inputs (sIPSCs), and can be stimulated to evoke excitatory (eEPSCs) and inhibitory synaptic currents (eIPSCs). (D) Intrinsic ion properties will interweave with the synaptic events to shape the output of the neuron. (E) The interrupted blood brain barrier in aspects of the NTS allow for circulating mediators and hormones to directly access the neurons. (F) Finally, the system is plastic and subject to long-term changes to affect autonomic reflexes over the long-term. (▼) stimulus artifact (●) PSCs.
ACKNOWLEDGMENTS

Many thanks to John Horowitz, Ph.D. and Jesse Joad, M.D. for their thoughtful insights and contributions and to National Heart, Lung, and Blood Institute Grant HL-60560.

REFERENCES


9 Nitroxergic Modulation in the NTS: Implications for Cardiovascular Function

Julian F.R. Paton, James Deuchars, Sheng Wang, and Sergey Kasparov

CONTENTS

9.1 Overview ......................................................................................................210
9.2 NO as a Neuro-Modulator — General Concepts and Current Views ......210
  9.2.1 Different Nitric Oxide Synthase (NOS) Isoforms in the Brain .....211
  9.2.2 Activation of nNOS and eNOS ............................................................212
    9.2.2.1 nNOS ..................................................................................212
    9.2.2.2 eNOS ..................................................................................212
  9.2.3 NO Targets within the Brain ...............................................................213
  9.2.4 Modulation of Synaptic Transmission in the CNS .........................213
9.3 NO and the NTS ..........................................................................................214
  9.3.1 Where Is the Nitrergic System in this Nucleus....................................214
    9.3.1.1 Localization of Nitric Oxide Synthesizing Neurones in the NTS ....214
    9.3.1.2 Nitrergic NTS Neurones and Their Co-Localization with Other Markers of Cell Phenotype 219
    9.3.1.3 Nitrergic NTS Neurones and Their Co-Localization with Transmitter Receptors 220
    9.3.1.4 Synaptic Input to nNOS Containing Neurones in the NTS ...221
    9.3.1.5 Origin and Neurochemistry of nNOS Immunoreactive Axons and Fibers in the NTS 222
  9.3.2 Functional Studies .............................................................................224
    9.3.2.1 Cardiac and Vascular Responses to NO in the NTS ......224
    9.3.2.2 NO in NTS and Modulation of Cardiovascular Reflexes 224
There is a wealth of information supporting the presence of nitric oxide synthase (NOS) isoforms in the nucleus tractus solitarii (NTS). This chapter summarizes the state-of-play regarding nitric oxide (NO) signalling and modulation of cardiovascular function within the NTS. We begin with a brief and general account of NO signalling within the central nervous system, which is followed by a more focused discussion of the data related to the NTS, including anatomical distribution of NO sources, colocalization of NOS with transmitters and receptors, as well as functional studies on NO modulation of cardiovascular variables and reflexes. We conclude that nitroxergic mechanisms are strongly associated with the classical transmitters—glutamate and GABA within the NTS. Although much progress has been made, a greater understanding of the physiological and pathophysiological actions of the nitroxergic system within the NTS will require further studies based on multi-disciplinary approaches with more subtle and sophisticated strategies. For example, cell-specific targeting using somatic gene transfer might help to reveal the mechanisms by which such a highly diffusible signalling molecule as NO can exert localized and specific responses. This remains an open question and a challenge for the future.

**9.2 NO AS A NEURO-MODULATOR — GENERAL CONCEPTS AND CURRENT VIEWS**

NO was originally discovered as endothelial hyperpolarizing factor, shown to be present within neurones and also released following activation of glutamatergic transmission within the brain. It is one of the smallest signalling molecules within the central nervous system. With its unique ability to traverse membranes, NO is capable of both intra- and inter- cellular signalling and hence may act as both an autocrine and paracrine messenger. In some brain regions, facilitatory and inhibitory modulation can occur on the same neurone and this appears to be concentration dependent. Figure 9.1 depicts a respiratory neurone recorded from the ventrolateral medulla onto which an NO donor (diethylamine NONOate) was picoejected. Low amounts of the donor (e.g., using low ejection pressures) depressed rhythmic neuronal firing. In contrast, when the ejection pressure was elevated neuronal activity was greatly augmented. This demonstrates the ability of NO to produce opposite...
effects on a single brainstem neurone. This could be attributable to either concentration dependent processes or two mechanisms that are spatially separated with the more distant one activated when higher amounts of NO donor are released. Alteration of neuronal excitability by NO may occur via presynaptic and postsynaptic mechanisms. To date, NO has been reported to modulate glutamate, \( \gamma \)-amino-butyric acid (GABA), acetylcholine, noradrenaline, serotonin, dopamine, histamine, and taurine transmitter systems (see Reference 118 for review). All these transmitters are abundant in the NTS (see, for example, Reference 76) and therefore potentially prone to modulation by NO. However, as will become evident, most research effort has concentrated on the interaction of NO with excitatory and inhibitory amino acid transmission.

9.2.1 Different Nitric Oxide Synthase (NOS) Isoforms in the Brain

NO generation requires activation of NOS. Three NOS isoforms have been cloned: neuronal or nNOS, endothelial or eNOS, and inducible or iNOS. All isoforms convert L-arginine to L-citrulline and NO. NOS activity depends on a number of factors; first, the availability of molecular oxygen; second, an electron donor is required (e.g., nicotinamide adenine dinucleotide phosphate or NADPH), and third, the cofactor, tetrahydrobiopterin, needs to be present. Finally, intracellular calcium is required for enzyme activation, which occurs through binding to calmodulin. Regarding iNOS, this enzyme is housed within macrophages and activated by cytokines but in this chapter we focus entirely on NO derived from neuronal and endothelial NOS (see Reference 32 for review).
The catalytic activity of nNOS and eNOS has been studied in vitro and may be quite different from that which occurs in vivo. Biochemical in vitro data suggest that eNOS (derived from peripheral tissues) might produce smaller amounts of NO than nNOS. For example, eNOS formed NO was estimated to be 60 to 140 nmol/min/mg. In contrast, nNOS purified form porcine brain produced NO at a rate of 900 nmol/mg/min (Reference 70) whereas 230 nmol/mg/min of NO was produced from recombinant nNOS. These data, however, are strikingly different from the value of 42 pmol/min/mg NO from rat forebrain nNOS. Parenthetically, macrophage iNOS generated 1000 to 1300 nmole/min/mg NO. Whether these in vitro NO production rates translate into different bioavailable concentrations for targets such as sGC in the brain is not clear. In a series of studies by Garthwaite’s group, ambient NO concentration in brain tissue was estimated to be 1 to 2 nM, comparable with the estimated EC50 for sGC. This contrasts with 2 to 10 times higher NO concentrations needed for the mitochondrial respiratory chain. One caveat with all these in vitro measures is that NO is assumed to be derived solely from nNOS without consideration of an additional supply from eNOS, which may occur in the brain and certainly does in the NTS. Another unknown is the efficacy of NO scavenging systems and how they influence bioavailability.

9.2.2 Activation of nNOS and eNOS

The intracellular mechanisms that lead to activation of nNOS and eNOS are distinct. These differences are discussed next for each isoform.

9.2.2.1 nNOS

NO synthesis in the brain is mainly regulated by the influx of Ca²⁺ through receptor-dependent channels, particularly following activation of NMDA receptors. The influx of Ca²⁺ rises the cytosolic Ca²⁺ concentration and leads to Ca²⁺-calmodulin binding to nNOS leading to its activation and release of NO. The coupling of NMDA receptor-mediated calcium influx and nNOS activation is postulated to be due to a physical coupling of the receptor and the enzyme by an intermediary adaptor protein, PSD95, through a unique PDZ-PDZ domain interaction between PSD95 and nNOS. In the case of nNOS, its PDZ domain interacts with a postsynaptic density protein PSD-95, whereas NMDA receptor can also bind with PSD95. Due to the proximity of NMDA receptors to the enzyme, nNOS is directly exposed to the flux of Ca²⁺ entering through the activated NMDA receptor. There are other potential regulators of the NMDA receptor/nNOS coupling. For example, CAPON, an nNOS-associated protein, which interacts with the nNOS PDZ domain through its C terminus, competes with PSD95 for interaction with nNOS. Thus, CAPON would reduce the accessibility of nNOS to NMDA receptor-mediated calcium influx, diminishing the capacity of nNOS to release NO.

9.2.2.2 eNOS

One of the best known mechanisms of eNOS activation requires release of Ca²⁺ from intracellular stores to activate calmodulin, and the resultant Ca²⁺-calmodulin
complex initiates eNOS dimerization leading to formation of the enzymatically active molecule (reviewed in Reference 41). An alternative mechanism has been suggested in vascular smooth muscle and endothelial cells where activation of phosphatidylinositol-3-OH-kinase (PI3K) leads to stimulation of the serine/threonine kinase Akt. Consequently, phosphorylation of eNOS on residue serine-1177 by Akt increases eNOS activity at resting levels of intracellular Ca\(^{2+}\). This mechanism is thought to be responsible for eNOS activation in the endothelium following shear stress and the presence of vascular endothelial growth factor both \textit{in vitro} and \textit{in vivo}.\textsuperscript{28,36} Finally, interactions with other proteins that regulate eNOS activity, such as heat-shock protein 90 (Hsp90\textsuperscript{39}) and calveolin-1\textsuperscript{41} have also been implicated in its regulation.

\textbf{9.2.3 NO TARGETS WITHIN THE BRAIN}

Once NO is released it can act on a number of targets but its primary intracellular receptor is soluble guanylyl cyclase (sGC), which is responsible for production of cyclic guanine monophosphate (cGMP). cGMP-dependent protein kinase G (PKG) phosphorylates a wide range of intracellular targets. However, because of its free radical property, NO is also able to react with superoxide producing peroxynitrite (OONO\(^{-}\)). Therefore, OONO\(^{-}\) must also be considered as a potential NOS-derived modulator of neurotransmission in both physiological and pathophysiological conditions (see Reference 104). Thus, it becomes important to demonstrate whether the downstream signalling of NO can be blocked by either a sGC antagonist, such as 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), or by removing OONO\(^{-}\) either using a scavenger, such as uric acid, or enhanced enzymatic activity of copper or zinc superoxide dismutase.

\textbf{9.2.4 MODULATION OF SYNAPTIC TRANSMISSION IN THE CNS}

NO can modulate both excitatory and inhibitory neurotransmission, often within the same region of the brain. To date, most information indicates that NO modulates glutamate and GABA release as its major effector in the CNS.\textsuperscript{104,118,119} Some studies have shown that the effect of NO on glutamate release can be bidirectional with lower concentrations of the NO donor — S-nitroso-N-penicillamine inhibiting this process and higher concentrations potentiating it,\textsuperscript{130} which may provide an alternative explanation to the data depicted in Figure 9.1. This is consistent with the bi-directional actions of cGMP agonists on glutamate release.\textsuperscript{134} There are some indications that NO can also alter NMDA receptor function but reports are somewhat inconsistent (for example, References 51 and 93).

NO and NO donors have also been shown to increase release of GABA, although it is not always possible to distinguish between direct stimulation of the pre-synaptic GABAergic release machinery within axonal terminals \textit{versus} a network effect that stimulates GABAergic neurones via excitatory circuits. Likewise, the effects of NO on GABA release can also be bi-directional with inhibitory and excitatory responses mediated by lower and higher concentrations respectively.\textsuperscript{118} It should be noted that NO may also modulate GABA\textsubscript{A} receptor function.\textsuperscript{121}
Finally, either NO or peroxynitrite are thought to modulate extracellular concentration of monoamines (and possibly also amino acid neurotransmitters) by acting on their transporters and re-uptake mechanisms.\textsuperscript{69,104}

All told, the bulk of the current information suggests that modulation of transmitter release is the key mechanism of the cGMP-mediated actions of NO under physiological conditions.

9.3 NO AND THE NTS

9.3.1 WHERE IS THE NITRERGIC SYSTEM IN THIS NUCLEUS?

Since the introduction of the NADPH diaphorase (NADPH-d) histochemical staining method for nervous tissue,\textsuperscript{146} its modification for use on aldehyde fixed material,\textsuperscript{128} and the assertion that it can be used for visualizing NOS activation,\textsuperscript{31,122} and most recently by the detection of nNOS mRNA using \textit{in situ} hybridization in neurones throughout the nervous system,\textsuperscript{25,50} many authors have reported widely distributed systems of NO producing neurones in many vertebrate species. The initial conclusions about extensive and prominent neuronal systems throughout the brain were based on NADPH-d technique,\textsuperscript{152} and subsequently refined using immunohistochemical hybridization (for example, References 20 and 54). These authors have all briefly described the system of NO neurones in the medulla oblongata, while others\textsuperscript{52,73,105,133} have studied these neurones in relation to specific areas of the medulla concerned with the control of particular autonomic functions. This section will review the findings with regard to the NTS. We will discuss:

- The location of nNOS-immunoreactive (nNOS-IR; Figure 9.2) neurones and NADPH-d positive neurones
- Co-localization of nitrergic neurones with other markers of cell phenotypes and neurotransmitter receptors
- Synaptic input to nNOS containing neurones
- The origin and neurochemistry of nNOS containing axons and fibers (Figure 9.2)

9.3.1.1 Localization of Nitric Oxide Synthesizing Neurones in the NTS

9.3.1.1.1 Relative Distribution of nNOS-IR and NADPH-Diaphorase Activity

Evidence has been presented for several areas of the nervous system that immunohistochemistry and NADPH-d techniques result in the visualization of the same neuronal populations.\textsuperscript{15,25,50,129} On the other hand, the NADPH-d activity related to NOS may represent only a fraction of the total diaphorase pool\textsuperscript{106,150} and there are several proteins that are distinct from nNOS but exhibit nitroblue tetrazolium reduction activity\textsuperscript{25} and so could lead to false positive identification of NADPH-d containing cells as nNOS containing.\textsuperscript{163} Thus, caution in interpretation of NADPH-d findings may be required.
FIGURE 9.2 Light microscope images showing the distribution of nNOS immunoreactivity in the NTS of the rat. A-C: Montages of low power light microscope images showing the distribution of nNOS immunoreactivity (detected using peroxidase immunohistochemistry) in the NTS at different rostro-caudal levels. A: At rostral levels, nNOS immunoreactive cells observed predominantly in the central (cen) subdivision. Labeled cells are also observed in the region of the tractus solitarius (TS) and in the medial subdivision of the NTS. B: At intermediate levels, nNOS immunoreactive cells are observed in the commissural, medial subnuclei and in the region of the tractus solitarius. C: At caudal levels of the NTS, nNOS immunoreactive cells are observed in the commissural (com) and medial (m) subdivisions and in the tractus solitarius. At all rostro-caudal levels, nNOS immunoreactive fibers and punctate staining indicative of nNOS immunoreactive terminals can be observed throughout the NTS and in the tractus solitarius. Approximate levels rostral (+) and caudal (−) to the obex are shown in the top right of each panel. (Adapted from Atkinson, 2002).6
Due to uncertainties regarding the relationship between nNOS and NADPH-d staining, we have previously compared the distribution of nNOS-IR and NADPH-d staining throughout the medulla of the cat and rat by double labelling sections and by examining series of adjacent sections. In dual labelled 50 µm vibratome sections, in which the NADPH reaction was performed prior to NOS immunolabeling, approximately 80% of NADPH positive cells showed nNOS-IR, and approximately 95% of nNOS-IR cells showed NADPH staining. In adjacent 30 µm sections at all transverse levels, there was a close correspondence between the positions of NOS-IR cells or fibers and NADPH-d positive cells or fibers in most anatomical divisions (including NTS) of both cat and rat. Groups of cells intensely or weakly stained by one method showed a similar staining intensity with the other method. A few regions showed slight discrepancies, i.e., external cuneate nucleus, hypoglossal nucleus, inferior olive, nucleus ambiguus, where a large number of weakly NADPH-d positive cells were sometimes found, but few nNOS-ir cells were seen. It can be concluded, therefore, that highly similar sets of neurones in the medulla are labelled by the two different techniques. This is consistent with a complimentary study in which the distribution pattern of NADPH-d localized cells were indistinguishable from neurones containing nNOS mRNA, as revealed by in situ hybridization or nNOS protein detected by immunohistochemistry (References 10 and 72 and Figure 9.2). Therefore, since the distribution of nNOS-IR and NADPH reactive neurones in the NTS are broadly similar they will be considered together here.

9.3.1.1.2 Distribution and Morphological Characteristics of nNOS Containing Neurones in the NTS

Neuronal NOS/NADPH staining in the NTS is characterized by many fibers, varicosities and groups of small to medium-sized neurones throughout the nucleus, the numbers and packing density of the cells increasing towards rostral levels (Figure 9.2). Such nitricergic neurones have been localized in the NTS with similar findings as summarized below (Table 9.1; Figure 9.2). These data refer specifically to rat NTS, but a similar distribution has been reported in the NTS of the squirrel monkey and parts of the human NTS. Chan et al. have found NADPH-d NTS neurones that were also responsive to arterial pressure rises suggesting that neurones mediating the baroreceptor reflex may contain nNOS (see Figure 9.3A).

9.3.1.1.3 eNOS in the NTS

In addition to nNOS, eNOS also plays a critical role in regulating cardiovascular variables in the NTS (see Section 9.3.2; Figure 9.4). An obvious source of eNOS is the endothelium. The information available suggests that this may contribute significantly to NO functions in the NTS: Gross et al. described microvascular specializations within NTS that included “an extraordinary capillary density” that was almost eight times that of adjacent area postrema — a highly vascular organ in its own right (Figure 9.4). This gives substantial substrate for eNOS generated NO in the NTS. In the same paper, the authors described that “neurons and their processes were found in a dense feltwork closely surrounding individual capillaries” implying limited diffusion distances for endothelial generated NO to target neuronal networks. However, with this impressive blood supply one wonders to what degree it also...
scavenges NO following its release from both endothelial and neuronal sources. Thus, while the endothelium of the vasculature is a major potential source of NO, the blood passing through it is likely to contribute significantly with its removal thereby reducing the potency and longevity of NO mediated effects.

A question is whether eNOS is also present in neurones as well as endothelial cells? eNOS has been detected in many diverse cell types throughout the body, including cardiac myocytes, astrocytes and glioma cells (for review see reference 78). However, currently the location in neurones remains contentious. For example, several groups have reported that eNOS can be detected in hippocampal pyramidal neurones using immunohistochemistry29,30,103,145 and galactosidase eNOS reporter in mice,145 while others have detected eNOS only in endothelial cells in the brain using in situ hybridization131 or immunohistochemistry.135,148 We detected eNOS in NTS neurones using immunohistochemistry.110 However, authors who detected eNOS protein in hippocampal neurones immunohistochemically29,30,103 subsequently reported that sensitive radioactive in situ hybridization in the hippocampus detected eNOS mRNA only in endothelial cells in the hippocampus.13 Considering the impor-

### TABLE 9.1

**Localization and Quantification of nNOS/NADPH Diaphorase Containing Neurones in the Nucleus Tractus Solitarius of the Rat**

<table>
<thead>
<tr>
<th>Subnucleus of NTS</th>
<th>Cell Number</th>
<th>Morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gelatinosus</td>
<td>7 ± 3</td>
<td>Few intensely labelled scattered neurones and many fibers;</td>
</tr>
<tr>
<td>Commissural</td>
<td>238 ± 36</td>
<td>Long moderately stained bipolar cells with processes that travel long distances laterally</td>
</tr>
<tr>
<td>Medial intermediate (0-0.75 mm rostral to obex)</td>
<td>123 ± 15</td>
<td>Small (10-15 µm diameter) lightly labelled triangular cells</td>
</tr>
<tr>
<td>Medial rostral (0.75-2 mm rostral to obex)</td>
<td>763 ± 155</td>
<td>Small, well stained cells with occasional multipolar processes</td>
</tr>
<tr>
<td>Ventral/ventrolateral</td>
<td>287 ± 82</td>
<td>Medium-sized, intensely labelled triangular and ovoid neurones with 2-4 prominent dendrites, forming clusters and extending in a band into the dorsal reticular area. At rostral levels these are densely packed with prominent, thick, contorted and varicose dendrites.</td>
</tr>
<tr>
<td>Central</td>
<td>NA</td>
<td>A large cluster of small, mostly ovoid neurones, some rather weakly labelled, with a high packing density and surrounded by a dense aggregation of fibers</td>
</tr>
<tr>
<td>Dorsal and interstitial</td>
<td>NA</td>
<td>Many small elongated bipolar neurones and fibers in a band extending over tractus solitarius (TS)</td>
</tr>
</tbody>
</table>

*a Cell numbers were quantified by Krowicki et al.72 Morphological descriptions are composites of those of Krowicki et al.72 and Batten et al.10*
Advances in Vagal Afferent Neurobiology

FIGURE 9.3 (A color version of this figure follows page 236.) nNOS in the NTS; association with the baroreceptor reflex pathway and glutamatergic neurotransmission. **A:** Fos expression (brown nuclear staining) in dorsolateral NTS neurones following intravenous injection of phenylephrine. Some of these arterial pressure-sensitive NTS neurones were positively stained for NADPH-diaphorase (purple cytoplasm) suggesting that some contain nNOS. Key: Open arrow: NADPH-diaphorase staining only; arrow head: arterial pressure-sensitive only; filled arrows: NADPH-diaphorase and arterial pressure-sensitive. (Data from Chan et al. 2000. With permission.)

**B:** Confocal image of fibers within the caudal NTS immunopositive for nNOS (blue fluorescence) and glutamate related transmission (green: glutamate vesicular transporter type 1 or Vglut1; red: Vglut2; magenta: nNOS and Vglut2). Note that nNOS was only co-localized with Vglut2 and that Vglut2 did not co-localize with Vglut1. (Data from Lin et al. 2004. With permission.)

**C:** Triple immunocytochemical detection for nNOS (green; C1), the AMPA receptor subunit – gluR1 (red; C2) and the NMDA receptor subunit – NMDAR1 (blue; C3). Triple labeled NTS neurones appear white-light blue (C4). Not all neurones were immunopositive for nNOS but those that were also contained NMDAR1 and gluR1. (Data from Lin and Talman 2002. With permission.)

**D:** Co-localization of the NMDA receptor subunit – NMDAR1 (red fluorescence; D1) with nNOS positive caudal NTS neurones (green; D2) appear yellow (D3). Almost all nNOS positive neurones contained the NMDAR1 whereas only a proportion of NMDAR1 neurones contained nNOS. (Data from Lin and Talman 2000. With permission.)
Nitroxergic Modulation in the NTS: Implications for Cardiovascular Function

9.3.1.2 Nitrergic NTS Neurones and Their Co-Localization with Other Markers of Cell Phenotype

9.3.1.2.1 Glutamate and GABA

Glutamate has been immunohistochemically co-localized with nNOS in NTS neurones (Figure 9.3). In the cat, neurones double-labelled for both nNOS activity and glutamate-immunoreactivity were observed occasionally in the NTS. In the rat, a different pattern of co-existence was observed where nNOS-IR cells were also found to be moderately glutamate-IR, particularly in the commissural, medial, ventral and ventrolateral subnuclei. A detailed study examining the extent of co-localization of nNOS and glutamate revealed that the percentages of nNOS-IR neurones that were also glutamate-IR differed among the sub-nuclei: central (83±13%), interstitial (64±26), dorsolateral (41±12), ventral (41±13), medial (20±9), and commissural (9±8%). These varying degrees of co-localization suggest that some nNOS neurones may contain another transmitter, possibly GABA, but there has yet to be conclusive proof from dual-labelling studies of such coexistence in the NTS.
Using antibodies to glutamate does not permit separation of neurones that use glutamate as a neurotransmitter from those where metabolic glutamate is labelled. However, immunohistochemistry used to localize vesicular glutamate transporters (VGluT) can be used to label glutamate within pre-synaptic terminals. In all subnuclei of the rat NTS nNOS fibers co-localize with VGLUT2, but not VGLUT1 (Figure 9.3B). However, while there is a strong chance that the source of at least some of these dual labelled fibers are NTS neurones, dual labelling in situ hybridization and nNOS immunohistochemical studies have yet to be reported in the NTS.

9.3.1.2.2 nNOS Co-Localization with Other Neuropeptides/Neuromodulators in the NTS

Neurones in the NTS that contain nNOS co-localize with few other markers of neuronal phenotype. Double labelling studies using either enzymatic or fluorescence methods on cat and rat brainstem sections demonstrated that very few NOS-containing neurones in the medulla were immunoreactive for the catecholamine synthesizing enzymes tyrosine hydroxylase (TH), dopamine β-hydroxylase (DBH), or phenylethanolamine N-methyl transferase (PNMT), even though they were extensively intermingled. These results are in general agreement with earlier findings. Similarly, of neuropeptides only somatostatin has been shown to co-localize with a few nNOS cells in the NTS, but such lack of labelling may represent the difficulties in localizing peptides in somata. This type of examination may be better performed using in situ hybridization to localize peptide producing cells. In addition, nNOS in the NTS did not co-localize with neurones containing the calcium binding proteins calbindin, calretinin or parvalbumin. To summarize, the evidence above suggests that the primary neurotransmitter co-existing with NOS in the NTS appears to be glutamate. Whether other transmitter substances co-exist with NOS remains to be fully established.

9.3.1.3 Nitrergic NTS Neurones and Their Co-Localization with Transmitter Receptors

nNOS has been found co-localized with NR-1 glutamate receptor subunit in various parts of the brain in numerous studies. In NTS, NO may act as a mediator of the cardiovascular responses to glutamate injections into the rat NTS consistent with the immunohistochemical localization of glutamate receptors on nNOS containing NTS neurones (Figure 9.3C and Figure 9.3D). These authors have demonstrated that the cell bodies and proximal dendrites of almost all nNOS neurones in the NTS contained the NR-1 subunit of the NMDA receptor. Thus, in NTS, as in other parts of the brain Ca entry via NMDA receptors might be one of the mechanisms of nNOS activation. Similarly, nearly all nNOS neurones in the NTS contained immunoreactivity for the glutamate AMPA receptor subunit GluR1 (Figure 9.3C). The high degree of co-localization in both cases suggested that AMPA and NMDA receptor subunits co-localized in the same nNOS positive neurones. This was confirmed by triple labelling immunofluorescence in which all nNOS-IR neurones in the NTS were shown to contain the NMDA NR1 and AMPA GluR1 receptor subunits (Figure 9.3D).
Since other receptors also gate calcium permeable pores these provide an additional avenue through which to activate nNOS. One such sub-family of receptors are the P2X receptors and P2X$_2$ receptor subunit immunoreactivity has been shown to be present in nNOS neurones of the NTS. The percentage of nNOS neurones that also contained the P2X$_2$ receptor subunit varied between NTS subdivisions: commissural (65±5%), sub-postremal (48±4%), and rostral (43±4%). If P2X$_2$ receptors can trigger nNOS-mediated NO release, they might be important for modulating energy-dependent neuronal activity in the NTS, a hypothesis that remains to be tested.

9.3.1.4 Synaptic Input to nNOS Containing Neurones in the NTS

Surprisingly there is little information regarding the origin or neurochemistry of nNOS containing synaptic inputs to neurones in the NTS. In one study vagal afferent fibers were anterogradely labelled from the nodose ganglion and nNOS-IR neurones identified as postsynaptic to these fibers using electron microscopy. Since the primary transmitter in vagal afferent fibers is likely to be glutamate, this is consistent with light microscopic identification of synaptic terminals apposing nNOS-IR structures that contain glutamate (Figure 9.3B) or the vesicular glutamate transporter VGluT2. Since all NTS nNOS-positive neurones also contain the NMDA receptor NR1, and NR1 containing neurones are postsynaptic to vagal afferent fiber terminals, the activation of nNOS neurones by vagal afferent fibers might result in the release of NO.

The link between vagal afferent fiber input and possible activation of nNOS may be important for mediation of cardiovascular reflexes. This is supported by functional data (as described below) and the finding that neurones in the NTS expressing the c-fos proto-oncogene in response to phenylephrine induced hypertension, also displayed diaphorase activity or nNOS mRNA (Figure 9.3A). Considering these links it would be interesting to fully analyze functionally and anatomically the relationship between baroreceptive nerve afferents and nNOS neurones in the NTS. Additionally, vagal afferent synaptic inputs to nNOS containing NTS neurones may also be involved in esophageal reflexes. Sensory vagal neurones carry information from the esophagus to a discrete part of the NTS — the central subnucleus (ceNTS). The ceNTS is also rich in nitrergic neurones. Histochemical localization of NADPH-d has revealed a cluster of positive neurones in the central subnucleus in a number of species, humans, rat, and cat. NOS-IR has also been found in the neurones of the ceNTS. These cells appear to receive information from esophageal afferents since nitrergic cells in the ceNTS are innervated by afferents labelled from the esophagus in both rat and rabbit. However, it should be noted that repetitive distension of the esophagus did not lead to c-fos expression in nitrergic neurones in the ceNTS, leaving the role for these nNOS positive neurones to be determined. Below we discuss further the origin of synaptic inputs to identified nitrergic neurones in the NTS.
9.3.1.5 Origin and Neurochemistry of nNOS Immunoreactive Axons and Fibers in the NTS

In addition to labelled somata, nNOS-IR and/or NADPH-diaphorase reactivity is also present in numerous axons and fibers throughout the NTS (Figure 9.2 and Figure 9.3B).\textsuperscript{10,72,73,80} Fiber labeling for nNOS-IR has been assessed as moderate intensity in the subpostremal, commissural, central and medial subnuclei while other subnuclei contained weak staining.\textsuperscript{80} A similar pattern has been described in other studies localizing nNOS using immunohistochemistry\textsuperscript{10,72,73,80} and/or NADPH diaphorase.\textsuperscript{10,72,73,80} Several studies have considered the source of these fibers. One possibility that has received a great deal of attention is that the nNOS originates from vagal afferent fibers, however, there are numerous other possible sources of nNOS in the NTS and both these are reviewed below.

9.3.1.5.1 Relationship between nNOS and Vagal Afferent Neurone Somata, Fibers, and Terminals

Evidence for the formation of NO by vagal afferent fibers is provided by both anatomical and functional studies showing that the cell bodies of vagal afferent fibers in the nodose ganglia contain NADPH-d\textsuperscript{2,102,124,165} NOS immunoreactivity\textsuperscript{3,7} and nNOS mRNA.\textsuperscript{76} Functionally, application of NO or NO donors to nodose ganglia neurones elicits a depolarization\textsuperscript{77} and increases cytoplasmic Ca\textsuperscript{2+} concentrations,\textsuperscript{127} suggesting that NO is able to be synthesized by, and cause an effect in, primary vagal afferent neurones. Peripheral vagal afferent fibers may also contain NOS, since NOS immunoreactive fibers in guinea pig cardiac ganglia disappeared following vagotomy.\textsuperscript{144} In addition, ligation of the vagus nerve distal to the nodose ganglion results in accumulation of NADPH diaphorase and tritiated nitro-L-arginine binding proximal to the ligature,\textsuperscript{34} which may either indicate transport of NOS along the nerve fibers heading centrally or may reflect response to injury. In other studies, the levels of NOS and NADPH-d staining following nodose ganglionectomy were shown to decrease in certain subnuclei of the NTS on the ipsilateral side.\textsuperscript{73,91,124} These changes were particularly marked in the gelatinosus, subpostremal, medial and interstitial subnuclei,\textsuperscript{124} and were interpreted as indicating a loss or down-regulation of NO synthesis in degenerating vagal afferent fibers. At the ultrastructural level, nNOS immunoreactivity has been observed in degenerating terminals in the NTS following nodose ganglionectomy.\textsuperscript{80} While each of these studies concluded that NOS was contained in central terminals of vagal afferent fibers, the evidence leading to these conclusions appears to be somewhat contradictory.

To directly test whether vagal afferent terminals in the NTS contained nNOS we anterogradely labelled vagal afferents and combined this with nNOS immunohistochemistry at the light and electron microscopic levels.\textsuperscript{7} In light of previous studies (described above) a major surprise was that we did not detect nNOS in vagal afferent fibers or terminals. It was unlikely that the localization of the anterograde tracer with diaminobenzidine blocked immunolocalization of antigens in the same terminals, as we had previously shown that the same dual labelling procedure is effective for localizing purine receptors in vagal afferent terminals.\textsuperscript{8,27}
The lack of nNOS in vagal afferent terminals in the NTS, yet the presence in nodose ganglion cells of nNOS protein and mRNA, and the various effects of NO on these neurones, suggests that NO produced by the neuronal isoform of NOS may be utilized by vagal afferent cell bodies as an intraneuronal messenger within the ganglion, but not transported to the central fibers and terminals. Such a conclusion was supported by our experiments, in which we specifically labelled vagal sensory neurones innervating the heart. Despite the fact that dual labelled nodose ganglion cells could be observed in these experiments, no cholerotoxin b labelled centrally projecting vagal axons in the NTS were found to contain nNOS-IR. As ligation studies have demonstrated a bi-directional transport of NADPH-d activity in the cervical vagus nerve distal to the nodose ganglion, it is possible that NOS could be transported from the ganglion cells down the peripheral axons of the afferent neurones, e.g., contributing to the plexus of NOS immunoreactive fibers innervating the cardiac ganglia of rodents.

Therefore, the evidence for the localization of nNOS in central vagal afferent terminals is controversial and even in studies where such co-localization has been detected the degree of co-localization was minimal. One possibility could be that nNOS-positive fibers could belong to glossopharyngeal or facial cranial nerve fibers, which also travel in the tractus solitarius and terminate in the NTS. However, this explanation is also unlikely because glossopharyngeal cell bodies in the petrosal ganglion are rarely NADPHd positive.

Therefore, the existence of nNOS-containing primary afferents in the NTS appears to be elusive such that the majority of nNOS positive fibers must originate from within the brain.

9.3.1.5.2 CNS Sources of nNOS Containing Terminals in the NTS

Since there are many nNOS producing neurones in the NTS, there is a high probability that these are the source for many of the nNOS containing fibers in the NTS. Indeed, injection of retrograde tracer into the caudal NTS results in labelled neurones in the rostral portion of the NTS that also contain nNOS. However, it is feasible that the labelling in neurones close to the injection site is due to dendritic uptake of tracer. It would, therefore, be preferable to identify the axonal projections of single labelled nNOS containing neurones in the NTS. Such details could be gleaned by intracellular labelling of single neurones subsequently identified as nNOS containing, followed by tracing of their axons similar to that conducted on calbindin, GABA or dopamine-beta-hydroxylase-immunopositive neurones in the NTS. Such studies would be significantly enhanced if the physiological properties of these neurones were functionally characterized such as can be determined in vivo or in the working heart brainstem preparation prior to immunohistochemical labelling.

Neurones in other regions of the CNS that contain nNOS and project to the NTS have been detected by injecting a retrograde tracer into the NTS and subsequently detecting which retrogradely labelled neurones contain nNOS. The caudal zone of the NTS was injected with retrograde tracer and nNOS detected in retrogradely labelled neurones in many of the areas of the medulla that are known to relay somatosensory and viscerosensory inputs to the NTS: the raphe nuclei, dorsal,
Advances in Vagal Afferent Neurobiology

intermediate and lateral reticular areas, spinal trigeminal and paratrigeminal nuclei and the external cuneate and medial vestibular nuclei. Isolated dual-labelled neurones were also scattered throughout most of the divisions of the reticular formation. In another study, the nNOS containing projection from the paratrigeminal nucleus to the dorsal vagal complex has also been detected. Similarly, neurones in the medial parvicellular subdivision of the paraventricular hypothalamic nucleus that project to the NTS were identified as nNOS containing.

9.3.2 FUNCTIONAL STUDIES

9.3.2.1 Cardiac and Vascular Responses to NO in the NTS

With the wealth of evidence supporting the presence of nitrergic mechanisms in NTS, it is not surprising that NO acting in this nucleus influences the cardiovascular system. We will first survey data describing changes in cardiovascular parameters evoked by activation or de-activation of NO release and then consider those studies in which NO was demonstrated to modulate reflex responses.

A variety of studies have shown that the precursor for NO, L-arginine, and multiple NO donors injected into the NTS produce bradycardia and a depressor response (see Table 9.2). In some studies, the depressor response was associated with an inhibition of renal sympathetic nerve activity (Table 9.2). Some evidence exists that this response can be prevented by blockade of sGC, suggesting that the response is due to NO rather than OONO-. More recently, eNOS has been over-expressed in the NTS to produce chronically elevated levels of NO, which also produced bradycardia and depressor responses and could be prevented by sGC inhibitors. In contrast, a variety of NOS inhibitors injected into NTS, as well as expression of antisense oligonucleotides for nNOS, produced an opposite pattern of response in the majority of studies (tachycardia and hypertension; see Table 9.2 for references). These exogenous interventions convincingly demonstrate that NO can excite neuronal networks influencing cardiovascular activity within NTS and plays a role in central regulation of arterial pressure both in normotensive and hypertensive animals. The finding that NO in NTS produces a depressor/bradycardia response does not automatically associate it with a role in baroreflex functions, as many reflexes can evoke this pattern of response. Indeed, these studies do not establish which reflex pathway(s) is (are) being affected or whether there are differences between the effects of NO released from nNOS versus eNOS activation. Further, neither do they demonstrate that these actions reflect the physiological effects of native NO released from its natural sources.

9.3.2.2 NO in NTS and Modulation of Cardiovascular Reflexes

NO modulation of the baroreceptor reflex at the level of the NTS has been studied most extensively. In contrast to the consistent findings of nitroxergic influences in NTS on arterial pressure and heart rate, the effects of NO on the baroreceptor reflex are inconsistent (Table 9.3): Some authors report that either blockade of NOS in the NTS or microinjection of NO donors failed to affect the baroreceptor reflex gain in both anaesthetized (rabbit, cat) and conscious animals (rat). Other studies
have shown that blocking NOS in the NTS significantly inhibited the baroreceptor reflex bradycardia evoked by intravenous injection of phenylephrine in anaesthetized rats. \(^9\) In stark contrast to these latter studies, other authors have described an increase in the baroreceptor reflex heart rate range when L-NMMA was microinjected into NTS, suggesting an attenuating role for NO on baroreflex function. \(^1\) Our work in unanaesthetized decerebrate rats supports the latter observation. Although we failed to observe any change following NTS microinjections of NOS inhibitors (L-NAME, L-NMMA, TRIM) in decerebrate unanaesthetized rats (see Table 9.3), \(^1\) we did find that L-arginine, sodium nitroprusside and a NO donor (diethylamine NONOate) all attenuated the baroreceptor reflex bradycardia (Figures 9.5A and 9.6A). \(^1\) Furthermore, this NO-induced baroreflex inhibition was blocked by 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), a sGC inhibitor, and hence mediated by NO (Figure 9.6B). \(^1\) If this is the case, one would predict that reducing NO production in the NTS would enhance cardiac baroreflex gain. This was confirmed in unrestrained conscious animals fitted with radio transmitters for remote recording of blood pressure. In these animals, adeno viral induced expression of a

**TABLE 9.2**

<table>
<thead>
<tr>
<th>Species</th>
<th>Agent(s) used</th>
<th>Response</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit</td>
<td>L-NMMA</td>
<td>↑BP ↑HR↑RSNA</td>
<td>Harada et al. 1993</td>
</tr>
<tr>
<td>Rabbit</td>
<td>L-arginine</td>
<td>No change</td>
<td>Harada et al. 1993</td>
</tr>
<tr>
<td>Rat (SD)</td>
<td>L-arginine</td>
<td>↓BP ↓HR ↓RSNA</td>
<td>Tseng et al. 1996</td>
</tr>
<tr>
<td>Rat (SD)</td>
<td>SNP; SIN-1</td>
<td>↓BP ↓HR</td>
<td>Vitagliano et al. 1996</td>
</tr>
<tr>
<td>Rat (SD)</td>
<td>L-arginine</td>
<td>↓BP ↓HR</td>
<td>Lo et al. 1997; 1998; 1999</td>
</tr>
<tr>
<td>Rat (SHR)</td>
<td>L-NMMA</td>
<td>No change</td>
<td>Pontieri et al. 1998</td>
</tr>
<tr>
<td>Rat (SD)</td>
<td>L-arginine; SNP</td>
<td>↓BP ↓HR</td>
<td>Lin et al. 1999</td>
</tr>
<tr>
<td>Rat (WKY)</td>
<td>nNOS antisense</td>
<td>↑BP</td>
<td>Maeda et al. 1999</td>
</tr>
<tr>
<td>Rat (WKY)</td>
<td>AdV eNOS</td>
<td>↓BP ↓HR</td>
<td>Sakai et al. 2000</td>
</tr>
<tr>
<td>Rat</td>
<td>NO(aq); NO donors</td>
<td>No change</td>
<td>Talman et al. 2001</td>
</tr>
<tr>
<td>Cat</td>
<td>L-Arg</td>
<td>↓BP</td>
<td>Wu et al. 2002</td>
</tr>
<tr>
<td>Rat (SHR)</td>
<td>AdV eNOS</td>
<td>↓BP ↓HR – SHR&gt;WKY</td>
<td>Hirooka et al. 2003a; 2003b</td>
</tr>
<tr>
<td>Rat (WKY)</td>
<td>AdV TeNOS</td>
<td>↓HR</td>
<td>Waki et al. 2003b</td>
</tr>
<tr>
<td>Rat (SHR)</td>
<td>AdV TeNOS</td>
<td>↓BP ↓HR</td>
<td>Waki et al. 2004</td>
</tr>
<tr>
<td>Cat</td>
<td>L-arginine</td>
<td>↓BP ↓HR</td>
<td>Wu et al. 2004</td>
</tr>
<tr>
<td>Rat (SHR)</td>
<td>AdV eNOS</td>
<td>↓BP ↓HR</td>
<td>Tai et al. 2004</td>
</tr>
</tbody>
</table>

*Note: Abbreviations: AdV, adenoviral vector-mediated over expression; BP, blood pressure; HR, heart rate; L-NMMA, N\(^G\)-monomethyl-L-arginine monoacetate; NOS, nitric oxide synthase (e, endothelial; n, neuronal); RSNA, renal sympathetic nerve activity; SD, Sprague-Dawley; SHR, spontaneously hypertensive rat; SNP, sodium nitroprusside; SIN-1, 3-morpholinosydnonimine; TeNOS, truncated mutant form of eNOS expressed to block endogenous eNOS; WKY, Wistar Kyoto rat. Literature is presented in chronological order.

\(^a\) Increase in HR only seen after sinoaortic denervation and bilateral vagotomy.
dominant negative protein to chronically block eNOS activity in the NTS enhanced spontaneous baroreflex gain.155

The reasons for the inconsistency between studies on baroreflex modulation by NO in the NTS (see Table 9.3) may relate to anaesthesia. Our studies109,110 and the complementary findings of Pontieri et al.117 were all performed without anaesthesia. Further, our studies obtained consistent data whether applying exogenous NO or blocking its endogenous supply in two distinct animal models that were both

<table>
<thead>
<tr>
<th>Species</th>
<th>Preparation Type</th>
<th>Action</th>
<th>Agent Used</th>
<th>Response</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit</td>
<td>Anaesth</td>
<td>Block NOS</td>
<td>L-NMMA</td>
<td>No ∆ in baroreflex</td>
<td>Harada et al. 1993</td>
</tr>
<tr>
<td>SHR</td>
<td>Conscious</td>
<td>Block NOS</td>
<td>L-NMMA</td>
<td>↑ range baroreflex</td>
<td>Pontieri et al. 1993</td>
</tr>
<tr>
<td>Cat</td>
<td>Anaesth</td>
<td>Block NOS</td>
<td>L-NNA</td>
<td>No ∆ in baroreflex</td>
<td>Zanzinger et al. 1995</td>
</tr>
<tr>
<td>Cat</td>
<td>Anaesth</td>
<td>supply NO</td>
<td>SNAP</td>
<td>No ∆ in baroreflex</td>
<td>Zanzinger et al. 1995</td>
</tr>
<tr>
<td>Rat</td>
<td>Anaesth</td>
<td>Block NOS</td>
<td>L-NMMA; L-NAME</td>
<td>No ∆ baroreflex gain</td>
<td>Lo et al. 1996</td>
</tr>
<tr>
<td>Rat</td>
<td>Decerebrate/unanesth</td>
<td>Block NOS</td>
<td>L-NAME; Block nNOS</td>
<td>L-NMMA; TRIM</td>
<td>Paton et al. 2001</td>
</tr>
<tr>
<td>Rat</td>
<td>Decerebrate/unanesth</td>
<td>Supply NO</td>
<td>L-Arginine; SNP; dea-NONOate</td>
<td>L-NMMA</td>
<td>Paton et al. 2001</td>
</tr>
<tr>
<td>Rat</td>
<td>Conscious</td>
<td>Block eNOS</td>
<td>AdV TeNOS</td>
<td>↑ baroreflex gain</td>
<td>Waki et al. 2003b</td>
</tr>
<tr>
<td>SHR</td>
<td>Conscious</td>
<td>Block eNOS</td>
<td>AdV TeNOS</td>
<td>↑ baroreflex gain</td>
<td>Waki et al. 2004</td>
</tr>
<tr>
<td>Rat</td>
<td>Anaesth</td>
<td>Block nNOS</td>
<td>AR-R 17477</td>
<td>↓ baroreflex</td>
<td>Talman and Dragon 2004</td>
</tr>
<tr>
<td>Rat</td>
<td>Decerebrate/unanesth</td>
<td>Block sGC</td>
<td>ODQ</td>
<td>No ∆ in baroreflex</td>
<td>Paton and Kasparov 2001</td>
</tr>
</tbody>
</table>

Note: Abbreviations: AdV, adenoviral vector-mediated over expression; anaesth, anaesthetized; AR-R 17477 is a neuronal specific NOS inhibitor; dea-NONOate, diethylamino NONOate — an NO donor; L-NAME, N G-nitro-L-arginine methyl ester hydrochloride; L-NMMA, N G-monomethyl-L-arginine monoacetate; L-NNA, N G-nitro-L-arginine; NOS, nitric oxide synthase (e, endothelial; n, neuronal); ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinazolin-1-one; SHR, spontaneously hypertensive rat; SNAP, S-nitroso-N-acetylpenicillamine; SNP, sodium nitroprusside; TeNOS, truncated mutant form of eNOS expressed to block endogenous eNOS; TRIM, 1-(2-trifluoromethylphenyl)imidazole.

Literature is presented in chronological order.
Nitroxergic Modulation in the NTS: Implications for Cardiovascular Function

227

un-anaesthetized. We would argue that data from a conscious unrestrained animal is likely to produce the most reliable and reproducible results.

There are reports of excitatory effects of L-arginine and sodium nitroprusside on the rate of discharge of NTS neurones recorded extracellularly in vitro and in vivo. We have observed membrane depolarization in our unpublished in vitro intracellular recordings but only when high concentrations (>100 nM) of NO were applied; lower concentrations failed to alter membrane potential of unidentified neurones but did modulate excitatory and inhibitory transmission within the nucleus. Regarding the latter, Figure 9.6A depicts that an NO donor given locally that depressed the synaptic excitatory response following baroreflex stimulation; the latter supports the notion that NO in the NTS inhibits baroreflex signaling within the NTS, but is without effect on the peripheral chemoreceptor reflex (Figure 9.5B), indicating reflex specificity.

FIGURE 9.5 NO modulation of baroreceptor reflex processing in the NTS is reflex specific. A: NTS microinjections of L-arginine (NO precursor) or a NO donor (diethyl amine NONOate) all produced a reversible depression of the cardiac component of the baroreceptor reflex. (Data from Paton et al. 2001a. With permission.) 110 B: In contrast to the baroreceptor reflex, microinjection of a NO donor (NONOate) into NTS sites known to mediate the cardiac component of the chemoreceptor reflex were ineffective in modulating the chemoreceptor reflex bradycardia. (Unpublished data from J.F.R. Paton and S. Kasparov.)
9.3.2.3 NO in NTS and Interactions with Other Transmitter Substances

Here, we discuss interactions between putative transmitters and NO in the NTS. Interactions can work reciprocally such that NO can alter efficacy of other transmitters and vice versa.

9.3.2.3.1 Glutamate

Within higher brain structures glutamatergic transmission, particularly NMDA receptor activation and associated increases in intracellular calcium concentration, results in NO generation. In the NTS, anatomical data support co-existence between nNOS and glutamate (see Section 9.3.1.2.1). At the functional level, an NO-glutamate interaction has been validated in numerous functional studies:
• While iontophoretic application of L-NAME affected neither spontaneous nor vagus nerve evoked potentials, it did decrease the firing response to (RS)-a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA; but not NMDA) receptor stimulation. Additionally, a NO donor (a NONOate) also enhanced the firing response to AMPA, but not NMDA, suggesting that NO facilitates AMPA transmission in the NTS.

• In microdialysis studies, NO in NTS stimulated glutamate release whereas NMDA and AMPA released NO. Interestingly, L-NMMA reduced basal glutamate levels whereas blockade of either NMDA or non-NMDA was without effect on NO levels suggesting endogenous NO release by glutamatergic mechanisms, but not vice versa.

• Lo et al. microinjected either glutamate or NMDA into the NTS and produced depressor and bradycardic responses that were blocked by prior injection of an NO synthase inhibitors. Similar studies were performed by Lin et al. in which a specific nNOS antagonist was successfully used, as well as ODQ.

• Conversely, administration of NMDA and non-NMDA receptor antagonists attenuated the depressor and bradycardic effect induced by NTS microinjection of L-arginine. Therefore, these results suggest that NO and glutamate receptors reciprocally interact within the NTS affecting cardiovascular regulation.

The mechanisms by which NO can promote glutamate (and other transmitters) release are not well understood. NO is thought to act via NO/sGC/cGMP pathway that may lead to release of intracellular Ca²⁺ as a primary event enhancing transmitter release. Alternatively NO may affect transmitter release machinery. There are mechanisms that can explain the glutamate-induced release of NO. As to the NMDA receptor-induced NO release, this likely stems from the influx of Ca²⁺ via NMDA receptor-operated channels. Influx of Ca²⁺ causes Ca²⁺-calmodulin binding to nNOS leading to its activation. There appears to be physical coupling of the NMDA receptor and nNOS by the postsynaptic density protein 95 (PSD95) through a unique PDZ-PDZ domain interaction. Due to the close proximity of NMDA receptors to nNOS this enzyme is thought to be directly exposed to Ca²⁺ influx. This interaction between nNOS and the NMDA receptor is kept in check by an nNOS-associated protein (so called CAPON) that interacts with the PDZ domain of nNOS through its C terminus and competes with PSD95 for an interaction with nNOS. Thus, excessive CAPON activity would suppress NMDA receptor-nNOS interactions thereby reducing NO release. Regulating CAPON activity, therefore, would be a method for controlling the nNOS-NMDA interaction. There are also other mechanisms which can control this process: for example, in cortical neurones NMDA-evoked NO release can be inhibited by serotonin acting via 5-HT₁A and 5-HT₂C receptors. Finally, recently it has been shown that in some brain areas, such as prefrontal cortex, NMDA receptors are unable to activate nNOS, but this can be achieved by blockade of GABAA receptors, suggestive of as yet an unknown mechanisms controlling nNOS activity.
9.3.2.3.2 GABA and Angiotensin II

We have demonstrated that low fentanyl doses of angiotensin II in the NTS attenuates the cardiac baroreceptor reflex and that this is blocked by non-isoform specific NOS antagonism.\textsuperscript{110} As described above, L-arginine and NO donors mimic the effects of angiotensin II in NTS by depressing the cardiac baroreceptor reflex gain; both the latter are ODQ-sensitive.\textsuperscript{112} A common link between NO and angiotensin II appears to be related to the GABA\textsubscript{A} receptor. First, antagonism of the GABA\textsubscript{A} receptor with bicuculline prevents the baroreceptor reflex depressant effects of angiotensin II in NTS.\textsuperscript{109} Second, the NO-mediated depression of the firing response of single NTS neurones activated by aortic baroreceptors can be nulled by local delivery of bicuculline.\textsuperscript{116} Third, angiotensin II can enhance evoked GABA-mediated inhibitory postsynaptic potentials in around 30\% of NTS neurones recorded intracellularly from medullary slices.\textsuperscript{60} Since angiotensin II does not increase the response to iontophoretically applied GABA, we suggested that its effect may be on pre-synaptic targets. Incidentally, NO-mediated facilitation of synaptic inhibition is not unique within cardiovascular neuronal networks and is found in the rostroventrolateral medulla\textsuperscript{51} and the hypothalamus.\textsuperscript{136}

9.3.2.3.3 NO-NMDA and GABA

As NO can interact with both glutamate and GABA neurotransmission in the NTS, predicting the outcome of injecting NO donors into NTS may be impossible. Although there is no data examining the interaction between NO, NMDA and GABA within NTS, such an interaction appears in the hypothalamus. Bains and Fergusson\textsuperscript{9} studied the actions of NO donors on neurones within the paraventricular nucleus of the hypothalamus \textit{in vitro}. They reported that an NMDA agonist elicited GABA-evoked IPSPs which were prevented by pretreating the slice with an NOS inhibitor. These data illustrate that NMDA receptor stimulation can, indirectly, modulate GABAergic mediated IPSPs via generation of NO. Thus, NO may serve to couple glutamatergic and GABAergic transmission and in this capacity provide a neutralizing influence to prevent neuronal overexcitability.

9.3.2.3.4 Other

Lo et al.\textsuperscript{88} have described an interaction in the NTS between adenosine and NO. Microinjection of adenosine into the NTS evoked a depressor/bradycardic effect that was attenuated by prior injection of NOS inhibitors. Conversely, the pressor response evoked by 5-HT\textsubscript{3} receptor stimulation was also attenuated by either NOS or sGC antagonism.\textsuperscript{132} Thus, both sympatho-excitatory and sympatho-inhibitory responses evoked from the NTS can be modulated by NO.

9.3.2.4 Making Sense from a Messenger that Is Diffuse

Over the years, authors have remarked about the viscerotopic organization of the NTS. Indeed, there is evidence for viscerotopic afferent termination in the nucleus,\textsuperscript{14} but significant overlap is recognized. In terms of the baroreceptor input, there seems to be some organization of aortic versus carotid sinus nerve inputs. Sawchenko and colleagues have demonstrated that aortic baroreceptors project mainly in regions
rostral to commissural NTS whereas the latter region seems to be home to carotid sinus afferents.19,20 Whether this has any functional correlate awaits testing. This topographical organization is greatly diluted when one compares the locality of physiologically characterized NTS neurones, including putative second order neurones.108 Even in the so called “cardiovascular NTS” (a term that is grossly misleading) neurones responding to stimulation of the peripheral chemoreceptors, subdiaphragmatic vagus nerve (i.e., abdominal afferents) as well as arterial baroreceptors are all found inter-mixed.108 This apparent loss of viscerotopographic representation at the neuronal level probably relates to the fact that most inputs land on dendrites that can extend many hundreds of microns from the cell body.27,111,114 With such inter-mixing of functionally or reflex distinct NTS neurones together with their tight packing one has to question whether it is possible for a diffusible modulator such as NO to exert specific actions on neuronal circuits mediating different reflex pathways? If not, are we to consider that the effects of NO are analogous to those of “volume transmission”? This appears not to be the case, rather NO can exert reflex specific effects. Figure 9.5 illustrates that selective actions of NO in the NTS can be demonstrated. In this example, microinjection of a NO donor depress the baroreceptor reflex bradycardia while the cardiac response to peripheral chemoreceptor stimulation remained unaffected (but could be quenched by hyperpolarizing agents given at the same site — not shown). The question arises as to how NO exerts specific effects in the NTS given that these neurones mediating these reflexes are essentially intermingled in the same areas of NTS,108 especially if NO affects targets in excess of 150 µm, as suggested from in vitro brain slice work.43,55 We propose that NO-mediated signalling is a tightly targeted process. There are several mechanisms which may act in concert to restrict the actions of NO and enable it to achieve cellular resolution specificity in spite of its ability to freely cross cellular membranes.

9.3.2.4.1 Putting the Source Next to the Target

When NO is released by nNOS activation it may occur in micro-domains that coincide with the location of neuronal membrane NMDA receptors. Supramaximal activation of NMDA receptors produces only 1-2 nM NO, which is comparable to EC50 of sGC.42,44,66 This suggests that it is highly unlikely that the concentration of NO released from nNOS micro-domains is restricted to adjacent neurones only. Several factors will actively reduce the spread of released NO. For example, NO scavenging, especially by haemoglobin contained in capillaries, will reduce NO spread. This may be a major factor in the NTS which has been described as a highly vascularized structure.45 In addition, an as yet unidentified cellular mechanism for NO inactivation may limit NO half-life in the brain to around 100 ms which also imposes strong restrictions on the maximal distance of NO signalling in vitro.43 Hence, it is likely that under physiological circumstance, nNOS-released NO only targets selected neurones superposed to nNOS microdomains. A good example of NO microdomains is that within a single myocardial cell where nNOS and eNOS operate to liberate NO to affect distinct biochemical processes in different parts of the same cell (for review, see Reference 113). In case of NO released from the endothelial cells in the brain, the situation might be somewhat different because the widespread capillary network in NTS (see Reference 45) could generate a diffuse
NO signal within the capillary lumen. How much of the eNOS-produced NO can actually cross the blood brain barrier to reach neurones is unclear and may relate to both blood flow and PaO₂ levels; the latter affecting haemoglobin’s affinity for NO.\textsuperscript{99} It is likely that on the brain side of the capillary NO concentration will drop very sharply due to the same cellular sinks mentioned above and, therefore, we postulate that the targets for eNOS-derived NO must be located very close to the capillary wall.

A possibility is that the type of NOS isoform might be related to a transmitter system. In support of this notion, Kano et al.,\textsuperscript{57} using dialysis techniques, infused NMDA into cortical structures while measuring GABA and glutamate efflux in both eNOS and nNOS knockout mice. They found that NMDA infusions in nNOS knockout animals produced greater GABA release compared to glutamate whereas glutamate concentrations were higher compared to GABA in eNOS knockout mice. They proposed that nNOS was associated with glutamatergic transmission (for which there is evidence in the NTS — see Sections 9.3.1.2.1 and 9.3.1.3 and Figure 9.3) and that GABAergic transmission was related to NO released from eNOS; the latter supports our data.\textsuperscript{109} Thus, the type of NOS isoform may offer specificity of effect of NO. nNOS and eNOS have very different spatial distributions within the NTS with nNOS confined to neurones with the majority of eNOS housed within the endothelium. It remains controversial as to whether eNOS also resides in glia and neurones but there have been neuronal sightings based on immunohistochemical data.\textsuperscript{31a,110,145}

9.3.2.4.2 Regulating sGC Responsiveness

The response of the target cell to NO may be a highly dynamic and regulatable process due to the characteristics of sGC: this may enable certain physiological responses to NO to be seen only under conditions when sGC becomes active. For example, sGC is negatively regulated by cytosolic Ca\textsuperscript{2+} and, therefore, cellular response to NO may depend on intracellular Ca\textsuperscript{2+} levels. sGC is relatively rapidly desensitized in a matter of seconds, which may also limit NO actions via cGMP pathway in cells exposed to sustained high NO concentrations.\textsuperscript{12} Intracellular ATP also plays a critical role in controlling sGC activation by NO. ATP inhibits sGC with a Ki of ~1mM suggesting that in cells maintaining normal physiological ATP concentration, sGS activity is inhibited by more than 50%.\textsuperscript{125} Thus, if metabolic stress or hypoxia leads to a decrease in ATP levels, cellular responses to NO might be potentiated.\textsuperscript{125} This mechanism might have added to the inconsistency of the data obtained with microinjections of NO donors and NOS inhibitors in the NTS as the responses could be dependent on brainstem oxygenation levels and ATP concentration. Regulation of sGC together with variations in the rate of cGMP breakdown and levels of phosphodiesterase activity may also provide a fundamental way for de-coding NO signals under both physiological and pathophysiological conditions and allow only some of the potential NO targets to respond to it at any given time.\textsuperscript{12}

9.3.2.4.3 Differential Sensitivity to NO within Excitatory and Inhibitory Synapses

NO signalling specificity may rely on a differential sensitivity of biochemical pathways in different neuronal populations to NO. For instance, is the machinery
regulating GABA release more sensitive to NO than that of glutamate? So far our intracellular data indicate that this is the case in the NTS. Evoked EPSPs were potentiated at lower concentrations of aqueous solutions of NO than evoked IPSPs in vitro.157

9.3.2.4.4 Inhibitory Synaptic Weight Differences between Reflex Circuits in NTS

The functional outcome of NO release in a complex nucleus such as NTS might depend on downstream differences in synaptic weights affecting distinct reflex circuits. In our experiments, NTS microinjections of small doses of pentobarbitone, only sufficient for potentiation of the effects of endogenous GABA, inhibited baroreflex-evoked bradycardia, but not chemoreceptor-evoked bradycardia.59 Thus, it is possible that the synaptic connectivity in NTS is organized in such a way that an increase in GABA release may have stronger inhibitory action on some neuronal pathways compared to others. If this is the case, then this difference could be easily amplified in the presence of NO.

9.3.2.4.5 Is the Response Really Mediated by NO?

Finally, as raised earlier, some actions ascribed to NO may actually result from OONO\(^-\), which can affect transmitter release independently of NO.104 Therefore, in conditions of either high concentrations of NO, superoxide or lack of oxygen, OONO\(^-\) will form. OONO\(^-\) may have completely different actions to NO affecting additional and/or different cellular targets. Therefore, comparisons between studies can only be made where the effects have been proven to be dependent on sGC activation. Figure 9.6B demonstrates that the NO-mediated depression of the cardiac baroreflex is mediated by sGC as the effects were completely abolished by ODQ.

9.3.2.5 Questioning Our Experimental Approach: Are We Being too Heavy Handed?

If NO signalling is tightly focused to adjacent cells or sub-cellular structures, the use of NO donors becomes questionable as these drugs are unable to provide the required degree of spatial accuracy and concentration. Indeed, the tissue concentration of NO from NO donors is completely unknown and this might introduce additional artefacts. An alternative is to use L-arginine to enhance NO synthesis by native NO syntheses. This should occur at the same sites of release as when endogenous NOS becomes activated by physiological stimuli. One caveat with L-arginine is that one has to assume that the activity of both eNOS and nNOS is normal and not disturbed by a particular experimental setting. Otherwise a spurious result may occur. It is also possible that the bioavailability of microinjected L-arginine for nNOS and eNOS could differ. In addition L-arginine might have actions unrelated to NO production: for example, both, L, and D-arginine had similar effects on pressure and renal sympathetic nerve activity when injected in NTS.97

In loss-of-function experiments, NO production may be inhibited using a variety of NOS inhibitors or NO can be removed using NO scavengers.141 Unfortunately, the specificity of many NOS blockers is far from perfect and they allow only some
degree of differentiation between eNOS and nNOS. When NOS blockers are used one has to presume that there is endogenous NO synthesis and that a lack of NO release will cause a change in a measured parameter. In our experiments in an unanaesthetized arterially perfused rat preparation, we did not observe any measurable effects of NOS blockers (L-NAME or L-NMMA) microinjected into the NTS on either baroreceptor or chemoreceptor reflexes, indicating an absence of endogenous NOS activity. However, both drugs antagonized the depressant action of angiotensin II on the baroreflex implying that angiotensin II released NO.109,110 Talman and Dragon142 reported that an nNOS inhibitor - AR-R 17477 microinjected into the NTS significantly increased mean arterial pressure without affecting heart rate, and reduced the gain of the baroreflex in anaesthetized rats. It is therefore possible that factors such as anaesthesia (which, among many other actions triggers release of large amounts of renin,65 and presumably angiotensin II), can lead to activation of NOS and, therefore, introduce a NO-dependent effect. The caveats described above could help explain the inconsistencies in the role of NO in baroreflex gain as illustrated in Table 9.3.

9.3.2.6 Gene Transfer Approaches to Studying Nitroxergic Mechanisms in the NTS

With the subtlety of the nitroxergic system in the NTS and the problems associated with conventional pharmacological approaches, what is the alternative? Recently, we and others have adopted viral gene transfer as a technique to disentangle the complexities of NO mechanisms in the NTS. Here, we discuss the pros and cons of the technique indicating potential pitfalls.

9.3.2.6.1 Over Expressing a NOS Isoform

eNOS was over expressed in the NTS using an adenoviral vector and measures of heart rate and blood pressure made.48,49,67,68 In this scenario, the spatial distribution of the expressed isoform will depend on the virus type and the promoter used. In the case of the aforementioned works, human cytomegalovirus (HCMV) promoter was used which we have shown causes preferential expression in the endothelium and glia, at least in NTS.61,62 Since it is unlikely that the infected cells will be unable to regulate activity of the ectopically expressed transgene, large quantities of NO will be produced. This will affect the spatial issues discussed above concerning source and target proximity. Thus, NO may reach new targets which it was unable to activate when released from native NOS activity. In conclusion, it seems that if the NO signalling does occur in microdomains, data obtained by ectopic expression of NOS isoforms will need to be re-evaluated.

9.3.2.6.2 Loss of Function

A way to evaluate NOS function may be to express a dominant negative protein to block NOS activity.58,115 We adopted this approach using adenoviral vectors.110 This critically depends on the specificity of the dominant negative molecule and the ability of the viral vector to express sufficient quantities of the dominant negative protein in cells that contain the native NOS isoform. There are a number of advantages of expressing dominant negatives:
• Endogenous activity is the only activity affected.
• It allows an assessment of the functional significance of different NOS isoforms (neuronal versus endothelial, for example).
• Chronic eNOS (or nNOS) down-regulation can be assessed in unrestrained, conscious animals that are not subjected to any additional experimental stress.

So far, in experiments with a dominant negative eNOS protein, we have observed an increase of the spontaneous baroreflex gain in normotensive animals\textsuperscript{154,155} without a change in resting levels of blood pressure. A similar experiment with the nNOS dominant negative would help to resolve some of the controversies around the role of nNOS in the NTS. Viral vectors would also allow targeting different cell types specifically (neurones vs. endothelium vs. glia), thereby allowing an analysis of the functional significance of different cellular components. This awaits testing.

9.4 CONCLUSION

We hope that this discussion has shed light on the subtleties of the nitroxergic system and thus the need to revise our experiments that take issues such as NOS isoform type, NO concentration, NO diffusion distance, and NO source versus target into consideration. It would be most helpful if we could establish whether NO release actually occurs in microdomains and how tight the coupling between the NO source and its target really is. It is also evident that understanding a role for NO in the NTS in terms of hypertension requires chronic studies. The use of long-term blood pressure recording, together with spatial and temporal regulation of gene expression, will be an important next step for unmasking physiological and pathophysiological roles of NO in this nucleus.

ACKNOWLEDGMENTS

We wish to thank the British Heart Foundation and the Biotechnology and Biological Sciences Research Council for their financial support. Sheng Wang is supported by an Overseas Research Scholarship.

REFERENCES


COLOR FIGURE 5.4 Biotin-conjugated dextran-tetramethylrhodamine (TMR-B) is compatible with neuronal stains, allowing for permanent visualization of vagal terminals and myenteric neurons. (A) An IGL (golden brown; TMR-B stained with a DAB reaction) is seen in close approximation to several neurons (light blue; stained with the putative pan-neuronal stain cuprolinic blue) within a myenteric ganglion. (B) TMR-B (golden brown) is also compatible with nicotinamide adenine dinucleotide phosphate diaphorase (NADPHd; dark blue) staining. In the myenteric plexus, neurons that produce nitric oxide synthase can be demonstrated histochemically in aldehyde-fixed tissue using a histochemical reaction for NADPHd. Note the disparate relationship of the IGLs to the NADPHd-positive and NADPHd-negative (unstained) neurons. Mouse stomach whole mounts; scale bar = 25 μm.
COLOR FIGURE 5.5 The durability of the TMR-B molecule makes it an excellent choice for lengthy staining protocols, such as immunocytochemistry, that typically require exposure of the tissue to harsh detergents such as Triton X-100. (A) An IGLE (dark brown) visualized using TMR-B stained with a DAB reaction can be seen in close association with several myenteric neurons (stained red using Vector NovaRED) positive for the calcium-binding protein calbindin, which was labeled using a polyclonal antibody to calbindin. (B) TMR-B can also be used in conjunction with both a histochemical reaction and an immunocytochemical reaction. An IGLE is labeled brown using the tracer TMR-B stained with DAB, while two different nonoverlapping phenotypes of myenteric neurons are labeled different colors. A nitric oxide synthase producing neuron is labeled dark blue using an NADPHd reaction, while a calbindin-positive neuron is labeled red using NovaRED. Mouse stomach whole mounts; scale bar = 25 µm.
COLOR FIGURE 9.3 nNOS in the NTS; association with the baroreceptor reflex pathway and glutamatergic neurotransmission. **A:** Fos expression (brown nuclear staining) in dorsolateral NTS neurones following intravenous injection of phenylephrine. Some of these arterial pressure-sensitive NTS neurones were positively stained for NADPH-diaphorase (purple cytoplasm) suggesting that some contain nNOS. Key: Open arrow: NADPH-diaphorase staining only; arrow head: arterial pressure-sensitive only; filled arrows: NADPH-diaphorase and arterial pressure-sensitive. (Data from Chan et al. 2000. With permission.)

**B:** Confocal image of fibers within the caudal NTS immunopositive for nNOS (blue fluorescence) and glutamate related transmission (green: glutamate vesicular transporter type 1 or Vglut1; red: Vglut2; magenta: nNOS and Vglut2). Note that nNOS was only co-localized with Vglut2 and that Vglut2 did not co-localize with Vglut1. (Data from Lin et al. 2004. With permission.)

**C:** Triple immunocytochemical detection for nNOS (green; C1), the AMPA receptor subunit – gluR1 (red; C2) and the NMDA receptor subunit – NMDAR1 (blue; C3). Triple labeled NTS neurones appear white-light blue (C4). Not all neurones were immunopositive for nNOS but those that were also contained NMDAR1 and gluR1. (Data from Lin and Talman 2002. With permission.)

**D:** Co-localization of the NMDA receptor subunit – NMDAR1 (red fluorescence; D1) with nNOS positive caudal NTS neurones (green; D2) appear yellow (D3). Almost all nNOS positive neurones contained the NMDAR1 whereas only a proportion of NMDAR1 neurones contained nNOS. (Data from Lin and Talman 2000. With permission.)
COLOR FIGURE 9.4 Presence of eNOS in the NTS. A: The NTS receives a rich supply of blood vessels that may reflect the high density of neuronal activity in this region of the brain. B: Double immunofluorescence for eNOS (yellow) and the angiotensin type 1 receptor (AT1R; red) are both present on capillaries running through caudal NTS. With the high capillary density, eNOS may significantly contribute to NO production in this nucleus. (Unpublished data from J.F.R. Paton, H. Waki, S. Yao, J. Deuchars, and S. Kasparov.)
COLOR FIGURE 10.2 Neuroanatomical studies of AVPNs innervating the airways of the ferret. (A) Coronal section showing the rostral nucleus ambiguus region (rNA) where the majority of AVPNs are located. (B) Higher magnification of the area (box in A), illustrating labeling in AVPNs following cholera toxin β subunit (CT-b) injections into the tracheal wall. Panels C-F are confocal microscopic images: (C) CT-b labeled AVPNs visualized with a fluorescein-conjugated secondary antibody (FITC, green). (D) ChAT labeling observed in rNA neurons is visualized with Texas Red-conjugated secondary antibody (TR, red). (E) Superimposed images overlapping CT-b (green) and ChAT (red) signals. Yellow color indicated colocalization of signals. (F) Higher magnification of the area (box in E). Scale bar= 1mm (A), 150 µm (B), 60 µm (C-E), and 20 µm (F).
COLOR FIGURE 10.6 Projections of locus coeruleus (LC) noradrenergic cells to AVPNs. (A) Schematic of a coronal section showing the location of pontine noradrenergic cell groups. (B) LC neurons were labeled with pseudo rabies virus-encoding green fluorescent protein (PRV-GFP) following injection of PRV-GFP into the upper lobe of the right lung. (C) TH-containing cells (red) were identified in the same section of the LC. (D) A subpopulation of LC neurons projecting to AVPNs innervating the lung is noradrenergic, as indicated by the arrows.
COLOR FIGURE 10.8 Confocal microscope images of α2A-adrenergic receptor subunit (α2A-AR) expression in AVPNs innervating the extrathoracic trachea. (A) AVPNs are identified with CTb using a fluorescein-conjugated secondary antibody (FITC, green). (B) Specific α2A-AR expression, visualized with a Texas Red-conjugated secondary antibody (TR, red), is observed on perikaryon and dendritic membranes of neurons within the rNA. (C) Superimposed images overlapping CTb-specific (FITC, green) and α2A-AR-specific (TR, red) signals. Yellow color indicated colocalization of signals. (D) Higher magnification of the area (box in C). In control experiments, there was no apparent cross-reactivity of the secondary antibodies. Bar = 40 µm (A-C); 20 µm (D).
COLOR FIGURE 10.10 (Upper panel) Confocal microscopic images showing specific expression of the 5-HT<sub>1A</sub> subunit by AVPNs in a ferret. CT-b labeled AVPNs are identified using a fluorescein-conjugated antibody (green). The 5-HT<sub>1A</sub> subunit, visualized by Texas Red-conjugated secondary antibody (red), was observed on the membrane of the perikaryon of neurons within the rNA. Superimposed images overlapping CT-b-specific (green) and specific 5-HT<sub>1A</sub> (red) signals. Yellow color indicated colocalization of signals. (Lower panel) An example of the effect of raphe neuron stimulation (L-glutamate, 4 nmol/80 nl) on serotonin release in the rostral ventrolateral medulla (rVLM) of an anesthetized cat. Release of serotonin following stimulation of raphe nuclei decreased airway smooth muscle tone and lung resistance (P<0.05). This response was diminished after blockade of 5-HT receptors within the ventrolateral medulla.

COLOR FIGURE 11.1 Confocal fluorescent microscopic image illustrating the receptor structure of an SAR located in the peripheral airway (diameter <180 µm) of a rabbit; the receptor was first identified by electrophysiological recording. (Panels A-D) Consecutive optical slices from the outside (A) to the inside (D) of the bronchiole wall. Smooth muscle bands can be seen in C. (E) projection image of a stack of the six optical sections (each 3.3 µm thick). Note that the axon is about 3 µm in diameter and the branch to the left low corner is about 1.5 µm. (Modified from Yu et al., 2003.)

185
**COLOR FIGURE 12.1** Schematic overview of the vagal afferent innervation to the gastrointestinal tract. Vagal afferents terminate in the muscle layers as intramuscular arrays (IMA) or intraganglionic endings (IGLE) associated with the enteric nervous system (ENS) and in the mucosa where they are exposed to different mechanical forces and chemical microenvironments. These afferents convey moment-to-moment information along modality-specific pathways that determine reflex activity, endocrine status through the hypothalamic-pituitary-adrenal (HPA) axis, behavioral responses affecting food intake and metabolic function, and that may influence perception either directly or by modulating descending influences that regulate spinal sensory pathways.

**COLOR FIGURE 12.6** Schematic representation of ion channels and receptors that determine afferent sensitivity.
COLOR FIGURE 16.2 NEP expression in a section of tracheal mucosa visualized by immunofluorescence with an FITC-conjugated specific antibody. In the airway epithelium NEP is concentrated in the basal cell layer, thus in a strategic position to cleave and inactivate the neuropeptides near their sites of release from the network of sensory nerve terminations concentrated in close association with the basal cells. Strong immunoreactivity can also be seen overlapping areas of Monastral red pigment extravasated from post-capillary venules after an injection of SP.
COLOR FIGURE 16.3 Mast cells/nerve interactions – Lung sections from weanling rats sacrificed 5 days after the intranasal inoculation of virus-free medium (A) or RSV suspension (B). Mast cells (arrows) were identified by immunohistochemistry using a monoclonal antibody specific for tryptase. An average sevenfold increase in mast cell density was found in the lung sections from RSV-infected rats compared to pathogen-free controls. These mast cells were always clustered in close vicinity of nerve fibers (arrowhead).
COLOR FIGURE 18.1 Schematic representation of the placement of the VNS Therapy generator and the lead connecting the generator to the left vagus nerve. (Courtesy of Cyberonics, Inc., Houston, TX.)

COLOR FIGURE 18.2 Stimulating electrodes attached to the vagus nerve. The top helical coil is the negative electrode. The middle coil is the positive electrode. The bottom coil tethers the other electrodes to nearby connective tissue. (Courtesy of Cyberonics, Inc., Houston, TX.)


10 Monoaminergic Modulation in the Brainstem: Implication for Airway Function

Musa A. Haxhiu, Constance T. Moore, and Prabha Kc

CONTENTS

Abstract .................................................................................................................. 248
10.1 Introduction .................................................................................................. 248
10.2 General Considerations ........................................................................... 248
10.3 Structural Characteristics of AVPNs ............................................................ 250
  10.3.1 Ultrastructural Characteristics of AVPNs ........................................ 252
  10.3.2 CNS Innervation of AVPNs ............................................................. 254
10.4 Central Monoaminergic Control of AVPNs ................................................ 255
  10.4.1 Catecholaminergic Neurons ................................................................ 255
    10.4.1.1 Neuroanatomical Studies .................................................. 257
    10.4.1.2 Microdialysis and HPLC Measurements ................................. 259
    10.4.1.3 The Adrenergic Receptor Family ..................................... 259
    10.4.1.4 Physiological Responses ................................................... 260
  10.4.2 Serotonergic Neurons ....................................................................... 261
    10.4.2.1 Neuroanatomical Studies .................................................. 261
    10.4.2.2 The Electrochemical Studies ............................................ 261
    10.4.2.3 The Serotonergic Receptors ............................................. 263
    10.4.2.4 Physiological Experiments ............................................... 264
10.5 Functional Role of Central Control of Cholinergic Outflow to the Airways ............................................. 264
10.6 Relevance of Central Monoaminergic Inhibitory Inputs to the AVPNs .... 265
10.7 Summary and Future Studies .................................................................... 267
Acknowledgments ................................................................................................. 267
References .............................................................................................................. 267
ABSTRACT

Central nervous system (CNS) regulation of airway functions involves integrated networks that funnel information to the controlled airway effector units, via the airway-related vagal preganglionic neurons (AVPNs), the final common pathway from the brain to the airways. Activity of AVPNs is regulated by signals from sensory receptors that travel with afferent ascending nerves to the CNS and the incoming inputs from CNS cell groups to these neurons. Changes in AVVPN discharge can be modulated by inhibitory pathways. Using conventional and transneural labeling techniques, and ultrastructural, molecular, and physiological approaches, it has been shown that brainstem neurons (noradrenaline- and serotonin-containing cells) exert inhibitory influences on AVPNs. Inhibitory effects are mediated via both wiring (synaptic) and volume (nonsynaptic) transmission. Down-regulation of central monoaminergic inhibitory influences that results in a shift from inhibitory to excitatory transmission may lead to a hyperexcitable state of AVPNs, an increase in cholinergic outflow and airway hyperreactivity.

10.1 INTRODUCTION

Over the last 25 years interest in autonomic nervous system regulation of the airways has dramatically increased. This reflects evidence suggesting that repeated exposure to the allergens and environmental irritants causes changes in neural pathways, setting the stage for airway hyperresponsiveness. Greater spontaneous and evoked airway responses that include vasodilation, fluid hypersecretion, and smooth muscle contraction may last for hours, suggesting that the central nervous system (CNS) is involved in setting a new, more reactive state of the airways.

10.2 GENERAL CONSIDERATIONS

Central nervous system control of airway functions (Figure 10.1, Level 4) involves integrated networks along neural axis that funnel information to the controlled airway effector units via the airway-related vagal preganglionic neurons (AVPNs) in the medulla oblongata. These cells represent the final common pathway from the brain to the airways via intrinsic tracheobronchial ganglia that are part of the network for automatic feedback control (Level 3). Each ganglion, located in close proximity to the effector systems, possesses a relatively large number of neurons that can be considered as an expanded parasympathetic preganglionic efferent motor system (Level 2). This structural organization could explain the strong effects of a relatively small number of vagal efferent fibers on coordinated reflex changes of the airway smooth muscle tone, submucosal gland secretion, and blood flow along the tracheobronchial tree. A similar arrangement has been described in parasympathetic control of the enteric tract. This unified concept does not exclude probability that some of the vagal preganglionic neurons also provide, to a lesser degree, direct innervation of airway epithelial cells and the alveolar interstitium of the lung, since ganglia are absent altogether from the bronchial subepithelial space and the most distal gas exchanging units.
Previously, autonomic ganglia were considered as relay stations for the transfer of information from the CNS to the periphery that can be activated by inputs from the AVPNs in response to local sensory stimuli. However, recent studies clearly indicate that signals transmitted through the preganglionic nerves are not simply relayed by autonomic ganglionic cells and postganglionic fibers to the airways, they are integrated, filtered, and modulated before reaching the airway neuroeffector sites. These fibers innervate sensory receptors and transmit information from the airways to the CNS. They modulate the activity of AVPNs through central multisynaptic pathways and they may affect function of effector organs via two ill-defined local pathways that include axon reflex responses (Level 1) and sensory innervation of intrinsic airway ganglia (Level 2).

In recent years, there have been several important advances in our understanding of peripheral neural control of airway functions. These include effects of exposures to environmental noxious agents and of inflammation upon functional and phenotypic changes of sensory neurons, intrinsic ganglionic cells, and airway effectors that may explain pathophysiological features of airway diseases.

The extensive network of vagal afferent fibers innervates the bronchopulmonary sensory receptors that are specialized for detecting changes in chemical, mechanical, or thermal stimulus energy. They originate from the bipolar airway vagal afferent neurons found within the nodose and jugular ganglia and participate in reflex events of a protective nature. Furthermore, the C-fiber afferents are also believed to be responsible for mediating local axon reflexes, the release of neuropeptides and neurogenic inflammation (Level 1). Neurotransmitters released by sensory fibers may also activate local intrinsic ganglia, which are present in large and central airways in all species studied, and may contain excitatory or inhibitory neurotransmitters.
The central fibers of these bipolar sensory neurons ascend in the vagus nerve and enter the brain stem through the solitary tract, synapsing on neurons within the nucleus of the solitary tract (NTS), considered as second order neurons, which in turn projects to AVPNs. Signals from the NTS neurons may augment or suppress the activity of AVPNs, depending on the nature of the stimulus and consequently released neurotransmitter(s) at the synapse (synaptic transmission) or in the vicinity of the AVPNs (volume transmission).

The changes in activity of AVPNs that occur in response to stimulation of sensory receptors by specific stimuli are preprogrammed for control of distinct functions of effector units and for the coordination of rapid reflex responses of multiple systems that are characterized by reproducible qualitative changes, mediated via relatively fixed “hardwired” connections within the reflex neuronal network. Recent studies showed that excitatory signals arising from bronchopulmonary afferents stimulate second order neurons within the NTS via release of glutamate that activates alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors expressed by these neurons. Processed information, via the glutamate-AMPA signal pathway, is transmitted to AVPNs, and from AVPNs to bronchopulmonary effector systems, causing an increase in blood flow, submucosal gland secretion, and smooth muscle tone. The strength and quantitative aspect of the excitatory reflexes can be modified by central mechanisms that need further investigation.

Despite methodological advances made in neuroanatomical tracing, receptor immunohistological techniques, and neurophysiological methods, understandably, less consideration has been given to central control mechanisms. This is, partly, due to the complexity of the central nervous system, numerous projections that may involve multiple anatomical sites, and different forms of functional interaction, as well as interplay between excitatory and inhibitory signaling pathways. Therefore, there are a number of important unanswered questions concerning the central control of vagal preganglionic neurons that provide cholinergic outflow to the airways. This chapter is focused on the recent findings of the central monoaminergic inhibitory control of AVPNs that modulate cholinergic outflow to the airways and discusses possible pathophysiological consequences of alterations of these pathways regulating airway homeostasis.

10.3 STRUCTURAL CHARACTERISTICS OF AVPNs

Vagal preganglionic neurons that generate cholinergic outflow to the airways can be viewed as central integrators of multiple excitatory and inhibitory inputs that connect the brain with the bronchopulmonary effector system. Studies using retrograde tracer techniques indicate that in mammals (Figure 10.2), the preganglionic motor neurons innervating the airways arises from the rostral nucleus ambiguus (rNA) and from the rostral portion of the dorsal motor nucleus of the vagus (DMV). Furthermore, findings of tracing studies imply that the majority of AVPNs have multilobar projections and via intrinsic ganglia may be involved in the innervation of multiple airway segments, thereby assuring the symmetry and simultaneity of the bronchomotor responses. In addition, results of retrograde tracings suggest that some of the preganglionic neurons may provide direct innervation to the airway
tissues, without interposition of intrinsic neurons. Although it has been shown that other effector systems, for example ciliary muscle, receive dual parasympathetic innervation, directly from preganglionic neurons and via ganglionic cells, our recent studies in ferrets do not support functionally operative direct communication between AVPNs and the airway smooth muscle, bypassing intrinsic cholinergic ganglia.

Studies using the double-labeling method that combines the retrograde tracer cholera toxin β subunit (CT-b) and immunohistochemistry for choline acetyltransferase...
Advances in Vagal Afferent Neurobiology

(ChAT), indicate that in ferrets, AVPNs innervating the trachea and the intrapulmonary airways are cholinergic in nature (Figure 10.2) and use acetylcholine as a neurotransmitter. These neurochemical findings support the physiological studies showing that stimulation of efferent fibers in the trunk of the vagus nerve produces pronounced contraction of the airway smooth muscle that is solely mediated via cholinergic mechanisms, as well as an increase in the activity of airway secretory glands. In addition, cholinergic transmission is involved in the reflex induced submucosal gland secretion.

Furthermore, virtually all vagal preganglionic neurons innervating the trachea and intrapulmonary airways are cholinergic and co-express vasoactive intestinal polypeptide (VIP), but not nitric oxide synthase (an enzyme involved in the generation of NO), indicating that acetylcholine and neuropeptide VIP are coexisting messenger molecules in AVPNs. The role of VIP in the regulation of cholinergic outflow to the airways is not known. VIP could be coreleased with ACh at the synapse with intrinsic ganglionic neurons and may enhance synaptic efficacy in airway ganglia.

Functionally, the AVPNs within the rNA play a greater role in generating the cholinergic outflow to airway smooth muscle than preganglionic cells of the DMV. Based on this observation it was suggested that DMV neurons projecting to the airways may innervate tracheobronchial secretory glands, and blood vessels. However, more recent studies showed that AVPNs within the rNA also mediate reflex increases in submucosal gland secretion and blood flow, supporting the notion that cholinergic innervation arising from AVPNs lacks target specificity.

10.3.1 Ultrastructural Characteristics of AVPNs

It has been suggested that the ultrastructure and synaptology of the different divisions of the nucleus ambiguus innervating the alimentary system may be associated with specific physiological functions. Recently, we used cholera toxin β-subunit conjugated to horseradish peroxidase, as a retrograde cell body tracer, and electron microscopy to define ultrastructural characteristics of the AVPNs. Retrogradely labeled AVPNs in the nucleus ambiguus were readily detectable in the electron microscope. Cell bodies of labeled AVPNs were around $32 \pm 1 \times 23.0 \pm 1.3 \mu m$ (mean ± SEM.) in size, with abundant cytoplasm and intracellular organelles, a round uninvaginated nucleus, occasionally showing a prominent nucleolus (Figure 10.3) and displayed both somatic and dendritic spines (Figure 10.4). Somato-somatic appositions or somato-dendritic appositions without intervening glial processes and dendritic “bundling” of tracheal AVPNs were not observed. The axons of these neurons were seldom labeled, but were usually myelinated (Figure 10.4).

Localization and the ultrastructural features of AVPNs differ from neurons which have been examined in the dorsal and ventral columns of the nucleus ambiguus innervating the alimentary system. By comparison, esophageal motoneurons, despite some similarities, can be distinguished from AVPNs located within the rNA by the presence of extensive somato-somatic and somato-dendritic appositions. Furthermore, they also display finger- and leaf-like somatic protrusions, which
partially wrap longitudinally oriented dendrites and axons, and dendritic bundling is prominent. These latter characteristics appear to be unique to the compact formation of the nucleus ambiguus and clearly differentiate these neurons from tracheal VPNs.

In summary, at the ultrastructural level, AVPNs innervating the extrathoracic trachea are clearly distinguished from pharyngeal, laryngeal or esophageal motor-neurons in other subdivisions of the nucleus ambiguus. These data are consistent with the hypothesis that differences in the ultrastructure and synaptology of the different divisions of the nucleus ambiguus may be associated with specific physiological functions.

FIGURE 10.3 A retrogradely labeled airway-related vagal preganglionic neuron (AVPN) in the nucleus ambiguus. The neuron is identified by the presence of the tetramethylbenzidine tungstate (TMB) crystalline reaction product (large arrows). Note the round nucleus and prominent nucleolus. The area inside the box is enlarged in the inset and shows an example of a substance P immunoreactive nerve terminal (T), indicated by an amorphous diaminobenzidine reaction product, forming an asymmetric synapse with the perikaryon. Unlabeled (non SP-ir) terminals (t) are identified for comparison. The calibration bars = 2 µm in the larger panel and 500 nm in the inset. (From Reference 88. With permission.)
Recently, using conventional and transneural labeling techniques, it has been shown that the innervation of vagal preganglionic neurons that regulate parasympathetic outflow to the airways arises from cell groups located in the brainstem, and from several higher brain regions. By comparing the CNS inputs to the parasympathetic preganglionic neurons that innervate intrapulmonary airways with a comparable study dealing with the projections to the tracheal parasympathetic preganglionic cells, common patterns of innervation are seen. AVPNs receive inputs from the cell groups located in the ventral aspect of the medulla oblongata, NTS, pons, mesencephalic, dorsal, lateral and paraventricular hypothalamic, and the amygdaloid nuclei. Hence, the parasympathetic preganglionic neurons that innervate the airways are controlled by networks of brainstem and suprapontine cell groups that

**FIGURE 10.4** Somatic, dendritic and axonic profiles of retrogradely labeled AVPNs. (A) The perikaryon contains abundant rough endoplasmic reticulum (RER) and somatic spines and forms axo-somatic synaptic contacts (small arrows). (B) A dendrite illustrating dendritic spines forming axo-dendritic synapses (small arrows). (C) Axons of AVPNs are myelinated (M). Unlabeled myelinated axons (m) are indicated for comparison. The calibration bar = 500 nm for all panels. (From Reference 88. With permission.)

### 10.3.2 CNS INNERRVATION OF AVPNs

Recently, using conventional and transneural labeling techniques, it has been shown that the innervation of vagal preganglionic neurons that regulate parasympathetic outflow to the airways arises from cell groups located in the brainstem, and from several higher brain regions. By comparing the CNS inputs to the parasympathetic preganglionic neurons that innervate intrapulmonary airways with a comparable study dealing with the projections to the tracheal parasympathetic preganglionic cells, common patterns of innervation are seen. AVPNs receive inputs from the cell groups located in the ventral aspect of the medulla oblongata, NTS, pons, mesencephalic, dorsal, lateral and paraventricular hypothalamic, and the amygdaloid nuclei. Hence, the parasympathetic preganglionic neurons that innervate the airways are controlled by networks of brainstem and suprapontine cell groups that
lie in the same regions known to be involved in central control of cardiovascular and respiratory systems, as well as in regulation of state dependent behavioral changes. Whether different responses are produced by single neurons capable of affecting different neural pathways or by a complex set of heterogenous neurons regulating these individual systems needs to be examined.

In the brainstem (medulla oblongata and pons), the most consistent labeling was found in the subsets of monoaminergic cell groups: serotonergic containing neurons within the raphe pallidus, raphe obscurus, and nearby parapyramidal nucleus, in the pontine noradrenergic neurons of the locus coeruleus and subcoeruleus, and within the A5 noradrenergic cell group. In this review the focus is centered on the evidence linking monoaminergic neurons to the central control of cholinergic outflow to the airways.

10.4 CENTRAL MONOAMINERGIC CONTROL OF AVPNs

Under normal conditions, reflexly induced elevation of bronchomotor tone and its decay partly depends upon inhibitory input to AVPNs. Recently, it has been shown that brainstem monoaminergic neurons that project to AVPNs (Figure 10.5) exert inhibitory influences on cholinergic outflow to the airways that is conveyed through specialized membrane junctions with soma or proximal dendrites (i.e., synaptic transmission), and nonsynaptically, using the extracellular space as a communication channel, i.e., volume transmission.

In the mammalian central nervous system, monoaminergic pathways represent key components of the reticular activating system and are implicated in diverse physiological functions, including autonomic, motor and behavioral state control. The main subgroups of catecholaminergic and serotonergic neurons are located in the brainstem. Monoamine levels released at targeted sites are regulated by specific transporters.

10.4.1 CATECHOLAMINERGIC NEURONS

Since the pioneering work of Von Euler demonstrating the presence of catecholamines in nerve terminals, numerous studies have shown the role of catecholamines in the regulation of autonomic functions. The major catecholamines used by the nervous system are dopamine, noradrenaline, and adrenaline. These substances are synthesized in the central nervous system from tyrosine, a dietary amino acid. Tyrosine is converted to dihydroxyphenylalanine (DOPA) through addition of a hydroxyl group to the catechol ring by the enzyme tyrosine hydroxylase (TH). The enzyme TH is the rate-limiting enzyme in catecholamine synthesis. DOPA is then converted to dopamine by decarboxylation of the amine group by the enzyme DOPA decarboxylase. Dopamine is converted to noradrenaline by the enzyme dopamine-beta-hydroxylase through the addition of a hydroxyl group to the carbon atom nearest to the catechol ring. Noradrenaline in the periphery, and to some extent in the CNS, is converted to adrenaline by methylation of the terminal amide group by the enzyme phenylethanolamine N-methyltransferase.
Advances in Vagal Afferent Neurobiology

The availability of antisera against specific enzymes involved in catecholamine synthesis and against specific catecholamines themselves, allowed for a more precise determination of catecholamine expression. In general, six main groups of catecholamine cells are recognized in the brains of vertebrates:

- A caudal rhombencephalic group (A1-A3/C1-C3)
- A rostral rhombencephalic group (A4-A7)
- A mesencephalic group (A8-A10)

FIGURE 10.5 The major noradrenergic and serotonergic cell groups that project to AVPNs as demonstrated in rats by a retrograde transneuronal labeling technique. A1, area of the A1 noradrenergic cell group; A2, area of the A2 noradrenergic cell group; A5, area of the A5 noradrenergic cell group; AP, area postrema; GiV, gigantocellular reticular nucleus, ventral part; LC, locus coeruleus; NA, compact portion of rostral nucleus ambiguus; ROB, nucleus raphe obscurus; RPa, nucleus raphe pallidus; VII, VII cranial nerve.

The availability of antisera against specific enzymes involved in catecholamine synthesis and against specific catecholamines themselves, allowed for a more precise determination of catecholamine expression. In general, six main groups of catecholamine cells are recognized in the brains of vertebrates:
As shown in Figure 10.5, the major noradrenergic inputs to the AVPNs arise from the A5 cell group, the locus coeruleus (LC) and subcoeruleus.\textsuperscript{42,46,107} An example of LC neurons that project to AVPNs innervating the lung is noradrenergic, as indicated by the arrows.

- A diencephalic group (A11-A15)
- An olfactory bulb group (A16)
- A retinal group (A17)

FIGURE 10.6  (A color version of this figure follows page 236.) Projections of locus coeruleus (LC) noradrenergic cells to AVPNs. (A) Schematic of a coronal section showing the location of pontine noradrenergic cell groups. (B) LC neurons were labeled with pseudo rabies virus-encoding green fluorescent protein (PRV-GFP) following injection of PRV-GFP into the upper lobe of the right lung. (C) TH-containing cells (red) were identified in the same section of the LC. (D) A subpopulation of LC neurons projecting to AVPNs innervating the lung is noradrenergic, as indicated by the arrows.

10.4.1.1 Neuroanatomical Studies

Neuroanatomical studies using double immunolabeling at the light and electron microscopic levels revealed the relationship of TH containing fibers with the AVPNs. The results showed that, in the ferret, catecholaminergic terminals with frequent varicosities are present in close proximity to the identified AVPNs. Several TH axons were encountered with both cell bodies and their dendrites. Figure 10.6 demonstrates electron microscopic examples of two axo-dendritic synapses between TH-immunoreactive varicosities with an identified AVPN dendrite and with an unlabeled
Advances in Vagal Afferent Neurobiology

dendrite. However, quantitative studies indicated that only 0.2% of the terminals that form synapses with tracheal AVPNs in the rNA are immunoreactive for TH. These ultrastructural findings suggest that the modulatory effects of norepinephrine on cholinergic outflow to the airways are mainly exerted by nonsynaptic actions. Similarly, cortical noradrenergic innervation is also mediated primarily by volume transmission. On the other hand, it has been shown that TH-immunoreactive nerve terminals in more caudal regions of the nucleus ambiguus form distinct synapses on the negative inotropic vagal preganglionic neurons projecting to the heart. To further support neuroanatomical studies, norepinephrine release within the rostral nucleus ambiguus was determined using microdialysis and high-performance liquid chromatography (HPLC, Reference 53) (Figure 10.7).

FIGURE 10.7 Catecholaminergic innervation of AVPNs. (A) Axons of TH-containing neurons in the rNA are unmyelinated (U). Myelinated (m) and unmyelinated (u) axons that are not TH-ir are shown for comparison. Bar = 200 nm. (B) A TH-ir dendrite (D) is readily identifiable by the presence of an amorphous diaminobenzidine reaction product (curved arrows). This dendrite forms a synapse (arrow) with a non-TH-ir nerve terminal (t). Bar = 500 nm. (C) A TH-ir terminal (T) containing several large dense core vesicles (LDC) forms a synapse (arrow) on a unlabeled dendrite (d). Unlabeled terminals (t) and large dense core vesicles (ldc) are identified for comparison. Bar = 500 nm. (D) TH-immunoreactive terminal (T) containing several LDCs forms an axodendritic synapse (small arrow) on the dendrite (D) of a retrogradely labeled (thick arrow) AVPN. Bar = 200 nm. (From Reference 53. With permission.)
10.4.1.2 Microdialysis and HPLC Measurements

Microdialysis and HPLC Measurements of neurotransmitter release can be used for studying chemical neurotransmission and neurochemical characterization of brain circuitry. Although this approach possesses high specificity, it lacks temporal resolution because of the long sampling times needed to accommodate the low flow rates of perfusate through the probe. Another possible limitation is that the size of the probe reduces the anatomic specificity of the field from which the dialysate is collected. However, the application of microdialysis sampling using probes dimensioned for a rat or a mouse, is more feasible in larger animals, such as a ferret. Furthermore, an ultrasensitive HPLC method for the determination of norepinephrine by electrochemical detection makes it possible to measure basal levels of norepinephrine in the brain in femtogram per microliter concentrations. Using this method in ferrets, we have shown that the extracellular norepinephrine levels within the rNA are around 28 ± 5 fg/µl. Stimulation of the LC and subcoeruleus region elicited significant increase in norepinephrine release within the rNA (80.6 ± 13.7 fg/µl P < 0.05; Figure 10.9). The data indicate that endogenously released norepinephrine acting via adrenergic receptors can affect activity of AVPNs.

10.4.1.3 The Adrenergic Receptor Family

The adrenergic receptor family that mediates the effects of catecholamines, epinephrine and norepinephrine, is composed of three subfamilies (α₁, α₂, and β) each containing a minimum of three distinct subtypes. Each subtype is coded by a separate gene and displays characteristic tissue distribution, regulatory properties, and drug specificities. As opposed to the α₁- and -adrenergic receptors, activation of the α2-adrenoreceptors (α2-ARs) by norepinephrine inhibits neuronal activity. The α2-ARs are divided into four subtypes, based primarily on radioligand binding characteristics in native tissue homogenates. The α2A-ARs, characterized by relatively high affinity for yohimbine and rauwolscine, are present in lower brain stem neurons, including catecholaminergic and serotonergic cells innervating the spinal cord. Furthermore, the α2A-ARs are expressed on glutamatergic nerve terminals, where their activation could inhibit glutamate release and excitatory synaptic transmission.

Recently, codistribution of α2A-AR-specific staining in identified AVPNs was examined. Studies using a Leica TCS-SP2 laser-scanning confocal microscope demonstrated that many retrogradely labeled AVPNs (Figure 10.8A) express α2A-AR-specific staining (Figure 10.8B), which was also observed on dendrites of the AVPNs. This is clearly observable in the overlay images (Figure 10.8C and Figure 10.8D), characterized by the yellowish perikarya due to the overlap of CTβ-specific (FITC, green) and α2A-AR-specific (TR, red) staining. These findings indicate that α2A-ARs that are densely present on the somata and dendrites of AVPNs, as a heteroreceptor, mediate central inhibitory effects of norepinephrine on AVPN activity and consequently on cholinergic outflow to the airways and airway smooth muscle tone.

In contrast to AVPNs, several cranial nerve motor nuclei, including those from which the hypoglossal nerve arises, express α₁-adrenergic receptors. Thus the
postsynaptic excitatory effects of norepinephrine on hypoglossal motoneurons must be primarily mediated by $\alpha_1$-adrenoceptors. Furthermore, norepinephrine acting via $\alpha_1$-adrenoceptors, probably through a decrease in postsynaptic leak $K^+$ conductance, increases the excitability of both the central respiratory command and spinal inspiratory output cells. Conceivably, one could hypothesize that release of norepinephrine from nerve terminals, a subset of which make synaptic contacts with AVPNs and their processes, will cause an inhibition of AVPNs via activation of $\alpha_{2A}$-ARs expressed by these cells. This would lead to withdrawal of cholinergic outflow to the airways and airway smooth muscle relaxation.

10.4.1.4 Physiological Responses

Physiological responses to norepinephrine release within the rNA were measured to determine the effects of stimulation of the noradrenergic LC and subcoeruleus cell
group on airway smooth muscle tone. The results proved that stimulation of norepinephrine containing cells that project to AVPNs, induces centrally mediated inhibition of cholinergic outflow to the airways and a consequent airway smooth muscle relaxation.\textsuperscript{53} An alternative explanation for airway smooth muscle relaxation could be an increase in sympathetic outflow. However, the airway dilation elicited by LC stimulation is resistant to \(\beta\)-adrenergic receptor blockade by propranolol, which antagonizes the effects of sympathetic nerve stimulation but does not affect the release of norepinephrine within the central nervous system induced by chemical activation of catecholaminergic neurons. The observed decrease of airway smooth muscle tone might also arise from a modulation of baroreceptor inputs. Changes in arterial pressure result in alterations of airway tone that are in the opposite direction,\textsuperscript{116} however, activation of LC neurons had no significant effect on arterial blood pressure.\textsuperscript{53}

It is possible that centrally released norepinephrine may activate GABAergic interneurons that project to vagal preganglionic cells innervating the airways, as in other brain regions, via activation of \(\alpha_1\)- and \(\beta\)-ARs.\textsuperscript{92} Our previous studies showed that AVPNs express GABA<sub>A</sub> receptors, and GABA inhibits cholinergic outflow to the airways.\textsuperscript{52,100,118} However, the changes that we observed cannot be explained solely by the activation of GABAergic mechanisms, because prior blockade of \(\alpha_{2A}\)-ARs within the rNA region significantly reduced the airway smooth muscle relaxation induced by LC stimulation, as shown in Figure 10.9, lower panel.\textsuperscript{53}

### 10.4.2 Serotonergic Neurons

There is increasing evidence suggesting that midline serotonin (5-hydroxytryptamine; 5-HT) producing cells play an important role in the central regulation of autonomic functions.\textsuperscript{39,78} A regulatory role is witnessed by findings that neurons of this system are localized in the medial aspect of the brainstem, the most primitive portion of the CNS; they develop in early ontogeny, and are largely conserved. Furthermore, their axonal projections and terminal arborizations invade almost the entire neuraxis, from the most caudal segments of the spinal cord to the frontal cortex.\textsuperscript{6,60–62}

#### 10.4.2.1 Neuroanatomical Studies

Neuroanatomical studies combining transneuronal labeling techniques and double immunocytochemistry methods showed that an extensive rostrocaudal network of 5-HT containing neurons within the raphe obscurus, pallidus, and magnus nuclei, the gigantocellular reticular nucleus, pars alpha, and the 5-HT containing cells of the parapyramidal region, project to AVPNs.\textsuperscript{42,46,107} In addition, double-immunolabeling studies demonstrated that 5-HT-ir fibers were concentrated within the rNA, in close proximity to the AVPNs.

#### 10.4.2.2 The Electrochemical Studies

The electrochemical studies employing carbon fiber electrodes and an IVEC-10 hardware and software system were used to define whether stimulation of midline neurons induces release of 5-HT within the rNA. This system runs on a
DOS-compatible computer that can rapidly detect and discriminate monoamine neurotransmitters such as serotonin. The results showed that microinjection of L-glutamate, but not buffered saline, into the caudal raphe, caused a significant increase in 5-HT (Figure 10.10, lower left panel). Release of 5-HT was associated with a withdrawal of cholinergic outflow to the airways.50

FIGURE 10.9 (Upper panel) Left: Typical HPLC chromatograms obtained from microdialysates collected from AVPNs within the rNA in a control state (baseline) and during repeated excitation of LC neurons (stimulation). Right: Average results (mean ± SEM; n = 8) of the concentration of norepinephrine in microdialysates collected during the control state and at different time points after cessation of chemical stimulation (horizontal bar). In three control animals, no stimulation was performed. (NA, noradrenaline. *P < 0.05). (Lower panel) Tracings of tracheal segment pressure (P\text{\textsubscript{\textit{tseg}}}, cmH\textsubscript{2}O) from a paralyzed, oxygen-ventilated ferret. In the control period (A), activation of LC neurons by L-glutamate (4 nmol/80 nl) induced a decrease in tracheal tone, which is expressed as a decrease in P\text{\textsubscript{\textit{tseg}}}. Bilateral microperfusion of yohimbine into the rNA diminished tracheal smooth muscle response to LC stimulation (B). (B) Average decrease in P\text{\textsubscript{\textit{tseg}}} (mean ± SEM; n = 8) induced by LC stimulation before (A) and after (B) microperfusion of \(\alpha_{2A}\)-adrenoreceptor blockers into the rNA region. (*P < 0.001).
10.4.2.3 The Serotonergic Receptors

The serotonergic receptors that mediate effects of 5-HT are expressed by neurons in many brain nuclei involved in regulation of motor and sensory responses as well as on behavior. Actions of serotonin are due to the existence of at least 15 different 5-HT receptor subtypes. For example, activation of 5-HT\textsubscript{2A} receptors induces cell depolarization, while activation of 5-HT\textsubscript{1A}R has an opposite effect. Hypoglossal and phrenic motoneurons and motor cells innervating pharyngeal and laryngeal muscles express 5-HT\textsubscript{2A}Rs. Double-immunolabeling studies demonstrated that the majority of identified AVPNs contain 5-HT\textsubscript{1A}R (Figure 10.10 upper panel, M).
Physiological experiments examining the effects of 5-HT antagonists on raphe stimulation-induced responses showed that bilateral topical applications or microinjections of methysergide in the rostral ventrolateral medulla blocked airway smooth muscle relaxation induced by raphe stimulation, indicating that serotonergic inputs acting via 5-HT$_{1A}$R induces central withdrawal of cholinergic outflow to the airways (Figure 10.10, lower right panel).

The 5-HT$_{1A}$ receptor functions postsynaptically. The most obvious effect of serotonin, mediated by 5-HT$_{1A}$ receptors, is membrane hyperpolarization. This membrane-delimited signaling mechanism involves a G protein of the Gi/Go family, opening of inwardly rectifying potassium channels, and inhibition of calcium currents. 5-HT$_{1A}$ receptors, also as auto receptors, regulate serotonin release. Alterations in serotonergic pathways may cause failure of homeostatic responses to life-threatening challenges (e.g., asphyxia, hypercapnia) during sleep. In children or adults, altered serotonergic pathways may contribute to the severity of obstructive sleep apnea syndrome and probably to nocturnal asthma.

10.5 FUNCTIONAL ROLE OF CENTRAL CONTROL OF CHOLINERGIC OUTFLOW TO THE AIRWAYS

Under normal conditions, an increase or a decrease in chemical drive, induces parallel changes in airway smooth muscle tone and breathing pattern, indicating the link between neuronal networks that control resistance of the tracheobronchial conduits and the respiratory drive to chest wall pumping muscles. Recently, the neuroanatomical basis for this integration have been studied, combining the retrograde tracer technique and the transneuronal labeling method. It was shown that a subset of bulbospinal cells that project to the phrenic nuclei also innervate the airway-related vagal preganglionic neurons, coupling inspiratory activity and parasympathetic outflow to the airways. Furthermore, physiological findings indicate that during normal breathing as well as during many evoked respiratory reflexes, cholinergic outflow to the airways changes in parallel with the inspiratory drive of the phrenic nerve. However, responses of the phrenic nerve and AVPNs need not to parallel each other. For example, chemical stimulation of pulmonary C-fiber receptors, or the aspiration reflex causes inhibition of inspiratory activity, but induces an increase in cholinergic outflow to the airways and consequently an elevation of airway smooth muscle tone. In contrast, activation of the midbrain periaqueductal gray induces a release of GABA within the rNA region and airway smooth muscle relaxation, but increases phrenic nerve activity. Furthermore, excitation of somatosensory fibers elicits an augmentation in respiratory output but a decrease in bronchomotor tone that cannot be explained by adrenergic or nonadrenergic noncholinergic (NANC) inhibitory influences. Taken together, these results indicate that AVPNs within the rNA form a distinct and identifiable cell group, the activity of which is regulated by multiple CNS nuclei, utilizing common and/or specific neuronal pathways.
During normal breathing and changes in chemical drive, airway smooth muscle tone and airway resistance manifest rhythmic fluctuations that are superimposed on baseline levels. Following hypocapnic apnea, characterized by a complete cessation of phrenic nerve discharge, the gradual increase in arterial PCO2, or step decrease in inspired O2, cause progressive increase in airway smooth muscle tone.26,27,45,99 The response commences prior to reappearance of phrenic nerve discharge. With the onset of rhythmic phrenic nerve firing, rhythmic oscillation of airway smooth muscle tone and airway resistance can be observed. 27,45,85,99 Within the respiratory cycle, the peak of airway smooth muscle tone and airway resistance appear in early expiration, in phase with the post inspiration inspiratory activity of the phrenic nerve.45 The elevation of airway smooth muscle tone, airway and tissue components of total lung resistance reduce the dead space, oppose distortion of the airways and most distal ventilatory units. Phasic oscillation with the peak in early expiration may serve to optimize gas exchange by reducing large fluctuations in functional residual capacity, and protect the airway from collapsing. Therefore, the central coordination of cholinergic activity to the airways with the respiratory drive and fluctuation of smooth muscle tone within the respiratory cycle keep airways functioning, maintaining stability through the changes within the respiratory cycle that optimizes gas exchange and work of breathing on a breath by breath basis.45

The assumption that central cholinergic outflow to the airways has protective values is recently supported by experimental findings showing that interruption of the nerve supply to the lungs (for instance after lung transplantation) abolishes the integration of bronchomotor and ventilatory activities, and, by increasing airway deformation, initiates fibroproliferative responses in the airway walls. This causes the structural changes that are analogous to those observed in bronchiolitis obliterans after lung transplantation. In addition, the destruction of vagal motor fibers leaves behind a surviving population of denervated intrinsic neurons, which may be a source of over expressed neurotransmitters and their receptors that enhance inflammatory processes, as shown in vagally denervated rats. 18

Certainly, the protective nature of the centrally mediated vagal tone to the airways is altered if stress-induced increase in cholinergic outflow is not shut down when no longer needed. Extended allergen exposure, inflammatory reaction to inhaled irritants, and changes in lung compliance may increase excitatory inputs to the NTS that in turn will activate the AVPNs inducing airway hyper-responses9,20,36,64,76,104,109,137 and sustained bronchoconstriction, which in part is due to impaired central inhibitory influences. Recently, it has been shown that the increase in cholinergic outflow, genetically determined, causes airway hyperresponsiveness. 31

10.6 RELEVANCE OF CENTRAL MONOAMINERGIC INHIBITORY INPUTS TO THE AVPNs

In vagally intact animals and humans, exposure to environmental pollutants like ozone and cigarette smoke, inhaled allergens, and bronchopulmonary viral infections often lead to airway hyperresponsiveness. This occurs due to an injury-induced local
release of substances that evoke increased neuronal activity in sensory afferents innervating the injured and inflamed airways. As in pain-related disorders, persistent stimulation of sensory fibers by these substances may lead to progressive enhancement of centrally mediated airway responses to stimuli of low intensity, which, under normal conditions, have no significant effect on airway function. This could be due to reduced inhibitory influences such as alterations in noradrenergic and serotonergic innervation. While changes in peripheral elements of the system and in NTS second order sensory neurons that subserve processing and responses to these stimuli have been partly characterized, alterations in central circuits that lead to an enhanced and sustained excitable state of AVPNs in response to excitatory inputs need further investigation.

Preliminary data of our ongoing studies, however, suggest that repeated exposures to antigen tend to diminish central inhibitory influences upon AVPNs, leading to central sensitization and airway hyperreactivity. Hence, alterations in inhibitory control of AVPNs may contribute to airway hyperreactivity. For example, exercise increases the levels of noradrenaline in the brain regions innervated by the LC, and in humans induces airway dilation due to inhibition of resting vagal tone, through the muscle reflex. Downregulation in central noradrenergic control of AVPNs may contribute to exercise-induced asthma.

Neural mechanisms are involved in fear and emotional distress enhanced the occurrence of bronchoconstrictive attacks. Previous receptor binding studies have shown that chronic psychosocial stress down-regulates the binding sites for α2-adrenergic as well as 5-HT1A receptor ligands in several brain sites. Although the receptor changes on AVPNs have not yet been analyzed, the stress-induced down-regulation of receptor expression on AVPNs may contribute to an imbalance in the excitatory and inhibitory inputs to AVPNs. In addition, chronic stress reduces expression of α2-adrenoceptor in glutamatergic neurons of the brainstem. Since glutamate is the main excitatory neurotransmitter involved in reflex bronchoconstriction, the stress-induced down-regulation in α2-adrenoceptor and 5-HT1A receptor expression in these neurons might augment airway bronchoconstrictive reflex responses via increased glutamatergic drive to the vagal preganglionic neurons innervating the tracheobronchial system.

The firing rate of noradrenergic and serotonergic neurons progressively changes across the sleep-wake arousal cycle; it is diminished in quiet sleep, abolished when entering active rapid-eye movement (REM) sleep, and is dramatically elevated during arousal. Therefore, reciprocal changes in airway smooth muscle tone may occur with fluctuations in the activity of monoaminergic cells that project to the AVPNs. This hypothesis is supported by findings showing that in humans, airway caliber also undergoes cyclic oscillations: it decreases at night and increases during the day. The fluctuations are greatly amplified in patients with nocturnal worsening of asthma and might be due in part to withdrawal of the inhibitory influences that, in turn, trigger the cascade of events that enhances airway narrowing and nocturnal worsening of bronchoconstriction.

In addition, sleep is associated with state-dependent hypotonia of the upper airway dilating muscles. Qualitatively different responses to norepinephrine or
serotonin of motoneurons that innervate upper airway dilating muscles, versus those that control cholinergic outflow to the airways, could be related to their simultaneous action on different serotonin receptor subtypes expressed by these motoneurons. Norepinephrine or 5-HT-induced withdrawal of cholinergic outflow to the airways may be partly mediated via facilitation of GABA-mediated inhibitory transmission to AVPNs. Recently, it has been shown that AVPNs are under tonic GABAergic inhibition, removal of which induces bronchostriction.100

10.7 SUMMARY AND FUTURE STUDIES

This review briefly summarized the recent findings on the central regulation of vagal preganglionic neurons as a final link between the CNS and the airways, with the emphasis on the role of central monoaminergic cell groups in regulation of AVPN activity, consequently, cholinergic outflow to the airways. Furthermore, we discussed possible pathophysiological consequences of alterations of these inhibitory pathways in regulation of airway functions.

Future studies are aimed to: 1) further characterize mechanisms of central sensitization and abnormalities of central processing of sensory inputs underlying neuropathology of airway hyper-reactivity; 2) define signaling pathways that mediate fear and emotional-distress-induced bronchoconstriction and dyspnea; and 3) better understand the role of central excitatory and inhibitory pathways influencing cholinergic outflow to the airways during behavioral changes (i.e., mechanisms of worsening of airway functions during sleep and shortly after brief exercise).

ACKNOWLEDGMENTS

This research was supported by grants from the National Institutes of Health (HL-50527 and 1U54NS39407). The authors wish to thank Drs. Serdia Mack and Martha Miller for reading the manuscript, and Ms. Lee A. Watson for secretarial support.

REFERENCES


Advances in Vagal Afferent Neurobiology


Part V

Organ-Specific Afferent Nerves
11 Bronchopulmonary Vagal Afferent Nerves

Lu-Yuan Lee and Bradley J. Undem

CONTENTS

11.1 Introduction ..................................................................................................279
11.2 Classification of Vagal Lung Afferents........................................................280
  11.2.1 Laryngeal Afferents............................................................................280
  11.2.2 Bronchopulmonary Afferents...........................................................281
    11.2.2.1 Slowly Adapting Pulmonary Stretch Receptors......................281
    11.2.2.2 Rapidly Adapting Pulmonary Stretch Receptors .................283
    11.2.2.3 Bronchopulmonary C Fibers ............................................285
  11.2.3 Other Types of Lung Afferents.........................................................289
  11.2.4 Difference in Afferent Phenotypes Related to Ganglion Origin ....289
11.3 Afferent Properties Altered by Pathophysiological Conditions in the Airways .................................................................290
  11.3.1 Acute Airway Injury .......................................................................291
  11.3.2 Chronic Airway Inflammation .......................................................292
11.4 Inflammatory Mediators Involved in Hypersensitivity of Lung Afferents..................................................................................294
  11.4.1 Prostaglandin E$_2$ ...........................................................................294
  11.4.2 Bradykinin .......................................................................................295
  11.4.3 Hydrogen Ion ..................................................................................296
  11.4.4 Adenosine .......................................................................................298
  11.4.5 Nerve Growth Factor (NGF)............................................................301
11.5 Conclusion ....................................................................................................302
References..............................................................................................................303

11.1 INTRODUCTION

Sensory nerves in the respiratory tract play an essential role in performing two primary functions of the respiratory control system: regulatory function and defense reflexes. The former is responsible for the maintenance of homeostasis, and regulates breathing pattern, bronchomotor tone, and other important airway functions (Chapter 15). The latter protects the lung, as well as the rest of the body, from potential health-hazardous effects caused by airborne particles and chemical irritants.
The afferent activities that arise from sensory terminals located in the lung and airways are conducted almost exclusively by branches of vagus nerves. These vagal afferent fibers innervate the entire respiratory tract ranging from larynx, trachea to lung parenchyma, and project to the nucleus tractus solitarius in the medulla (Chapter 8). These sensory nerves all together are termed vagal lung afferents in this chapter, and represent approximately one fifth of the total number of afferent fibers in the vagus nerves. Although a large number of afferent fibers innervating the pulmonary vasculature are known to course through the sympathetic nerves via the white rami communicants to the spinal cord, and their cell bodies are located in the thoracic (T1-T6) dorsal root ganglia (DRG), the specific role of these “sympathetic afferents” in the regulation of respiratory functions remains unclear.

Several extensive reviews on the physiological functions and properties of these sensory receptors have been published previously. This chapter is intended to summarize briefly the relevant background information and to focus primarily on recent significant findings of the afferent properties of vagal bronchopulmonary afferents and the mechanisms underlying the hypersensitivity of these afferents. The neural pathways and reflex functions of these afferents are described in detail in Chapters 10 and 15.

11.2 CLASSIFICATION OF VAGAL LUNG AFFERENTS

11.2.1 LARYNGEAL AFFERENTS

In view of the strategic location and critical role of larynx in the protective and regulatory functions of breathing, it should not be surprising to learn that the larynx is supplied by a substantially larger number of sensory nerves per unit of luminal surface area than anywhere else in the entire respiratory tract. The larynx is innervated by the recurrent laryngeal nerves and by the internal and external branches of superior laryngeal nerves. The cell bodies of these laryngeal afferents are located in the vagal sensory ganglia, with a majority of them in the nodose ganglion. Afferent properties and functional characteristics of these receptors have been extensively studied and clearly documented. Sant’Ambrogio and coworkers have classified the laryngeal afferents that exhibit respiratory-related activities into three major categories based upon their sensory modality. Cold receptors sense the drop in temperature. Because the drop of laryngeal luminal temperature is proportional to the magnitude of inhaled airflow, these receptors are sensitive to inspired flow and were, therefore, initially named “flow” receptors. Pressure receptors detect either negative (collapsing) or positive (distending) transmural pressure in the larynx during respiration. They represent the majority of the laryngeal afferents and probably play a major role in the reflex regulation of the laryngeal caliber and central respiratory drive. Drive receptors are activated by the contraction of laryngeal muscles during respiratory movements. The activities of all three types of receptors are conducted in myelinated afferents that represent the majority of the afferent fibers innervating the larynx.
A smaller percentage of the laryngeal afferents has no or irregular spontaneous activity that is unrelated to the respiratory cycles. They consist of both myelinated and unmyelinated afferents. It has been shown that these receptors have distinct sensitivity to chemical and mechanical stimulation and are responsible for eliciting the protective reflex responses (e.g., apnea, cough, etc.) against inhaled irritants. Some of these afferents can be activated by high concentration (>8%) of CO₂. These receptors are also stimulated when they are exposed to the solution lacking permanent anions (e.g., chloride ion) either by topical application or by aerosol, which may be partially responsible for the apneic response to inhalation of distilled water aerosol in humans. The transduction mechanism(s) is not fully understood, but the responses seem to be evoked by either abnormal ionic compositions or low osmolality of the liquid layer lining the laryngeal mucosal surface.

11.2.2 Bronchopulmonary Afferents

There are three major types of vagal afferents arising from endings located in the lower airways and lungs: slowly adapting pulmonary stretch receptors (SARs or stretch receptors), rapidly adapting pulmonary stretch receptors (RARs or irritant receptors), and bronchopulmonary C fibers (Juxta-pulmonary capillary receptors or J receptors). More in-depth descriptions of the afferent properties and reflex regulatory functions of these receptors have been published in several reviews. Afferent discharge from the first two types of receptors is conducted by myelinated A fibers, whereas the last one is conducted by nonmyelinated (C-) fibers in the vagus; these afferents can be identified by generally accepted and clearly defined criteria. In addition, there are a significant percentage of vagal afferents that do not seem to fall into any of these categories. Recent studies have further revealed new information concerning the classification of these receptors, which has modified or expanded the previous knowledge about functional profiles of these different receptor types.

11.2.2.1 Slowly Adapting Pulmonary Stretch Receptors

Afferent activity and functional characteristics of SARs were first described by Adrian in 1933. The discharge pattern of SARs usually exhibits distinct respiratory phasic modulation; their activity increases during inspiration and decreases during expiration. The most commonly accepted criterion for identifying these receptors is a low (<0.7) adaptation index in response to a constant-pressure or constant-volume lung inflation. The earlier observations suggested that the main stimulus to SARs is the volume expansion of the lungs. Thus, it was somewhat surprising to note that the distribution of these receptors is predominantly in the conducting airways where the volume expansion is relatively limited. Indeed, more recent studies have demonstrated that the transluminal pressure (or wall circumferential tension) in the airways, rather than the volume expansion, is the actual stimulus to these receptors. Thus, the SAR’s activity is also increased as a result of bronchoconstriction. The transduction mechanism involved in activating these receptors is not fully understood. Immunohistochemical and anatomical evidence strongly
suggest the linkage between the SAR sensory terminal and airway smooth muscles.\textsuperscript{151,179,185} Using immunohistochemical staining of Na\textsuperscript{+}/K\textsuperscript{+}-ATPase, Yu and coworkers\textsuperscript{185} recently demonstrated that single SAR receptor contains multiple terminals innervating separate receptor fields; some terminal knobs are in contact with smooth muscles and others are not (Figure 11.1). They further suggested that the unit activity of a SAR is determined by the “interaction and integration” of the signals from these terminals.\textsuperscript{186} Still, little is known about the transduction mechanisms and receptor channels mediating the signal transduction at the nerve terminals. Recent advances in the studies of mechanosensory transduction channels, the family of degenerin proteins, in invertebrates\textsuperscript{60} have shown a promising possibility that may eventually lead to a better molecular characterization of the receptor apparatus and identities of the proteins responsible for detecting the pressure changes by the sensory terminals of SARs.

Although SARs are generally considered to be pure mechanoreceptors and relatively insensitive to chemical stimuli, their activity during eupneic breathing has been shown to be inhibited by increasing CO\textsubscript{2} partial pressure either in the pulmonary arterial blood or in the alveolar gas.\textsuperscript{151,152} This inhibitory effect of CO\textsubscript{2} is believed to result from production of hydrogen ions, which in turn activate the 4-aminopyridine (4-AP)-sensitive potassium channels.\textsuperscript{116} SARs are also stimulated by veratridine, and the response can be blocked by ouabain, a Na\textsuperscript{+}-K\textsuperscript{+} ATPase inhibitor, or flecainide, a sodium channel blocker, suggesting the involvement of voltage-sensitive sodium channels.\textsuperscript{117}

**FIGURE 11.1 (A color version of this figure follows page 236.)** Confocal fluorescent microscopic image illustrating the receptor structure of an SAR located in the peripheral airway (diameter <180 µm) of a rabbit; the receptor was first identified by electrophysiological recording. (Panels A-D) Consecutive optical slices from the outside (A) to the inside (D) of the bronchiole wall. Smooth muscle bands can be seen in C. (E) projection image of a stack of the six optical sections (each 3.3 µm thick). Note that the axon is about 3 µm in diameter and the branch to the left low corner is about 1.5 µm. (Modified from Yu et al., 2003.)\textsuperscript{185}
11.2.2.2 Rapidly Adapting Pulmonary Stretch Receptors

RARs are located along the entire tracheobronchial tree with a higher density in the larger airways, particularly at the branching points. The majority of RARs exhibit polymodal sensitivity. Hyperinflation of the lungs is a very effective stimulus to RARs, and their response to a given level of lung inflation is enhanced by increasing the rate of inflation. The response of RARs to lung inflation adapts rapidly and usually has an adaptation index of greater than 0.8. Recent finding by Matsumoto et al. showed that the adaptation rate of RARs was reduced significantly by 4-AP, suggesting that the rapid adaptation of these receptors to the maintained stimulation (lung inflation) may be related to activation of the potassium channels. Similarly, 4-AP applied directly to the nerve terminals activated the mechanical-sensitive afferents that were previously quiescent by inhibiting the potassium channels in isolated guinea pig airways. The receptor protein(s) involved in the RAR signal transduction has not been identified. Amiloride at the dose sufficient to block the Epithelial Sodium Channels (ENaCs), members of the degenerins superfamily, failed to block the transduction of mechanical stimulation of RARs in isolated guinea pig trachea.

A high percentage of these receptors, in contrast to SARs, are not activated during eupneic breathing. Moreover, the majority of RARs is consistently stimulated by deflation of the lung (below the resting lung volume), and thus they were also described as deflation receptors. Recent studies have demonstrated that an increase in the lung stiffness is an effective and consistent stimulus to RARs (Reference 84); this may provide a partial explanation for the paradoxical responses of RARs to both inflation and deflation of the lungs since deflation of the lungs is known to induce atelectasis and reduction in lung compliance. In a series of studies, Kappagoda and coworkers have shown that RARs are consistently activated by an increase of fluid filtration into the interstitial space in the lung; mild increases in the interstitial fluid volume and pressure were generated in their studies by independently manipulating various components of the Starling forces governing fluid exchange across the pulmonary capillary wall (e.g., mitral valve lesion, pulmonary lymphatic obstruction, etc.). The enhanced activity of RARs may be responsible for initiating the reflex response to pulmonary venous congestion and edema. RARs can also be activated by application of a small volume of hypoosmotic solution (e.g., distilled water) into the airway lumen; whether this action is secondary to the change in the interstitial fluid volume due to osmosis remains to be determined. An increase in the interstitial fluid volume can also result from the action of nitric oxide, an endogenous free radical gas that increases the microvascular permeability and fluid flux into the perivascular space in the airway tissue. Thus, activation of pulmonary C fibers has been shown to produce a secondary stimulation of RARs (Reference 82) via the release of substance P and other tachykinins, which can, in turn, trigger the release of nitric oxide from endothelial cells (Figure 11.2).

The identity of RARs was first defined based on their discharge characteristics in response to lung inflation, but they are often stimulated by inhaled irritants and it was their sensitivity to chemical irritants that led to their more publicized name irritant receptors. For example, it has been shown that RARs can be
**FIGURE 11.2**

A

- **a**
  - AP
  - ABP (mmHg)
  - 75
  - 0

- **b**
  - AP
  - ABP (mmHg)
  - 75
  - 0

B

- **Before L-NMMA–PBS**
  - [Substance P] (nmol kg⁻¹)
  - Control
  - 0.89
  - 1.57
  - 2.78

- **After L-NMMA–PBS**
  - [Substance P] (nmol kg⁻¹)
  - Control
  - 0.89
  - 1.57
  - 2.78

RAR activity (impulses s⁻¹)

- **Before L-NMMA–L-Arg**
  - [Substance P] (nmol kg⁻¹)
  - Control
  - 0.89
  - 1.57
  - 2.78

- **After L-NMMA–L-Arg**
  - [Substance P] (nmol kg⁻¹)
  - Control
  - 0.89
  - 1.57
  - 2.78

Control 0.89 1.57 2.78

**RAR activity (impulses s⁻¹)**

- **Before L-NMMA–PBS**
  - [Substance P] (nmol kg⁻¹)
  - Control
  - 0.89
  - 1.57
  - 2.78

- **After L-NMMA–PBS**
  - [Substance P] (nmol kg⁻¹)
  - Control
  - 0.89
  - 1.57
  - 2.78

Control 0.89 1.57 2.78
Bronchopulmonary Vagal Afferent Nerves

stimulated by various irritant gases such as ammonia, sulfur dioxide, cigarette smoke, etc.\textsuperscript{121,178,179} Several investigators have suggested that the response of RARs to chemical stimuli may result from the secondary effect of these irritants on the mechanical properties of the airways and lungs because of the slow onset and long-lasting stimulation.\textsuperscript{9,11,34,69,82,94,173} However, convincing evidence of the chemosensitive property of RARs has been described in several instances; for example, cigarette smoke exerts an immediate stimulatory effect on RARs (latency < 1 second) before any change of bronchomotor tone takes place.\textsuperscript{94,95} In addition, a subset of RARs that have a scant or irregular baseline activity and are generally not activated by lung deflation has been identified in several species.\textsuperscript{10,69,94,166} The sensory endings of these RARs are believed to locate near the airway epithelium.\textsuperscript{147} Their receptor behaviors are consistent with those of the traditional “irritant receptors,”\textsuperscript{145,146,179} exhibiting distinct sensitivity to inhaled irritants and frequently found at the branching points of central airways (e.g., carina). Even though this subtype of RARs seems to be small in number, their reflex action and role in regulating the respiratory defense function should not be overlooked.

Activation of RARs by inhaled irritants has profound effects on the regulation of airway protective functions, which are discussed in detail in Chapter 15. More definitive evidence is needed to differentiate their sole effect from that mediated through the stimulation of bronchopulmonary C-fiber endings since many of these irritants stimulate both of these receptors simultaneously.\textsuperscript{32,69,179}

11.2.2.3 Bronchopulmonary C Fibers

Bronchopulmonary C fibers represent >75% of the afferent fibers in the pulmonary branch of the vagus nerve.\textsuperscript{3,81} It is well recognized that the afferent activity arising from these C-fiber endings plays an important role in regulating the airway functions in both physiological and pathological conditions.\textsuperscript{34,107} The Coleridges and their coworkers have demonstrated that these afferents can be subdivided into two major groups based upon their anatomic locations in the respiratory tract and circulatory accessibility.\textsuperscript{34} Pulmonary C fibers are those arising from the endings located in the
Advances in Vagal Afferent Neurobiology

lung parenchyma (including pulmonary capillaries) and small intrapulmonary airways receiving blood supply from the pulmonary circulation, whereas bronchial C fibers are those with endings located in the medium- and large-size airways receiving blood perfusion primarily from bronchial circulation. However, in view of the substantial vascular anastomosis between bronchial and pulmonary circulations, it seems conceivable that certain C-fiber endings located in the intrapulmonary airways may receive blood supply from either or both of these circulatory routes, depending on the experimental condition (e.g., airway pressure, lung volume, pulmonary and systemic arterial pressures, etc.). Both bronchial and pulmonary C fibers exhibit polymodal sensitivity, they can be activated by mechanical means such as light probing or hyperinflation of the lungs. It was suggested that pulmonary C fibers are more sensitive to mechanical stimuli (e.g., lung inflation), whereas bronchial C fibers have greater chemosensitivity, although it is difficult to evaluate the difference quantitatively because the actual level of stimulus at the receptor terminal cannot be accurately measured. Interestingly, a recent study has revealed that bronchial and pulmonary C-fiber afferents arise from different ganglion origins and represent different phenotypes (Section 11.2.4), which may offer the explanation for the noticed difference in the sensory modality between these two groups of C fibers.

The exquisite sensitivity to chemical irritants (e.g., acid, nicotine, capsaicin, etc.) and certain endogenously released autacoids (e.g., prostaglandin, bradykinin, etc.) is undoubtedly the most prominent characteristic of bronchopulmonary C-fiber afferents. Immunohistochemical studies clearly illustrated the presence of C-fiber sensory endings containing tachykinins or calcitonin gene-related peptide (CGRP) in the mucosa of all sizes of airways in various species including humans. These nerve endings display extensive axonal arborization that either extends into the space between epithelial cells or forms network-like plexus immediately beneath the basement membrane of epithelium (Figure 11.3). The superficial locations of these nerve endings in the airway lumen and the potent biological activities of these neuropeptides suggest an important role of these afferents in regulating the airway responses to inhaled irritants.

When pulmonary C-fiber endings are activated by chemical irritants inhaled into the lungs or by circulated autacoids, action potentials are conducted through the vagus nerves to the commissural subnucleus of the nucleus tractus solitarius and elicit the classical pulmonary chemoreflexes, characterized by the triad of apnea, bradycardia and hypotension, along with delayed tachypnea and reflex bronchoconstriction mediated via the vagal efferent pathway. On the other hand, selective stimulation of bronchial C-fiber endings by chemical irritants injected directly into the bronchial circulation or inhaled into the airways elicits reflex bronchoconstriction, rapid shallow breathing and hypersecretion of mucus. It is believed that activation of either type of these endings can evoke dyspneic sensation, airway irritation and cough, which are the typical responses to the disturbances caused by inhaled irritants or airway inflammation.

Neuropeptides such as tachykinins and CGRP are synthesized in many, but not all, of the cell bodies of pulmonary C neurons in nodose and jugular ganglia, transported and stored in the sensory terminals in many species, including man; these sensory neuropeptides are known to act on a number of effector cells in the...
respiratory tract (e.g., airway and vascular smooth muscles, cholinergic ganglia, inflammatory cells, mucous glands). When bronchopulmonary C-fiber endings are activated, in addition to the reflex responses described above, the impulses may also propagate antidromically to other peripheral branches via the axonal ramifications and trigger the release of tachykinins from the sensory endings (Figure 11.4), eliciting the “axon reflexes” (Chapter 16). These peptides can then produce additional local effects such as bronchoconstriction, protein extravasation, airway mucosal edema, and inflammatory cell chemotaxis. Indeed, these responses in the tracheobronchial tree could not be abolished by bilateral vagotomy after inhalation of various irritant chemicals known to activate C-fiber endings or by electrical stimulation of distal ends of cut vagus nerves in guinea pigs. Sustained and intense stimulation of these endings can lead to the development of “neurogenic inflammatory reaction” in the airways.

A number of ligand- and voltage-gated ion channels and pharmacological receptors have been identified on the sensory terminals of C fibers as well as in the cultured C neurons innervating the airways and lung. The overall electrophysiological and pharmacological properties and excitability of each pulmonary sensory neuron are regulated by the functional expression and interplay of these receptor proteins. One prominent feature of pulmonary C-fiber afferents is the expression of the transient receptor potential vanilloid type 1 receptor (TRPV1), a member of the
TRP channel family,²⁵ at the sensory terminal.⁹⁹,¹⁶⁹ Because capsaicin, the major pungent ingredient of hot peppers and a derivative of vanillyl amide, is a potent and selective activator of the TRPV1 receptor, “capsaicin-sensitive lung afferents” are often considered synonymous with “pulmonary C fibers.” Other ligand-gated ion channels expressed on the pulmonary C-fiber neurons include acid sensing ion channels (ASICs), 5-hydroxytryptamine subtype 3 receptor (5-HT₃), P2X₃ purinoceptor, etc.⁹⁹,¹⁶⁹ A number of voltage-sensitive channels are known to play pivotal roles in regulating the excitability of these neurons. For example, it has been clearly demonstrated how the Ca²⁺-dependent K⁺ current that gives rise to a slow after-hyperpolarization (IₘAHP) can alter the resting membrane potential and discharge frequency in isolated nodose C neurons.³⁵,¹⁷² Furthermore, IₘAHP and other voltage-sensitive current species, such as the tetrodotoxin-resistant sodium current (TTX-R I₉Na), can be modulated by various endogenous mediators (for details see Section 11.4). A recent report has illustrated that the expression of TTX-R I₉Na is three-fold greater in isolated pulmonary capsaicin-sensitive neurons than those insensitive to capsaicin,¹⁰⁰ which may explain why the responses mediated through the axon reflex cannot be abolished by TTX in isolated airways.¹¹⁴

FIGURE 11.4 Schematic illustration of the distribution pattern of vagal C-fiber afferent endings and their potential interaction with other cell types in airway mucosa. E, erythrocyte, L, leukocyte, TKs, tachykinins; N, neuroepithelial body. See text for further explanation.
11.2.3 Other Types of Lung Afferents

The classification of lung afferents described above is generally accepted by investigators, but it is obviously not completely applicable in some instances. For example, a significant percentage of the lung mechanoreceptors fall into the intermediate type between SARs and RARs based upon their responses to lung inflation and adaptation indexes.\cite{10,178,187} Bergren reported four subtypes of SARs in rat lung\cite{10}; receptors discharge exclusively during the inflationary or the deflationary phase of the respiratory cycle, and those discharge bi-phasic but mostly during the inflationary phase or the deflationary phase. Their adaptation index also changed substantially at different levels of inflation pressures.\cite{10} Similar but to a less extent of the heterogeneity of SAR discharge pattern has also been reported in other species such as rabbits and cats.\cite{176,178,187}

Cough reflex is one of the most effective defense functions against inhaled irritants and is elicited exclusively via activation of vagal afferents. However, the “cough receptor” that initiates the reflex action has not been unequivocally defined; the question as to which type of airway afferents is responsible for eliciting the cough reflex has been debated among the scientists over decades.\cite{34,181} A recent report by Canning and coworkers\cite{20} has shown that a subset of A\textsubscript{δ} afferents innervating the trachea and larynx are the receptors primarily responsible for triggering the cough reflex elicited by the upper airway irritation in guinea pigs. Their cell bodies are located exclusively in nodose ganglia and their action potentials are conducted through thin myelinated fibers in the recurrent laryngeal nerve of the vagus with an average conduction velocity of \( \sim 5 \text{ m/sec} \). They can be activated by acid and punctate mechanical stimulation applied to the upper airways, but are not sensitive to either capsaicin or bradykinin, unlike the bronchopulmonary C fibers. Furthermore, they appear to be different from the traditionally defined RARs because they conduct action potential at a much slower velocity than intrapulmonary RARs, and they are not activated by lung inflation or bronchoconstriction.

Although sensitivity to capsaicin is considered a characteristic feature of bronchopulmonary C-fiber endings, some C-fiber afferents are not activated by capsaicin even at a relatively high dose.\cite{69} Furthermore, afferent properties of the same type of receptor may vary substantially between different species. For example, phenylidiguanide and phenylbiguanide are potent stimuli of pulmonary C fibers via an activation of the 5-HT\textsubscript{3} receptor, a ligand-gated cation (Na\textsuperscript{+})-selective channel, in cats and rats,\cite{34,107} but their actions either do not exist or are almost negligible in dogs.\cite{34} In addition, sensory receptors in the lung may undergo phenotypic switch under certain pathophysiological conditions (for details see Section 11.3.2).

11.2.4 Difference in Afferent Phenotypes Related to Ganglion Origin

In common with other cranial nerves, there are two distinct sensory ganglia situated along the cervical aspect of the vagus nerves. As the vagus emerges from the cranium there is a gangliform enlargement termed the jugular ganglion. After it exits the jugular foramen, the vagus is joined by the cranial portion of the accessory nerve,
where it then enlarges into a second ganglion termed the nodose ganglion. The jugular and nodose ganglia are also referred to as the superior and inferior vagal ganglia, respectively. The neurons within these two ganglia have distinct embryological origins. The neurons in the nodose ganglia arise from the epibranchial placodes, whereas the neurons in the jugular ganglia (and dorsal root ganglia) arise from the neural crest (see Chapter 1 for more details).

Visceral afferent nerve fibers in the vagus are often considered *a priori* to arise from nodose ganglion neurons. The contribution of jugular ganglion neurons to visceral afferent innervation has been a relatively unstudied area. The relative contribution of jugular and nodose neurons to vagal afferent innervation of the respiratory system has been analyzed in rats, guinea pigs, and ducks. In each case the innervation appears to be derived nearly equally from neurons within nodose and jugular ganglia. In rats and guinea pigs the neuropeptides containing respiratory vagal afferent neurons are found mainly in the jugular ganglia. Electrophysiological studies have demonstrated that intrapulmonary stretch receptors, (RAR and SAR) in guinea pigs, rabbits, and cats are derived from nodose ganglion neurons. Bronchopulmonary C fibers, however, arise from both jugular and nodose neurons. The nociceptive C fibers with receptive fields in the larynx, trachea and large bronchi are nearly uniformly jugular ganglion nerves. Jugular neurons also project capsaicin-sensitive Aδ fibers to the extrapulmonary airways. By contrast, both jugular and nodose neurons project C fibers with receptive fields within the guinea pig lungs. Consistent with the observation that nearly all neuropeptides containing vagal neurons innervating the airways are situated in the jugular ganglia, the bronchopulmonary jugular C-fiber neurons contain substance P and CGRP, whereas only a subset of nodose C-fiber neurons contain neuropeptides. In addition to neurochemical differences, there are also pharmacological distinctions between jugular and nodose C fibers in the lungs. Classical C-fiber stimulants such as bradykinin and capsaicin stimulate both nodose and jugular C-fiber terminals in the lungs. By contrast, the nodose C fibers are vigorously activated by purines such as ATP (via P2X receptors), whereas jugular C fibers are not. Similarly, serotonin and adenosine selectively stimulate only the nodose C-fiber population, via 5-HT3 and A1 receptors, respectively. How the classification of vagal C-fiber phenotypes based on their cell body location in placodal vs. neural crest ganglia corresponds to the more traditional bronchial vs. pulmonary C-fiber classification remains to be determined.

11.3 AFFERENT PROPERTIES ALTERED BY PATHOPHYSIOLOGICAL CONDITIONS IN THE AIRWAYS

Increasing evidence indicate that vagal bronchopulmonary afferents play an important part in the manifestation of certain airway diseases. As described earlier, activation of C-fiber endings and RARs elicits centrally mediated reflex responses such as reflex bronchoconstriction, and also evokes dyspneic sensation, airway irritation, and cough. Furthermore, it is well documented that the sensitivity of these airway
afferents can be enhanced by injury or inflammation of airway mucosa during both acute and chronic airway diseases.70,103,172 Thus, when the excitability of these afferents is enhanced, a given level of bronchoactive challenge will generate a greater intensity of afferent discharge, which consequently causes a more severe bronchoconstriction mediated through both the cholinergic reflex pathway and local release of tachykinins. Indeed, symptoms involving activation of these afferents, such as cough, bronchoconstriction and dyspnea, are commonly reported in patients suffering from various airway inflammatory diseases.

11.3.1 Acute Airway Injury

Acute airway mucosal injury and inflammation has been induced in several experimental models for studying the pathogenic mechanisms of airway hyperresponsiveness (AHR). One widely accepted model involves transient exposure to ozone (0.7–3.0 parts per million (ppm), 1 to 2 h), an environmental air pollutant, which induces AHR to inhaled histamine or acetylcholine in various species including humans.72,73,102 The augmented bronchoconstriction is mediated through both cholinergic and tachykinergic pathways, suggesting an involvement of bronchopulmonary C-fibers. Indeed, ozone-induced BHR was consistently accompanied by symptoms of coughing and dyspneic sensation in human subjects.72 Coleridge and coworkers first demonstrated that brief exposure to ozone (2 to 3 ppm) stimulated bronchial C-fibers in dogs.33 Ho et al. further showed that the responses of pulmonary C-fibers to lung inflation and chemical stimulation (capsaicin, lactic acid) were markedly enhanced for a sustained period (45 to 80 min) after their baseline activity returned to control following exposure to ozone (3.0 ppm; 30 min) in rats.70 Similar patterns of sensitization were also observed when acute airway mucosal damage was generated in other experimental conditions. For example, airway hyperreactivity accompanied by mucosal injury could also be induced in guinea pigs by exposure to toluene diisocyanate (TDI; 1 to 2 ppm, 1 to 2 h),62,153 an industrial air pollutant known to induce asthma in man after prolonged exposure.133 The TDI-induced hyperresponsiveness to acetylcholine was attenuated by treatment with tachykinin antagonists or by depletion of tachykinins with capsaicin pretreatment, suggesting the involvement of bronchopulmonary C-fiber afferents.153,165,182

Airway hyperresponsiveness and mucosal inflammation are the most prominent pathophysiological features of airway allergic reaction. Both acute and delayed hypersensitivity of the sensory nerves developed in the sensitized airways following the allergen challenge, and their pathogenic mechanisms have been well-documented50,122,170,171 (Section 11.3.2). Hyperventilation with cold dry air is another experimental model that has been shown to generate acute airway epithelial injury and inflammation in various animal species as well as in humans.55,135 Breathing cold air is known to increase the bronchomotor response to inhaled irritants in asthmatics and healthy subjects,4,43,165 and a possible involvement of stimulation of bronchopulmonary C fibers and release of tachykinins in the airways has been reported.158,184 However, direct evidence and types of airway afferents involved have not been established in this model.
Taken together, all these observations suggest that the excitability of bronchopulmonary C-fiber endings is enhanced during acute airway mucosal injury and inflammation.

11.3.2 CHRONIC AIRWAY INFLAMMATION

Many of the symptoms of asthma, chronic bronchitis, and chronic obstructive pulmonary disease can be attributed to inappropriate vagal sensory nerve activity. These common afflictions are often associated with irritating sensations, constant coughing, a thirst for air (dyspnea), and excessive parasympathetic reflex activity. Although much has been learned about the pharmacological effects of specific inflammatory mediators on vagal afferent nerves, the mechanisms underlying vagal afferent neuromodulation by chronic airway inflammation remain poorly understood.

The three most common experimental models of airway inflammation are allergic inflammation, inflammation caused by environmental pollutants, and inflammation induced by respiratory virus infections. Animal models most often used in these studies are guinea pigs, rats, and, more recently, mice.

Guinea pigs are very susceptible to allergic (type 1 immediate hypersensitivity reactions). They can be actively sensitized to an antigen such as ovalbumin by simple intraperitoneal injection of the antigen. This leads to a humoral immune response and the production of reaginic antibodies (IgE and in the guinea pig, IgG1). After about three weeks the animal will be sensitized such that Fc receptors on tissue mast cells will be bound with ovalbumin-specific antibodies. At this point, administering the ovalbumin by aerosol results in an antigen-antibody dependent mast cell activation and, ultimately, airway eosinophilic inflammation with features similar to that seen in asthma. Animals can also be passively sensitized by intravenous or intraperitoneal injections with serum or purified antibodies taken from actively sensitized animals. The animals will be passively sensitized within 48 hours of the injection.

Allergic inflammation is associated with an increase in the amount of sensory neuropeptides such as tachykinins and CGRP in the airways. This can be attributable at least in part to an increase in expression of preproCGRP and preprotachykinin mRNA in the vagal sensory neurons. In addition to increases in sensory neuropeptides production, guinea pigs with allergically inflamed airways have increased parasympathetic reflex activity, and show a heightened sensitivity to cough.

Allergic inflammation may cause a phenotypic switch in the tachykinergic innervation of the airways. Under normal conditions, virtually all of the tachykinin containing neurons innervating the guinea pig airways, as in other tissues, are small diameter, neurofilament-negative, capsaicin-sensitive C-fiber neurons. As early as 1 day following allergen inhalation, however, as many as 20% of the large diameter, NF-positive neurons began producing substance P and CGRP. This sensory nerve phenotype corresponds with low-threshold mechanosensitive A-fibers. Such a qualitative change in tachykinergic innervation may influence airway physiology in two ways. First, if the low-threshold mechanosensors store tachykinins in their peripheral terminals, it would change the site within the airway wall at which tachykinins may be released during axon reflexes in the lungs. This could lead to a qualitative change in the characteristics of the neurogenic inflammation in the lungs. Second, if sensory
neuropeptides are released from the central terminals of low-threshold mechanosensors, this could lead to increases in synaptic efficacy in the NTS through the processes of central sensitization. Experiments have not yet been designed that would address either of these hypotheses.

The idea that allergic inflammation can lead to enhanced synaptic transmission in the CNS (central sensitization) gains support from studies carried out on nonhuman primates. When monkeys were repeatedly exposed to aerosol allergen (house dust mite), there was a noted increase in excitability of neurons in the nucleus tractus solitarius.27 The number of spikes evoked by a prolonged suprathreshold current injection was about five-fold greater in allergen-challenged vs. control (filtered air exposed) monkeys.

As discussed earlier (Section 11.2.2 and Section 11.3.1), inhaled pollutants such as sulfur dioxide, cigarette smoke, and ozone can directly modulate afferent nerve activity.109 Chronic treatment with ozone inhalation has also been used as model to study the effect of irritant-induced inflammation on vagal afferent nerve activity.83 The RAR activity in guinea pigs exposed to 0.5 ppm of ozone for 8 h/day for 1 week was markedly enhanced above that observed in control animals treated with filtered air. Inhalation of ozone or TDI has also been associated with increases in preprotachykinin gene expression in airway sensory neurons.182,183

Respiratory virus infections cause symptoms of the common cold. A more serious consequence of respiratory virus infections is the severe exacerbation of bronchial asthma. In fact, the vast majority of children hospitalized for their asthma are found to have concurrent viral infections of their respiratory tract.59 Most respiratory viruses infect the cells of the airway epithelium, a site rich in nociceptive-like sensory nerves. The mechanism by which respiratory viruses modulate airway sensory nerves to evoke sneezing, coughing, sore throat, etc. is poorly understood.

The most common viruses used to inflame the respiratory tract of laboratory animals are Sendai virus (parainfluenza virus) and respiratory syncytial virus (RSV). Inoculation of guinea pigs with Sendai virus causes a classical airway inflammation that peaks in about three to four days and persists for about one week. This treatment is associated with a hyperreflexive state, and a marked increase in the response to tachykinins released from bronchopulmonary C-fiber endings.18,144 Tachykinins are metabolized in the airways mainly by neutral endopeptidase (NEP). Part of the increased response to C-fiber activation in virally treated animals may be secondary to an inhibitory effect of the viral treatment on NEP activity.80 Parainfluenza virus also leads to a phenotypic switch in tachykinergic innervation of the airways similar to that described above in allergen-inflamed airways. Within three days of viral infection neurons projecting low-threshold mechanosensitive A-fibers begin producing substance P.22 In allergen-treated guinea pigs, this effect lasts only about 24 to 48 hours,50 whereas in virally treated animals the switch in tachykinergic phenotype is more persistent, lasting more than 2 weeks. In rats treated with RSV, there is a substantial increase in nerve growth factor (NGF) production in airway epithelial cells.77 Increases in NGF by airway epithelium may contribute to increases in sensory nerve excitability and neuropeptides gene expression.79,182
11.4 INFLAMMATORY MEDIATORS INVOLVED IN HYPERSENSITIVITY OF LUNG AFFERENTS

Although the mechanisms underlying the inflammation-induced hypersensitivity of airway afferents are not fully understood and likely vary between different experimental conditions, the sensitizing effects of certain inflammatory mediators released in the airways are believed to play a major part. The signal transduction pathways are likely to vary among different mediators. Some mediators (e.g., 5-HT) act directly on certain ligand-gated ion channels present on the neuronal membrane, whereas other autacoids (e.g., bradykinin) alter channel functions via intracellular second-messenger signaling pathways. It is also possible that certain mediators may evoke both these effects on the neuronal membrane, but at different time points after the initial challenge. The involvement of some of these mediators in modulating directly and/or indirectly the sensitivity of airway sensory nerves under various pathophysiological conditions has been extensively documented in the literature. Some of them have drawn special interest in recent studies and are described in more detail in the following sections.

11.4.1 PROSTAGLANDIN E2

It has been shown that O3-induced AHR is prevented by pretreatment with indomethacin, indicating a potential role of cyclooxygenase metabolites of arachidonic acid. PGE2, a potent autacoid derived from arachidonic acid metabolism through the enzymatic action of cyclooxygenase and PGE synthase, has been considered one of the primary candidates. The airway epithelium, which is the main target of initial assault by the inhaled irritants, is also the major cellular source of this autacoid. A recent report indicates that expression of proteinase-activated receptor 2 (PAR-2), a receptor for mast cell tryptase, is markedly upregulated in the airway epithelium during mucosal inflammation, and activation of PAR-2 triggers the release of PGE2 from epithelium. Inhalation of PGE2 aerosol also induces reflex bronchoconstriction in asthmatic patients, despite its potent direct dilating effect on airway smooth muscles. Furthermore, inhaled PGE2 enhances the sensitivity of the cough reflex elicited by capsaicin in humans, suggesting a PGE2-induced sensitization of pulmonary C-fiber afferents. Indeed, this contention is supported by a recent observation; exogenous PGE2 at a low dose does not cause any systemic effects, but markedly enhances the excitabilities of pulmonary C fibers to chemical stimulants and to lung inflation in anesthetized rats. However, whether the sensitizing effect of PGE2 is generated by its direct action on the sensory nerves, or by a secondary effect of PGE2 on other target cells (e.g., vascular smooth muscles) in the airways was not known.

In cultured vagal (nodose and jugular ganglia) pulmonary sensory neurons identified by retrograde labeling with fluorescent tracer and neuronal activity measured by the perforated patch-clamp technique, PGE2 (1 µM) markedly increased the whole cell current density and the number of action potentials evoked by either current injection or chemical stimulants (Figure 11.5). PGE2 increased the magnitude of peak current and caused a hyperpolarizing shift of the current-voltage
activation curve obtained by depolarizing voltage steps. The sensitizing effect of PGE2 could also be mimicked by butaprost (Figure 11.5), a selective agonist of the EP2 prostanoid receptor, or by a direct activation of adenylyl cyclase or protein kinase A (PKA), and prevented by pretreatment with inhibitors of adenylyl cyclase or PKA. Taken together, these studies suggest that PGE2 activates the Gs protein-coupled EP2 prostanoid receptor present on the membrane of C neurons, which upon activation increases the enzyme activity of adenylyl cyclase. Presumably, the resulting rise in cAMP may then stimulate PKA, which in turn enhances the neuronal excitability by increasing the phosphorylation of both ligand-gated channels and voltage-sensitive channels; the former includes the TRPV1 and 5-HT3 receptors, and the latter includes the TTX-R INa. Based upon the studies in DRG nociceptive C neurons, other voltage-sensitive currents that may be involved in the PGE2-induced airway hypersensitivity include a delayed rectifier-like outward potassium current and a calcium-dependent potassium current, I_{sAHP}.

11.4.2 BRADYKININ

Bradykinin, a nonapeptide derived from kininogen precursors, is produced upon the activation of tissue or plasma kallikreins by tissue damage, viral infection and other
inflammatory reactions. Increased concentrations of kinins in the bronchoalveolar lavage is found in patients suffering from asthma attack or anaphylaxis. Inhalation of aerosolized bradykinin causes bronchoconstriction in asthmatics but not in normal subjects, and the effect is reflex in origin because it can be attenuated or prevented by ipratropium bromide. Bolus injection of relatively high doses of bradykinin has been shown to stimulate bronchial C-fiber afferents and some RARs, but not pulmonary C fibers in dogs. Its effect on RARs was thought to be secondary, at least in part, to the cardiovascular actions of bradykinin. Kajekar and coworkers recently reported in an isolated preparation of guinea pig trachea and bronchus that bradykinin activates the sensory terminals of both C- and Aδ-afferents innervating these airways. Similarly, inhalation of bradykinin aerosol also causes bronchoconstriction and stimulates both RARs and pulmonary C fibers in anesthetized guinea pigs; the response of the former but not the latter is drastically attenuated by a pretreatment with isoproterenol to prevent the bronchoconstriction. Furthermore, a distinct difference in the sensitivity to bradykinin was found between the sensory nerves originating from the jugular and nodose ganglia; the nodose C-fiber afferents exhibited a lower sensitivity. This difference may be related to the different phenotypes of neurons in different ganglion origins (see Section 11.2.4).

Bradykinin is also known to be a potent algesic agent, and can sensitize visceral and somatic nociceptors. Similarly, bradykinin has been shown to enhance the sensitivity of C-fiber afferents in isolated guinea pig airways, but it does not alter the response of Aδ afferents to hypertonic saline in the same preparation. Although the mechanisms underlying this action are still not fully understood, it is believed to involve activation of the G protein-coupled bradykinin B2 receptors. Activation of the B2 receptor leads to stimulation of phospholipase C and production of diacylglycerol (DAG), which in turn activates the protein kinase C (PKC); pretreatment with the B2-receptor antagonist or the PKC inhibitor effectively attenuates the effect of bradykinin on nociceptive afferents. Recent studies further revealed another potential sensitizing pathway: activation of the B2 receptor leads to production of prostaglandin I2 in the sensory neurons, which may contribute to a bradykinin-induced inhibition of I5AHP and sensitization of these neurons (Figure 11.6). Furthermore, an involvement of TRPV1 channel in the bradykinin-induced increase in the excitability of these sensory neurons has been reported in several recent studies. One possibility is that modulation of the TRPV1 current by bradykinin is mediated through a direct action of PKC on the TRPV1 receptor. Carr et al. in a recent study demonstrated that B2 receptor-mediated effect of bradykinin on airway C fibers is mediated through the production of lipoxygenase products that, in turn, activate TRPV1 receptors. Indeed, bradykinin has also been shown to induce the production of 12-lipoxygenase metabolites of arachidonic acid in DRG neurons.

11.4.3 HYDROGEN ION

It is well documented that the concentration of hydrogen ion increases in the extracellular fluid of inflamed and ischemic tissues. In patients during asthmatic attack, the pH of the airway vapor condensate of exhaled gas is reduced to 5.23, as compared to 7.65 in healthy individuals. This abnormally low airway pH returns to normal
after anti-inflammatory therapy,\textsuperscript{78,93} suggesting the tissue inflammation as the origin of airway acidosis. Extracellular acidification can activate nociceptors, the counterpart of bronchopulmonary C-fiber and A\textdelta afferents in other organ systems, in various species.\textsuperscript{12,143,161} Indeed, inhalation of acidic aerosol elicits cough and reflex bronchoconstriction in humans,\textsuperscript{87,156} suggesting that stimulation of lung afferents is involved. Fox et al.\textsuperscript{54} demonstrated that C-fiber afferents innervating the guinea pig trachea were stimulated when the isolated airway was perfused with acidic buffer at pH of 5.0. In contrast, tracheal A afferents were not activated by acid buffer under the same condition. Similarly, lactic acid, produced endogenously in large quantity during anaerobic tissue metabolism, stimulates pulmonary C-fiber afferents in a dose-dependent manner (pulmonary venous blood pH: 7.09 to 7.29) in anesthetized rats, and hydrogen ions are primarily responsible for the action.\textsuperscript{75} The acidic stimulation of C-fiber afferents was abrogated by capsazepine,\textsuperscript{54} a selective antagonist of the TRPV1 channel, which is in partial agreement with the finding that hydrogen ion can modulate the properties of the TRPV1 channel.\textsuperscript{25} However, it is also possible

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure11_6.png}
\caption{Effect of tranylcypromine (TCP), a prostacyclin synthase inhibitor, on the actions of bradykinin (BK) in an acutely isolated guinea-pig nodose neuron. Control, a slow after-hyperpolarization (AHP\textsubscript{slow}) produced by four action potentials. BK (100 nM), superfusion with BK substantially diminishes the magnitude of AHP\textsubscript{slow}. TCP (10 \textmu M), superfusion with a Locke solution containing TCP has little effect on the AHP\textsubscript{slow}, but its presence in the local solution abrogated the action of 100 nM BK (BK + TCP). TCP (10 \textmu M) + PGI\textsubscript{2} (1 \textmu M), addition of 1 \textmu M PGI\textsubscript{2} to the TCP-containing Locke solution resulted in a block of the AHP\textsubscript{slow}. Wash, the AHP\textsubscript{slow} returned to near control values after superfusion with drug-free Locke solution. Action potentials were clipped. Insets below the AHP\textsubscript{slow} are electronic voltage transients produced by 160 ms, 100 pA rectangular current pulses. Resting membrane potential was –62 mV. (From Weinreich et al., 1995.)\textsuperscript{177}}
\end{figure}
that certain autacoids (e.g., lipooxygenase metabolites, etc.) are released from the surrounding tissue upon the action of hydrogen ion, which in turn can activate the TRPV1 receptor on the nerve terminals. A recent study using a similar isolated airway-nerve preparation showed that both C-fiber and Aδ afferents could be activated by acidification, dependent upon the magnitude, duration, and rate of acid application. A rapid and transient application of acid stimulated both RARs and C fibers, probably by activation of the ASICs that are proton-gated amiloride-sensitive Na+ channels. In contrast, a slow and sustained reduction in pH stimulated only the C fibers via an activation of TRPV1 since the effect was almost completely blocked by capsazepine.

In addition to the stimulatory effect, acidification is also known to induce hyperalgesia by sensitizing nociceptors in the peripheral tissue. Electrophysiological recording further demonstrated that acid enhanced the stimulatory effect of capsaicin on DRG neurons. Acidification of lung tissue occurs commonly even under normal physiological conditions; for example, an increase in CO₂ concentration in the alveolar gas can lead to acidification of pulmonary blood and interstitial fluid. This usually occur when the CO₂ production is exceedingly high (e.g., during strenuous exercise) or when the CO₂ elimination from the lungs is hindered (e.g., in obstructive airway diseases). In a recent study, when pH in the pulmonary venous (left atrial) blood was reduced from 7.40 to 7.17 by administering CO₂-enriched gas mixture (15% CO₂, balanced air) into the lung for 30 sec, the responses of pulmonary C-fiber afferents to various chemical stimulants (capsaicin, phenylbiguanide, adenosine) were markedly elevated (Figure 11.7). This sensitizing effect of CO₂ is believed to be mediated through the action of hydrogen ion, and not CO₂, on the terminal membrane of C fibers because the sensitization was significantly attenuated by infusion of HCO₃⁻, which prevented the CO₂-induced acidosis in the pulmonary venous blood (Figure 11.8). Interestingly, the same degree of acidosis did not alter the sensitivities of RARs and SARs to lung inflation. The involvement of ASICs in the observed effect of CO₂ can probably be ruled out because of the relatively mild reduction in pH in this study. It was postulated that the sensitizing effect of hydrogen ion on pulmonary C-fiber terminals might involve an inhibition of the inward rectifier K⁺ (Kir) channels by the intracellular acidification. An increase in intracellular H⁺ concentration has also been shown to modify other types of voltage-sensitive ion channels, including the T-type Ca²⁺ channel, delayed rectifier K⁺ channels and certain subtypes of Na⁺ channels. These voltage-sensitive channels may collectively lead to an increase in membrane excitability and contribute to the generator potential when membrane depolarization occurs as a result of activation of ligand-gated channels by chemical stimulants.

11.4.4 Adenosine

Adenosine is a purine nucleoside product of ATP metabolism, and is produced by virtually all metabolically active cells, particularly when the energy demand cannot be matched by oxygen supply such as during tissue ischemia or inflammation. Published evidence has strongly suggested the involvement of adenosine as an
Bronchopulmonary Vagal Afferent Nerves

Inflammatory mediator in the pathogenesis of airway hyperresponsiveness. \(^{125,137}\) In support of this hypothesis, high concentrations of adenosine have been detected in the bronchoalveolar lavage fluid of subjects with asthma\(^ {46}\) and the plasma level of adenosine doubles after receiving antigen challenge in asthmatic patients.\(^ {115}\) Adenosine is also commonly used as a drug administered by intravenous bolus injection for the treatment of supraventricular tachycardia. Intravenous injection of adenosine has been reported to cause dyspnea, chest discomfort, and bronchospasm in

**FIGURE 11.7** (Panel A) Experimental records illustrating the effect of alveolar hypercapnia (HPC) on afferent responses to right atrial injection of capsaicin (Cap, 0.5 µg/kg) of a pulmonary C fiber arising from an ending in right lower lobe in an anesthetized, open-chest rat. Upper panel, control response; middle, response during transient HPC (15% CO\(_2\), balance air; 30 s) which was initiated \(\sim 20\) s prior to the capsaicin injection; lower, recovery (10 min after termination of HPC). Capsaicin solution (volume: 0.2 ml) was injected as a bolus into the right atrium at the arrow. Note that the spontaneous respiratory movement and systemic hypotension during HPC could be detected by the P\(_t\) signal in the middle panel. (Panel B) Group data showing the effect of HPC on averaged pulmonary C-fiber and cardiovascular responses to capsaicin injection. Fiber activity (FA) was measured in 0.5-s intervals in each fiber. Capsaicin (Cap, 0.25 to 1.0 µg/kg) was injected into right atrium at time zero. Open circle, control response; closed circle, response during transient HPC that was initiated 20-25 s prior to the capsaicin injection and terminated 5-10 s after the injection. Data represent means ± SE of 27 fibers from 21 rats. AP, action potentials; P\(_t\), tracheal pressure; ABP, arterial blood pressure; HR, heart rate. (Modified from Gu and Lee, 2002.)\(^ {64}\)
Advances in Vagal Afferent Neurobiology

Patients; the adenosine-induced bronchoconstriction can be attenuated by muscarinic receptor antagonists, suggesting that it is mediated through cholinergic reflex and that stimulation of pulmonary afferents by adenosine is likely involved. A recent study reported by Hong et al. showed that right atrial bolus

FIGURE 11.8 (Panel A) Effects of transient alveolar hypercapnia (HPC) on pulmonary C-fiber responses to injections of capsaicin (Cap), phenylbiguanide (PBG) and adenosine (Ado) in anesthetized rats. ∆FA (FA: fiber activity) was measured as the difference between the peak FA (averaged over 2-s intervals) and the baseline FA (averaged over 10-s intervals) in each fiber. Open bars, control responses; closed bars, responses during transient HPC (15% CO₂, balance air; 30 sec); hatched bars, responses during recovery (~10 min after termination of HPC). Numbers of C fibers studied were 27, 13, and 12 for Cap, PBG, and Ado, respectively. Data represent means ± SE. * Significantly different (P < 0.05) from control response. (Panel B) Effect of transient HPC (15% CO₂, balance air; 30 s) on pulmonary C-fiber response to right atrial injection of capsaicin (0.5 to 1.0 µg/kg), arterial blood pH (pHa) and partial pressure of CO₂ (PₐCO₂) before (closed bars) and during (open bars) infusion of NaHCO₃ (1.36 to 1.82 mmol/kg/min; 35 s). Data represent means ± SE of 10 fibers from 10 rats in the upper panel, and 9 rats in the middle and lower panels. * Significant difference between control and HPC. † Significant difference between the corresponding data of before and during infusion of NaHCO₃. (Modified from Gu and Lee, 2002.)
injection of a therapeutic dose of adenosine (320 µg/kg) evoked a distinct stimulatory effect on vagal pulmonary C-fiber terminals in rats.\textsuperscript{74} Furthermore, the response showed a long latency (3 to 18 s) and was frequently followed by a recurrent stimulation, further revealing a possibility of sensitization of these afferents. Indeed, a follow-up study by Gu and co-workers further demonstrated that slow infusion of a lower dose of adenosine (40 µg/kg/min for 90 sec) that did not produce significant changes in the basal cardiovascular conditions induced a pronounced and reversible sensitizing effect on the pulmonary C-fiber responses to chemical stimulation and lung inflation in rats.\textsuperscript{65} The potentiating effect of adenosine infusion was completely prevented by pretreatment with DPCPX (100 µg/kg), a selective antagonist of the adenosine A\textsubscript{1} receptor, but unaffected by DMPX (1 mg/kg), an A\textsubscript{2} receptor antagonist, or MRS 1191 (2 mg/kg), an A\textsubscript{3} receptor antagonist. This potentiating effect was also mimicked by CPA (0.25 µg/kg/min for 90 s), a selective agonist of the adenosine A\textsubscript{1} receptor. Thus, their study clearly indicated that the sensitizing effect of adenosine on the sensory endings is mediated primarily through the adenosine A\textsubscript{1} receptor. Whether this sensitizing effect of adenosine on the C-fiber endings is generated by a direct activation of the A\textsubscript{1} receptor expressed on the neuronal membrane of these sensory terminals or involves releases of other mediators from intermediate cells could not be determined in their study. For example, adenosine has been postulated to degranulate mast cells and release histamine and other autacoids,\textsuperscript{51,65} which can in turn sensitize C-fiber afferents.\textsuperscript{106} On the other hand, Middlekauff et al.\textsuperscript{120} have demonstrated that adenosine inhibited the slow after-hyperpolarization that developed following action potentials in a large percentage (∼50%) of vagal afferent C neurons, and consequently increased the neuronal excitability of these afferents. This inhibition of after-hyperpolarization by adenosine is probably mediated by the adenosine A\textsubscript{1} receptor-mediated attenuation of the voltage-dependent Ca\textsuperscript{2+} currents in rats.\textsuperscript{120} Their finding seems to support the notion that adenosine contributes to the development of airway hyper-responsiveness, and activation of the adenosine A\textsubscript{1} receptor plays a role in this action. It should also be pointed out that adenosine injected at comparable doses failed to stimulate either C or A∆\textsubscript{3} afferents innervating the heart\textsuperscript{131} and skeletal muscles.\textsuperscript{143} Whether this discrepancy is due to the expression of different adenosine receptors on the various sensory terminals or the absence or paucity of certain intermediate cell types targeted by adenosine in these other tissues is not known.

11.4.5 Nerve Growth Factor (NGF)

NGF is a member of neurotrophin family and has been known for decades for its important influence on the growth and maintenance of certain specific subsets of peripheral sensory neurons as well as the cholinergic nuclei of the basal forebrain during development and maturation.\textsuperscript{111,164} Recent investigations have further revealed its important role as an inflammatory mediator during acute tissue injury; NGF is synthesized and released from inflammatory cells such as eosinophils, mast cells, and macrophages, in addition to the fibroblasts and epithelial cells at the site of injury.\textsuperscript{110,111,127,150,157} Indeed, in patients with asthma or other types of airway inflammatory reactions, there is a pronounced increase in the NGF level in the serum and
Advances in Vagal Afferent Neurobiology

bronchoalveolar lavage fluid.\textsuperscript{15,128} In guinea pigs, administration of NGF induces airway hyperresponsiveness to histamine that involves increased release of tachykinins from airway sensory nerves.\textsuperscript{39} It is known that administration of NGF in somatic tissues induces both acute and long-lasting hyperalgesic effects, and increases tachykinin synthesis in DRG nociceptive neurons.\textsuperscript{41,112,155} A hyperinnervation of the airway by the tachykinin-containing sensory fibers was found in transgenic mice that over-expressed NGF in Clara cells in the lung, accompanied by a pronounced airway hyperresponsiveness to capsaicin inhalation challenge.\textsuperscript{76} NGF has also been shown to lead to a phenotypic switch in the airway sensory neurons, in addition to increasing the synthesis and release of tachykinins; expression of tachykinins has been induced by injection of NGF into the tracheal wall in approximately 10\% of the large-diameter (presumably A\(\delta\) neurons) nodose neurons that innervate the trachea and normally do not contain tachykinin.\textsuperscript{79} This finding is consistent with the suggestion that NGF plays a part in the allergen-induced phenotypic change in the airway sensory neurons\textsuperscript{171} (Section 11.3.2). In cultured DRG neurons or frog oocytes co-expressing TRPV1 and TrkA tyrosine kinase receptors, NGF increases the neuronal sensitivity to capsaicin, hydrogen ion and thermal stimulation\textsuperscript{30,155}; the responses to these stimuli seem to be linked specifically to a modulatory effect on the TRPV1.\textsuperscript{30} Whether NGF also alters the excitability of other ligand-gated channels in nociceptive neurons is unclear.\textsuperscript{13} This hyperalgesic action of NGF on DRG neurons is believed to be mediated through the G-protein-coupled TrkA receptors, which in turn activates mitogen-activated protein kinase and phospholipase C (PLC)-\(\gamma\) signaling pathway.\textsuperscript{30,58} Furthermore, these studies suggest that PLC activation is the primary pathway in the NGF-induced potentiation of TRPV1 channel.\textsuperscript{30} However, whether NGF sensitizes bronchopulmonary C neurons via an activation of the same signaling pathway remains to be determined.

11.5 CONCLUSION

Vagal bronchopulmonary receptors are classified into three major types of afferents based upon generally accepted criteria. However, it is apparent from the information presented in this chapter that each of these major afferent types, particularly the C fiber and the RAR, consist of a heterogeneous group of sensory nerves with a wide spectrum of electrophysiological and neurochemical properties, and exhibiting multiple sensory modalities. Additional subsets of lung afferents characterized by their specific physiological and pharmacological profiles continue to be identified. As the new methods and technologies of cell and molecular biology were made available to the investigators, our knowledge about the properties and functions of these afferents has been substantially advanced in the last decade and will certainly continue to expand. In particular, more emphasis seems to be placed on identifying receptor proteins and channels expressed on the terminal membrane, understanding the signal transduction mechanisms, and investigating the pharmacological properties of these afferents.

Vagal lung afferents are known to play an important role in regulating the overall respiratory function under normal physiological conditions. Even more important, recent studies began to reveal their critical involvement in the manifestation and
pathogenesis of various airway diseases. However, fundamental knowledge about the transduction mechanisms and the biological factors that regulate the receptor sensitivity in each type of these lung afferents is still lacking, which remains the major challenge to the investigators in this field. A better understanding of the electrophysiological and pharmacological properties of these sensory nerves at the cellular and molecular levels should help to develop new therapeutic strategies for alleviating the various symptoms (e.g., cough, bronchospasm, etc.) associated with hypersensitivity of these afferents.

REFERENCES


12 Vagal Afferents Innervating the Gastrointestinal Tract

Michael J. Beyak and David Grundy

CONTENTS

12.1 Introduction ................................................................................................. 315
12.2 Morphological Features of the GI Sensory Innervation ............................ 316
12.3 Muscle Mechanoreceptors .......................................................................... 318
  12.3.1 Regional Differences in Vagal Mechanosensitivity ......................... 319
  12.3.2 The Question of Threshold ............................................................. 320
  12.3.3 Sensitization of Vagal Mechanoreceptors ..................................... 322
  12.3.4 Chemical Modulation of Mechanosensitivity ............................... 323
12.4 Voltage and Ligand-Gated Ion Channels on GI Vagal Neurons .............. 323
  12.4.1 Voltage-Gated Sodium Channels ................................................... 324
  12.4.2 Voltage-Gated Potassium Channels ............................................. 325
  12.4.3 Inflammatory Modulation of Voltage-Gated Channels ............ 326
12.5 Ligand-Gated Ion Channels ........................................................................ 327
  12.5.1 5-HT3 ............................................................................................... 327
  12.5.2 P2X Receptors ................................................................................ 328
  12.5.3 Ionotropic Excitatory Amino Acid Receptors ............................... 329
  12.5.4 TRPV1 and Other TRP Channels .................................................. 329
  12.5.5 ASIC Channels ............................................................................... 330
12.6 Mucosal Chemoreceptors ........................................................................... 331
12.7 Sensory Signal Transduction ...................................................................... 331
12.8 Behavioral Aspects of Vagal Afferent Signals ........................................... 332
12.9 Cytokines and Illness Behavior ................................................................. 335
  12.9.1 5-HT, Nausea and Vomiting ........................................................... 336
  12.9.2 Sensation: Mediation vs. Modulation .......................................... 338
12.10 Conclusion .................................................................................................. 339
References .............................................................................................................. 339

12.1 INTRODUCTION

The abdominal vagus conveys a vast amount of sensory innervation from the gastrointestinal tract to the brainstem. Sensory innervation to the GI tract is also provided
by spinal afferents that differ both morphologically and functionally from their vagal counterparts. These differences are consistent with the widely held belief that vagal and spinal afferents process different aspects of sensory information and, in particular, that spinal afferents convey nociceptive information, while vagal afferents mediate brainstem reflexes involved in the control and coordination of gastrointestinal function. However, it is clear that vagal information is disseminated widely in the CNS to regions involved in behavioral mechanisms relating to food assimilation and metabolic control and contributes to satiety, anorexia, and nausea. Moreover, the overlapping properties of certain subpopulations of spinal and vagal afferents suggest that both may contribute to pain processing. The aim of this chapter is to review the properties of GI vagal afferents in relation to their role in reflexes, behavior, and visceral sensation.

12.2 MORPHOLOGICAL FEATURES OF THE GI SENSORY INNERVATION

The subdiaphragmatic vagal nerve trunks innervate the upper gastrointestinal tract and accessory organs including the liver, pancreas, and biliary tree. The dorsal and ventral vagal trunks divide soon after passing through the diaphragm with hepatic, celiac, and gastric branches supplying these various regions.

Detailed fiber counts at the level of the diaphragm have demonstrated that afferent fibers outnumber efferent fibers by between 7- and 16-to-1 across a range of species. The abdominal vagus is therefore a predominantly sensory nerve and a vast amount of sensory information is conveyed to the brainstem. These afferents are predominantly C-fibers or A-delta fibers that supply the entire length of the GI tract, although the density of innervation is highest in the proximal gut and declines towards the distal bowel where pelvic afferents take over. This chapter will focus on afferents supplying the esophagus, stomach, and small bowel.

The sensory cell bodies are located mainly in the nodose ganglia, but others in the jugular ganglia may also supply the GI tract. Recent data suggests that nodose and jugular sensory neurons supplying the cervical esophagus may be differentiated on the basis of chemosensitivity to ATP and mechanical responses to distension, leading to speculation that they may subserve different physiological functions.

The jugular and nodose ganglia are derived from the neural crest and epibranchial placodes respectively during embryonic development and project centrally to the brainstem and peripherally to their terminations in the organs they innervate. Within the brainstem, vagal afferents terminate mainly in the nucleus tractus solitarius (nTS), although some afferents project into the dorsal motor vagal nucleus (DMVN) where they make monosynaptic connections with vagal motorneurones. Other afferent terminals project into the area postrema (AP), a region of the brainstem that integrates peripheral and central signals involved in triggering nausea and vomiting. From the nTS, second order neurones project a short distance into the dorsal motor vagal nucleus to complete the vago-vagal circuits through the brainstem that help control and co-ordinate gut function. Other second order neurones
Vagal Afferents Innervating the Gastrointestinal Tract

Vagal afferents innervating the gastrointestinal tract ascend to higher regions of the brain including the hypothalamus and limbic system, where they modulate autonomic function and behavior\(^{162}\) (Figure 12.1).

The sensory cell bodies synthesize the transmitters and enzymes for their synthesis that the nerve terminal utilizes in the process of synaptic transmission. Glutamate is a major excitatory transmitter at first order synapses in the nTS, but other transmitter substances exist in the brainstem including acetylcholine,
catecholamines, neuropeptides (especially tachykinins), and GABA. The cell bodies also express a range of receptors and ion channels that determine afferent sensitivity in the periphery and regulate synaptic transmission in the brainstem.

The terminal distribution of these vagal afferents in the bowel wall has been recently reviewed and is also discussed elsewhere in this volume. Morphologically, two distinct and characteristic patterns of terminal distribution can be identified within the gut wall. One population of vagal afferent fibers terminate in the muscularis externa and form endings either in the muscle layers or in the myenteric plexus, sandwiched between the longitudinal and circular muscle layer. The second population of vagal afferents make endings in the mucosal lamina propria where they are positioned to detect material absorbed across the mucosal epithelium or released from epithelial and subepithelial cells, including enterochromaffin and immunocompetent cells (see Figure 12.1).

Muscle and mucosal afferent endings have multimodal sensitivity responding to mechanical, chemical and thermal stimulation. However, their sensitivity is shaped by their surroundings within the bowel wall and thus endings in the muscle and mucosa have markedly different properties. Those within the muscle layers of the gut wall respond to distension and contraction. Mucosal afferents do not respond to distension or contraction but are exquisitely sensitive to mechanical deformation of the mucosa such as might occur with particulate material within the lumen. These are considered to be distinct sub-populations. Muscle endings continue to respond after removal of the mucosa while mucosal endings are sensitive to brief exposure to luminal anaesthetic. There is however, a report of endings in the ferret esophagus and stomach that respond to both modes of stimulation but this is not a general feature across all species.

### 12.3 MUSCLE MECHANORECEPTORS

Terminals in the muscle have been described as intramuscular arrays, consisting of several long (up to a few millimeters) and rather straight axons running parallel to the smooth muscle bundles and connected by oblique or right angled short connecting branches. Also running within the muscle are interstitial cells of Cajal (ICC), which have been shown to form a functional link between the motor innervation and the muscle. ICC have also been shown in intimate contact with sensory nerve terminals as revealed by the expression of TRPV1 (References 140 and 186) and anterograde labelling from the nodose. Mice with a mutation in the kit signalling pathway necessary for normal ICC development have altered gastric reflex function implicating ICCs in sensory signal generation although this needs to be examined more directly using electrophysiological approaches. There is also evidence that the pattern of vagal afferent innervation is altered in the mutant mouse, leading to the suggestion that ICCs may provide a scaffold for the sensory innervation.

The intramuscular arrays have been suggested to be the in-series tension receptor ending reflecting the property of some vagal afferents to respond both to passive stretch and active contraction of the muscle. However, recent in vitro studies that are described in detail elsewhere in this volume have mapped hot spots of
mechanosensitivity to morphologically identified endings within the guinea-pig esophagus and stomach found no relationship with IMA. Instead, mechanosensitivity was associated with a second type of ending, intraganglionic lamina ending or IGLEs, that are located in the myenteric plexus. These endings form basket-like structures surrounding myenteric ganglia and it is has been proposed that at this site the endings are exposed to stress and strain generated by muscle stretch and contraction. If this is the case, then vagal mechanoreceptors, which function as in-series tension receptors, may detect tension from an in-parallel location. IGLEs may also respond to chemical stimuli such as acetylcholine and ATP, raising the possibility that these endings also play a key sensory role in detecting release of mediators from within the synaptic neuropil of the myenteric ganglia or surrounding tissues. However, evidence that such chemosensory mechanisms contribute to mechanotransduction is lacking.

A wealth of early literature from Paintal and Iggo onward has demonstrated that vagal afferents convey physiologically relevant signals from the GI tract to the brainstem. This information originates in sensory endings in the gut wall with terminal distribution patterns consistent with a role in signaling mechanosensory information from the muscle. However, it is clear that these endings possess multimodal sensitivity, which is ultimately determined by ion channels and receptors on the sensory endings. However, because of the association between vagal endings and other structures within the gut wall, the sensitivity of these afferents is often modulated by factors that dissipate and distribute the stimulus energy within the tissue.

The most obvious example of this is seen with the multitude of factors that influence muscle tone. Iggo coined the term “in-series tension receptor” to describe the property of vagal afferents to the stomach. The single fibers he recorded from the vagus responded to tension generated both from stretch or distension and when the muscle contracted. This sensitivity is comparable to that seen in Golgi tendon organs while the “in parallel” muscle spindle responds to stretch, but these are unloaded during contraction. However, unlike with Golgi organs the sensitivity of vagal afferents to tension is exquisite. Contractions generating intraluminal pressure rises of a few mmHg or less, are associated with trains of afferent action potentials in vagal afferents that encode the timecourse and force of contractile activity. Vagal afferents recorded in vitro, so that tension across the tissue can be tightly controlled, respond to forces as small as 1g. Clearly, any stimulus that alters muscle tone will influence vagal afferent firing and the literature is complicated by a variety of studies that have failed to monitor or control effects on motility.

12.3.1 REGIONAL DIFFERENCES IN VAGAL MECHANOSENSITIVITY

Vagal afferents supplying the esophagus, stomach, and small intestine respond similarly to stretch and contraction although regional differences in motor function help shape the stimulus-response function of the vagal endings supplying different regions. In the stomach, this is dramatically illustrated by comparing the response of afferents supplying the proximal and distal stomach. The properties of muscle mechanoreceptors in the corpus reflect the reservoir function of this region. With
the stomach empty, they generate a low frequency irregular spontaneous activity, which increases dramatically when the volume of the stomach is increased within the physiological range of gastric filling.\textsuperscript{3,22,92}

They, therefore, effectively monitor the degree of gastric filling. However, being tension receptors, they can also signal increases and decreases in muscle tone, the latter occurring during receptive or adaptive relaxation.\textsuperscript{22} Such relaxations ensure that the stomach can fill either during food ingestion or following retropulsion from the intestines prior to vomiting with minimal increases in pressure. LOS pressure prevents reflux into the esophagus but this barrier is held only 10 to 30 mmHg above gastric pressure. The stomach is therefore a low-pressure system and the vagal afferents innervating this region are tuned to signal information over these low ranges in distending pressure.

In contrast, the properties of receptors in the antrum reflect the function of this region as a muscular pump. Being less distensible than the corpus, with much of a meal being accommodated in the latter, antral mechanoreceptors do not readily respond to increases in gastric volume, but instead generate a burst of impulses as each wave of contraction passes over the receptive field.\textsuperscript{3} The amplitude and waveform of the contraction are reflected in the rate and duration of discharge in the mechanoreceptors, while the velocity and direction of propagation of the contraction will be encoded in the discharge from adjacent receptors. Intestinal mechanosensitive afferents behave in a similar way.\textsuperscript{41}

When gastric distension is performed at a rate matching that observed when an animal eats, then the ability of the stomach to relax in order to accommodate the stimulus will have an obvious bearing on the response profile. Distension of the stomach, for example using a balloon system or with saline following esophageal ligation, to levels that exceed the normal adaptive capacity of the proximal stomach lead to antral distension and an associated maintained activation of afferents from this region\textsuperscript{3} (See Figure 12.2). Afferents supplying the corpus also encode these supraphysiological patterns of distension but the functional relevance is unclear, although there is evidence that meal distribution between antrum and corpus may be disturbed under certain circumstances and these may generate aberrant signals in vagal afferents leading to symptoms of dyspepsia.\textsuperscript{161,180,181}

12.3.2 The Question of Threshold

High threshold afferents have not been described in the vagus (i.e., thresholds in the noxious range >30 mmHg), which clearly distinguishes vagal and spinal nociceptors.\textsuperscript{62} In contrast the presence of low threshold vagal afferents has been frequently described in the esophagus, stomach, and small bowel of a wide variety of animal species including sheep, cat, dog, ferret, opossum, guinea-pig, rat, and mouse.\textsuperscript{3,41,47,91,134,137,167,174} The sensitivity of esophageal IGLEs is also consistent with low thresholds for activation.\textsuperscript{198} Similarly, the low threshold component of the mesenteric afferent response to distension of the rat jejunum is absent when vagal afferents are eliminated from the bundles by prior vagotomy.\textsuperscript{25} Sengupta’s study\textsuperscript{167} of the stimulus response function in the opossum is especially significant because they directly compared vagal and spinal afferents supplying the same region of
esophagus. Vagal afferents had only low-threshold characteristics while afferents encoding into the noxious range were exclusive to spinal nerves.

In contrast, the potential for vagal afferents supplying to stomach to encode noxious levels of stimulation has recently been described\(^\text{132,133}\) and is important because this sensitivity implies a role in nociception rather than in physiological control. Supraphysiological can apply to the rate of distension as well as magnitude. Rapid (phasic) distension or stretch gives rise to afferent responses of greater magnitude than those to slow filling. At the onset of such distension, wall tension is highest, exceeding that normally accounted under physiological circumstances.\(^\text{3,22,37,132}\) This gives rise to a transient afferent response that declines rapidly along with wall tension as the stimulus is accommodated.\(^\text{199,200}\) This dynamic sensitivity of the endings reflects their ability to respond to changes in tension that exceed those encountered physiologically.

Vagal afferents with low activation thresholds but responses that continue to encode levels of distension that when applied in humans give rise to pain have been described.\(^\text{132}\) Wide dynamic sensitivity, a term adopted from the literature on the sensitivity of dorsal horn neurones, describes this pattern of low threshold but broad response profile and while this term is not universally accepted in the field, it is a useful shorthand to distinguish between two populations of low-threshold afferent: those which saturate being low threshold and those that continue to encode beyond the physiological range being wide dynamic range. Vagal afferents with wide-dynamic sensitivity have been described recently in the rat stomach.\(^\text{132,133}\) Thresholds were typically around 5 mmHg but up to 18 mmHg and varied dependent upon the

---

**FIGURE 12.2** Single fiber recording from a vagal afferent supplying the rat stomach. The inset shows the raw “neurogram,” while a sequential rate histogram shows the frequency of action potentials at baseline and following distension with saline achieved by raising the height of the fluid reservoir connected to the gastric cannula. The numbers refer to the height of the reservoir above the stomach in cm. Note that the threshold occurred between 2 and 4 cm H\(_2\)O and the response maximum at 8 cm H\(_2\)O. (Modified from Jiang W and Grundy D. *J Auton Nerv Syst* 78: 99–108, 2000.)
duration of the distension, implicating accommodation as a determining factor. Afferents responded in a graded fashion to pressures above threshold, an observation that might implicate vagal afferents in nociception. However, whether this sensitivity has any functional relevance is difficult to envisage since the stomach has its own pressure release valve in the LOS. Levels of intragastric pressure are unlikely to exceed LOS pressure unless they arise ahead of a peristaltic antral contraction propelling fluid toward and through the pylorus. Nevertheless, gastric distension to 80 mmHg, a level far in excess of what might be considered physiological, stimulates c-Fos expression in the brainstem that is reduced but not abolished by vagotomy, data interpreted as supporting a role for vagal afferents in the processing of noxious visceral stimuli, perhaps by contributing to the affective-emotional component of visceral pain. Colonic distension also gives rise to c-Fos not only in brainstem nuclei (nTS and rostral ventrolateral medulla) but in higher regions involved in pain processing including the periaqueductal gray, amygdale, hypothalamus, and thalamus. Interestingly, only the brainstem expression was lost after perivagal capsaicin. Thus while activation of colonic vagal afferents is represented in the brainstem it is spinal afferents that are the source of higher levels of brain activation.

12.3.3 Sensitization of Vagal Mechanoreceptors

The sensitivity of spinal afferents is not fixed but threshold and gain can be altered during inflammation and injury. Such peripheral sensitization leads to hypersensitivity and hyperalgesia. Similar sensitization has been described for vagal afferents. The implication being that these sensitized endings may lead to altered motility or sensations arise from the GI tract, for example during dyspepsia.

A battery of mediators can influence the sensitivity of spinal afferents but their role in vagal afferent sensitization is yet to be determined. Such substances are usually released under conditions of inflammation, injury, or ischemia from a plethora of cell types e.g., platelets, leukocytes, lymphocytes, macrophage, mast cells, glia, fibroblasts, blood vessels, muscle, and neurones. Each of these specific cells (e.g., mast cells) may release several of these modulating agents, some of which may act directly on the sensory nerve terminal, while others may act indirectly following release of other agents from other cells in a series of cascades. These mediators produce their effects on visceral afferent nerves by three distinct processes. The first process is direct activation, which generally involves the opening of ion channels present on the nerve terminals. Acid may be one such substance since when applied at concentrations that cause tissue injury there is an increase in c-Fos in the brainstem and elsewhere in the CNS. The second process is sensitization, which may occur in the absence of a direct stimulation but which usually results in afferent hyperexcitability to both chemical and mechanical modalities. Prostaglandins play a pivotal role in the sensitization of spinal afferents and there is some evidence that they may have a similar on vagal afferents. The third process is altering the phenotype of the afferent nerve, for example through alterations in the expression of mediators, channels, and receptors, or modulating the activity of these by changing the ligand-binding characteristics or coupling efficiency of other receptors. NGF is typical of such a mediator in spinal afferents. However, while TrkA
receptor expression is abundant on dorsal root ganglia, it is relatively absent on nodose neurones where BDNF and GDNF are considered to have a prominent trophic influence.96

12.3.4 CHEMICAL MODULATION OF MECHANOSENSITIVITY

In addition to the chemical mediators that sensitize responses to mechanical stimulation, there are additional substances that result in inhibition of mechanosensitivity in the GI tract. The best studied of these is GABA acting through the GABA(B) receptor. Page and Blackshaw135 demonstrated that the GABA(B) agonist baclofen dose dependently inhibited responses of mucosal afferents to von Frey hair stimulation and also inhibited responses of tension/mucosal endings to circumferential stretch. Further studies showed that GABA(B) receptors are present on nodose ganglion neurons and that GABA(B) agonists inhibit gastric vagal afferent responses to distention.169 These observations may have relevance to transient lower esophageal sphincter relaxations, which underlie gastro-esophageal reflux, as these can be inhibited by baclofen, a GABA(B) agonist.

Somatostatin may also modulate mechanosensitivity in response to distention of the colon and small intestine. However these effects seem to be limited to spinal fibers, since vagotomy did not prevent an inhibitory effect of the somatostatin agonist octreotide on distention evoked mesenteric afferent discharge, however it did prevent the reduction in basal firing rate induced by octreotide, suggesting that intestinal vagal fibers other than mechanosensors are inhibited by octreotide.25

The literature on the effect of opioids on vagal mechanosensors is controversial. In the intestine, µ and δ agonists directly excite mesenteric afferents, an effect that is largely blocked by chronic vagotomy, and which occurs in a subset of mechanosensitive units.51 On the other hand, in the stomach, µ and δ agonists had no effect on basal firing rate or vagal responses to gastric distention, while a δ agonist attenuated responses to gastric distention, however this effect was not blocked by a selective antagonist, and only partially antagonized by high doses of naloxone, raising questions about the mechanism of action.133 Thus the action of opioids on GI vagal afferents may differ between different organs or under different experimental conditions.

There exist preliminary reports regarding the effects of activation of metabotropic glutamate receptors (mGluR3 receptors) on vagal afferents as well as galanin.136 In the upper GI tract these cause inhibition of responses to von Frey hair stimulation and mechanical stretch. These data taken together suggest that there exists a complex system of neuromodulators capable of attenuating responses of vagal afferent nerves to distention.

12.4 VOLTAGE AND LIGAND-GATED ION CHANNELS ON GI VAGAL NEURONS

The vagal afferents innervating the gastrointestinal tract express a wide variety of voltage-gated ion channels, endowing them with the ability to express a broad range of firing patterns. In addition they contain a vast number of receptors, enabling the detection of chemical mediators released by enteroendocrine cells and inflammatory
cells, as well as the potential to detect luminal contents. A number of these receptors
directly activate vagal afferents, through the opening of ligand-gated ion channels.
Furthermore, gastrointestinal vagal afferents are unique, in that their terminals are
located in an environment where there is a vast complexity of intrinsic innervation,
i.e., the ENS. Thus the expression of a number of neurotransmitter receptors on GI
vagal afferents raises the possibility of communication between the extrinsic and
intrinsic nervous systems of the gut. In addition, a wealth of evidence is emerging
that voltage and ligand-gated ion channels are altered in a variety of disease states
in both somatic and visceral primary afferent nerves, and that these changes may
underlie the abnormalities in sensory function seen in circumstances such as inflam-
mation or nerve injury.

12.4.1 VOLTAGE-GATED SODIUM CHANNELS

The voltage-gated sodium currents in primary sensory neurons can be grouped
broadly into those sensitive to nanomolar concentrations of the puffer fish (Fugu sp.)
toxin tetrodotoxin (TTX) and those resistant to TTX. These ion channels play a
critical role in action potential electrogenesis, and in determining the types of firing
pattern that can be sustained by these cells. In terms of GI – projecting vagal
neurons, sodium currents have been examined in only nodose ganglion neurons
projecting to the rat stomach. In these studies, nodose ganglion cells were retro-
gradely labelled by injection of tracer into the stomach wall and subsequent whole
cell patch clamp recordings were performed on labelled neurons. Both TTX sensitive
and TTX-resistant currents were found in most nodose neurons. The TTX sen-
sitive current was a low-threshold, rapidly inactivating current, with slow repriming
kinetics. The TTX-resistant current was more slowly inactivating, had a higher
threshold for activation, and had rapid repriming kinetics (See Figure 12.3). The
TTX-resistant current also is subject to slow, use dependent inactivation, which can
be modulated by a variety of mediators and pharmacological agents. The molec-
ular identities of the voltage-gated Na+ channel subunits in DRG sensory neurons
have been well studied. The slow inactivating TTX - resistant current is mediated
by the Na\textsubscript{v} 1.8 (SNS) channel while the majority of the TTX sensitive current is
carried by the Na\textsubscript{v} 1.7 channel and to a lesser extent Na\textsubscript{v} 1.6 (References 48, 188,
and 189) and Na\textsubscript{v} 1.3, especially after axotomy. A TTX – resistant channel with
persistent inactivation kinetics has also been identified and designated Na\textsubscript{v} 1.9. Recently in mouse nodose ganglia mRNA for the Na\textsubscript{v} 1.8 channel has been
demonstrated. Apart from this report there has been no immunohistochemical or
molecular characterization of the voltage-gated sodium currents in nodose neurons,
however, the available biophysical data agrees with the predominant channels being
similar to those found in DRG neurons, i.e., the TTX-R Na\textsubscript{v} 1.8 and TTX-S Na\textsubscript{v}
1.9 current. A sodium current with persistent kinetics has not yet been identified in
vagal sensory neurons.

How might the expression of a variety of Na\textsubscript{v} affect firing in sensory neurons?
TTX-resistant action potentials have been observed in cell bodies, but not in nerve
fiber recordings. Insights into this question have been provided by studies using both
modelling and gene deletion models. Modelling studies in nodose,163 and DRG
neurons\textsuperscript{152} suggest that the TTX-R current contributes to sustained firing, and that neurons expressing this channel may be slowly adapting, likely a consequence of this channel’s rapid repriming kinetics. In addition, coexpression of this channel with the much lower threshold TTX-S current, allows overall for a lower threshold for activation. Furthermore, the relatively heterogeneous properties of the TTX current seen in nodose neurons could contribute to a broad range of action potential morphologies and consequent firing patterns.\textsuperscript{163}

\subsection*{12.4.2 Voltage-Gated Potassium Channels}

Voltage-gated potassium channels are essential in the setting of membrane potential, firing threshold and repolarizing the cell after an action potential.\textsuperscript{80} Potassium channels are among the largest family of ion channels, with voltage-gated potassium channels (K\textsubscript{v}) having several different subtypes. Macroscopic currents in stomach-projecting

\textbf{FIGURE 12.3} Voltage-gated inward sodium currents present in rat gastric nodose neurons. (Top) Rapidly activating and inactivating TTX-sensitive currents. (Bottom) The majority of gastric nodose neurons also express a TTX-resistant current that is more slowly activating and inactivating. (Modified from Bielefeldt K, Ozaki N, and Gebhart GF. \textit{Gastroenterology} 122: 394–405, 2002. With permission.)
nodose neurons have been studied in the rat (See Figure 12.4). These studies have demonstrated the presence of potassium currents with inactivating (I_A type) and sustained (I_K or delayed rectifier type) kinetics in gastric nodose ganglion neurons. The I_A is inhibited at least in part by 4-aminopyridine (4-AP), and dendrotoxin. That dendrotoxin inhibits the inactivating current suggests the presence of Kv 1.1, 1.2 and 1.6 channels, which is supported by the presence of mRNA for these channels in unselected nodose neurons. Zagarodnyuk et al. used nerve fiber recording techniques to characterize pharmacologically the roles of various potassium channels in controlling both spontaneous and stretch activated firing rates in esophageal vagal afferents. Both dendrotoxins and 4-AP resulted in an increase in spontaneous firing and the magnitude of stretch evoked responses suggesting that Kv 1.1, 1.2, and 1.6 are important modulators of vagal afferent activity, at least in the esophagus. In addition, it appears that modulation of Kv currents may be a mechanism whereby inflammation modulates neuronal excitability in GI spinal and vagal sensory neurons. The latter study showed a reduction in A-type potassium currents in nodose neurons following gastric ulceration, changes which would be expected to lead to an increase in excitability, as has been shown in visceral DRG neurons.

Further studies have examined the presence of calcium activated potassium channels (K_Ca) vagal neurons. These channels can be divided based on their single channel conductances into small (2 to 20 pS; SK_Ca), intermediate (20 to 100 pS; IK_Ca) and large conductance (200 to 400 pS; BK_Ca). It has been suggested that in a variety of neurons these channels may regulate the refractory period and the post spike after hyperpolarization. One study has specifically examined the role of different types of potassium channels/currents present in esophageal vagal afferent nerve fibers. However, the selective BK_Ca channel blocker iberiotoxin, or the selective SK_Ca channel blocker apamin, had no effect, suggesting that calcium activated channels play little role in regulating the excitability of stretch sensitive esophageal vagal afferent neurons. However, it is known that in unselected nodose neurons, there is evidence from single channel recordings that BK_Ca and IK_Ca channels are present and can modulate excitability. In addition, in airway C-fiber vagal afferents, opening of BK_Ca channels significantly reduces responses to chemical stimuli. Perhaps in other gastrointestinal regions or in mucosal afferents, calcium-activated potassium channels play a role.

12.4.3 Inflammatory Modulation of Voltage-Gated Channels

It is increasingly clear from a number of studies in DRG neurons innervating both somatic and visceral structures that inflammation can modulate the activity of a number of these channels. In the bladder and gastrointestinal tract, DRG neurons innervating the ileum and colon are made hyperexcitable (lowering of current threshold and increase in tonic firing) by inflammation and these changes in excitability are accompanied by reductions in potassium currents (See Figure 12.4) and increases in sodium currents, in particular the TTX resistant sodium current. Similar alterations in sodium and potassium currents in nodose neurons innervating
Vagal Afferents Innervating the Gastrointestinal Tract

The stomach have been observed. Thus modulation of these key determinants of neuronal excitability (voltage gated ion channels) appears to be an important mechanism whereby disease states may modulate sensory afferent traffic from the GI tract. Alterations in spinal afferents clearly have implications for pain produced by inflammatory diseases of the GI tract (e.g., inflammatory bowel disease). On the other hand, a conclusive role for vagal afferents in nociception has yet to be demonstrated, but it is interesting to speculate that hyperexcitability of vagal afferents may at least in part underlie phenomena such as nausea, vomiting and anorexia that accompany these diseases.

12.5 LIGAND-GATED ION CHANNELS

12.5.1 5-HT3

5-hydroxytryptamine is an amine neurotransmitter whose greatest stores are found in the gastrointestinal tract. One of its receptor subtypes 5-HT3 is a ligand-gated

---

**FIGURE 12.4** Voltage-gated outward potassium currents in rat gastric nodose neurons evoked by depolarizing voltage steps to +40 mV. (A) Note two currents separated by manipulating the holding potential. At a holding potential of −110mV, two components are present, an inactivating current and a sustained one. At a holding potential of −30mV only the sustained component is seen (Iₖ). Digital subtraction of the two traces reveals the inactivating component (Iₐ). (B) Induction of experimental ulcers with acetic acid causes a reduction of the magnitude of the Iₐ, which would be expected to lead to increased excitability. (Modified from Dang et al. *Am J Physiol Gastrointest Liver Physiol* 286: G573–G579, 2004. With permission.)

---

The stomach have been observed. Thus modulation of these key determinants of neuronal excitability (voltage gated ion channels) appears to be an important mechanism whereby disease states may modulate sensory afferent traffic from the GI tract. Alterations in spinal afferents clearly have implications for pain produced by inflammatory diseases of the GI tract (e.g., inflammatory bowel disease). On the other hand, a conclusive role for vagal afferents in nociception has yet to be demonstrated, but it is interesting to speculate that hyperexcitability of vagal afferents may at least in part underlie phenomena such as nausea, vomiting and anorexia that accompany these diseases.

12.5 LIGAND-GATED ION CHANNELS

12.5.1 5-HT3

5-hydroxytryptamine is an amine neurotransmitter whose greatest stores are found in the gastrointestinal tract. One of its receptor subtypes 5-HT3 is a ligand-gated
ion channel. This receptor is of particular interest clinically given the efficacy of 5-HT3 antagonists as anti-emetics, and the clinical use of serotonin modifying drugs in the treatment of functional GI diseases. There is abundant evidence that 5-HT acts on gastrointestinal vagal afferent terminals or cell bodies. Blackshaw and Grundy demonstrated using in vivo single fiber recording techniques, that mucosal receptors in the upper GI tract responded to 5-HT, an effect that was blocked by prior administration of granisetron, a 5-HT3 receptor antagonist. This response was also blocked by mucosal administration of lidocaine suggesting that 5-HT was acting on mucosal endings. Further studies demonstrated no effect of 5-HT on mechano-sensitive units in the upper GI tract, whose endings are thought to lie within the muscular layer. Similarly, in the small intestine, Hillsley et al. showed that 5-HT caused a biphasic increase in afferent discharge, the first phase blocked by 5-HT3 receptor antagonism, and mucosal lidocaine, whereas the second phase was blocked by 5-HT2A receptor blockade, and was limited to single units which responded to mechanical stimuli (suggesting that this effect was secondary to increases in motility). The mucosal afferent component was completely eliminated by chronic vagotomy. Further evidence for the importance of 5-HT3 mediated signalling is given by the presence of mRNA for the 5-HT3 receptor in nodose ganglion cell bodies. At the cellular level, 5-HT has been shown to activate an inward current in isolated nodose neurons, which is mimicked by 2-methyl 5HT, and antagonized by the selective 5-HT3 receptor antagonists ondansetron and bemesetron. Ion substitution experiments show this to be predominantly a Na+ and K+ conductance. Taken together these experiments indicate that 5-HT3 receptors, located on gastrointestinal mucosal vagal afferents may be an important modulator of activity. Because of the putative role of 5-HT in sensing luminal contents (see later), these mechanisms may have implications in the regulation of nutrient intake. Recent evidence also indicates perturbation in the 5-HT signalling system in both ulcerative colitis and IBS, raising important questions about its role in disease states.

12.5.2 P2X RECEPTORS

Extracellular ATP is recognized as a potent activator of a variety of sensory neurons, and acting through the ligand gated ion channels, the P2X receptors, has been shown to play an important role in nociceptive signalling. In recent years it has become apparent that purinergic receptors also exist on a variety of visceral, including gastrointestinal afferent nerve terminals and cell bodies. Dense immunoreactivity for P2X2 and P2X3 receptors is seen on mouse and guinea pig vagal IGLEs. Electrophysiological studies using whole-cell patch clamp techniques have demonstrated ATP-gated currents indicating the presence of P2X2 and P2X2/3 receptors on nodose ganglion cell bodies. In the esophagus and stomach, the P2X receptor agonist, α, β-methylene ATP (α, β-meATP) activates vagal afferents in the mouse and guinea pig. The ATP sensitive units had both mucosal and mechanosensitive receptive fields. In the intestine, mesenteric afferents are excited by P2X agonists, independent of effects on motility. It has been proposed that ATP may be a mediator of neural responses to distention of a hollow viscus. Indeed there is evidence for this in spinal afferents innervating the bladder, where P2X antagonists
or deletion of the P2X2/3 genes attenuates afferent responses to distention. A similar result has been shown in the colon, where P2X receptor antagonists inhibit distension induced responses in the pelvic nerve, and that distention was associated with the release of ATP from the mucosa. Because of the close proximity of IGLE like endings to enteric nerves and the possibility that the latter are a source of ATP, Zagarodnyuk et al. examined the role of endogenous P2X stimulation on mechanosensitivity. They demonstrated that α,β-mATP (a P2X agonist) excites mechanosensitive IGLE type endings, however they could not demonstrate an effect of P2 receptor antagonism on stretch induced firing, concluding that endogenous ATP release did not play a role in mechanotransduction. These experiments were however performed on in vitro tissue, with the mucosa removed. Thus the possibility remains that the source of ATP is the mucosa, rather than enteric nerves. Nonetheless, the above experiments provide strong evidence that ATP can modulate activity of both mucosal and intramuscular vagal endings in the GI tract. Whether ATP is released under physiological or conditions of tissue injury and subsequently acts on vagal afferents remains to be demonstrated. However, experiments performed in the ferret, show that esophagitis reveals a sensitizing effect of P2X agonists on responses to mechanical esophageal stimulation, not seen in the control situation. Thus, it appears that purinergic signalling may also be important in the GI tract under some inflammatory conditions as well. Studies indicating that P2X receptor expression, and ATP gated currents are increased by inflammation in somatic spinal afferents support this idea.

12.5.3 Ionotropic Excitatory Amino Acid Receptors

It has recently been recognized that not only are the receptors for excitatory amino acids expressed on CNS neurons, they are expressed in primary afferent endings and cell bodies in the periphery as well. mRNA for ionotropic glutamate receptors is found in both vagal and spinal afferent neurons, the latter possibly being implicated in visceral nociception. Recent work by Sengupta et al. has demonstrated that vagal afferent responses to antral distention are inhibited by NMDA and AMPA receptor antagonists. The source of endogenous glutamate that acts on these neurons is unclear, but it is possible that it is released from enteric neurons or derived from food sources. Evidence for the latter exists in that NMDA receptor blockade increases food intake, and Glutamate receptors may thus emerge as important modulators of vagal sensitivity and possibly modulators of food intake.

12.5.4 TRPV1 and Other TRP Channels

Capsaicin, the pungent ingredient in chilli peppers, activates small unmyelinated sensory nerve fibers via stimulation of the vaniloid receptor TRPV1. TRPV1 is a ligand-gated nonselective cation channel. The actions of capsaicin on GI vagal afferent nerves have been appreciated for a number of years and the presence of immunoreactivity for TRPV1 receptors on gastrointestinal vagal afferent nerves has recently been demonstrated. In the rat and ferret upper GI tract, capsaicin excites
about 30% of vagal afferent nerve fibers, and excites equal portions of chemosensitive and mechanosensitive endings.7,23

The physiological role of the TRP channels in vagal afferents is still somewhat unclear. It is known however that these channels are important in temperature and acid sensation. For example the TRPV1 channel is activated by pH less than 5.5, and also by noxious heat. Furthermore, subthreshold heat or acid stimuli can potentiate the responses to other stimuli. Given that the upper GI tract is exposed to extremely acidic conditions and also that it is the only visceral region exposed normally to temperatures outside the range of normal body temperatures, these channels may be of some importance in signaling of these stimuli via vagal or spinal afferents. Acid is clearly capable of activating GI vagal afferents21 however the role of TRPV1 in this is unknown. Recent data have also suggested the intriguing possibility that fatty acids, specifically oleoylethanolamine (OEA), which is structurally related to anandamide, can activate the TRPV1 channel, while earlier experiments have implicated OEA in suppressing food intake, via activation of vagal afferent nerves, raising the possibility that TRPV1 is a sensor of GI satiety signals.1 Conclusive experiments to demonstrate these roles have yet to be performed.

In addition to the capsaicin receptor, TRPV1, there exist a variety of other TRP channels that may be of importance in temperature sensation. A recent report has provided evidence that nodose neurons innervating the upper gut express mRNA for the putative temperature sensitive channels TRPV1, TRPV2, TRPV4, TRPN1, and TRPM8, and that intracellular calcium increases in these neurons in response to cooling, heating, capsaicin or icilin, indicating that there are functional temperature sensitive channels present in vagal afferent neurons.202 The function of these TRP channels in the gut is unclear, however it has long been recognized that temperature can modulate motor reflexes in the esophagus179,202 and activation of these temperature sensitive channels may be a mechanism by which this occurs. In addition recent data indicate that TRPV4 is a mechanosensitive channel,173,202 and therefore one might postulate a role for it in GI mechanosensation.

12.5.5 ASIC CHANNELS

ASIC channels are part of the Deg/ENac family of ion channels which also include amiloride sensitive channels in the kidney and the channels important in touch sensation in the nematode C. elegans. These channels can be activated by pH and blocked by amiloride and there is increasing evidence that they may be important in mechanotransduction. There are 4 ASIC channel subunits which combine to form functional heteromeric channels.105 These channels have been demonstrated to be present in nodose baroreceptor neurons, both in their cell bodies and nerve terminals, and are likely important in transducing pressure changes from the aortic arch.49,50 There is indirect evidence that these channels play a role in vagal gastrointestinal; mechanosensation. Zagarodnyuk et al.199,200 have demonstrated that in the guinea pig esophagus, the amiloride analogue benzamil inhibits stretch evoked firing201 and a recent preliminary report suggests that ASIC channel transcripts are present in nodose neurons innervating the GI tract.203 Experiments using analogues of amiloride as ASIC / ENac blockers should be interpreted with caution however, since these
drugs may have non-specific actions such as inhibition of TTX-resistant sodium currents in sensory neurons. Nonetheless, the presence of these channels in vagal sensory neurons suggests a potential role in sensory transduction in the GI tract and clearly requires further study.

12.6 MUCOSAL CHEMORECEPTORS

Mucosal terminals are most abundant in the proximal duodenum becoming relatively sparse in the distal small intestine. Vagal afferent fibers penetrate the circular muscle layer and submucosa to form networks of multiply branching axons within the lamina propria of both crypts and villi. Terminal axons are in close contact with, but do not penetrate the basal lamina and are thus in an ideal position to detect substances including absorbed nutrients and mediators released from both epithelial cells and other cells within the lamina propria. The mucosal mechanoreceptors are characterized by low thresholds to mechanical stimuli such as stroking with a fine brush, relatively rapid adaptation to continuous stimulation and in most cases sensitivity to a variety of chemical stimuli (polymodal receptors).

As discussed earlier, there is an enormous range of chemical mediators that influence the sensitivity of vagal afferents. Electrophysiological, immunocytochemical and molecular biological techniques have revealed the functional expression of receptors to various mediators on the cell bodies of visceral sensory neurones in the nodose ganglia or on their processes in the gut wall. This may be functionally relevant for mucosal endings associated with enteroendocrine cells, macrophages or mast cells in the lamina propria and may provide the basis for transduction of luminal signal or sensory modulation associated with intestinal immune responses. However, because of the complexity of the enteric innervation and the reflex consequences for motility, secretion, blood flow, immune function, etc. arising from enteric neural influences, the signals generated within vagal endings may be secondary to these events and not arise through direct activation. There is, therefore, considerable uncertainty in the literature on vagal afferent sensitivity that arises from a failure to distinguish primary from secondary activation.

12.7 SENSORY SIGNAL TRANSDUCTION

Mediators that produce a direct stimulation of visceral sensory nerve endings may do so as part of a discrete sensory signalling pathway. In this case, the afferent neurone does not respond directly to a stimulus, but following the release of a mediator from a primary sense cell. Examples of these cells, which effectively act as principal sensory transducers, are enterochromaffin (EC) cells, which release 5-hydroxytryptamine (5-HT) and enteroendocrine cells that release CCK. The basic principle for both is that they represent secondary sense cells in the gastrointestinal mucosa which are strategically positioned to monitor luminal composition. These cells have an apical tuft of microvilli which is proposed to “taste” the luminal contents and in response to an appropriate stimulus release the contents of storage granules across the baso-lateral membrane to stimulate afferent terminals in close
proximity. Electrophysiological evidence suggests that 5-HT and CCK act on distinct sub-populations of vagal mucosal afferent nerves (Figure 12.5). As such, this sensitivity represents an example of a high-fidelity, modality-specific signal transduction pathway. This mechanism, more than likely, functions in the detection of moment-to-moment changes in luminal composition and operates, in the main, below the level of consciousness.

12.8 BEHAVIORAL ASPECTS OF VAGALafferent SIGNALS

It has long been known that nutrients and other luminal chemicals can provoke motor and secretory responses that help coordinate digestion and absorption. Pavlov (1902) considered that intestinal nutrients stimulated vagal afferents that served to reinforce the cephalic signals that drove gastric acid secretion. While secretin, gastrin, and CCK were classically described as gastrointestinal hormones that regulated secretion and motility, it is now apparent that endocrine mediators such as CCK can also act locally at the site of release on the terminals of vagal sensory endings and that this underlies many of the physiological actions of the endogenous mediator. There is evidence that secretin may also act in a similar manner.
Vagal Afferents Innervating the Gastrointestinal Tract

333

the case of CCK, there is overwhelming evidence that it contributes not only to vago-vagal reflex mechanisms but also plays a crucial role in feeding behavior following transmission of sensory signals from the nTS to the hypothalamus. Other satiety and orexigenic mediators including leptin, ghrelin, orexin are found in the gastrointestinal tract, released during fasting or upon feeding, and act on receptors expressed on the terminals of vagal afferent endings. The concept of the vagus nerve as an integrator of feedback signals that modulate feeding behavior and metabolic function is therefore becoming well established.

CCK was the first satiety hormone to be shown to influence the firing of vagal afferents. Since the feeling of fullness may arise from gastric distension the earlier observations focused on the effect of CCK on gastric mechanosensitive endings leading to the suggestion that the response of gastric mechanoreceptors to stretch can be influenced by CCK released when nutrients enter the duodenum. Gastric afferent activity is stimulated by CCK both in vivo and in vitro but at concentrations delivered intravenously or close arterially that are unlikely to be observed postprandially. At lower plasma levels CCK has been shown to sensitize the afferent’s response to stretch leading to enhanced discharge in brain stem neurons consistent with an action of CCK on gastric mechanosensitive afferents. This sensitivity is suggested to underlie the satiety effect of CCK with gastric afferents signaling both the quantity of ingested material through distension and its quality in terms of nutrient density following its delivery to the duodenum. However, as discussed above the proximal stomach relaxes to accommodate food during ingestion and in addition, following nutrient entry into the duodenum, gastric tone is inhibited further as part of the mechanisms that inhibit gastric emptying. As tension receptors, gastric vagal afferents may therefore be minimally active during normal gastric filling and emptying. In this respect gastric tone is inhibited in response to CCK and vagal mechanoreceptors can be off-loaded as the stomach relaxes. This observation is consistent with data which suggests that inhibition of food intake by gastric distension and intestinal nutrients is mediated by independent mechanisms and that the action of endogenous CCK on satiety is not via an endocrine action but through local paracrine mechanisms.

Another target for CCK action is on the terminations of intestinal mucosal afferents which lie in close proximity to the enteroendocrine cells, which release CCK in response to luminal nutrients and which might be implicated in afferent signal transduction. Good evidence is available that CCK acts in such a fashion. Thus, it has been shown that a subset of vagal mucosal afferents are exquisitely sensitive to CCK acting on the CCKα receptor. These same CCK-sensitive afferents are stimulated by luminal application of protein digestion products and both the response to exogenous CCK and response to luminal protein are blocked by a CCKα receptor antagonist. CCK release is also stimulated by fatty acids above a critical chain length of 12 C-atoms and there is a wealth of literature on the mechanisms controlling CCK release. In this respect, long (C18) and short chain fatty acids (C4) both stimulate mesenteric afferents supplying the rat jejunum but only the former is mediated via CCK.

The CCKα receptor exists in 2 different affinity states (high and low) that can be differentiated by their sensitivity to CCK-JMV-180 (a high affinity agonist and
Studies on isolated nodose neurons suggested that the low- and high-affinity receptors are linked to different calcium signal cascades. The paracrine mediated action of CCK on intestinal vagal afferents leading to pancreatic secretion is via the high affinity receptor while it is the low-affinity receptor that features in satiety. Gastric vagal afferents are also inhibited by CCK-JMV-suggesting that the high-affinity receptor is predominantly expressed on vagal sensory neurons. Afferent fibers appear to be functionally tuned to respond to CCK since those responding to 5-HT3 receptor activation (see later section) do not respond to CCK and vice versa, afferents responding to CCK do not respond to 5-HT while 5-HT3 receptor-activated afferents do not respond to CCK (Figure 12.6).

There is some evidence of a post-absorptive sensor for glucose and the early literature on “gluoreceptors” is reviewed elsewhere. Mei demonstrated effects of both absorbable and nonabsorbable sugars on vagal afferent activity while the ability of phlorhizin to block the effect of luminal glucose would suggest active transport is necessary for signaling to occur. Hepatic afferents may also sense glucose following absorption into the portal vein.

There is also evidence that other macronutrients may act by a postabsorptive mechanism. Melone and Mei demonstrated that long-chain fatty activated vagal afferents in the cat. While CCK as a contributing factor was not examined in this study, it is intriguing that long-chain fatty acids are preferentially steered toward chylomicron formation during absorption and preventing chylomicron formation using Pluronic L-81, a nonionic hydrophobic surfactant, attenuates some of the reflex and behavioral responses to luminal lipid. Both chylous lymph and apolipoprotein A-IV (released by enterocytes during chylomicron formation) activate vagal

**FIGURE 12.6 (A color version of this figure follows page 236.)** Schematic representation of ion channels and receptors that determine afferent sensitivity.
afferents, an effect blocked by CCKα receptor blockade implying that CCK release is an integral part of lipid signaling by a direct action on CCK release or CCK release following absorption.

The expression of CCKα receptors on nodose ganglion neurons is consistent with its role in sensory signal transduction. That these same neurons also express orexin receptors (OX-R1) and leptin receptor implies that other signals from the GI tract known to modulate feeding behavior may do so by acting at the level of the vagal afferent nerve terminal.29,30 A functional interaction between leptin and CCK has been shown in both cat intestinal mechanoreceptors60,61 and rat gastric afferents.73,184 In both species, leptin could either augment or inhibit afferent firing but the latter reversed after treatment with CCK. What is unclear from these studies is the extent to which motor response may have contributed to these findings especially since micromolar concentrations of peptide delivered close arterially would likely exceed that of the endogenous ligands. There is also evidence that leptin-stimulated pancreatic secretion is mediated by CCK release and an action on vagal afferents expressing CCK1 receptors.73 In contrast orexin (albeit a high doses) has been shown to inhibit the response of rat mesenteric afferents to CCK.29 Ghrelin, another appetite stimulating peptide located in the stomach has also been shown to inhibit vagal afferent firing in contrast to the stimulatory action of CCK.45 While its receptor GHS-R is present on nodose neurons that project to the stomach,160 Thus, a complex picture of receptor interactions at the level of the vagal sensory nerve terminal is emerging but their role in reflex control and feeding behavior is yet to be determined. Also uncertain is the extent to which other gut endocrine signals including GRP, neuropeptide Y, and PYY also influence afferent sensitivity.

12.9 CYTOKINES AND ILLNESS BEHAVIOR

The mucosal epithelium is the first line of defense against pathogen bacteria and antigenic material. Elements of the gut immune system are part of the surveillance system that guards against such harmful material and there is increasing evidence that vagal afferents are part of the mechanism for rapid detection and brainstem signaling following activation of a local inflammatory response.67 In this respect both mast cells and resident macrophages are closely associated with vagal afferent terminals and a bidirectional communication has been proposed. Immune cells release or generate a variety of mediates that potentially could lead to afferent interaction. These include amines like 5-HT and histamine, proteases, prostanoids and cytokines including IL1β and TNFα.102

Intestinal anaphylaxis has been used as a model for mast cell-mediated signaling and leads to pronounced brainstem Fos activation,34,104,166 which can be attenuated by treatment with 5-HT3 and histamine H1 receptor antagonists. Mesenteric afferent responses in ovalbumin sensitized to luminal antigen give rise to a dramatic increase in firing within 30 seconds a response which is again attenuated by serotonergic and histaminergic receptor antagonism.93 Mast cell proteases may also contribute to the signaling from mast cells since PAR activating peptides are powerful stimuli of mesenteric afferent bundles.103 However, the functional importance of PARs awaits the development of specific receptor antagonists.
Recognition of the cellular components of harmful bacteria generates a macrophage-driven cytokine cascade that is referred to as an acute phase response and drives a local inflammatory reaction, as well as generating behavioral responses known as sickness behavior that include fever, anorexia and hyperalgesia. Lipopolysaccharide (LPS) from gram negative bacteria is used experimentally to trigger an acute phase response including hyperalgesia. High doses of LPS are lethal and protection from lethality has been used as an experimental model to investigate modulation of immune responses. Clinically this is equivalent to the events that follow breakdown in the gastrointestinal protective mechanisms when large numbers of bacteria translocate the intestinal mucosa. One dramatic example of when this occurs is during multi-organ failure in critical care patients. Major trauma with hypoperfusion of the GI tract leads to a breakdown in the protective mechanisms and bacteria cross the mucosa epithelium triggering a major systemic inflammatory response.

The macrophage driven cytokine cascade following LPS administration gives rise to an increase in circulation IL-1β and TNF-α. These cytokines orchestrate both the local inflammatory response and the CNS consequences that are manifested as illness behavior. These CNS consequences appear to involve both direct effects of circulating cytokine and activation of afferent inputs to the central circuits that regulate temperature, feeding behavior and pain modulation. Thus brain fos expression following LPS or IL-1β have been shown to be attenuated following procedures that interrupt the afferent traffic to the brainstem and spinal cord in the vagus and spinal nerves respectively, although this finding remains controversial. Such observation suggests that afferent neuron excitability is augmented following treatment with LPS and a number of studies have investigated modulation of afferent sensitivity in response to cytokines such as IL-1β.

LPS administration leads to a biphasic increase in afferent sensitivity. An immediate effect (with secs) may reflect a direct effect on the afferent nerve terminals while a gradually developing increase which peaks with one to two hours may arise following cytokine activation. The rapidity of such signaling indicates the speed by which such information is transferred to the CNS. Descending vagal influences may in turn regulate the course of the inflammatory response.

12.9.1 5-HT, Nausea and Vomiting

5-Hydroxytryptamine (5-HT) is widely distributed within the wall of the gastrointestinal (GI) tract where it has the potential to modulate the enteric reflex circuits that control motor and secretory responses to stimuli from the gut lumen. In this respect, 5-HT is implicated in the mechanisms that detect the physical and chemical nature of luminal contents to bring about responses that are appropriate for the digestive needs of the individual. Enterochromaffin (EC) cells in the epithelial lining are believed to function as secondary sense cells, transducing luminal stimuli via the release of 5-HT from the basolateral membrane of the cells onto afferent nerve terminals in their vicinity. Another potential source of 5-HT are the mucosal mast cells which, in some rodent species, is released together with histamine and proteases when these cells are triggered as part of an intestinal anaphylactic reaction.
cells are also in close proximity to the terminals of both intrinsic and extrinsic afferent nerve endings. Thus, 5-HT released either during normal digestive activity or following immune cell activation, has the potential to modulate the activity of those nerve circuits involved in local reflex control\textsuperscript{11} and others that convey information to the central nervous system.\textsuperscript{81,204} Experimental recordings from mesenteric nerve bundles as they emerge from the bowel wall in anaesthetized rats have revealed distinct subpopulations, some of which powerfully respond to exogenous 5-HT. One population generates a brief but powerful burst of action potentials in response 5-HT, while another population responds more slowly and less intensely.\textsuperscript{84} These two populations can be characterized further on the basis of their anatomical location, physiology and pharmacology. The former project into the intestinal mucosa and, in addition to a direct sensitivity to 5-HT, respond to luminal chemicals such as acid while the latter respond to mechanical distension and contractions, properties indicative of muscle afferents. Furthermore, the early transient response is mimicked by 5-HT\textsubscript{3} receptor agonists and abolished by 5-HT\textsubscript{3} receptor antagonists, while the slow sustained response appears to be secondary to a contractile response mediated by 5-HT\textsubscript{2A} Receptors present on the intestinal smooth muscle. They also follow different pathways to the CNS since the 5-HT\textsubscript{3} receptor-mediated response is a property of vagal afferents while the secondary activation can occur after the surgical elimination of vagal afferent populations by truncal vagotomy.\textsuperscript{81} The sensitivity of mucosal afferents to both 5-HT and acid raises an important question with respect to the potential role of EC cells in signal transduction. Could these cells be the transducers of luminal chemicals in the same way as that proposed for intrinsic afferents?\textsuperscript{10,100} If this were the case, then the response to acid should be sensitive to treatment with 5-HT\textsubscript{3} receptor antagonists. However, the response to 150 mM hydrochloric acid was insensitive to granisetron, implying that there may be multiple transduction processes involved in the stimulation of these fibers. Similar observations have been made previously with respect to vagal mucosal afferent fibers in anaesthetized ferrets.\textsuperscript{19} Thus, 5-HT and acid may act independently to activate vagal mucosal afferent fibers and this postulate is consistent with the view that visceral sensory neurones are polymodal.

One way in which endogenous 5-HT may activate these vagal mucosal afferents is observed with cancer chemotherapy agents such as cisplatin. Nausea and vomiting are major side-effects of both cancer radio- and chemotherapy. 5-HT\textsubscript{3} receptor antagonists can markedly attenuate these side effects and a wealth of evidence suggests that these agents act at the level of the vagal afferent nerve terminal.\textsuperscript{2} Animals treated with the anti-neoplastic agent, cisplatin, show an elevation in mesenteric afferent activity within 10 to 15 min of administration, which can be reversed upon treatment with 5-HT\textsubscript{3} receptor antagonists.\textsuperscript{83} Thus clearly there is the potential for endogenous 5-HT to modulate vagal afferent traffic and this may explain the effectiveness of 5-HT\textsubscript{3} receptor antagonists as antiemetics for radio- and chemotherapy.
12.9.2 Sensation: Mediation vs. Modulation

There is a wealth of evidence to support the view that vagal afferents play a role in pain modulation by influencing descending spinal pathways that regulate synaptic input to the dorsal horn. Vagal afferents also influence the HPA axis, which in turn influences immune mediated hyperalgesia. These aspects are dealt with elsewhere in this volume (Janig; Chapter 17). Here we will consider evidence that suggests that vagal afferents may directly mediate symptoms of nausea, discomfort, and pain, or contribute to the affective/emotional components of visceral pain.

Indirect evidence to support this view arises from a number of sources. The first is that vagal afferents respond to nociceptive mechanical and chemical stimulation and this leads to brainstem representation of nociceptive signals (see above). Second order neurones ascend from the nTS to various CNS areas involved in affective responses if not to thalamic regions that project to the sensory cortex. The wealth of this sensory information relates to physiological, moment-to-moment activity from throughout the proximal GI tract. The pattern of activity may reflect all is well during the course of normal digestion and would rapidly signal when motor function is disrupted. A classic example of disorder motor function occurs during the prodromal phase of emesis when retroperistaltic contractions return material to the stomach, which in turn relaxes prior to expulsion. These prodromal motor patterns are vagally driven and are associated with feelings of nausea. In animals, vagal stimulation rapidly evokes behaviors consistent with nausea. Vagal stimulation is also becoming routine for patients with intractable epilepsy with no reports of these patients experiencing pain. However, the stimulus intensity used for such treatment may be insufficient for C-fiber activation.

Attempts to correlate nausea with motor disturbances in humans have proved inconclusive. Many individual who experience vection-induced nausea have minimally delayed gastric emptying while others with little nausea have profound motor disruption. Disordered motility and nausea may be epiphenomenon rather than cause and effect. Vagotomy, also leads to disrupted gastric accommodation and symptoms of nausea, early fullness, bloating, and discomfort collectively referred to as postvagotomy syndrome. Since the vagal afferent input is compromised by this surgical procedure, then clearly postvagotomy symptoms must arise from non-vagal inputs to the CNS. Similar symptoms in nonulcer dyspepsia and diabetic neuropathy may arise through similar mechanisms.

Spinal injury patients have markedly blunted sensory experience from their colon and rectum suggesting that spinal afferents are the major route for sensation from the distal bowel. Comparable studies following gastric and esophageal stimulation have yet to be performed. However, such studies may be difficult to interpret because of vagal afferents that project into the cervical cord that are implicated in angina pain.

The answer to the role of vagal afferents in sensory perception may lie in functional imaging studies in which sensory experience and brain activation can be correlated. However, only recently has the resolution necessary to determine
brainstem activation been achieved\textsuperscript{176} and has yet to be applied to gastrointestinal afferents.

\section*{12.10 CONCLUSION}

The gastrointestinal tract is endowed with a rich vagal afferent innervation, which clearly is capable of sensing mechanical events in the gut wall as well as chemical mediators. Furthermore, the complex enteric microenvironment containing immune cells, microbes, endocrine cells, and intrinsic enteric nerves increases the complexity of stimuli, which may impact on GI vagal afferent pathways. These afferent nerves play key roles in homeostasis in terms of food intake, motility, and nutrient assimilation, as well as protective mechanisms such as emesis. Emerging evidence also indicates a role for gut vagal afferents in a variety of disease states, such as local gut injury or inflammation as well as systemic inflammation such as occurs in sepsis. Further work is needed to define the role of vagal afferents in nociceptive processing, to characterize the changes occurring in primary vagal afferents in disease states, and to examine the potential for interaction between the rich intrinsic innervation of the gut and extrinsic vagal afferents.

\section*{REFERENCES}

\begin{enumerate}
\item Ahern GP. Activation of TRPV1 by the satiety factor oleoylethanolamide. \textit{J Biol Chem} 278: 30429+30434, 2003.
\item Berthoud HR, Lynn PA, and Blackshaw LA. Vagal and spinal mechanosensors in the rat stomach and colon have multiple receptive fields. \textit{Am J Physiol Regul Integr Comp Physiol} 280: R1371–R1381, 2001.
\end{enumerate}


Advances in Vagal Afferent Neurobiology


Vagal Afferents Innervating the Gastrointestinal Tract


Advances in Vagal Afferent Neurobiology


13 Cardiac Vagal Afferent Nerves

Harold D. Schultz

CONTENTS

13.1 Introduction ..................................................................................................351
13.2 Morphological Characteristics of Cardiac Vagal Afferents .......................352
13.3 Electrophysiological Characteristics of Cardiac Vagal Afferents .......... 353
  13.3.1 Single-Fiber Recordings ..................................................................353
  13.3.2 Patch-Clamp Recordings .................................................................354
  13.3.3 Classifications of Sensory Function ..............................................355
  13.3.4 Mechanosensitive Cardiac Vagal Afferents ..................................355
  13.3.5 Cellular Mechanisms of Mechanotransduction in Cardiac Vagal
          Afferents ...........................................................................................359
  13.3.6 Chemosensitive Cardiac Vagal Afferents ..................................360
  13.3.7 Cellular Mechanisms of Chemotransduction in Cardiac Vagal
          Afferents ...........................................................................................362
13.4 Cardiac Vagal Afferent Function in Pathophysiological States ..............363
  13.4.1 Myocardial Ischemia and Reperfusion ............................................363
  13.4.2 Heart Failure ....................................................................................365
  13.4.3 Hypertension ....................................................................................366
  13.4.4 Diabetes Mellitus .............................................................................367
13.5 Summary ......................................................................................................368
References ..............................................................................................................369

13.1 INTRODUCTION

The heart possesses a complex sensory innervation of mechanically and chemically sensitive endings that send action potentials to the central nervous system via vagal and sympathetic afferent pathways. These inputs impose reflex influences on sympathetic and parasympathetic outflow to control cardiac and hemodynamic function. In addition, sensory input is relayed to neurons in intrinsic cardiac ganglia that may serve a “local circuit” role in the coordination of parasympathetic and sympathetic activity to various regions of the heart. This chapter will discuss only the sensory innervation of the heart that sends impulses to the nucleus tractus solitarius in the medulla via the vagus nerves. This focus, however, is not intended to downplay the
importance of cardiac sympathetic afferent neurons or sensory neurons in cardiac ganglia as components of an intricate sensory network that coordinates reflexes from the heart. The electrophysiology of sensory neurons in these other pathways has been summarized in other excellent reviews.1–4

Cardiac vagal afferent function has been defined traditionally by the reflex responses evoked by mechanical and chemical stimuli applied to the heart. Thus, mechanical stretch of receptors in the veno-atrial junction in either atria, but most prominent in the left atrium, evokes the Bainbridge reflex consisting of a reflex sympatho-excitation to the heart to cause tachycardia, and a sympatho-inhibition to the kidney to increase urine flow.5 Stimulation of mechanoreceptors in the left ventricle by distension of the chamber or injection of veratridine into the coronary circulation evokes the Bezold-Jarisch reflex, a widespread sympatho-inhibition causing hypotension and bradycardia similar to that evoked by the arterial baroreceptors.6 Stimulation of chemically sensitive vagal afferent endings in the ventricles and atria by intracoronary or epicardial application of compounds such as capsaicin, phenylbiguanide, prostaglandins, and bradykinin also evokes a Bezold-Jarisch effect, sometimes regarded as the coronary chemoreflex.7

The complexity of the entire cardiac sensory network consisting of multiple sensory modalities, divergent afferent pathways, and competing central influences on autonomic outflow makes it difficult to predict the functional significance of the vagal afferent innervation from the heart in any given situation. Nevertheless, the first step toward a thorough understanding of the function of these afferent nerves is to understand how they encode mechanical and chemical (metabolic) information from the working heart. The purpose of this review is to summarize important background information, recent advances, and future goals in our understanding of the electrophysiology of these cardiac vagal afferents.

13.2 MORPHOLOGICAL CHARACTERISTICS OF CARDIAC VAGAL AFFERENTS

The principle that “form subserves function” underscores the importance of appreciating the neuroanatomy and morphology of the sensory nerves innervating the heart. Unfortunately, little is known about the structure of most cardiac sensory endings. Much more is known about the ultra-structure of vagal afferent endings in the atria than that in the ventricles simply because the thin walls of the atria facilitate histological examination. An abundance of easily identifiable nerve endings are located in the atrial endocardium as end-nets and as various forms of complex unencapsulated endings.8–10 The complex unencapsulated endings are generally connected to large myelinated (A-) nerve fibers, which can be identified electrophysiologically as mechanoreceptors.10 Their structure is similar to that of other mechanoreceptors (pulmonary slowly adapting stretch receptors and aortic baroreceptors) that respond to tissue deformation or stretch.8 These complex unencapsulated endings are found in highest density near junctions of the great veins and atria, and the coronary sinus.8–10
In addition to the complex unencapsulated endings, a network of fine-branched neurites (end nets) form extensive anastomoses of many fibers, which are located throughout the endocardium of both the atria and ventricles.\textsuperscript{8,9,11} Unfortunately the structure of these end nets provides little insight to their function. It is tempting to speculate that this network is connected to the nonmyelinated (C-) and small myelinated (A\textsubscript{\delta}-) fiber neurons that are the major proportion of vagal afferent fibers arising from both the atria and ventricles.\textsuperscript{12} Studies using immunofluorescent staining of tachykinins and CGRP as markers of C-fiber sensory nerves confirm an abundance of C-fibers in the atrial endocardium and myocardium.\textsuperscript{13} But as yet, there is no reliable information about the terminal structures attached to these cardiac vagal C-fibers.

The ultra-structures of vagal afferent endings in the ventricles have not been identified other than the network of endocardial end nets as described previously. However, sensory nerve fibers expressing CGRP and tachykinins are present in the ventricles, most prominently along the perivascular innervation of coronary vessels, but also throughout the ventricular wall.\textsuperscript{13} Despite the lack of morphological evidence, electrophysiological recordings provide convincing evidence that vagal sensory afferents innervate most regions of the ventricles and pericardium.\textsuperscript{14–16}

It had been thought that sensory nerves do not innervate myocytes until a recent study by Cheng et al. described two types of ending, “varicose-intercalated” and “parallel-elongated,” that make intimate contact with cardiac muscle fibers in the atria.\textsuperscript{9} Of note was that some afferent fibers appeared to branch out with one or more of these endings in the myocardium and with collateral branches of complex unencapsulated endings in the endocardium. Thus, it appears that there can be heterogeneity of endings from a single fiber, both with respect to terminal structures and their location. This anatomical polymorphism is supported by electrophysiological studies that indicate that many cardiac vagal afferents fibers exhibit polymodal sensitivities.\textsuperscript{14–16}

Another interesting and important observation made by Cheng et al. was that some vagal afferent fibers arise from dense pericellular terminals encompassing small intensely fluorescent (SIF) cells in the cardiac ganglia.\textsuperscript{9} The function of SIF cells in cardiac ganglia is debated, but the speculation is that they serve as chemoreceptors, neurosecretory cells, or interneurons.\textsuperscript{17} The sensory characteristics of the vagal afferent nerves associated with SIF cells in cardiac ganglia is not known and should be addressed.

13.3 ELECTROPHYSIOLOGICAL CHARACTERISTICS OF CARDIAC VAGAL AFFERENTS

13.3.1 SINGLE-FIBER RECORDINGS

A variety of techniques have been used to describe the electrophysiological properties of vagal sensory endings in the heart. The gold standard from the earliest experiments to the present has been the extracellular recording of single-fiber action potentials from fine nerve bundles dissected from the distal cut end of the vagus nerve. A slightly modified method used by some has been to insert a fine unipolar
electrode into the nodose ganglion to record action potentials from the afferent cell bodies. The receptive field of the afferent fiber being recorded must be confirmed to arise from the heart through a series of tests such as mechanical manipulation of the cardiac chambers, injection of certain chemicals into the coronary circulation or application to the epicardium, or mapping of the surface of the heart with a stimulating electrode or fine probe.

The type of fiber being recorded (A-, Aδ-, or C-fiber) can be determined by measuring the conduction velocity (CV) or velocity of propagation of action potentials along the fiber path. Myelinated (A-) fibers exhibit fast CVs > 8-10 M/s, whereas nonmyelinated (C-) fibers exhibit very slow CVs < 2.5 M/s. Thinly myelinated (Aδ-) fibers exhibit intermediate CVs between the two extremes. The relative proportion of A- to C-fiber afferents in cardiac tissue is difficult to ascertain. Histological examination of cardiac branches of the vagus nerve indicate that C-fiber afferents predominate from the heart. Electrophysiologically, however, it is more difficult to record single action potentials from C-fibers, and thus, estimates based upon single fiber recordings often overestimate the proportion of A-fibers. But, even by such standards, it is much more difficult to find A-fiber afferents in the ventricles than in the atria.

When summarizing the accumulated information that has been gained from these studies, it is important to recognize an inherent limitation. The ability to find afferent vagal fibers arising from the heart requires random sampling of literally hundreds of afferent fibers in the vagus nerves. It is easy to overlook afferents from the heart that are not easily recognized because they either are silent at rest or do not respond to a specific test stimuli used by an investigator to identify a cardiac fiber. As a result, investigators tend to find what they are looking for in afferent recordings. Nevertheless, these types of experiments have provided valuable insight into the function of vagal sensory afferents in the heart because the discharge characteristics of identified cardiac afferents correlate well with known reflex responses that occur under similar conditions. Despite this wealth of information, we cannot yet assume that we have sufficient knowledge of the sensory properties of all vagal afferent nerves in the heart.

13.3.2 Patch-Clamp Recordings

The ultimate goal of revealing the mechano- and chemo-transduction processes in the afferent endings in the heart requires definition of the signaling pathway between the stimulus and the specific ion channels in the cell membrane that influence its electrical excitability. Recording voltage potentials or ionic currents generated across the membrane of isolated cell bodies from the nodose ganglion are recent techniques that are providing important information about the membrane characteristics of these cardiac neurons. Cell bodies in the nodose ganglion that innervate the heart can be identified or tagged by fluorescent labels applied to the cardiac tissue, which are then taken up by the afferent terminals and transported to the cell bodies. The nodose ganglion is removed and cell bodies dispersed in vitro. The tagged cardiac cells thus can be easily identified for recording by patch-clamp techniques.
Although this method allows one to identify and record from nodose neurons that innervate the heart, the technique does not allow one to differentiate to any certain degree the type or location of the sensory terminals within the heart that were projecting to the cell bodies. These cell bodies in the nodose ganglion also project presynaptic terminals to the nucleus tractus solitarius in the brainstem that are likely to have membrane characteristics quite divergent from those of the sensory terminals. Thus, it cannot be assumed that membrane function at the level of the cell body reflects that which occurs in the sensory terminal. Unfortunately, it is not yet technically possible to isolate (and in the case of C-fibers even identify) the sensory terminals themselves from cardiac tissue to allow patch-clamp procedures at the level of the sensory receptor. Nevertheless, some insight can be gained from patch-clamp recordings of the cell body when membrane characteristics of the somata can be correlated with the sensory discharge characteristics of the afferent neuron.

13.3.3 Classifications of Sensory Function

Traditionally sensory endings in the heart, vasculature, and lungs have been classified as either mechanoreceptors or chemoreceptors based upon their discharge patterns. Many vagal afferents from the heart, particularly the C-fiber afferents, overtly respond to both mechanical and chemical stimuli and are regarded as polymodal. On the other hand, while many afferent endings only respond directly to mechanical stimuli and are regarded as mechanoreceptors, their mechanosensitivity often can be shown to be altered by the chemical environment. Conversely, afferents regarded as chemoreceptors may find their chemical sensitivity markedly altered by mechanical changes occurring in the chamber wall. Thus, while it is utilitarian to assign discreet categories of mechanosensitivity, chemosensitivity, and polymodal sensitivity to afferent populations, the functional characteristics of all afferent endings in the heart is much more likely to represent a complex interaction of their mechanical and chemical environment.

13.3.4 Mechanosensitive Cardiac Vagal Afferents

Using the single fiber technique, Paintal in 1953 classified atrial mechanoreceptors according to their pattern of discharge in relation to the atrial pressure wave. Type A receptors (not to be confused with the term A-fiber) are those that discharge in phase with the “a” wave of atrial systole; type B receptors discharge during the “v” wave of atrial filling (Figure 13.1). Type AB receptors have characteristics of both type A and B but are not as commonly found. The afferent nerve fibers connected to type A, B, and AB receptors exhibit fast conduction velocities (> 10 m/s) and thus are A-fibers. The ratio of the two types of receptors in atrial tissue differs from species to species. There are more type B than type A in dogs, and more type A than type B in monkeys.

Blood volume expansion and hemorrhage increase and decrease respectively atrial type B receptor activity in proportion to the amplitude of the v wave. Activity from these receptors thus appears to be a fairly accurate indicator of the
FIGURE 13.1 Examples of electrophysiologic recordings from afferent vagal fibers with mechano- and chemosensitive nerve endings in the dog heart. (Upper traces) Two A-fiber mechanoreceptors (Type A and Type B) from the left atrium. (Middle trace) A coronary mechanoreceptor. (Lower traces) A C-fiber mechanoreceptor and a chemosensitive ending from the left ventricle. Note that the mechanically sensitive endings exhibit pulse-modulated discharge. C-fiber afferents exhibit a lower frequency range and more irregular discharge patterns than A-fibers. (Panel A) Afferent responses to intracoronary injection of capsaicin (1 µg/kg at mark). The chemosensitive C-fiber ending in the left ventricle (lower trace) rapidly responds to capsaicin with a volley of impulses. A-fiber mechanoreceptors in the atria and ventricles (upper panels) are unresponsive to the chemical stimulus. Also illustrated is a C-fiber mechanoreceptor in the left ventricle that does not respond to chemical stimulation. (Panel B) Afferent responses to aortic occlusion. The atrial type B receptor, but not type A, markedly increases activity in response to the elevation of left atrial pressure (upper traces). Similarly, the coronary mechanoreceptor (middle trace) responds to the increase in coronary (arterial) pressure, and the C-fiber ventricular mechanoreceptor responds to the increase in left ventricular pressure (lower trace). The chemosensitive C-fiber ending in the lower trace is unresponsive to the pressure changes. (Panel C) Topographical maps of regions of the heart (light gray) innervated by the various types of afferent vagal endings illustrated to their left. Atrial receptors are located in highest numbers around the atrio-venous junctions and lateral walls of the atria. Coronary mechanoreceptors are found near the origin of the left coronary arteries. Ventricular C-fiber endings are found throughout the ventricles, but are more numerous in the posterior wall of the left ventricle in dog. (Dark gray = areas of more profuse innervation). Neurographic recordings were corrected for conduction delay in the afferent fibers to illustrate synchronization with cardiac pressures and ECG. Abbreviations: ECG, electrocardiogram; LAP, left atrial pressure; AP, arterial pressure; LVP, left ventricular pressure; LCX, left circumflex coronary artery; LAD, left anterior descending coronary artery; RCA, right coronary artery.
volume of the atrium. Atrial type B receptors slowly adapt to a step change in atrial pressure, and their pressure sensitivity resets to a higher threshold and operating range when subjected to a sustained elevation in atrial pressure. Increases in heart rate at any given pressure decrease the number of impulses generated per beat in type B receptors, but do not alter the impulse activity per unit time. The endings appear to act as distortion receptors, as discharge frequency is proportional to wall strain. Their activity is modulated in spontaneous breathing animals concurrent with atrial transmural pressure changes due to swings in intrathoracic pressure during breathing.

Unlike type B receptors, type A receptors do not change their firing rate in response to volume expansion or hemorrhage. They increase activity in response to increasing heart rate (atrial contraction) and inotropic state, and thus appear to be coupled to active tension of the atrial wall.

Considerable controversy still exists whether atrial type A and B receptors represent different receptor types that give rise to different reflex responses. Histological evidence is convincing that the receptor terminals are the same, and they respond identically to dynamic sinusoidal mechanical stretch of the atrial tissue. Other evidence suggests that the only difference between the type A and B receptors is the location in the atrium, and either type can change its firing pattern to the other type under certain hemodynamic states and atrial locations. Nevertheless, it is still unknown whether the differing afferent signals arising from atrial type A and B receptors is processed differently by the CNS.

Both atria are also innervated by C-fiber mechanoreceptors, and based on fiber population in the vagus nerve, probably significantly outnumber the A-fiber afferents in most species. The receptive fields for these C-fiber afferents do not appear to differ significantly from that of their A-fiber counterparts, concentrated at the atrial-venous junctions, but they can be found throughout the atria including the atrial septum and appendages. The discharge pattern of C-fiber atrial afferent fibers is much more variable than that of A-fibers. Many exhibit a discharge pattern similar to either type A or type B atrial receptors but at lower and more irregular discharge frequencies and higher thresholds to pressure. Their spontaneous activity is lower than that in A-fibers, and many are inactive at normal atrial pressures. In addition, their sensitivity to pressure changes is much less than that of A-fibers. Some C-fiber endings respond to distension or distortion of atrial tissue with only brief bursts of activity. Evidence suggests that these low-frequency C-fiber afferents do not reset their pressure sensitivity to a higher operating point in response to a sustained increase in atrial pressure, a typical response characteristic of A-fiber mechanoreceptors. Many of these C-fiber afferents also respond to application of chemicals such as capsaicin, a specific stimulus to chemically sensitive C-fiber afferents, or phenylbiguanide (PBG) a serotonin agonist. These chemical effects cannot be linked to mechanical changes in the atrial tissue.

A- and C-fiber mechanosensitive afferents have been recorded from the left ventricle of several species. Most ventricular A-fiber mechanosensitive afferents appear to innervate the coronary vessels since most respond to changes in coronary pressure, but not ventricular pressure. The discharge characteristics of these “coronary” mechanoreceptors, a pulse modulated activity that becomes continuous...
at higher pressures, resemble in many respects that of aortic baroreceptors (Figure 13.1). As with aortic baroreceptors, mean discharge frequency rather than instantaneous frequency encodes pressure changes in coronary baroreceptors.38 One important distinction between coronary and aortic baroreceptors is the observation that coronary baroreceptors do not acutely reset their operational range or sensitivity with sustained changes in pressure.39

A-fiber endings have been recorded also in the endo- and epicardial regions of the posterior wall near the atrioventricular groove and in the region of the coronary sinuses.38,40 They were reported to have a greater sensitivity to changes in LVP, much higher discharge frequencies, and more discrete receptive fields than their C-fiber counterparts.

The preponderance of afferent fibers arising from the left ventricle appear to be C- and Aδ-fibers. Despite sparse histological evidence, ventricular C-fiber mechanoreceptors have been identified electrophysiologically in all depths of the ventricular wall, with considerable discrepancy as to whether they are found in highest numbers in the epicardium, endocardium, or myocardial regions,34–41 and it is often difficult to precisely locate the receptive field, particularly for those endings lying deep in the myocardium. At least in dogs, it appears that they are more numerous on the posterior wall of the left ventricle.42 C-fiber afferents that appear to arise from the coronary vessels have been mentioned in passing, but have not been carefully characterized. Far fewer afferents have been found to terminate in the right ventricle than the left,33,34,43 in keeping with the weak reflex responses to distension of the right ventricle.44

There are widely differing reports on the pattern of discharge of the ventricular C-fibers, which probably reflects the heterogeneity of the afferents and, to a certain extent, the selection criteria used in different studies.34–36,45 These afferents generally have very low or no resting discharge,34–36,45 which can be influenced by thoracic pressure in spontaneous breathing animals.46 Many respond to ventricular contraction with a pulsatile discharge (Figure 13.1), whereas others have a discharge that is totally random.34 Because of the wide variability in the manner in which the ventricular afferents respond to chamber distension, it is difficult to categorize these mechanosensitive afferents as a class. However, in general, most appear to be much more sensitive to increases in diastolic pressure than systolic pressure, even though they generally discharge during systole.45

Increases in inotropic state induced by electrical stimulation of the efferent cardiac sympathetic nerves or administration of sympathomimetic drugs such as isoproterenol increase discharge of many ventricular C-fiber afferents.35,36,47 Conversely, a reduction in inotropic state with beta adrenergic antagonists decreases afferent activity.47 However, changes in ventricular contractility are usually accompanied by changes in diastolic and systolic pressure that make it difficult to sort out an inotropic influence.

The reflex effects of these cardiac mechano- or “volume” receptors are well described in several excellent review articles, and will not be detailed here.5,16,48–50 There are notable differences in reflex effects evoked from mechanoreceptors in the atria vs. the ventricles, and the functional significance of the ventricular
mechanoreceptors has recently been called into question.\textsuperscript{50} In general, however, cardiac mechanoreceptors serve to maintain normal pressures in the cardiac chambers via regulation of blood volume, cardiac output, and arterial pressure.

Paradoxically, a small proportion of ventricular afferents that respond to increased ventricular pressures are also stimulated by a reduction in ventricular filling (to decrease diastolic and systolic pressure) when combined with a high inotropic state.\textsuperscript{51} Atrial mechanoreceptors have not been reported to exhibit this behavior. It had been widely speculated for some time that these ventricular afferents contribute to the vasovagal reaction (profound hypotension, and bradycardia) in response to hypovolemia. The electrophysiological evidence is scant, and requires further investigation. Even the original report by Thoren concedes that only a small population of the recorded left ventricular mechanoreceptors (< 20%) were capable of being stimulated by decreased cardiac filling, and then only in the presence of a large increase in inotropic state of the ventricle.\textsuperscript{51}

13.3.5 CELLULAR MECHANISMS OF MECHANOTRANSDUCTION IN CARDIAC VAGAL AFFERENTS

The mechanism(s) responsible for mechanotransduction in stretch-sensitive afferent endings from the heart remain largely undefined. Some evidence has been gained from studying aortic baroreceptor neurons that may parallel that in cardiac afferents, particularly those of coronary baroreceptors, which resemble aortic baroreceptors in afferent activity. Mechanical deformation of nodose cell bodies from aortic afferents activates Ca\textsuperscript{++} influx through stretch activated ion channels (SAC) since the effect can be blocked by gadolinium (Gd\textsuperscript{+++}), a nonspecific SAC channel blocker.\textsuperscript{52} Single channel patch-clamp recordings verified that these nodose baroreceptor cells possess a Gd\textsuperscript{+++} sensitive, nonspecific cation channel that demonstrates opening events activated by negative pressure applied to the patch pipette.\textsuperscript{53} This channel may be linked to the degenerin/epithelial Na\textsuperscript{+} channel (DEG/ENaC) family of proteins that are known to function in mechanotransduction in nematodes.\textsuperscript{54} βENaC and γENaC proteins were found to be expressed in aortic baroreceptor neurons.\textsuperscript{54} And the DEG/ENaC channel blocker amiloride has been shown to block mechanically-induced depolarization of mechanosensitive nodose neurons but does not attenuate the action potential discharge evoked by current injection.\textsuperscript{55} This mechanically induced depolarization was not blocked by tetrodotoxin, a voltage-gated Na\textsuperscript{+} channel blocker.

The role of ENaC in the mechanosensitivity of cardiac afferents is uncertain. In a recent study, epicardial application of benzamil (an amiloride analogue) attenuated the reflex renal sympatho-inhibition to blood volume expansion.\textsuperscript{56} In isolated cardiac neuron, however, neither benzamil nor amiloride affected the Gd\textsuperscript{+++}-sensitive depolarizing current evoked by hypoosmotic stretch of the nodose neurons.\textsuperscript{56} This study also showed that benzamil impaired C-fiber conduction by an unknown mechanism, which could explain the discrepancy between the reflex and patch-clamp results. Thus, the role of ENaC in exclusively mediating mechanosensory transduction in cardiac vagal afferent endings is suspect.
13.3.6 CHEMOSENSITIVE CARDIAC VAGAL AFFERENTS

In general, vagal A-fiber mechanoreceptors from the atria and ventricles do not appear to exhibit overt responsiveness to endogenous chemical mediators. Although they can be stimulated by agents that influence neuronal ion channels such as veratrum alkaloids (an activator of voltage gated Na⁺ channels) and tetraethylammonium chloride (TEA, an inhibitor of voltage gated K⁺ channels), they are refractory to capsaicin and PBG, substances used to stimulate chemosensitive C-fiber sensory afferents.

Although these mechanosensitive A-fiber afferents are not stimulated outright by chemical mediators, the possibility that endogenous mediators play an autocrine or paracrine role in altering their pressure sensitivity is deserving of further exploration. Parallel studies on aortic and carotid sinus baroreceptors have indicated that a number of circulating hormones and locally produced factors modulate baroreceptor activity. Prostacyclin enhances baroreceptor sensitivity, an effect linked to inhibition of Ca⁺⁺ activated K⁺ currents in these neurons. Nitric oxide decreases baroreceptor pressure sensitivity through a cGMP-independent inhibition of voltage-gated Na⁺ channels. The mechanism is thought to involve nitrosylation of the channel proteins. Oxygen radicals and a platelet-derived factor are also known to impair baroreceptor sensitivity through mechanisms that remain to be elucidated.

By contrast to the A-fiber afferents, one or more of a variety of chemical mediators overtly stimulates many if not most of the C-fiber vagal afferents from the heart. One may even wonder whether the number of different compounds found to stimulate these afferents is limited only by the number of investigations. Examples of these compounds include bradykinin, cyclooxygenase products, H⁺ ion, adenosine, histamine, serotonin, substance P, angiotensin II, reactive oxygen species, thromboxane, and nitric oxide.

In general, most of the endogenous compounds found to activate cardiac vagal afferent endings are capable of being produced within cardiac tissue and are consistent with a function to signal stress (e.g., ischemia) or trauma to the myocardium. Stimulation of chemically sensitive endings in the heart evokes the depressor Bezold-Jarisch reflex (a.k.a. coronary chemoreflex) characterized by bradycardia, hypotension, and active cholinergic coronary vasodilatation. The functional importance of this reflex is thought to be protective to the ischemic myocardium. Thus, the reflex bradycardia, decreased contractility, and peripheral vasodilatation decrease the work of the heart and reduce oxygen demand of the compromised myocardium. Furthermore, the cholinergic-mediated increase in coronary blood flow would increase oxygen delivery.

These chemically sensitive afferents generally exhibit sparse and irregular activity or no activity at rest. Many show little or nor responsiveness to large changes in cardiac distension, whereas others could be considered mechanoreceptors. They generally respond to chemicals with rapid bursts of activity that can vary in duration from a few seconds with capsaicin (Figure 13.1) to many minutes with bradykinin. Considerable variability also exists from fiber to fiber in their
relative sensitivity to various chemical mediators. In fact, occasionally afferent fibers can be found that are inhibited by a chemical mediator. These differences exemplify the extensive heterogeneity of sensory modalities of these C-fiber afferents thatfraughts their classification.

Capsaicin has been used in studies as a tool to identify chemically sensitive C- (and Aδ-) fiber endings in the heart. Capsaicin is a xenobiotic ligand for the vanilloid type 1 receptor (VR1 or TRPV1), a TRP (transient receptor potential) nonspecific cation channel with a high permeability to Ca++. Using immunohistochemical labeling, TRPV1 can be seen in an intricate network of thin fibers in the rat myocardium, primarily near the epicardial surface and more sparsely within the myocardium. No TRPV1 staining could be seen in the endocardium or atrial wall. The study, however, did not differentiate between vagal and sympathetic afferent pathways.

The endogenous ligand for the cardiac TRPV1 receptor is not known. Recent studies have shown that several products of lipoxygenases and epoxygenases can directly activate the TRPV channels. In this regard, Sun, Wang, and Schultz have shown that the lipoxygenase and epoxygenase pathways contribute to activation of cardiac vagal afferents during ischemia. Capsaicin-sensitive endings in the heart also respond to a variety of other mediators including bradykinin, cyclooxygenase products, and oxygen radicals. Schultz and Ustinova have shown oxygen reactive species stimulate cardiac chemosensitive vagal afferents via TRPV1, which may play an important role in activation of these afferents endings during myocardial ischemia and reperfusion (see below). Recently, the endocannabinoid anandamide has been proposed to function as an endogenous agonist of the TRPV1 receptor, but its role in cardiac afferent function has not been explored.

Vanilloid-sensitive nerves have two distinct subdivisions, a peptidergic population, characterized by the coexpression of TRPV1 with neuropeptides in their sensory terminals, and a purinergic one, coexpressing the adenosine triphosphate (ATP)-gated ion channel P2X₃. Activation of TRPV1 triggers vesicular release of neuropeptides from the peptidergic sensory terminals. Nerve endings containing CGRP and SP are found throughout the cardiac tissue as discussed earlier. These neuropeptides evoke potent coronary vasodilation and decrease cardiac contractility and may serve a cardioprotective role to facilitate blood flow and energy conservation. Indeed, we have shown that capsaicin-sensitive nerve endings in the heart liberate neuropeptides during ischemia and thereby facilitate postischemic recovery of the heart.

The serotonin analogue, phenylbiguanide (PBG), is also used to stimulate C-fiber cardiac vagal afferents in many species. This effect is mediated through 5-HT₃ receptors. Serotonin release from platelets recently has been shown to play a major contribution to activation of cardiac vagal afferents neurons in response to thromboxane. Another recent study has indicated that in the rat heart, a majority (75%) of PBG sensitive C-fiber afferents in the heart also were activated by modest increases in left ventricular end diastolic pressure (LVEDP) of 4-8 mm Hg, and thus could be classified as bimodal. This population of afferents contrasts with the population of capsaicin–sensitive C-fiber endings in the rat heart that were shown to be largely unresponsive to even large changes in LVEDP. Thus it would appear
that the 5-HT₃ receptor is more closely linked to mechanoreceptor function in these cardiac afferents than the TRPV₁ receptor.

It can be shown that various endogenous chemical mediators released from or delivered to cardiac tissue can act synergistically or antagonistically to alter the sensitivity of cardiac vagal C-fiber afferents. For example, prostaglandins and adenosine enhance afferent C-fiber responsiveness to bradykinin. On the other hand, 5-HT₃ receptor activation inhibits conductance in stretch-activated ion channels in mechansensitive cardiac nodose neurons and impairs reflex responses to volume expansion. Thus, it appears that function of these chemosensitive and mechanosensitive C-fiber afferents in various physiological or pathological situations is governed by a balance of chemical and mechanical signaling events. As yet, we have only a rudimentary understanding of these complex interactions at the cellular level.

### 13.3.7 Cellular Mechanisms of Chemotransduction in Cardiac Vagal Afferents

The signal-transduction pathways in cardiac chemosensitive neurons and their modes of interaction have not yet been delineated at the cellular and molecular (ion channel) level. A recent study by Thompson et al. exemplifies the potential difficulties that lie ahead in these types of studies. When these investigators recorded from cardiac vagal neurons in situ, they found that local application of adenosine to the terminal in the heart evoked activation of the neurons. By contrast, when the cell bodies in the nodose ganglion were excised and studied in vitro, adenosine reduced the excitability of these cardiac neurons. These results illustrate that signaling mechanisms can be quite different between the sensory terminal and the cell body, particularly with regard to chemical mediators.

Voltage-gated K⁺ currents are likely to play a role in regulating the excitability of chemosensitive cardiac vagal afferents. BaCl₂ and TEA, nonspecific voltage sensitive K⁺ channel blockers, applied to the receptive fields in cardiac tissue were shown to activate these vagal afferents. Kv1.1, Kv1.2, and Kv1.6 PCR products have been identified in rat nodose ganglion cells. In current-clamp experiments of these nodose cell bodies, α-dendrotoxin, a specific toxin for the KV₁ family, lowered the threshold for initiation of discharge in response to depolarizing current steps, reduced spike after-hyperpolarization, and increased the frequency pattern of discharge of A- and C-fiber type neurons. These effects were greater in C-fiber neurons.

A- and C-fiber neurons in the nodose ganglion can be differentiated on the basis of their Na⁺ currents. A-fiber neurons express a single rapidly inactivating tetrodotoxin (TTX)-sensitive Na⁺ current, whereas C-fiber neurons coexpress this TTX-sensitive current and a slowly inactivating TTX-resistant Na⁺ current. Using this criteria, Doan and Kunze have observed that both A- and C-fiber type nodose neurons from neonatal rat exhibit a hyperpolarizing activated cation (Na⁺) current (I₉), which contributes an inward current at the resting membrane potential. The magnitude of this I₉ in A-fiber neurons is substantially larger than that in C-fiber neurons. An inward rectifier K⁺ current (Iₖᵣ⁺), which can contribute to setting the resting membrane potential in neurons, could not be identified in these neurons. Thus modulation of
Cardiac Vagal Afferent Nerves

IH may play an important role in regulating the resting membrane potential and excitability of cardiac afferent neurons, particularly A-fiber neurons, which in the heart are primarily mechanoreceptors. It would be of great interest to learn whether chemical modulation of pressure sensitivity in these neurons could be mediated through this channel.

Nodose C-fiber neurons sensitive to bradykinin are known to exhibit a slow postspike hyperpolarization that appears to be mediated by Ca\(^{++}\) dependent K\(^{+}\) channels.\(^{88}\) This slow after-hyperpolarization dramatically reduces the excitability of the neuron to depolarizing stimuli. The slow after-hyperpolarization is found in about a third of rabbit nodose C-fiber neurons, is carried by a small conductance K\(^{+}\) channel activated by increases in intracellular [Ca\(^{++}\)], is voltage independent, and is inhibited by increases in intracellular [cAMP].\(^{89}\) Ca\(^{++}\) influx through N type Ca\(^{++}\) channels to activate Ca\(^{++}\) release from the endoplasmic reticulum selectively elicits the slow after hyperpolarization current.\(^{90}\)

Undem and Weinreich have shown that a number of mediators including serotonin, histamine, several prostanoids, leukotrienes, and bradykinin, inhibit after-hyperpolarization in nodose C-fiber afferents and increase their excitability.\(^{91}\) In addition, activation of bradykinin receptors was shown to increase production of PGI\(_2\) in the nodose ganglion, which in turn inhibited the slow after-hyperpolarization to increase excitability.\(^{92}\) The signaling pathway for these events remains to be determined.

Middlekauff et al. have shown that adenosine also inhibits slow after-hyperpolarization in C-fiber vagal afferent neurons from rabbits.\(^{93}\) The effect was shown to be mediated by the A\(_{2}\) receptor, which stimulates cAMP production. Thus, in rabbits, A\(_{2}\) receptor mediated activation of cAMP attenuates slow after-hyperpolarizations and enables C-fiber nodose neurons to fire rapidly and repeatedly in the presence of other excitatory stimulants. In other studies in rat cardiac nodose neurons, these investigators demonstrated that adenosine inhibited the Ca\(^{++}\) current through N type Ca\(^{++}\) channels via A\(_{1}\) receptors.\(^{94}\) Thus in rats, A\(_{1}\) receptor-mediated inhibition of Ca\(^{++}\) influx attenuates the slow after-hyperpolarization current to increase excitability of cardiac C-fiber afferents.

It is important to note that many of these studies did not differentiate nodose neurons by type or location of their sensory terminals. Nevertheless, their results may provide important clues to sensory function in cardiac vagal afferents. These studies also emphasize the growing evidence that a number of different mechanisms exist that may influence the excitability of cardiac vagal afferent neurons. Much more work is needed in these areas to provide insight into the chemotransduction process in these cardiac vagal afferents.

13.4 CARDIAC VAGAL AFFERENT FUNCTION IN PATHOPHYSIOLOGICAL STATES

13.4.1 MYOCARDIAL ISCHEMIA AND REPERFUSION

Myocardial ischemia is a potent stimulus to excitation of cardiac vagal sensory endings.\(^{95-98}\) Temporary occlusion of the coronary arteries can be shown to stimulate
both mechanically and chemically sensitive cardiac vagal afferents. The response of the mechanosensitive afferent fibers probably results from wall motion abnormality and increased filling pressures in the cardiac chambers due to impairment of cardiac pump function and arrhythmias. Activation of cardiac vagal mechanoreceptors is reported to be modest in comparison to that of chemosensitive endings during ischemia.

A number of chemical mediators are known to be released from ischemic cardiac tissue and have been shown to contribute to activation of chemosensitive vagal endings in the heart. These mediators include (not exclusively) bradykinin, prostaglandins, reactive oxygen species, adenosine, substance P, K+, and H+ ions. The relative importance of these various chemical mediators is unclear. It is likely that the contribution of various mediators is related to the time course and extent of ischemia and location of the afferent ending in relation to the ischemic zone. Ustinova and Schultz found that arachidonic acid metabolites contribute to activation of chemosensitive vagal afferent endings in the rat heart at the onset of ischemia and that oxygen radical production exerts a secondary effect after more prolonged ischemia. They also observed that cyclooxygenase-mediated activation was localized to afferent endings within the ischemic zone, whereas oxygen radical activation was more widespread to endings in and surrounding the ischemic area. In other studies, Thompson et al found that cardiac vagal afferent responses to coronary occlusion could be blocked by a P1-purinergic receptor antagonist in the pig heart. Others propose that serotonergic pathways via thromboxane production and platelet activation may also be important in stimulating cardiac vagal afferents via during ischemia.

A recent study has shown that cardiac afferent cell bodies in the nodose express acid-evoked currents (ASICs), but the magnitude of this current was about 10-fold smaller than that of cardiac sympathetic afferents. On the other hand the nodose neurons exhibited a larger ATP-evoked current than that in sympathetic afferent neurons.

Presently, there is little consensus on the hierarchy of chemical mediators responsible for activation of chemosensitive cardiac vagal afferents during ischemia, an issue confounded by the diverse heterogeneity of these afferent endings and possible species differences. It is also likely that cardiac vagal afferent responsiveness during ischemia results from a synergistic interplay among several mediators.

Much less attention has been given to afferent responses during reperfusion of the ischemic myocardium. Clinical studies have shown that reperfusion of the ischemic myocardium by thrombolysis in patients with acute myocardial infarction is associated with bradycardia and hypotension. That these cardio-depressor effects during reperfusion are also the result of stimulation of sympathetic-inhibitory reflexes mediated by these vagal afferents from the left ventricle can be supported by afferent recordings. Chemosensitive afferent endings were shown to be stimulated briskly at the onset of reperfusion of the ischemic rat ventricle. Nerve endings inside and outside of the ischemic zone were stimulated, but those within the ischemic zone were most prominently affected. The reperfusion stimulation was prevented by antioxidants, deferoxamine, or dimethylthiourea, and thus likely to be mediated by hydroxyl radicals. The ability of oxygen radicals to stimulate cardiac vagal afferent endings was mediated through the TRPV1 receptor.
Although functional denervation of regions within and distal to an infarct does occur following chronic transmural myocardial infarction, the functional integrity of afferent fibers is unaffected for up to 12 hours following the onset of an acute transmural infarct. Even following global ischemia with ventricular fibrillation, compound action potentials can be generated in C-fibers for up to 2 hrs, A\(\delta\)-fibers for up to 4 hrs, and A-fibers for at least 12 hours. Thus afferent endings are capable of signaling events within an ischemic area of the heart for several hours even under anoxic conditions.

### 13.4.2 Heart Failure

Clinical and animal studies confirm that, in general, cardiovascular mechanoreflexes, including arterial, atrial, and ventricular mechanoreflexes, are significantly blunted in congestive heart failure (HF). Although the mechanisms responsible for mechanoreflex abnormalities in HF are not completely understood, it is clear that mechanoreceptor endings themselves exhibit a depressed sensitivity to changes in pressure. Studies by Gilmore and colleagues have shown that the reduced sensitivity of atrial mechanoreceptors in dogs with HF is accompanied by a decrease in compliance of the atrial wall and morphological changes in the receptor endings. In addition to cardiac remodeling, changes may also occur in the cellular processes within the nerve endings themselves. For example, the reduced pressure sensitivity in arterial baroreceptors during HF is mediated by an increase in Na\(^+/\)K\(^+\) ATPase activity in the baroreceptor neurons leading to membrane hyperpolarization and enhanced postexcitatory depression of baroreceptor activity. This effect can be reversed by digitalis glycosides to inhibit Na\(^+/\)K\(^+\) ATPase activity. Indeed, the cardiac glycoside ouabain has been shown to increase the pressure sensitivity of left atrial stretch receptors in dogs.

Unlike the mechanoreflexes, evidence suggests that the cardiac vagal chemoreflex is potentiated in dogs with HF. We examined the discharge characteristics of chemosensitive endings in the left ventricle in dogs after chronic ventricular pacing as a model of low-output HF. We found that resting discharge of cardiac vagal fibers in HF dogs was normal. However, the afferent response to bradykinin was significantly enhanced in HF dogs. The B\(_2\) receptor antagonist HOE-140 inhibited this effect. This enhanced sensitization of the cardiac vagal chemosensitive afferents appeared to be receptor-specific because there was no enhancement of the afferent response to capsaicin.

Increased levels of cyclooxygenase products appears to mediate sensitization of the cardiac chemosensitive endings to bradykinin in HF. Prostaglandin production is known to be elevated in cardiac tissue during HF. This effect is supported by known interactions of prostaglandins to potentiate both afferent and reflex responses evoked by stimulation of cardiac vagal endings with bradykinin.

The natriuretic peptides and vasopressin are elevated in HF and may also play a role in sensitization of cardiac vagal afferents. Vasopressin has been shown to enhance the pressure sensitivity of both C- and A-fiber left ventricular vagal afferents. The influence of ANP and BNP of cardiac vagal afferents is less clear. ANP and BNP enhance the reflex bradycardia in response to activation of both cardiac
vagal mechano- and chemoreceptor endings.\textsuperscript{114,115} Furthermore, the sympatho-inhibitory effect of ANP is preserved in HF rats.\textsuperscript{116}

Although it is known that the reflex effects of ANP are dependent on afferent input from vagal C-fibers, the locus of action of ANP (and BNP) in this reflex arc remain unknown. It is generally assumed that ANP/BNP stimulate cardiac vagal C-fiber afferents, but no electrophysiological evidence has been reported to substantiate this claim. On the contrary, a study by Deliva and Ackerman indicates that ANP confined to the heart from the within the pericardial sac was not capable of eliciting a sympathoinhibitory response.\textsuperscript{117} Our own observations also have suggested that neither intravascular nor topical application of ANP to the heart are capable of overt activation of cardiac vagal C-fiber afferents.\textsuperscript{116} However, it may be possible that these natriuretic peptides enhance the excitability of these cardiac afferents to other chemical and mechanical stimuli that must occur concomitantly to reveal activation of the afferents. In this regard, Deliva and Ackerman provide evidence that the reflex effects of ANP are abolished by 5-HT3 receptor antagonists.\textsuperscript{117} Nevertheless, without direct electroneurographic evidence, one must use caution in the interpretation of the reflex studies, since the natriuretic peptides could influence autonomic function via central effects and effects on efferent ganglionic neurotransmission.\textsuperscript{118}

Undoubtedly, many yet unexplored factors also may contribute to altered cardiac vagal chemosensory function in HF in addition to prostaglandins and vasoactive peptides. The role of oxidative stress and other metabolic changes in HF is of obvious concern that awaits future exploration. Of these, one important factor that must be taken into consideration is the influence of ventricular distension on cardiac chemosensory function. In a recent study, Wang and co-workers have found that acute volume expansion enhances cardiac sympathetic afferent reflex responses to epicardial application of bradykinin.\textsuperscript{119} The mechanism of this interaction between wall stress and chemosensory function in the heart is not yet clear, but may be related to increased O\textsubscript{2} demand in response to the heightened workload.

### 13.4.3 Hypertension

Abnormalities in cardiac baroreflex function in hypertension are well documented, but there is considerable variability in the degree and direction of these changes.\textsuperscript{120–125} While there is general agreement that the arterial baroreflex is impaired and/or reset to higher pressures in most forms of hypertension, the reflex function of cardiac mechanoreceptors in hypertensive animals is variable. This variability is likely to be dependent upon the type and duration of hypertension, the species, and other confounding variables such as the degree of cardiac hypertrophy/remodeling and humoral/hormonal factors. In addition, there is very little direct electrophysiological data from cardiac vagal afferents in hypertensive animals to support results from reflex studies.

In renal hypertensive dogs and spontaneously hypertensive rats, there is a resetting of atrial receptors to a higher pressure threshold.\textsuperscript{126,127} Yet, in the rat study, Thoren and colleagues found that the reflex renal sympathetic nerve responses to volume expansion were augmented despite resetting of atrial receptors.\textsuperscript{128} This discrepancy between afferent and reflex function was reconciled when they observed
that the compliance of the left atrium was decreased in the hypertensive rats, thus raising resting atrial pressure, and enhancing the pressure changes in response to any given volume load. Studies in humans tend to support these findings since unloading of cardiac baroreceptors with lower-body negative pressure evokes preserved or augmented reflex responses in hypertensive patients. Yet, other studies have shown that impairment of reflex function from the heart is closely correlated with myocardial remodeling and cardiac hypertrophy secondary to hypertension. Studies in humans tend to support these findings since unloading of cardiac baroreceptors with lower-body negative pressure evokes preserved or augmented reflex responses in hypertensive patients. Yet, other studies have shown that impairment of reflex function from the heart is closely correlated with myocardial remodeling and cardiac hypertrophy secondary to hypertension. Sodium balance may also play an important role in influencing the sensitivity of cardiac afferents in hypertensive states. Findings suggest that a high-salt diet sensitizes cardiac vagal reflexes to volume expansion in Dahl rats resistant to salt-induced hypertension, but impairs reflex responses to volume expansion in the salt sensitive Dahl rats. The mechanism of this salt-induced plasticity in cardiac mechanoreceptor function is not known. The effects of hypertension on cardiac vagal chemosensitive endings are less well defined. The reflex sympatho-inhibition in response to stimulation of chemosensitive ventricular vagal afferents with epicardial nicotine was markedly impaired in renal hypertensive rabbits. Similarly the reflex sympatho-inhibition to prolonged 5-HT₃ receptor activation with PBG was depressed in DOCA-salt hypertensive rats, but the reflex evoked by brief bolus injection of the drug was normal in the hypertensive animals. In addition, the ability of ANP to enhance the cardiac reflex effects of 5-HT is not present in hypertensive rats with cardiac hypertrophy. Such studies, however, do not allow one to differentiate afferent function from possible central alterations in the control of sympathetic outflow in hypertension. Unfortunately, electroneurographic recordings from chemosensitive vagal afferents from the heart in hypertensive animals have not been reported.

13.4.4 Diabetes Mellitus

Autonomic neuropathies in diabetes mellitus contribute to the development of silent myocardial ischemia, hypertension, and cardiac arrhythmias that place the patient at risk of sudden cardiac death. Consistent with these clinical signs of generalized peripheral autonomic neuropathy, it is likely that the function of cardiac afferent endings is depressed in diabetes. Studies have shown that the reflex sympatho-inhibitory response to a volume load to the heart are impaired in STZ-induced diabetic rats, a model of type I diabetes and in obese Zucker rats, a model of type II diabetes. It is not yet known whether this impairment of the cardiopulmonary baroreflex originates from abnormal mechanoreceptor function in cardiac vagal afferent neurons or elsewhere in the reflex arc. However, the diabetic rats have less compliant atrial tissue, which may reduce stimulation of the volume receptors to volume load. The possibility of altered atrial mechanoreceptor sensitivity in diabetes needs to be assessed. In regard to cardiac chemoreceptor function, we have shown that the Bezold-Jarisch reflex response to stimulation of cardiac chemosensitive endings is significantly blunted in STZ-induced diabetic rats. Diabetic rats with insulin replacement do not exhibit impaired cardiac chemoreflexes. In afferent studies, cardiac vagal
afferent C-fiber activity in response to epicardial capsaicin and bradykinin was significantly blunted in diabetic rats. Chronic vitamin E treatment effectively prevented these cardiac afferent and reflex defects in diabetic rats without altering resting blood glucose or hemodynamics. These results indicate that the cardiac vagal chemoreflex is markedly depressed in STZ-induced diabetic rats and that oxidative stress is the major underlying mechanism causing impairment of the chemosensitive and reflex properties of cardiac sensory nerve endings in this condition.

In patch-clamp experiments, we found that peak current densities for both TTX-r and TTX-s I\textsubscript{Na} were suppressed in cardiac nodose chemosensitive neurons from diabetic rats, but TTX-r I\textsubscript{Na} was suppressed to a greater degree than TTX-s I\textsubscript{Na}. Extracellular superoxide dismutase or tiron acutely enhanced TTX-r I\textsubscript{Na} but not TTX-s I\textsubscript{Na} in the diabetic neurons. We further showed that TTX-r Na\textsubscript{v1.8} (slow) but not TTX-r Na\textsubscript{v1.9} (persistent) was depressed in the diabetic neurons, and that superoxide anion radicals generated by xanthine/xanthine oxidase significantly reduce Na\textsubscript{v1.8} TTX-r I\textsubscript{Na} but not Na\textsubscript{v1.9} TTX-r I\textsubscript{Na} in normal cardiac vagal neuron. These studies indicate that superoxide-mediated impairment of Na\textsubscript{v1.8} TTX-r I\textsubscript{Na} in cardiac chemosensitive vagal neurons contributes to the impaired responsiveness of these sensory afferents in the diabetic state. These observations also illustrate that Na\textsubscript{v1.8} TTX-r channels are redox sensitive, which may influence cardiac C-fiber function in other conditions in which oxidative stress occurs.

13.5 SUMMARY

The heart is innervated by an array of sensory nerve endings that project impulses to the central nervous system via the vagus nerves. Mechanosensitive endings encode filling volume of the atria and ventricles, heart rate, and force of muscle contraction, and function in the ongoing regulation of blood volume, cardiac output, and arterial pressure. Chemically sensitive endings respond to a variety of endogenous chemical mediators, and appear to function to protect myocardial tissue during periods of stress. However, we must be careful not to become too complacent with these simple and straight-forward definitions of purpose. The sensory characteristics of all afferent endings in the heart represent a complex interaction of their mechanical and chemical environment that may have profound consequences on reflex function for any given physiological or pathophysiological condition.

Recordings of impulses from “single fibers” in the vagus arising from the heart have provided important information about the manner in which these afferents encode their cardiac environment in a variety of conditions. This chapter highlights the abundance of existing knowledge derived from such recordings, but it is also clear that we are far from having a complete picture of the wide spectrum of types of sensory endings that innervate the heart and their responses to any number of factors, particularly in pathophysiological states such as heart failure, hypertension, and other conditions that affect cardiac performance. The need for continuing to pursue these types of studies remains strong.

In order to more fully understand the signal transduction process that occurs in these cardiac sensory endings, it will be necessary to define the cellular processes that govern the excitability of the receptor membrane. Recent attempts to record the
membrane properties of isolated cell bodies from the nodose ganglion have revealed characteristics of Na+, K+, and Ca++ channels that may be functionally important at the sensory receptor level. At present, however, little is known about ion channel function in nodose neurons that innervate the heart. The challenge will be to dedicate more of these types of studies to cardiac sensory neurons, to find ways to better correlate the functional properties of ion channels in the cell body to those at the sensory terminal, and, ultimately, to analyze the sensory terminal itself.

REFERENCES

Advances in Vagal Afferent Neurobiology


Part VI

Vagal Reflexes and Sensation
14 Vagal Afferent Nerve Stimulated Reflexes in the GI Tract

Jyoti N. Sengupta and Reza Shaker

CONTENTS

14.1 Introduction ..................................................................................................380
14.2 Esophageal and Supra-Esophageal Reflexes ..................................................380
  14.2.1 Esophago-UES Contractile Reflex (EUCR) ........................................381
  14.2.2 Pharyngo-UES Contractile Reflex (PUCR) ......................................382
  14.2.3 Laryngo-UES Contractile Reflex (LUCR) .......................................384
  14.2.4 Esophago-Glottal Closure Reflex (EGCR) .......................................384
  14.2.5 Pharyngo-Glottal Closure Reflex (PGCR) .......................................385
  14.2.6 Pharyngo-Esophageal Inhibitory Reflex (PEIR) .............................386
  14.2.7 Pharyngeal Swallows (PS) ................................................................387
  14.2.8 Pharyngo-LES Reflex Relaxation (PLESR) ....................................388
  14.2.9 Laryngo-LES Reflex Relaxation (LLESR) ......................................388
  14.2.10 Esophago-LES Reflex Relaxation (ELESR) .................................388
  14.2.11 Laryngo-Gastric Reflex Relaxation (LGRR) .................................389
  14.2.12 Pharyngo-Gastric Reflex Relaxation (PGRR) ...............................389
  14.2.13 Esophago-Gastric Reflex Relaxation (EGRR) ...............................389

14.3 Gastric Reflexes ...........................................................................................390
  14.3.1 Gastro-LES Reflex Relaxation (GLRR)/Transient LES Relaxation (TLESR) ..............................................................390
  14.3.2 Gastro-Gastric Reflex .................................................................391
  14.3.3 Gastro-Pyloric Reflex Relaxation (GPRR) ....................................391
  14.3.4 Gastro-Duodenal Reflex (GDR) ....................................................391
  14.3.5 Gastro-Colonic Reflex (GCR) ......................................................392

14.4 Intestinal Reflexes .......................................................................................392
  14.4.1 Duodeno-Gastric Reflex Relaxation (DGRR) ..................................392

14.5 Conclusion ....................................................................................................393

References ..............................................................................................................393
14.1 INTRODUCTION

The thoracic and abdominal viscera are dually innervated by vagal and spinal primary sensory afferent fibers. It is generally thought that vagal afferent fibers transmit nonpainful sensations to the central nervous system, whereas the spinal afferent fibers are mostly involved in nociceptive transmission. Although vagal primary afferent fibers elicit sensory perceptions, major functions of these afferent fibers are to serve as afferent limbs of several regulatory reflexes including viscero-visceral, viscero-vascular, and broncho-pulmonary reflexes. The proportion of primary sensory afferent fibers in the vagus constitutes approximately 80 to 90% of the total number of axons\textsuperscript{7,29,38,41,58,62,84} that extensively innervate the thoracic and abdominal viscera. In the gastrointestinal (GI) tract, afferent innervations spanning from the pharynx to transverse colon\textsuperscript{14,32} give rise to several vago-vagal reflexes to regulate airway protection, deglutition, gastric accommodation, gastric emptying, humoral secretion, nausea, vomiting and satiety. These reflexes function in orthograde as well as in anterograde manner.

The majority of vagal sensory afferent fibers are pseudounipolar cells with their cell bodies located in the nodose and jugular ganglia. In the cat, the majority of the large diameter afferent fibers in the vagus nerve are cardiovascular and pulmonary, whereas GI afferent fibers show a differential pattern of distribution of myelinated and unmyelinated fibers along the GI tract of the cat.\textsuperscript{83} The proportion of unmyelinated fibers increases from the oral to aboral end of the GI tract. A similar distribution pattern was also documented in the ferret\textsuperscript{7} and in the rat.\textsuperscript{104} For the majority of vagal reflexes processing occurs in the nucleus tractus solitarius (NTS) in the brainstem. The second-order neurons of NTS richly project to the dorsal motor nucleus of vagus (DMN) and to nucleus ambiguas (NA) to influence the excitatory and inhibitory preganglionic motoneurons. Several neuropeptides including glutamate (excitatory), thyrotropin-releasing hormone (excitatory), neuropeptide Y (excitatory), norepinephrine (inhibitory), and γ-aminobutyric acid (inhibitory) are involved in complex reflex functions.

14.2 ESOPHAGEAL AND SUPRA-ESOPHAGEAL REFLEXES

There are several vagally mediated reflexes that are triggered by mechanical and chemical stimulation of the pharynx, larynx, and esophagus. These reflexes are believed to protect the esophagus and supra-esophageal areas (i.e., pharynx and larynx) against the entry of gastric contents by enhancing the upper esophageal sphincter (UES) pressure, closing the vocal cord and introitus to the trachea, clearing the contents from the pharynx and esophagus. In addition to these stimulatory reflexes, mechanical stimulation of the larynx, pharynx, and esophagus can induce relaxation of the lower esophageal sphincter and proximal stomach (fundus and corpus).

The former group of reflexes includes:

- Esophago-UES contractile reflex (EUCR)
- Pharyngo-UES contractile reflex (PUCR)
• Laryngo-UES contractile reflex (LUCR)
• Esophago-glottal closure reflex (EGCR)
• Pharyngo-glottal closure reflex (PGCR)
• Secondary esophageal peristalsis
• Pharyngeal swallow (PS)

The latter group of reflexes include:

• Pharyngo-esophageal inhibitory reflex (PEIR)
• Pharyngo-LES relaxation reflex (PLESR)
• Laryngo-LES relaxation reflex (LLESR)
• Esophago-LES relaxation (ELESR)
• Esophago-UES relaxation reflex
• Laryngo-gastric reflex relaxation (LGRR)
• Pharyngo-gastric reflex relaxation (PGRR)
• Esophago-gastric reflex relaxation (EGRR)

14.2.1 ESOPHAGO-UES CONTRACTILE REFLEX (EUCR)

The UES is the barrier that protects against the entry of gastric contents into the aerodigestive tract by maintaining a high pressure between the esophagus and pharynx. At rest, the high pressure of UES is generally maintained by the tonic contraction of the cricopharyngeus (CP) muscle. This tonic contraction is abolished during dynamic conditions such as swallowing, belching, and vomiting. The cervical esophagus and inferior pharyngeal constrictor muscles contribute to the closing of the UES. The degree of UES pressure is quite variable during sleep and is significantly lower in the elderly compared with young individuals. In human volunteers, UES pressure increases significantly during esophageal distension (ED), instillation of water into the esophagus and gastroesophageal reflux events. This has also been documented in experimental animals by recording electromyographic (EMG) response of the CP muscle to esophageal stimulation. It has been shown that EUCR is primarily an extrinsic vagally mediated reflex and afferent limb of the reflex lies in the vagus nerve. Bilateral cold block of the cervical vagus or transection of the thoracic vagus nerve abolishes the reflex. Recent studies have also documented that cervical transection between CP and the upper esophagus does not abolish ED-induced EUCR, suggesting that intrinsic neural plexus of the esophagus does not contribute to this contractile reflex. Interestingly, EUCR is more reproducibly activated by localized distension of the proximal than distal esophagus. Distension of the esophagus by air on the other hand, results in relaxation of the UES in the proximal esophagus and contraction in the distal esophagus in the majority of trials. Intraesophageal instillation of local anesthetic (2% lidocaine) or removal of the mucosal layer does not affect the slow ED-induced EUCR suggesting that distension-sensitive muscle afferent fibers are involved in activation of this reflex.
14.2.2 PHARYNGO-UES CONTRACTILE REFLEX (PUCR),

**Figure 14.1**

Mechanical (touch and pressure) stimulation to the mucosa of the naso-, hypo-, and laryngo- pharynx of the cat results in the contraction of the CP muscle indicative of an increase in pressure of the UES.\(^82\) Similarly, in humans stimulation of pharynx with minute amounts of water increases the resting tone of the UES.\(^126\) Application of local anesthetics (2% lidocaine) on the mucosa of the pharynx blocks the contractile responses of CP muscle to pharyngeal stimulation, but not to its response to esophageal distension.\(^82\) This observation indicates the stimulation of PUCR by pharyngeal mechanical stimulation and involvement of pharyngeal mucosal mechanoreceptors in eliciting this reflex.

It is most likely that PUCR may assist in controlling the swallowing process or in the coordination of swallowing during respiration. It has been documented that during swallow the UES contracts briefly before the relaxation of the UES to pass the bolus down through the pharynx.\(^59,61\) The head of the bolus moves through the pharynx ahead of the oral tongue pumps, reaching the hypopharynx before the pharyngeal peristalsis begins.\(^27,34\) The bolus does not proceed and is not forced through the UES even with large bolus volume until the UES or CP muscle actively relaxes at the appropriate time during the swallow process. The PUCR may help to maintain a closed UES, thereby arresting the bolus momentarily until the peristaltic pressure wave reaches the hypopharynx. The PUCR before the UES relaxation possibly helps to transfer the bolus from the oral cavity to the esophagus in an orderly fashion. Disturbances of smooth transition of bolus from oral to pharyngeal phases of swallowing could cause dysphagia. PUCR may also help to prevent pharyngo-esophageal flux of air during inhalation. It is also possible that this contractile response of the UES may counteract the inhibitory deglutitive reflexes, thus preventing pharyngeal reflux of gastric and esophageal contents. This reflex may also be initiated during esophago-pharyngeal reflux events by contact of regurgitated materials with pharyngeal mucosa preventing continued reflux into the pharynx.

In the cat, transection of glossopharyngeal nerves (GPN) abolishes the pharyngeal stimulation-induced PUCR, but not ED-induced EUCR, suggesting that the afferent limb of PUCR is mediated by mechanosensitive sensory afferent fibers in the GPN.\(^82\) On the other hand, transection of hypo-nodal vagus has no effect on PUCR, but abolishes EUCR, indicating that recurrent laryngeal nerve (RLN) has no function in PUCR. Studies have shown that the threshold volume for triggering the PUCR is significantly larger in the elderly compared with the young.\(^112\) Similarly, in patients with laryngitis attributed to reflux disease the threshold volume for triggering the PUCR is significantly larger than normal control subjects.\(^137\) However, the post-triggered maximum increase in UES pressure is the same for both groups, indicating that the contractile properties of UES muscles and motoneurons innervating these muscles remain unaffected in laryngitis patients, but the afferent limb becomes less responsive. Smoking adversely affects stimulation of PUCR.\(^36\) The threshold volume for triggering the PUCR is significantly larger in smokers compared with nonsmokers.
FIGURE 14.1 Pharyngo-UES contractile reflex. Effect of pharyngeal water injection on upper esophageal sphincter (UES) resting pressure. (A) Rapid-pulse injection of 0.2 ml of room-temperature water resulted in augmentation of UES pressure from 55 to 137 mmHg. As seen, this augmentation persisted for 22 sec until it returned to baseline after a dry swallow (DS). (B) Slow continuous infusion of room-temperature water also resulted in an increase of preinfusion pressure of 80 to 200 mmHg measured 3 sec before a swallow occurred. Esop: esophageum, EMG: electromyogram. (From Am J Physiol 273 [Gastrointest Liver Physiol 36]:G854–G858, 1997. With permission.)
14.2.3 Laryngo-UES Contractile Reflex (LUCR)

Stimulation of the larynx induces a brief closure of the introitus to the trachea by adduction of the vocal cords and arytenoids; a vago-vagal laryngeal adductor reflex.\textsuperscript{140a} Since the UES and larynx are both innervated by the vagus, it is conceivable that stimulating the larynx may induce contraction of the UES. In humans, stimulation of the larynx by air stimulation such as an air-pulse with two seconds of duration applied to the interarytenoid area induces a significant increase in UES resting pressure,\textsuperscript{60} which persists until the subject swallows. The UES pressure change can be observed only when air pulse produces mucosal deflection. The afferent limb of LUCR is primarily laryngeal mechanosensitive afferent fibers in the internal branch of the superior laryngeal nerve (SLN) and the efferent limb via the vagus and RLN. Although the baseline UES pressure in the elderly is significantly lower than in the young, the ratio of pressure change to laryngeal stimulation is similar between the two groups. Thus, it suggests the absence of age-related alteration of vagal efferent fibers innervating the UES and CP muscle. However, the threshold for eliciting LUCR is significantly greater in elderly subjects compared with young individuals indicating a loss of sensory function.

14.2.4 Esophago-Glottal Closure Reflex (EGCR)

The basic functions of the glottis are protective, respiratory, and phonation. Although the UES contractile reflexes (EUCR, PUCR, and LUCR) are the first line of defense in preventing the gastric contents from entering the supraesophageal areas, a large volume of gastroesophageal reflux (GER) may overwhelm the UES pressure to cause a potential threat of aspiration. Under this condition closure of the glottis is a second line of defense for protection of the airway. The function of the EGCR is to induce contractions of the glottal adductor muscles, including the cricothyroid (CT), lateral cricoarytenoid (LCA), and interarytenoid (IA) muscles, in response to stimulation of the esophagus (Figure 14.2). Studies in humans and cats have shown that rapid ED, as well as generalized distension of the esophagus by air injection, produces EGCR.\textsuperscript{123,127} This ED-induced glottal closure reflex indicates that the activation of distension-sensitive mechanoreceptors in the vagus nerve serves as the afferent limb of this reflex. However, the involvement of other sensory neurons in the esophagus cannot be ruled out. It has been shown that acid in the esophagus can also induce EGCR, suggesting an involvement of acid-sensitive chemoreceptors in evoking EGCR.\textsuperscript{141} Interestingly, EGCR can be elicited more frequently by distension of the proximal than mid- or distal esophagus. This could be due to differential distribution and phenotype (i.e., more rich in Ca\textsuperscript{++} binding proteins in the proximal vagal afferent fibers) of the vagal afferent fibers innervating the different regions of the esophagus.\textsuperscript{67,139} Another possible explanation for this phenomenon is that the proximal esophagus also receives innervation from RLN.\textsuperscript{66} Therefore, stimulation of the richly innervated proximal esophagus by two branches of vagus (cervical vagus and RLN) can readily activate EGCR. Studies in the cat have shown that the mid-collicular decerebration does not affect the ED-induced EGCR indicating that EGCR is primarily mediated by the brainstem.
Vagal Afferent Nerve Stimulated Reflexes in the GI Tract

14.2.5 PHARYNGO-GLOTTAL CLOSURE REFLEX (PGCR),

FIGURE 14.3

Like PUCR, stimulation of the pharyngeal mucosa with minute amounts of water induces a brief vocal cord adduction in humans and cats. It is known that the glottal closure reflex is mediated through RLN. The PGCR and glottal closure during pharyngeal swallow share the RLN motoneurons for the adduction of the glottal muscles. Bilateral transection of GPN abolishes this reflex, but reflexive swallow and its associated glottal closure remains unaffected. Therefore, it is suggested that the afferent limb of PGCR is exclusively in the GPN. The physiological function of this reflex is believed to be the triggering of the glottal closure when a portion of the oral bolus is spilled into the pharynx inadvertently during the preparatory phase of swallowing. In addition, this reflex may be triggered during reflux of gastric content into the pharynx, thereby preventing aspiration by closing the introitus into the trachea.

FIGURE 14.2 Esophagoglottal closure reflex. An example of electromyographic recording from interarytenoid and lateral cricoarytenoid muscles during a 2.5 cm middle esophageal balloon distension before (A) and after (B) bilateral cervical vagotomy. As seen, EMG activities induced by balloon distension are completely abolished after bilateral cervical vagotomy. (From Am J Physiol 266 [Gastrointest Liver Physiol 29]: G147–G153, 1994. With permission.)
The reflex deteriorates with aging and is absent in some patients with UES dysphagia. Studies show that in the elderly the threshold volume of water for triggering the PGCR is significantly larger compared with the young. Similarly, the threshold volume for triggering PGCR during rapid injection was significantly larger in chronic smokers. In smokers, acute smoking abolished this reflex during slow water injection. This observation implicates that loss of PGCR may contribute to the development of reflux related respiratory complications in smokers.

**14.2.6 Pharyngo-Esophageal Inhibitory Reflex (PEIR)**

There are two types of esophageal peristalsis depending on the mode of their initiation — primary and secondary. The primary peristalsis is generally induced by swallow and the secondary peristalsis is initiated in response to local esophageal stimuli. Secondary esophageal peristalsis plays an important role in the clearance of residual ingested materials and refluxate from the stomach. This function is particularly important for preventing acid from reaching the airway. It has been
shown in humans and experimental animals that pharyngeal mechanical (air distension or water injection) stimulation produces a general inhibition of both primary and secondary peristalsis; PEIR.\textsuperscript{12,13,71,111,135} The inhibition of esophageal peristalsis due to PEIR is not followed by generation of a new peristaltic pressure wave. This phenomenon is different from inhibition of an ongoing peristaltic wave by a closely performed second swallow, which results in inhibition of peristalsis due to the first swallow and generation of peristalsis by the second. The threshold volume (0.8mL) for eliciting the PEIR in humans and anesthetized cats is very similar.\textsuperscript{12,71} PEIR occurs in both the striated and smooth muscle portion of the esophagus. However, it has been found that the inhibition is relatively stronger in the striated muscle compared with smooth muscle.\textsuperscript{13,71} This difference could be due to the nature of innervation of these two types of esophageal muscles. The peristalsis of striated muscle portion of the esophagus is entirely dependent on extrinsic innervation, whereas the smooth muscle portion of the esophagus is more autonomous due to its innervation by intrinsic nerve plexi.

In the cat, it has been documented that PEIR is intensity-dependent; the stronger the pharyngeal stimulation the greater the inhibition of the secondary peristalsis.\textsuperscript{71} In addition, the PEIR is very specific to pharyngeal stimulation, since the stimulation of the nasopharyngeal area does not produce any inhibition of peristalsis. Transection of GPN markedly, but not completely, attenuates PEIR. Transection of SLN reduces the number of pharyngeal stimulation-induced inhibitions of esophageal peristalsis. Therefore, it is concluded that the afferent pathways for the PEIR are both in the GPN and SLN, although it is predominantly via GPN. Although pharyngeal stimulation in SLN transected cats produces less inhibition of peristalsis compared with GPN transected cats, electrical stimulation of the central cut-end of SLN produces a profound inhibition of esophageal peristalsis. The exact reason for this differential response is not known. It is suggested that electrical stimulation of SLN induces swallow, which in turn inhibits the peristalsis (i.e., deglutitive inhibition). It is also probable that pharyngeal stimulation by injecting water may not be an adequate stimulus to activate all sensory afferent fibers in the SLN and, therefore, may not exhibit robust reflex inhibition of peristalsis. Studies have shown that pharyngeal mucosal mechanoreceptors primarily elicit PEIR, which can be readily blocked by applying lidocaine on pharyngeal mucosa.\textsuperscript{71,135}

14.2.7 PHARYNGEAL SWALLOWS (PS)

Swallowing is usually a voluntarily act, but may also occur subconsciously. In addition, mechanical stimulation of the pharynx stimulates reflexive pharyngeal swallow that is irrepressible.\textsuperscript{93,94,100} A pharyngeal reflexive swallow is similar to a voluntary swallow in all aspects except that it does not involve the movement of the anterior tongue; therefore, it is not associated with lingual peristalsis.\textsuperscript{127} Pharyngeal reflexive swallow is believed to protect the airway from aspiration by a) closing the vocal cords and approximating the closed arytenoids to the base of the epiglottis, b) epiglottal descent and antero-superior excursion of the larynx, and c) by clearing the pharyngeal cavity by pharyngeal peristalsis and posterior tongue thrust. Pharyngeal reflexive swallow may be triggered by inadvertent entry of food into
the pharynx during the preparatory phase of swallowing or by entry of refluxate into the pharynx during reflux events. The threshold volume for triggering this reflex is significantly higher in the elderly compared with the young, smokers compared with nonsmokers, and alcoholics versus nonalcoholics. Acute IV infusion of alcohol increases the threshold volume for triggering this reflex.

### 14.2.8 Pharyngo-LES Reflex Relaxation (PLESR)

PLESR is the first barrier that prevents the entry of gastric contents into the esophagus. This barrier is maintained by generation of a high resting tone of the LES muscle. There are several peripheral factors that alter the LES pressure. LES reflex relaxation occurs orthogradely to stimulation of the pharynx (pharyngol LES reflex relaxation), larynx (laryngol LES reflex relaxation) and the esophagus (esophagol LES reflex relaxation) and retrogradely by gastric distension. These reflexes will be discussed in the following sections. In human studies and animal studies, it has been documented that intrapharyngeal instillation of minute amounts of water produces a prolonged LES relaxation. The threshold volume of water when injected rapidly is significantly less than slow continuous instillation. PLESR is more prevalent in elderly when compared with younger subjects. In the postprandial period, occurrence of PLESR associated with acid reflux is less (6%) in younger volunteers compared with a significantly higher occurrence (44%) in the elderly subjects and results in more reflux events.

### 14.2.9 Laryngo-LES Reflex Relaxation (LLESR)

Like PLESR, laryngeal stimulation by air pulse induces LES relaxation, which is independent of swallowing and esophageal peristalsis. The stimulation of the epiglottis and arytenoids produces a higher incidence of LES relaxation compared with the base of the tongue. The magnitude of relaxation differs significantly between the three anatomical sites, with greater relaxation occurring at the epiglottis and arytenoids compared with the base of the tongue. It is suggested that stimulation of the laryngo-pharyngeal mechanoreceptors mediate LES relaxation.

### 14.2.10 Esophago-LES Reflex Relaxation (ELESR)

ED induces isolated LES relaxation in humans and experimental animals. In achalasia patients, ED fails to produce LES relaxation. However the phasic contraction of the esophagus proximal to balloon distension exhibits a normal response. Thus, it suggests that extrinsic innervation of the esophagus is not affected in achalasia and it is likely that there is a loss of intrinsic inhibitory neurons in the LES. It is widely accepted that ELESR is a vago-vagal reflex phenomenon. The afferent limb of this reflex is undoubtedly vagal sensory afferent fibers projecting to the nucleus tractus solitarius (NTS). In the rat, it is more specifically in the region of NTS centralis. It is most likely that esophageal distension-sensitive muscle afferent fibers play a major role in the activation of ELESR.
14.2.11 LARYNGO-GASTRIC REFLEX RELAXATION (LGRR)

The stomach actively relaxes before food enters the stomach. This phenomenon is known as receptive relaxation. It is known that mechanical stimulation of the pharynx (pharyngo-gastric) and distension of the esophagus (esophago-gastric) induces gastric relaxation. The SLN sensory fibers that innervate the taste buds on the laryngeal surface of the epiglottis and larynx respond to mechanical and chemical stimuli. Interestingly, these afferent fibers are exquisitely more sensitive to water than any other chemical solution. It has been shown that these water-sensitive SLN-afferent fibers are involved in diuresis, drinking, and cardiovascular reflexes. Recent studies have reported that the administration of water into the larynx and epiglottis or electrical stimulation of the central cut-end of the SLN in rats inhibits gastric motility. Transection of cervical vagi, but not the spinal cord (T3-T4), prevents development of this inhibition. This finding suggests that the afferent limb of laryngo-gastric inhibitory reflex is exclusively vagally mediated. The laryngeal afferent stimulation possibly inhibits the preganglionic vagal motor neurons to inhibit the gastric motility. In a subsequent study, Kobashi et al. documented that electrical stimulation of the central cut-end of the SLN inhibits the tonic firing of the neurons in the intermediate dorsal motor nucleus of the vagus (DMV) of the rat. These investigators have also shown that systemic application of atropine blocks water stimulation-induced inhibition of gastric motility, suggesting the involvement of cholinergic pathways.

14.2.12 PHARYNGO-GASTRIC REFLEX RELAXATION (PGRR)

Pharyngeal distension in anesthetized dogs produces relaxation of the fundus and corpus. However, the motility of antrum remains unaffected. Anesthesia of the pharynx by 2% xylocaine or bilateral cervical vagotomy abolishes relaxation of the proximal stomach.

14.2.13 ESOPHAGO-GASTRIC REFLEX RELAXATION (EGRR)

ED induces reflex relaxation of the proximal stomach. This reflex requires an intact vago-vagal connection between the esophagus, brainstem, and stomach. The majority of the vagal afferent fibers from the esophagus project to the central part of the nucleus tractus solitarius (NTSc), which acts as a relay station for EGRR. It has been shown that the bilateral electrolytic lesion of NTSc abolishes ED-induced gastric relaxation. It is thought that activation of esophageal vagal afferent fibers produces inhibition of tonically active preganglionic motor neurons in the dorsal motor nucleus of the vagus (DMN) to produce relaxation of the proximal stomach. It has been shown that noradrenergic, but not nitrergic, neurons in the NTSc contribute in the modulation of EGRR. Microinjection of norepinephrine into the DMN can mimic this reflex.
14.3 GASTRIC REFLEXES

Stimulation of gastric mechano- and chemo-sensitive vagal afferent fibers evoke at least five anterograde and retrograde reflexes that alter the tone of the LES, pyloric sphincter, antrum motility, pylorus, duodenum, and colon. These reflexes are:

- Gastro-LES reflex relaxation (i.e., TLSER induced by gastric distension)
- Gastro-gastric reflex (GGR)
- Gastro-pyloric sphincter reflex relaxation (GPRR)
- Gastro-duodenal reflexes and
- Gastro-colonic reflex

The majority of mechanosensitive vagal afferent fibers innervating the stomach exhibit a spontaneous ongoing firing and respond to distensions and contractions. The tonic firing of these afferent fibers constantly regulate the gastric motility. The mechanical distension during food ingestion alters the firing of these afferent fibers. Similarly, any changes in the chemical environment of the stomach by nutrients and acid induces alteration in the motility pattern of the stomach, which is primarily due to activation of mucosal mechano/chemo- and chemo-sensitive afferent fibers. The integrative processing of all vago-vagal gastric reflexes takes place in the NTS.

14.3.1 GASTRO-LES REFLEX RELAXATION (GLRR)/TRANSIENT LES RELAXATION (TLESR)

Gastro-esophageal reflex mostly occurs due to transient lower esophageal sphincter relaxation (TLSER), which is not associated with swallowing or esophageal distension. Although the underlying mechanism for TLSER is not fully known, it has been shown that distension of the proximal stomach, including the cardia, plays a major role in inducing TLSER in humans, dogs, and ferrets. TLESR in response to gastric distension is a vago-vagal reflex and sympathetic pathways play no major role in its elicitation. A recent study in humans suggests that stimulation of stretch-sensitive vagal afferent fibers is involved in triggering TLSER. However, the contribution of chemosensitive mucosal afferent fibers in stimulation of TLSER cannot be ruled out. In dogs, ingestion of nutrients with a pH of 1.5 produces a two-fold increase in the number of TLESRs compared with nutrients with a pH of 5. This result indicates that pH-sensitive mucosal vagal afferent fibers may be involved in triggering TLSER. The frequency of TLESRs can be pharmacologically attenuated by GABA_B receptor agonists, cannabinoids and NMDA receptor antagonists. It has been proposed that attenuation of mechano-transduction properties of gastric vagal afferent fibers by these agents plays a critical role in reducing TLESR. However, the effects of these agents on brainstem neurons modulating the TLESR cannot be excluded.
14.3.2 GASTRO-GASTRIC REFLEX

Contraction and relaxation of different parts of the stomach closely coordinate to store (i.e., receptive relaxation) and expel (i.e., propagatory contractions) the gastric food content. Mechanical and chemical stimulation of the stomach elicit local reflexes that are dependent on extrinsic vagal innervations. In the ferret, gastric inflation with 50 ml of saline evoked large antral contraction. Bilateral cervical vagotomy, but not splanchnicotomy, markedly reduced the gastric distension-induced antral contractions. The corpus and antrum exhibit coordinated contraction in order to propagate ingested food toward the pylorus. It has been shown that distension of the corpus increases the frequency and amplitude of contractions of the antrum. On the other hand, lowering of the antral pressure enhances the contractions of the corpus, to propagate contents to the antrum. These observations suggest that activation or inhibition of vagal distension-sensitive afferent fibers reflexly influence the tonic firing of vagal motoneurons to regulate motility of different parts of the stomach. It has also been shown that selective mechanical distension of a particular portion of the stomach can also influence the motility of the same part. For example, distension of the corpus and antrum evokes contractions of corpus (corpo-corporal reflex) and antrum (antro-antral reflex), respectively. Chemosensitive vagal afferent fibers also play a role in gastric reflexes. It has been shown that instillation of 0.1N HCl (pH 1.2) into the stomach significantly inhibits motilin-induced gastric contraction, suggesting that the activation of mucosal chemosensitive afferent fibers reflexly inhibits gastric contraction to prevent acid to enter the duodenum.

14.3.3 GASTRO-PYLORIC REFLEX RELAXATION (GPRR)

Like UES and LES, the pyloric sphincter maintains a tone to regulate gastric emptying and to prevent reflux of duodenal contents into the stomach. The pyloric sphincter pressure is maintained under the influence of extrinsic (primarily by the postganglionic vagus nerve) and intrinsic myenteric neurons. The gastric distension during ingestion of solid food and liquid induces increased antral contraction and relaxation of the pyloric sphincter. The relaxation of pyloric sphincter following gastric distension is necessary for expelling the gastric contents into the duodenum. This gastric distension-induced reflex relaxation of the pyloric sphincter is a vagovagal reflex, which can be abolished by subdiaphragmatic vagotomy, but not by spinal transection. The relaxation occurs due to activation of postganglionic nitric oxide containing neurons. It has been shown that in hyperglycemia, gastric emptying of the rat is markedly affected by inhibiting GPRR. The inhibition of GPRR by glucose is thought to be mediated by hypothalamic NPY 1 receptors, since systematic administration of this peptide blocks the GPRR. GPRR can be restored by intracerebroventricular injection of selective NPY1 receptor antagonist (BIBP 3226).

14.3.4 GASTRO-DUODENAL REFLEX (GDR)

There are conflicting reports about GDR. In in vitro gastroduodenal preparation with intact celiac plexus of the rabbit, gastric distension inhibits duodenal motility.
Denervation of celiac plexus abolishes the gastric distension-induced duodenal inhibition, suggesting that the reflex arc is via the prevertebral ganglia. However, in the human prolong distension of the fundus at subconscious intensity increases duodenal motility. These differences in results are difficult to explain, since the experimental models and species are different. It is possible that different types of reflexes may be elicited due to stimulation of different parts of the stomach.

### 14.3.5 Gastro-Colonic Reflex (GCR)

Clinical observations indicate that there is often an urge for defecation following food intake. Studies have reported that there is an increase in motor activity of the rectosigmoid colon after the meal. This post-prandial change in contractile response of the colon is known as gastro-colonic reflex (GCR). In the dog, electromyographic (EMG) recordings from the colon show an immediate change in colonic motility after ingestion of food. It is believed that this reflex is triggered by activation of antral mechanoreceptors during distension and activation of intestinal chemoreceptors in the presence of nutrients in the small intestine. The reflex pathway of GCR has not been studied systematically in humans. However, in experimental animals (dogs, ferrets and rats) that receive vagal efferent innervation in the proximal colon, GCR is most likely a vago-vagal reflex. In the ferret, electrical stimulation of the central cut-end of the thoracic communicating branch (i.e., connecting dorsal and ventral vagal trunk) evokes colonic motility. Cooling of vagal trunks (ventral and dorsal) caudal to stimulating electrode completely abolishes electrical stimulation-induced contractile response of the colon, suggesting that spinal motorneurons are not the efferent limb of GCR.

### 14.4 Intestinal Reflexes

Several reflexes originate from different parts of small and large intestine that regulate gastric emptying and intestinal transit. These reflexes are 1) duodenogastric reflex relaxation, 2) ileal brake, 3) jejunal brake, 4) ceco-gastric reflex relaxation and 5) recto-gastric reflex relaxation. Of all these reflexes, the neural pathway of duodenogastric reflex relaxation (DGRR) has been studied extensively and it is known to be a vago-vagal reflex. The neural pathways of other reflexes are less defined and the contribution of the vago-vagal pathway is completely delineated. Therefore, the following section will only describe DGRR.

#### 14.4.1 Duodenogastric Reflex Relaxation (DGRR)

Mechanical and chemical stimulation of the duodenum induces gastric relaxation. In conscious dogs, distension of the duodenum induces gastric relaxation. The subdiaphragmatic vagal cooling or chronic vagal transection abolishes duodenal distension-induced gastric relaxation.

Like mechanical distension, acid, lipids, and chylomicron components in the duodenum can induce DGRR and decrease antral contractility. Inhibition of gastric motility by the acid in the duodenum prevents further acid from
entering the duodenum. It is believed that acid activates the duodenal mucosal afferent fibers either by directly stimulating the afferent fibers or indirectly by releasing secretin and CCK. Similarly, lipids and chylomicrons in the duodenum produce DGRR by activating the vagal mucosal afferent via CCK\textsubscript{A} receptors. Blocking the secretin by secretin antiserum and CCK\textsubscript{A} receptors by CCK\textsubscript{A} receptor antagonist devazipide can prevent the acid-induced gastric inhibition. Studies have also shown that these vagal chemosensitive afferent fibers are also sensitive to capsaicin. Desensitization of these fibers by capsaicin prevents acid-induced DGRR.

14.5 CONCLUSION

There are a large number of anterograde and retrograde vago-vagal reflexes that provide a means for change in function of a given site of the GI tract by alteration of luminal pressure and chemical balance of a distant organ. While most of these reflexes are well-defined and extensively studied under different conditions, a few have been reported recently and await either confirmation or better delineation. Some of these reflexes seem to work counter to normal physiologic functions, such as LES relaxation reflexes induced by pharyngeal and esophageal stimulation. Others enhance or regulate physiologic functions, such as esophago-UES, pharyngo-UES contractile, esophagoglottal closure reflexes, or the duodenogastric reflex. Over- or under-responsiveness of any of these reflexes can potentially contribute to abnormalities of gastrointestinal motor function.

REFERENCES


15 Reflexes Initiated by Activation of the Vagal Afferent Nerves Innervating the Airways and Lungs

Brendan J. Canning and Stuart B. Mazzone

CONTENTS

15.1 Introduction .................................................................................................. 404
15.2 Overview of the Vagal Afferent Innervation to the Airways ...................... 404
  15.2.1 Mechanoreceptors ............................................................................ 404
  15.2.2 Nociceptors....................................................................................... 405
15.3 Vagally Mediated Respiratory Reflexes and Cough.................................... 406
  15.3.1 Methods for Studying Vagal Afferent Control of Respiration and Cough .................................................................................................................. 406
    15.3.1.1 Conscious Animals .................................................................... 407
    15.3.1.2 Anesthetized Animals ........................................................... 408
  15.3.2 The Role of Vagal Afferents in the Control of Breathing............... 409
    15.3.2.1 Mechanoreceptors ............................................................. 409
    15.3.2.2 Nociceptors ....................................................................... 410
  15.3.3 The Role of Vagal Afferent Nerve Subtypes in Regulating the Cough Reflex .................................................................................................................. 411
    15.3.3.1 Mechanoreceptors ............................................................. 411
    15.3.3.2 Nociceptors ....................................................................... 412
15.4 Vagally Mediated Autonomic Reflexes ....................................................... 412
  15.4.1 Morphology of Airway Autonomic Innervation.................................... 412
  15.4.2 Monitoring Vagally Mediated Autonomic Reflexes in the Airways .................................................................................................................. 413
    15.4.2.1 Monitoring Reflex Regulation of Airway Smooth Muscle ................. 413
    15.4.2.2 Monitoring Reflex Regulation of Mucus Secretion........... 415
15.4.2.3 Monitoring Reflex Regulation of Pulmonary and Bronchial Blood Flow .......................................................... 415
15.4.3 Vagal Afferent Nerve Mediated Autonomic Reflexes in the Airways .............................................................................................................. 416
15.4.3.1 Autonomic Reflexes Regulating Airway Smooth Muscle Tone .................................................................................. 416
15.4.3.2 Autonomic Reflexes Regulating Glandular Secretion .... 417
15.4.3.3 Autonomic Reflexes Regulating the Bronchial Vasculature ........................................................................ 419
15.4.3.4 Cardiovascular Reflexes Initiated by Activation of Airway Vagal Afferent Nerves .................................................. 420

References........................................................................................................................................................................ 420

15.1 INTRODUCTION

Studies in animals and in humans have revealed an essential role for vagally derived bronchopulmonary afferent nerves in the regulation of homeostatic and defensive respiratory and autonomic reflexes. These reflexes are thought to protect the airways and lungs, optimize the work of breathing, maintain airway patency, and thus sustain adequate respiration in response to continually changing demands for gas exchange. The specificity and precision of respiratory and autonomic reflexes depends on the selective recruitment of airway vagal afferent nerve subtypes in response to a wide variety of physiologic and pathophysiologic chemical and mechanical stimuli. Once activated, these afferent nerves initiate stereotypical reflex responses that have facilitated their classification.

The afferent innervation of the airways and lungs has been described in detail elsewhere in this book (see Chapter 11). In this chapter, we will briefly describe the attributes of airway vagal afferent nerve subtypes that determine the reflex effects initiated upon their activation. We will also describe methods for studying respiratory and autonomic reflexes.

15.2 OVERVIEW OF THE VAGAL AFFERENT INNERVATION TO THE AIRWAYS

15.2.1 MECHANORECEPTORS

Rapidly (RAR) and slowly (SAR) adapting stretch receptors, the two primary classes of mechanoreceptors innervating the airways and lungs, are differentiated by their adaptation to sustained lung inflations.1,2 These myelinated afferents can be further differentiated by their activity during eupnea and by their response to lung deflation and by stimuli (e.g., bronchospasm) that alter airway pressures or mechanics.

Mechanoreceptors not readily fitting into a classification scheme consisting only of RARs and SARs also innervate the airways and lungs. Cough receptors, for example, recently described in the extrapulmonary airways of guinea pigs, are highly sensitive to punctate mechanical stimulation, acids, and low chloride solutions but
Reflexes Initiated by Activation of the Vagal Afferent Nerves 405

Unlike RARs and SARs, are not activated by distending pressures, airway collapse or stretch.\textsuperscript{3–7} Also unlike RARs and SARs, then, cough receptors are likely quiescent during eupnea, only becoming active in the presence of stimuli that evoke cough. Such vagal afferents likely innervate the airways of other species that cough such as dogs, cats, and humans,\textsuperscript{8–12} but have not yet been formally described in the published literature.

In most species, RARs and SARs are found primarily in the intrapulmonary airways and display some baseline activity during tidal breathing. RAR terminations are more concentrated in the conducting airways, whereas SARs terminate in the conducting airways but also in and around the gas-exchanging units of the lung.\textsuperscript{1,2,10,13–15} The cough receptors described in guinea pigs are found almost exclusively in the extrapulmonary airways.\textsuperscript{7}

The structures of the peripheral terminals of bronchopulmonary mechanoreceptors are poorly described. This is due in part to the fact that there is marked overlap in the termination sites of the various vagal afferent nerve subtypes innervating the airways but also because specific histochemical markers for these various subtypes are lacking. Species differences may also exist. In dogs, for example, SARs are abundant in the trachea while few innervate the extrapulmonary airways of guinea pigs, rats, rabbits, or cats.\textsuperscript{1,7,13,16} Based on their characteristic responses to lung stretch and smooth muscle contraction, however, it is reasonable to speculate that intrapulmonary RARs and SARs may directly innervate airway smooth muscle or align with the longitudinal or circumferential axis of the airways. Studies in rabbits have described such structures that appeared to correspond to the terminals of SARs.\textsuperscript{15} Comparably elegant studies of RAR nerve terminals have not been completed. Recent studies in guinea pigs have described a population of highly organized neuronal structures in the airway mucosa that likely correspond to the terminals of cough receptors.\textsuperscript{6,17} These terminals were found between the epithelium and the smooth muscle layer, an observation that is consistent with the lack of effect of epithelial removal or airway smooth muscle contraction on the responsiveness or excitability of these afferent nerves.\textsuperscript{3,18}

15.2.2 Nociceptors

Vagal nociceptors innervate the airways from the larynx to the terminal bronchioles and are defined by their responsiveness to noxious endogenous (e.g., bradykinin) and exogenous (e.g., capsaicin) chemical stimuli. Airway nociceptors are mostly unmyelinated, conducting action potentials in the C-fiber range (1 m/sec), but faster conducting A\textsubscript{δ} nociceptors have been described.\textsuperscript{4,19} Subtypes of airway afferent C-fibers have also been identified based on their differential distribution in the airways and lungs and/or their differing embryologic origins.\textsuperscript{20–22} Subtypes may also differ in their neurochemistry and responsiveness to chemical stimuli.\textsuperscript{21,22} In general, afferent C-fibers are quiescent in the normal, uninflamed airways, relatively unresponsive to lung inflation, collapse or stretch, and contribute little to the ongoing regulation of tidal breathing. Rather, nociceptors recruited by inhaled irritants or during airways inflammation likely play a critical role in regulating and/or initiating defensive airway and respiratory reflexes.\textsuperscript{19,21}
In rats and in guinea pigs, a major subpopulation of afferent C-fibers expressing the neuropeptides substance P, neurokinin A, and calcitonin gene-related peptide has been described.\textsuperscript{4,22–29} Immunohistochemical staining for these neuropeptides, along with creative tracing techniques, have been used to localize C-fiber terminations in the airways and lungs of guinea pigs and rats. These studies reveal that nociceptors innervate structures throughout the airway wall and lungs, but are particularly prominent in the airway epithelium, where they form a dense, poorly organized plexus beneath and between the epithelial cell layers lining the airways.\textsuperscript{24,26–28} Structures comparable to the neuropeptide containing fibers in the epithelium of guinea pigs and rats have been localized to the mucosa of other species, but in general, express far less neuropeptide immunoreactivity.\textsuperscript{29–33} Whether these structures correspond to the peripheral terminals of C-fibers in these species and where nociceptors that do not express the neuropeptides terminate in the airways of guinea pigs and rats is unknown.

It is tempting to speculate that the poorly organized but extensive epithelial terminations of at least a subpopulation of nociceptors place these vagal afferent nerves in an ideal position for detecting and responding to the external environment and to epithelial damage and inflammation, but render them less sensitive than RARs and SARs to the internal environment and the mechanical forces associated with breathing. This is consistent with the primary role of airway nociceptors in defensive reflexes, their modest activity during eupnea, their relative insensitivity to lung volume changes, and thus their limited role in homeostatic reflexes. Such speculation, however, is premature, given our lack of knowledge about the terminations of other afferent nerve subtypes, including subtypes of airway nociceptors.

15.3 VAGALLY MEDIATED RESPIRATORY REFLEXES AND COUGH

15.3.1 METHODS FOR STUDYING VAGAL AFFERENT CONTROL OF RESPIRATION AND COUGH

Three basic preparations are used to study vagal control of respiration: 1) indirect measures of breathing in awake animals, 2) direct measures of airway pressures or flows in spontaneously breathing anesthetized animals, or 3) electrophysiological measures of respiratory nerve or muscle activity in anesthetized and paralyzed animals. Deciding which preparation to use is governed by the desired end-point of the experiment. Each approach has advantages and disadvantages and all are limited by the difficulty of identifying stimuli that are selective for activating or inhibiting vagal afferent nerve subtypes. Assessing the role of vagal afferent subtypes to a specific respiratory reflex response thus often requires multiple approaches and creative experimental designs. Moreover, prior, coincident, or parallel studies of the responsiveness of afferent nerves to the stimuli used to evoke reflexes should also be completed such that physiological responses can be attributed to specific changes in afferent nerve subtype activity.
15.3.1.1 Conscious Animals

The easiest way to study breathing in most small animal species involves noninvasive (plethysmography) measures of respiration in conscious, freely moving, or lightly restrained animals. This method can be used to monitor the magnitude of inspiratory and expiratory efforts, as well as the timing of events during the respiratory cycle. Experimenting with conscious animals is particularly useful for pharmacological studies of the cough reflex (Figure 15.1). Such experiments are mostly limited to guinea pigs, however, as rabbits, rats, and mice have an unpredictable or absent cough response under normal conditions, and species such as cats are rarely studied in the conscious state.

**FIGURE 15.1** Representative traces showing respiratory movements in conscious and anesthetized animals. (A) Respiratory activity in a conscious, freely moving guinea pig monitored using plethysmography. Repetitive coughing was evoked following the introduction of bradykinin (10mg/ml) into the chamber (Mazzone, and Canning, unpublished data). (B) Changes in tracheal pressure associated with spontaneous respiration in an anesthetized guinea pig. Application of a bolus of 10 mM citric acid to the extrathoracic tracheal mucosa evoked a single cough response, characterized by a brief augmented inspiratory effort followed by a large, forceful expiration (Mazzone, and Canning, unpublished data; see also Reference 17). (C) Changes in tracheal pressure associated with spontaneous respiration in an anesthetized rat. Application of a bolus of 10 μM capsaicin to the extrathoracic tracheal mucosa evoked a brief period of apnea (Mazzone, and Canning, unpublished data). (D) Chest wall movements (measured using a calibrated impedance converter) in an anesthetized rat. Microinjection of the capsaicin analogue, resiniferatoxin (200 pmol), into the commissural nucleus of the solitary tract evoked a brief period of apnea (Mazzone, and Geraghty, unpublished data). Scale bars = 2 minutes.
15.3.1.2 Anesthetized Animals

Using noninvasive approaches limits the experimental manipulations that can be performed to determine the specific mechanisms and neural pathways underlying evoked respiratory reflexes. In these instances, a more appropriate means of monitoring respiration and cough may involve using anesthetized animals. Two methods are commonly used to monitor respiratory activity in anesthetized animals. The first involves direct measurements of airway pressure or airflow via an implanted tracheal cannula. Under these conditions, animals are allowed to breathe spontaneously via the cannulated portion of the airway while tidal and evoked changes in inspiratory and expiratory pressures and flow are measured at the cannula (described in detail in Reference 34). Inspired air should be warmed and humidified in these experiments to approximate the warming and humidifying function of the nose. This method can be used to study defensive respiratory responses evoked by punctate mechanical or electrical stimulation of the airway mucosa, or chemical stimuli applied topically to the airway mucosa or administered intravenously. Reflexes that can be studied under these conditions (with differing ease among species) include alterations in respiratory rate, tidal volume, inspiratory and expiratory timing and cough.\textsuperscript{7,38–45} Obviously, an ability to differentiate the various reflexes evoked is critical (Figure 15.1).

The specific neural pathways mediating an evoked response can be discerned by selective nerve denervations, drug pretreatments (either peripherally or centrally administered), and stimulus localization experiments. Studying reflexes such as cough in anesthetized, spontaneously breathing animals has the added advantage that the portion of airway by-passed by the tracheal cannula can be superfused with buffer and pharmacologically isolated from the rest of the airways.\textsuperscript{7,46} By adding appropriate antagonists to the tracheal perfusate in this design, it is even possible to evoke systemic responses via intravenously administered agonists without direct effects on the trachea.\textsuperscript{46} But using anesthetized animals for studying cough also has disadvantages as, unlike in conscious animals, anesthetized animals do not cough to many known tussigenic stimuli (such as capsaicin and bradykinin); see below.\textsuperscript{7,42,44,45,49} Whether this relates to the depressive effects of anesthesia on some cough pathways or the involvement of cortical centers in the manifestation of certain types of cough is presently unknown. Nevertheless, cough can be readily evoked in anesthetized guinea pigs, dogs, and cats by mechanically probing or electrically stimulating the airway mucosa, topically applied citric acid or inhalation of water or acetone vapor.\textsuperscript{7,41,48,49} In addition, while capsaicin and bradykinin do not evoke cough in anesthetized animals, both agents sensitize cough (evoked electrically or by citric acid applied topically to the tracheal mucosa), providing an alternative means to study regulation of cough by vagal nociceptors.\textsuperscript{49}

The second approach for studying respiratory control in anesthetized animals involves electrophysiological assessment of respiratory nerve (e.g., phrenic) or muscle (e.g., diaphragm) activity in paralyzed, artificially ventilated animals (Figure 15.2). Mechanical ventilation allows respiration to be tightly controlled, enabling ventilatory maneuvers that would otherwise be impossible in freely breathing animals, such as sustained lung inflations, lung collapse, and controlled alterations in breathing depth and rate with any desired gas composition.\textsuperscript{13,50–52} The ability
Reflexes Initiated by Activation of the Vagal Afferent Nerves

Mechanical ventilation also allows surgical access to thoracic structures such as the large pulmonary and bronchial vessels, thoracic vagus nerves, lungs, heart, esophagus, and related structures. Although it is possible to discern cough-like phrenic nerve responses following stimulation of the airway mucosa in anesthetized and paralyzed animals, phrenic nerve activity as an indicator of ventilatory control is the most common use of this method (Figure 15.2). In either case, the relationship between the measured responses (e.g., phrenic nerve activity) and the end organ responses predicted in a freely breathing animal may not always be clear. It is also reasonable to assume that the highly contrived nature of mechanical ventilation, with its fixed rates, volumes, and end-expiratory pressures, likely alters subsequently evoked reflexes in some way.

15.3.2 The Role of Vagal Afferents in the Control of Breathing

15.3.2.1 Mechanoreceptors

Studies by Hering and Breuer were the first to show the respiratory modifying effects of lung inflations and deflations. Subsequent studies by Head suggested the presence of pulmonary vagal afferent nerve subtypes with opposing actions on respiratory control. These observations, which have been widely replicated and
studied mechanistically, indicated that tonically active airway vagal afferent nerves reflexively modify the duration and intensity of inspiratory and expiratory efforts. It is now well established that activation of RARs (by deflation, or inflation in the absence of SAR activity) increases inspiration (leading to hyperpnea), whereas stimulation of SARs (by lung inflation) results in inspiratory termination (the Hering-Breuer inflation reflex). These opposing actions of RARs and SARs regulate the timing and rate of the respiratory cycle (Figure 15.3). RAR activation secondary to airways obstruction can also initiate sighs or augmented breaths, thereby opening collapsed airways and ensuring adequate ventilation.

As RARs and SARs evoke opposing effects on respiration, it follows that the excitatory and inhibitory responses are generated by different subpopulations of second (or higher) order neurons in the reflex pathway. Indeed, distinct neurons in the nucleus of the solitary tract receive excitatory glutamatergic input from SARs and RARs. In rats, some RARs also innervate inspiratory neurons in the dorsal respiratory group. RAR relay neurons and dorsal respiratory group inspiratory neurons likely stimulate respiration. By contrast, the neurons receiving input from SARs (pump cells) inhibit (via GABAergic mechanisms) brainstem neurons controlling respiration, including RAR relay neurons. Although the brainstem neurons receiving input from RARs and SARs likely receive complex regulatory inputs from other brainstem neurons regulating breathing, the properties of these distinct integrating neurons provide the foundation for differential regulation of respiration by RARs and SARs.

15.3.2.2 Nociceptors

Acute activation of cardiopulmonary nociceptors by intravenous or right atrial injection of capsaicin or the 5HT3 receptor-selective agonist phenylbiguanide evokes a
characteristic apnea, hypotension, and bradycardia (the Bezold-Jarisch reflex), suggesting that nociceptor activation may acutely inhibit respiratory drive (Figure 15.1).\textsuperscript{19,21,61} Brainstem injections of capsaicin in rats (to activate the central terminals of vagal nociceptors) also indicate that nociceptor activation may inhibit respiratory drive\textsuperscript{43} (Figure 15.1). But changes in respiration evoked by bronchopulmonary nociceptor stimulation may be related to the degree or rate of activation or the species under study.\textsuperscript{19,21,40,42} In guinea pigs, threshold doses of capsaicin applied to the tracheal mucosa or bradykinin given via inhalation tend to increase respiratory rate, whereas higher doses evoke respiratory slowing and apnea. Central injections of capsaicin in guinea pigs increase breathing rate.\textsuperscript{7,49} The responses evoked may also be related to the population of nociceptors that are activated. In rats, for example, the apnea evoked by nociceptor activation is reportedly abolished by laryngeal denervation.\textsuperscript{62}

15.3.3 THE ROLE OF VAGAL AFFERENT NERVE SUBTYPES IN REGULATING THE COUGH REFLEX

15.3.3.1 Mechanoreceptors

It has long been held that RARs are the primary afferent nerve subtype that regulates cough.\textsuperscript{2,8,9,37,47,63,64} This notion has prevailed despite the fact that stimuli that robustly activate RARs (e.g., bronchoconstrictors) are often poor tussigens.\textsuperscript{2,7,37,65,66} RARs in many species are also active throughout the respiratory cycle, but cough is evoked only by very specific stimuli.\textsuperscript{2,13} These observations call into question the role of the classically defined RARs in cough.

Studies in guinea pigs have identified a mechanically sensitive vagal afferent nerve subtype that is distinct from RARs and SARs and that plays an essential role in regulating cough. Unlike most RARs and SARs, which are found in the intrapulmonary airways and lungs, cough receptors are distributed primarily in the extrapulmonary airways.\textsuperscript{1,2,7,64} In anesthetized guinea pigs, electrical stimulation or mechanical probing of the tracheal mucosa, or acute application of acids (such as citric acid) or low chloride solutions to the tracheal mucosa activates the cough receptors and initiates coughing\textsuperscript{7,49} (Figure 15.1B). Coughing is not evoked by capsaicin, bradykinin, or other nociceptor stimulants via this pathway, although such stimuli may enhance cough.\textsuperscript{49}

Central integration of cough receptor input has not been studied. It seems likely, however, that input from RARs and SARs modulates cough receptor dependent cough. For example, SAR activation induced by respiratory preloading increases expiratory efforts during cough.\textsuperscript{67,68} Conversely, blocking SAR activity with inhaled sulfur dioxide attenuates coughing.\textsuperscript{69} Bolser and colleagues\textsuperscript{70} have proposed a role for SARs in the facilitation of inputs into a basic central cough pattern generator. Others have speculated about the inhibitory effects of SAR activation on the cough reflex.\textsuperscript{71} Recently, House et al.\textsuperscript{41} reported that inhalation of histamine or allergen initiated hyperpnea but no coughing in anesthetized dogs, with the hyperpnea likely attributable to stimulation of RARs. Subsequently, evoked cough responses (induced by repeated mechanical stimuli delivered to the tracheal/bronchial mucosa) were
markedly increased in frequency, suggesting that coincident RAR activation facilitates coughing.

15.3.3.2 Nociceptors

Capsaicin and bradykinin aerosols are very effective at evoking cough in conscious animals and in humans. Since capsaicin and bradykinin are reasonably selective for activating airway nociceptors, it seems likely that nociceptor activation can initiate coughing (Figure 15.1A). However, the neural mechanisms involved in nociceptor-dependent cough have proved difficult to elucidate, since bradykinin- and capsaicin-evoked cough (but not cough evoked by mechanical or electrical stimulation of the airway mucosa — likely dependent upon cough receptor activation) is abolished by anesthesia. It is unclear why anesthesia prevents nociceptor-dependent cough when other nociceptor-dependent reflexes (and the cough receptor-dependent cough) remain intact. Perhaps nociceptor activation is just threshold for evoking cough in conscious animals, but subthreshold in anesthesia. Alternatively, nociceptor-dependent cough may require the conscious perception of airway irritation. Elucidating the role of nociceptors in cough is made more difficult by the observation that acute activation of nociceptors with high doses of capsaicin may actually inhibit mechanically evoked cough. This data would seem to fit with the known inhibitory effects of nociceptors on respiration and may further support the hypothesis that nociceptor-dependent responses are governed by the degree of afferent activation.

While nociceptor activation does not evoke cough in anesthetized animals, their activation may facilitate coughing. Coincident activation of airway nociceptors (using threshold doses of capsaicin or bradykinin) reduces the threshold and enhances the magnitude of cough receptor-driven coughing in guinea pigs. Cough can also be sensitized by coincident activation of esophageal nociceptors. The facilitatory effect of nociceptors on the cough reflex appears to occur at central sites. Although the exact mechanisms that allow nociceptors to sensitize cough are unclear, evidence suggests that cough receptors and nociceptors converge onto common brainstem neuronal subpopulations that drive cough. In this scenario, nociceptor activation provides central input that is insufficient to evoke coughing, but sensitizes these relay neurons and reduces their threshold for activation by other afferent nerve inputs. A comparable synergistic interaction between airway mechanoreceptors and nociceptors regulates airway smooth muscle tone.

15.4 VAGALLY MEDIATED AUTONOMIC REFLEXES

15.4.1 MORPHOLOGY OF AIRWAY AUTONOMIC INNERVATION

Both sympathetic and parasympathetic nerves innervate the airways. Sympathetic nerves primarily innervate the pulmonary and bronchial vasculature, while airway parasympathetic nerves innervate the vasculature but also the mucus glands and the airway smooth muscle. Consistent with the models originally proposed by Hillarp, little discernible specialization is apparent on the effector cells adjacent to
airway postganglionic autonomic nerves. Morphological analyses also reveal little change in nerve fiber densities in the smooth muscle from the trachea to the bronchioles.\textsuperscript{82,83} The neurochemistry of these nerve fibers may, however, differ considerably in the large and small airways.

Preganglionic parasympathetic nerves arising bilaterally from nucleus ambiguus (nA) and the dorsal motor nucleus of the vagus nerve (dmnX) innervate airway parasympathetic ganglia.\textsuperscript{46,84–92} Preganglionic nerves regulating airway sympathetic innervation emanate from thoracic ventral roots to innervate thoracic and cervical sympathetic ganglia. Preganglionic parasympathetic neurons from nA regulate parasympathetic cholinergic outflow to airway smooth muscle and glands in dogs and guinea pigs.\textsuperscript{46,47,88} The roles of preganglionsics arising from dmnX are unknown.

Airway parasympathetic ganglia containing as few as one to over 100 neurons are randomly and sparsely dispersed primarily in a serosal nerve plexus of the airways.\textsuperscript{31,83,93–96} Fewer ganglia are associated with intrapulmonary airways than the extrapulmonary airways, and no ganglia are associated with bronchioles. Based on the effects of ganglionic blockade and vagotomy, preganglionic input appears to be essential for the end-organ effects mediated by airway parasympathetic nerves.\textsuperscript{79,97} Preganglionic input may however, be modulated by other nerves innervating the airway ganglia, including afferent nerves and perhaps adjacent parasympathetic ganglia neurons.\textsuperscript{96,98,99} There is little evidence for sympathetic innervation of airway parasympathetic ganglia.\textsuperscript{25,100,101}

Many neurotransmitters, in addition to acetylcholine and noradrenaline, have been localized to autonomic nerve terminals in the airways. These transmitters have multiple effects on the end organs of the airways, and their role as neurotransmitters or neuromodulators has been confirmed in many instances.\textsuperscript{79} Studies in animals indicate that noncholinergic parasympathetic transmitters are not coreleased with acetylcholine from a single population of postganglionic parasympathetic nerves. Rather, an entirely distinct parasympathetic pathway regulates airway noncholinergic nerve activity.\textsuperscript{83,93,102–104} Preganglionic nerve subtypes and reflexes differentially regulate these distinct parasympathetic pathways.\textsuperscript{102,105–107} Circumstantial evidence indicates a similar arrangement of the parasympathetic innervation of human airways.\textsuperscript{94,108,109}

15.4.2 Monitoring Vagally Mediated Autonomic Reflexes in the Airways

15.4.2.1 Monitoring Reflex Regulation of Airway Smooth Muscle

Techniques for monitoring vagally mediated autonomic reflexes in the airways have been around for well over a century. Measurements of airway mechanics are most often the least invasive, and thus studies of reflex bronchospasm preceded studies of neuronal regulation of airways mucus secretion and bronchial blood flow by many decades. Early methods and results have been reviewed in detail elsewhere.\textsuperscript{110,111}

There are several methods for monitoring airway mechanics in conscious, anesthetized, or anesthetized and paralyzed animals. In spontaneously breathing animals, airway mechanics can be monitored either by whole-body plethysmography or, using
more invasive procedures, by monitoring tidal volume, airflow, and transpulmonary pressure.\textsuperscript{112–114} In anesthetized and paralyzed animals sustained by fixed-volume ventilation, airway inflation pressures or a variety of measures of airway mechanics can be monitored.\textsuperscript{34,115} Other methods for monitoring reflex-mediated alterations in airway mechanics (primarily in larger animals) include collateral airways resistance measured through a wedged bronchoscope and roentgenograms.\textsuperscript{116–118}

There are advantages and disadvantages to each of the methods used for monitoring whole lung mechanics. For example, in conscious guinea pigs, a substantial portion of resistance to airflow arises in the upper airways.\textsuperscript{119} Comparable problems are associated with whole-body plethysmography in conscious, unrestrained mice.\textsuperscript{120} These confounding variables make it challenging to attribute changes monitored using these methods to vagally mediated reflex effects on airway smooth muscle. The role of secretions in altering resistance to airflow is also problematic in measures of whole-lung function used to monitor reflex changes in airway smooth muscle tone. In mice, for example, allergen-induced hyperresponsiveness is associated with a marked increase in mucus secreting cells lining the airways.\textsuperscript{121,122} Mucus hypersecretion would likely obstruct the airway and would confound measures of airway mechanics used to study reflex regulation of airway smooth muscle tone.\textsuperscript{123}

There are important differences in the nature of gas movement through the airways in freely breathing and mechanically ventilated animals also worth noting. In conscious animals, expansion of the thorax surrounding the lungs creates a negative transpulmonary pressure gradient resulting in lung inflation, while lung deflation occurs through lung compression upon elastic recoil of the thorax, airways, and lungs. Lung compression may be actively facilitated or entirely passive in nature. Tidal volumes, respiratory rate, and residual volumes can change dramatically during spontaneous breathing depending on the demands for gas exchange and changes in airway caliber.\textsuperscript{124} By contrast, tidal volume, residual volumes (assuming there is no gas trapping), and respiratory rate are held constant during mechanical ventilation. A positive pressure introduced into the airways by a mechanical ventilator inflates the lung while lung deflation occurs only through recoil of the thorax and inflated lung. Although not quantified in any study to date, it seems likely that these very different models for inflation and deflation of the lung have a profound effect on the airway reflexes evoked.

Because many of the stimuli used to reflexively alter airway smooth muscle tone can directly affect airway mechanics and because of the limitations of measuring whole lung mechanics described above, methods for monitoring changes in smooth muscle tone \textit{in situ} in an isolated segment of the trachea have been developed.\textsuperscript{46,125–128} The primary advantages of this approach are that the effects on airway tone can be directly attributed to effects on smooth muscle and that the reflex responses evoked can be physically separated from the stimuli evoking the reflex. Another advantage is the ability to isolate physiologically and pharmacologically the trachea by introducing drugs selectively to the trachea through the tracheal mucosa or the isolated tracheal vasculature.\textsuperscript{46,128} It is also possible to combine techniques to simultaneously monitor airway smooth muscle tone, mucus secretion, and alterations in airway vascular tone.\textsuperscript{88} As neurally mediated alterations in tracheal smooth muscle tone accurately reflect autonomic tone throughout the airways,\textsuperscript{89,97,127} and because these
Reflexes Initiated by Activation of the Vagal Afferent Nerves

measures are typically more sensitive than the relatively blunt measures of whole-lung mechanics available, these isolated tracheal segments are optimal for studying vagal reflexes (Figure 15.4).

15.4.2.2 Monitoring Reflex Regulation of Mucus Secretion

Methods for measuring reflex regulation of airway mucus secretion are limited to studies of the extrathoracic trachea (Figure 15.4 and Figure 15.5). These methods can involve histological evaluation of mucus secretion post-mortem in excised tissues, measuring glycoconjugate release into the airway lumen, direct visualization of glandular secretion, or observation of hillock formation on the airway mucosa. A critical evaluation of the advantages and disadvantages of these methods is not possible, as only a few studies of nerve-mediated mucus secretion have ever been published. A number of factors likely contribute to the infrequent study of reflex regulation of mucus secretion, including the difficulty of the methods, their poor sensitivity, and the observation that, in general, stimuli that evoke reflex bronchospasm (e.g., RAR or C-fiber stimulation, cigarette smoke, bronchospasm, mechanical stimulation of the airways, hypoxemia) also evoke mucus secretion.

15.4.2.3 Monitoring Reflex Regulation of Pulmonary and Bronchial Blood Flow

The first direct measures of bronchial vascular tone occurred long after the initial studies of reflexes regulating mucus secretion and airway smooth muscle tone. These pioneering and most subsequent studies of neuronal regulation of bronchial blood flow are limited to species that are large enough (sheep, dogs, pigs) so that catheters can be introduced into a bronchial artery. More recently, laser
Doppler methods have been introduced that allow for measurements of blood flow in extrapulmonary airways of all species.\textsuperscript{153,159–163} More sophisticated methods for monitoring bronchial vascular function, including high magnification bronchovideomicroscopy and magnetic resonance imaging, have been developed but have not been used to study neuronal control of the vasculature.\textsuperscript{164,165}

15.4.3 VAGAL AFFERENT NERVE MEDIATED AUTONOMIC REFLEXES IN THE AIRWAYS

15.4.3.1 Autonomic Reflexes Regulating Airway Smooth Muscle Tone

Postganglionic parasympathetic nerves innervate airway smooth muscle from the trachea to the terminal bronchioles. When activated, airway parasympathetic-cholinergic nerves initiate marked contractions of airway smooth muscle throughout the airway.

**FIGURE 15.5** Airway parasympathetic nerve mediated, reflex-induced mucus secretion in the trachea of a dog. Mucus accumulates on the surface of the trachea (A) before and (B) after lung deflation (a stimulus of RARs) and bilateral microinjection of drug vehicle into the nucleus ambiguus. In the lower panels, mucus secretion was monitored (C) before and (D) after lung deflation following bilateral microinjection of the glutamate receptor antagonist CNQX into the nucleus ambiguus. (Reproduced from Reference 88. With permission.)
Refluxes Initiated by Activation of the Vagal Afferent Nerves

Reflexes Initiated by Activation of the Vagal Afferent Nerves

Tree. Sympathetic innervation of airway smooth muscle in humans and in several other species is either sparse or nonexistent. Thus, although human airway smooth muscle expresses abundant β-adrenoceptors (primarily β<sub>2</sub>-adrenoceptors), direct evidence of functional sympathetic (adrenergic) innervation of human airway smooth muscle is lacking. It seems likely therefore, that hormonal catecholamines are the primary ligand for the β-adrenoceptors expressed on human airway smooth muscle. 79

The only functional relaxant innervation of airway smooth muscle in many species including humans is provided by the parasympathetic nervous system. 79 Vagally mediated relaxations of airway smooth muscle are mediated by the gaseous transmitter nitric oxide (NO, synthesized from arginine by neuronal NO synthase) and peptides such as VIP, PACAP, and PHI. These nonadrenergic, noncholinergic relaxant responses can be evoked in airways from the trachea to the small bronchi.

Many stimuli initiate reflex alterations in airway parasympathetic-cholinergic nerve input to airway smooth muscle. 79 The homeostatic role of these reflexes is not readily apparent, but they may serve to optimize the efficiency of gas exchange, and may facilitate clearance mechanisms during cough by regulating airflow velocity. Bronchoconstrictors such as histamine, prostaglandin D2, leukotrienes, and even methacholine, for example, can initiate both cough and reflex bronchospasm. 19,21,50,86,87,118,174,176 Nociceptor stimulants such as capsaicin, bradykinin, hypertonic saline, and acid also initiate reflex bronchospasm and cough. 19,21,63,72,73,117,126,157,166,167 Other stimuli initiating reflex bronchospasm include chemoreceptor stimulation, esophageal afferent nerve stimulation, and upper airway afferent nerve stimulation. 75,79,107,125,127 (Figure 15.6, Figure 15.7, and Figure 15.8).

Stimuli initiating reflex bronchodilatation through activation of airway noncholinergic parasympathetic nerves include stimulants of RARs and airway nociceptors (e.g., histamine, bradykinin, capsaicin, serotonin). 46,79,105,107,166,168,169 By contrast, chemoreceptor stimulation has little or no effect on noncholinergic-parasympathetic nerve activity. 105,107 Activating skeletal muscle afferents (as might happen during exercise) as well as SAR activation by deep inspiration or sustained lung stretch (which may also happen during exercise) also initiate reflex bronchodilation, primarily by withdrawing baseline cholinergic tone. 127,166,170–172

Reflexes initiating alterations in airway sympathetic nerve activity are poorly described. 173 Nevertheless, adrenoceptors and endogenous catecholamines are known to regulate airway smooth muscle tone. 153,174,175 The relative contribution of hormonal and neuronal sources of the catecholamines is not clear in any species. 79

Regardless of the autonomic nerves recruited to initiate reflex bronchodilatation, a critical experimental design consideration is the need for a sustained constriction of the airways prior to reflex activation such that dilatation can be evoked. It is also critical that baseline cholinergic tone be blocked, as a withdrawal of cholinergic tone in the airways cannot be distinguished from an actively mediated relaxation of airway smooth muscle (Figure 15.6 and Figure 15.7).

15.4.3.2 Autonomic Reflexes Regulating Glandular Secretion

Airway glands are regulated primarily by the parasympathetic nervous system. Acetylcholine is the main transmitter regulating airway glandular secretion, but other
FIGURE 15.6 Baseline cholinergic tone and reflex-induced increases in cholinergic tone measured in the guinea pig trachea in situ. Airway smooth muscle tone was measured isometrically, while the lumen of the trachea was continuously perfused with warmed, oxygenated Krebs bicarbonate buffer. (A) Airway parasympathetic-cholinergic nerves are tonically active, producing a stable contraction that is dependent upon ongoing preganglionic nerve activity arising from nucleus ambiguus. Microinjecting glutamate receptor antagonists CNQX and AP-5 into nucleus ambiguus virtually abolishes baseline cholinergic tone (measured by adding atropine to the tracheal perfusate). (B) Coincident with the bronchospasm evoked by intravenously administered histamine (which activates rapidly adapting receptors), cholinergic tone in the trachea is increased. The histamine H1 receptor antagonist pyrilamine to the tracheal perfusate prevents the direct effects of histamine on the trachealis. Subsequent addition of atropine to the tracheal perfusate reverses baseline cholinergic tone and prevents the histamine-induced reflex, while having no effect on the bronchospasm evoked by histamine. TT: tracheal tension, PIP: pulmonary inflation pressure, ABP: arterial blood pressure.

(Reproduced from Reference 46. With permission.)
peptide neurotransmitters may regulate mucus secretion. Sympathetic nerves play little or no role in mucus secretion and transmitters associated with sympathetic nerves have subtle, if any, effect on secretion, but they may regulate parasympathetic nerve activity. In reflexes initiating parasympathetic nerve-dependent mucus secretion are induced by many of the same stimuli of airway nociceptors and RARs that initiate reflex bronchospasm. It thus seems possible that the post-ganglionic parasympathetic nerve regulating mucus secretion and smooth muscle tone in the airways are derived from the same subpopulations of airway parasympathetic nerves.

15.4.3.3 Autonomic Reflexes Regulating the Bronchial Vasculature

Sympathetic and parasympathetic nerves regulate bronchial vascular tone. Sympathetic nerves mediate vasoconstriction through the actions of noradrenaline and neuropeptide Y, while parasympathetic nerves mediate vasodilation by the actions of acetylcholine, NO, and perhaps VIP and related peptides. Reflex regulation of bronchial vascular tone is poorly described, in large part due to the difficulty with which the bronchial vasculature is studied. Airway nociceptor stimulation is, however, known to initiate parasympathetic reflex dilatation of the

---

**FIGURE 15.7** Chemoreceptor stimulation and lung stretch reflexively regulate airway smooth muscle tone in the cat. Intravenous injection of sodium cyanide activates the carotid body, which reflexively increases blood pressure (upper trace), increases pulmonary inflation pressure (upper middle trace), increases airway smooth muscle tone in the trachea (middle trace), and increases inspiratory efforts (lower middle trace, measured through phrenic nerve activity). Airway CO₂ levels are monitored continuously (lower trace). Increasing end-expiratory pressure, which activates SARs, has the opposite effect on airway smooth muscle tone and inspiratory activity. (Reproduced from Reference 127. With permission.)
Advances in Vagal Afferent Neurobiology

15.4.3.4 Cardiovascular Reflexes Initiated by Activation of Airway Vagal Afferent Nerves

Activation of airway vagal afferent nerves is also known to have effects on heart rate, systemic blood pressure, and pulmonary vascular tone. These reflexes may serve to optimize respiration and gas exchange. Activation of airway nociceptors initiates bradycardia, and coronary artery dilation and dilatation of the pulmonary arteries. By contrast, activation of SARs produces a tachycardia, perhaps through a withdrawal of parasympathetic tone. Extrapulmonary airway vagal afferent nerves may induce vasodilatation and a decrease in blood pressure by reducing sympathetic nerve activity.

REFERENCES


Refluxes Initiated by Activation of the Vagal Afferent Nerves


Reflexes Initiated by Activation of the Vagal Afferent Nerves


Reflexes Initiated by Activation of the Vagal Afferent Nerves


Axon Reflex and Neurogenic Inflammation in Vagal Afferent Nerves

Giovanni Piedimonte

CONTENTS

16.1 Introduction ..................................................................................................432
16.2 Tachykinin Peptides, Receptors, and Peptidase ........................................432
   16.2.1 Peptides ............................................................................................432
   16.2.2 Receptors ..........................................................................................433
   16.2.3 Peptidases .........................................................................................433
16.3 Distribution and Function ............................................................................434
   16.3.1 Peptides ............................................................................................434
   16.3.2 Receptors ..........................................................................................435
   16.3.3 Peptidases .........................................................................................436
16.4 Effects of Tachykinins in the Airway ..........................................................437
   16.4.1 Plasma Extravasation and Neutrophil Adherence ...........................437
   16.4.2 Blood Flow .......................................................................................438
   16.4.3 Bronchomotor Tone .........................................................................439
   16.4.4 Mucociliary Clearance .....................................................................440
   16.4.5 Cough ...............................................................................................440
16.5 Neurogenic Inflammation in the Pathophysiology of Airway Diseases .....441
   16.5.1 Respiratory Infections ......................................................................441
   16.5.2 Neuroimmune Interactions ...............................................................442
   16.5.3 Age-Dependency ..............................................................................443
   16.5.4 Bronchomotor Tone .........................................................................443
   16.5.5 Post-Viral Inflammation ..................................................................444
   16.5.6 Neuroimmunomodulation ................................................................445
   16.5.7 Mast Cells ........................................................................................446
   16.5.8 Neurotrophins ..................................................................................447
   16.5.9 The RSV-Asthma Link ....................................................................449
16.6 Therapeutic Strategies ..................................................................................450
   16.6.1 Glucocorticoids ................................................................................450
16.1 INTRODUCTION

Local stimulation of vagal polymodal nociceptive afferent neurons (C-fibers) in visceral tissues results in a series of coordinated biological responses aimed at expelling the noxious agents and starting the inflammatory and immune response. This constellation of events is referred to as neurogenic inflammation and is due to the local release of peptide neurotransmitters stored in terminal varicosities of these nerves. As a matter of expediency, the work reviewed in this chapter pertains primarily to the respiratory tract. It should be noted, however, that the fundamental principles underlying neurogenic inflammation are similar irrespective of the organs and tissue in which it occurs.

The first part of this chapter focuses on the receptors and enzymes involved in the regulation of the biological responses to inflammatory neuropeptides in the respiratory tract, their distribution, function, and effects. The second part reviews the experimental evidence of the involvement of neuropeptides in the cellular and molecular mechanisms of airway inflammation. In particular, this section focuses on the effects of respiratory infections on sensory innervation of the airways at different developmental stages and explores the pathophysiologic manifestations of these effects, such as changes in vascular permeability and bronchial responsiveness occurring during and after the infection.

Also reviewed are the interactions between the sensorineural pathways and different effector cell types involved in immune and inflammatory responses, such as lymphocytes, monocytes, and mast cells, and the critical role of neurotrophic factors in the coordination and modulation of these interactions. A new model deriving from these data is discussed that could explain some of the unique phenotypic features of asthma in childhood.

In the last part, attention is directed toward the possible therapeutic strategies aimed at controlling neurogenic inflammatory responses.

16.2 TACHYKININ PEPTIDES, RECEPTORS, AND PEPTIDASE

16.2.1 Peptides

Mammalian tachykinins include SP, neurokinin A (NKA), neurokinin B (NKB), and the N-terminal extended forms of NKA (neuropeptide K, NPK; neuropeptide γ- NPγ). Their agonist activity is associated with the shared carboxy terminal domain (Phe-X-Gly-Leu-Met-NH₂), whereas the amino terminal domains confer binding selectivity for different receptor subtypes.1 Two distinct preprotachykinin (PPT) genes control the synthesis of tachykinin peptides.2 While NKB is the only product
from the PPT-B gene, the PPT-A gene directs the synthesis of several different peptides. As a result of alternative RNA splicing, three different mRNA transcripts are produced from the PPT-A gene: the $\alpha$PPT-A mRNA can encode only SP, whereas the $\beta$PPT-A and $\gamma$PPT-A mRNAs can encode both SP and NKA. Post-translational processing of the $\beta$PPT-A mRNA product can yield NKA or NPK, while $\gamma$PPT-A mRNA can yield NKA or NP$\gamma$.\textsuperscript{3,4} Thus, by virtue of the alternative mRNA splicing and post-translational processing mechanisms, the two tachykinin genes generate a family of peptides with substantially diverse biological properties.

### 16.2.2 Receptors

Three mammalian neurokinin (NK) receptors have been cloned and their deduced protein sequences with seven putative trans-membrane spanning helices is that typical of G protein-coupled receptors.\textsuperscript{5} Their activation results in a rise in cytosolic calcium ion ($Ca^{2+}$) concentration mediated by the phosphatidylinositol pathway.\textsuperscript{6} Each of the tachykinin peptides can act as a full agonist on all three receptors, if present at sufficiently high concentrations. However, SP, NKA (also NPK and NP$\gamma$), and NKB, display preferential affinity for NK$\textsubscript{1}$, NK$\textsubscript{2}$, and NK$\textsubscript{3}$ receptors, respectively.\textsuperscript{7} The poor selectivity shown by naturally occurring tachykinins is probably due to sequence homology at the carboxy terminus, since synthetic agonists obtained by structural modifications of this domain exhibit high selectivity for individual receptors.

### 16.2.3 Peptidases

The biological effects of tachykinins are modulated by enzymes expressed on the surface of the target cells (Figure 16.1). This structural arrangement allows these enzymes to degrade peptide agonists in the proximity of their receptors, thus limiting their activity. Most neurogenic inflammatory responses are modulated to a large extent by two membrane-bound peptidases, the neutral endopeptidase (NEP; also called enkephalinase, common acute lymphocytic leukemia antigen, CALLA, EC 3.4.24.11) and the angiotensin converting enzyme (ACE; also called kininase II, dipeptidyl carboxypeptidase I, peptidyl dipeptidase A, EC 3.4.15.1). The best evidence of the pivotal role played by NEP and ACE in the control of neuropeptide actions is that inhibition by selective blockers, such as phosphoramidon\textsuperscript{8} and captopril,\textsuperscript{9} respectively, potentiates neurogenic inflammatory responses.

NEP and ACE share several structural characteristics. They both are zinc metalloproteins with variable degree of glycosylation, anchored to different cell types by a single hydrophobic membrane-spanning domain, with their catalytic site facing the extracellular milieu. Although mainly membrane-bound, low concentrations of both NEP and endothelial ACE are found in soluble form in body fluids. The Zn$^{2+}$ ion is an essential component of the catalytic site and is the common target of highly potent and selective inhibitors that employ phosphate (phosphoramidon), thiol (thiorphan, captopril), or carboxyalkyl (enalapril, lisinopril) Zn-coordinating groups.

NEP cleaves internal peptide bonds at the amino side of hydrophobic amino acids and ACE cleaves carboxy-terminal di- or tri-peptides. Thus, SP is cleaved at
Advances in Vagal Afferent Neurobiology

the Gln6-Phe7, Phe7-Phe8, and Gly9-Leu10 bonds by NEP,\textsuperscript{10,11} and at the Phe8-Gly9 and Gly9-Leu10 bonds by ACE.\textsuperscript{11} The principal catabolic fragment generated by enzymatic cleavage of SP (SP\textsubscript{1-9}) is biologically inactive.\textsuperscript{12,13} Unexpectedly, the tachykinins NKA and NKB, which share the same carboxy-terminal sequence of SP, are hydrolyzed by NEP but not by ACE.\textsuperscript{14,15} Due to the different efficiency of peptidases at cleaving different substrates, the rank order of potency of neuropeptides may depend not only on receptor affinity and density but also on the presence of peptidases on the target cells. For example, before the NEP inhibitor leucine-thiorphan, the rank order of tachykinin potency on airway smooth muscle contraction is: NKA > SP > NKB. After leucine-thiorphan, the rank order of potency changes to: NKA = NKB > SP.\textsuperscript{16}

16.3 DISTRIBUTION AND FUNCTION

16.3.1 PEPTIDES

The sensory nerves of the upper\textsuperscript{17} and lower\textsuperscript{18} respiratory tract of several species, including humans, contain SP-immunoreactive axons. The terminal varicosities of these unmyelinated C-type fibers are particularly abundant in the airway epithelium and around mucosal arterioles.\textsuperscript{19} In addition to SP, C-fibers also contain NKA, NPK, and possibly NP\textsubscript{γ},\textsuperscript{20} all very potent agonists of NK\textsubscript{2} receptors and more potent bronchoconstrictor agents than SP.\textsuperscript{21,22} A nontachykinin peptide also contained in C-fibers is the calcitonin gene-related peptide (CGRP),\textsuperscript{23,24} which is a potent vasodilator in the skin\textsuperscript{25} and gastrointestinal tract,\textsuperscript{26} but does not seem to have a direct

FIGURE 16.1 Interactions between peptides, receptors, and membrane-bound peptidases. The biological effects of substance P (SP) and other tachykinins are mediated by specific neurokinin (NK) receptors and modulated by enzymes expressed on the surface of the target cells. This structural arrangement allows these enzymes to degrade peptide agonists in the proximity of their receptors, thus limiting their activity.
effect on microvascular permeability and blood flow in the airways. A mixture of these sensory neuropeptides is co-released within the airway mucosa upon afferent stimulation by a variety of physical and chemical stimuli. Other peptide mediators are contained in efferent autonomic nerves, both parasympathetic (vasoactive intestinal polypeptide, VIP; peptide histidine-isoleucine, PHI) and sympathetic (neuropeptide Y, NPY). This complex peptidergic network represents a third nervous pathway serving the airways in parallel to the cholinergic and adrenergic components: the noncholinergic-nonadrenergic nervous system (NANC).

Of the SP produced in the cell bodies of the dorsal root ganglia and of the nodose ganglion, 90% is transported into the peripheral varicosities of afferent C-type fibers and travels to the lungs within the vagus nerve. Thus, SP acts both as the transmitter of nociceptive information from the lungs to the CNS and as a potent inflammatory mediator at the peripheral terminal. Upon sensory nerve stimulation, the action potential is conveyed to the spinal cord, but also spreads to the peripheral collaterals branching in the airway epithelium and around airway vessels and smooth muscle fibers, resulting in a cascade-like amplification of the original stimulus (the “axon reflex”).

Release of neuropeptides from afferent C-fibers can be induced in experimental models by electrical stimulation or by pharmacological stimulation by capsaicin (8-methyl-N-vanillyl-6-nonenamide). This pungent principal of plants of the Capsicum family exerts a dual action selectively on C-fibers: its acute effect is the Ca$^{2+}$-dependent release of neuropeptides from the granules stored inside the nerve varicosities; the chronic effect of exposure to high concentration of capsaicin is the permanent desensitization to capsaicin itself and other stimuli. These effects of capsaicin are mediated by binding to the vanilloid-like transient receptor potential potential channel type 1 (TRPV$_1$), a calcium-permeable channel expressed predominantly by C-type unmyelinated sensory neurons. This receptor responds to a variety of microenvironmental stimuli (chemical irritants, heat, acid pH) by co-releasing multiple neurotransmitters, including substance P, neurokinins, CGRP, vasoactive intestinal peptide (VIP), and somatostatin. Because multiple stimuli can converge on the same TRPV channel, these channels also function as the site of integration of diverse physical and chemical signals toward the same transduction pathway. This property of capsaicin to release and subsequently deplete peptides from sensory nerve endings has been pivotal in understanding the distribution and physiological roles of the tachykinins. Additional information has been obtained from the use of the inorganic dye Ruthenium red as a blocker of the capsaicin-sensitive cation channel and, more recently, with capsazepine, which is thought to be a selective antagonist of the capsaicin receptor on sensory nerves.

### 16.3.2 Receptors

Autoradiographic studies with radiolabeled ligands have visualized SP-binding sites in the respiratory tract of rats, guinea pigs, rabbits, and humans. However, the localization of specific NK receptor subtypes has been difficult because of the poor selectivity of radioligands. On the basis of the biological effects caused by selective agonists, or blocked by selective antagonists, it can be inferred that NK$_1$ receptors,
having a pivotal role in neurogenic inflammatory responses, are predominantly expressed on endothelial cells, vascular smooth muscle cells, submucosal glands, and circulating leukocytes. The NK₂ receptors, affecting bronchoconstriction and facilitating pulmonary cholinergic neurotransmission, are expressed at the highest density on airway smooth muscle cells, on neurons in the parasympathetic autonomic ganglia, and on postganglionic neurons. Finally, NK₃ receptors have been found in the CNS, in the gastrointestinal tract, and in the vascular smooth muscle, but they have yet to be identified in the respiratory system.

16.3.3 Peptidases

The lungs contain the highest levels of both NEP and ACE activities in the body.⁴⁵,⁴⁶ Both peptidases are also present in tracheal homogenates, but in markedly reduced concentrations.⁴⁷ Immunohistochemical staining and enzyme assays have revealed the presence of NEP in different substructures within the respiratory tract, including the alveolar epithelium,⁴⁸ tracheal epithelium,¹⁶,⁴⁹ tracheal smooth muscle,¹³,¹⁶ and submucosal glands.⁵⁰ ACE seems to be concentrated along the luminal surface of the vascular endothelium.⁴⁸,⁵¹,⁵² Coexpression of ACE and NEP has been reported in arterial and venous endothelium.⁵³ In sections of rat trachea, immunoreactive NEP can be seen overlapping areas of Monastral pigment extravasated after an injection of SP (Figure 16.2). This pigment is a suspension of particles that leave the bloodstream only through the gaps that form along the endothelium of postcapillary venules in response to inflammatory mediators. The particles of pigment that extravasate remain trapped within the vessel wall, thereby labeling the sites of extravasation.⁵⁴ Thus, NEP is co-expressed with ACE⁵⁵ in the postcapillary venular endothelium, the elective site of mediator-induced extravasation and leukocyte adhesion.

As described in the previous section, the substrate specificity of NEP and ACE is largely overlapping. However, because both peptidases are membrane-bound, these enzymes can act only in the proximity of the cell surfaces where they are expressed. Thus, their selectivity and efficiency in vivo is restricted by the geometrical factors that determine the accessibility of the enzyme to potential substrates. ACE, which is predominantly an endothelial peptidase, modulates the biological activity of blood-borne substrates either injected⁵⁶–⁵⁸ or endogenously released.⁵⁹ In contrast, NEP, although expressed in the endothelium, seems to play a more relevant physiological role in the extra-vascular compartments. For example, in the airway epithelium NEP is concentrated in the basal cell layer (Figure 16.2), thus in a strategic position to cleave and inactivate the neuropeptides near their sites of release from the network of sensory nerve terminations concentrated in close association with the basal cells.¹⁹ In fact, removal of the airway epithelium has been shown to potentiate the contractile activity of tachykinins on the airway smooth muscle.¹⁶,⁶⁰,⁶¹ Furthermore, the changes in airway resistance produced by aerosolized SP⁶² or tracheal infusion of capsaicin⁶³ are modulated exclusively by NEP, indicating that this peptidase represents the main barrier against tachykinins released within or in proximity to the epithelial layer. However, even after removal of the airway epithelium, inhibitors of NEP still potentiate the bronchomotor effects of endogenous and
exogenous tachykinins. Thus, as neuropeptides diffuse from release sites to their receptors, the enzymatic cleavage by NEP on the surfaces of the target cells reduces the concentration of active peptide.

16.4 EFFECTS OF TACHYKININS IN THE AIRWAY

16.4.1 Plasma Extravasation and Neutrophil Adherence

Electrical stimulation of the cervical vagus nerve or pharmacological stimulation of sensory nerves with capsaicin results in complex changes in the tracheal microvasculature of rodents, leading to increased permeability to plasma macromolecules and increased number of neutrophils adhering to the endothelium. Both components of this neurogenic inflammation are blocked by selective antagonists of the NK$_1$ receptor subtype, and thus are mediated by the natural NK$_1$ ligand, SP. SP is known to cause complex changes in the endothelium of postcapillary venules, which represent the elective targets of inflammatory mediators. SP opens gaps in the endothelial lining, presumably by inducing contraction of endothelial cells. SP also promotes endothelial-leukocyte interaction via increased expression of adhesion molecules. Both effects are probably due to SP-induced mast cell degranulation with consequent release of tumor necrosis factor α (TNF-α) and cysteinyl leukotrienes.

Selective NK$_1$ receptor antagonists reduce the exudative response to a variety of inhaled irritants that are known to trigger asthma episodes in humans. For example, NK$_1$ antagonism inhibits airway plasma leakage induced by cigarette
smoke. Aerosolized hypertonic saline, which reproduces the pathophysiologic mechanism of exercise-induced asthma, also causes plasma extravasation in the rat airways that is abolished by pharmacologic sensory denervation with capsaicin or by pretreatment with an NK$_1$ receptor antagonist. NK$_1$-mediated plasma extravasation occurs in sensitized guinea pigs after challenge with aerosolized ovalbumin. The importance of the NK$_1$ receptor in the organization of pulmonary inflammatory reactions has been highlighted more recently by the observation that gene-targeted deletion of this receptor in mice prevents the acute inflammatory responses associated with immune complex-mediated lung injury, including plasma extravasation and neutrophil influx. These studies expand an already large body of evidence indicating that SP and other neuropeptides play an important modulatory role upstream from other inflammatory pathways in the respiratory tract and suggest that SP may play a role in the inflammatory processes affecting the distal airways and the lung parenchyma, whereas most previous studies had focused on the large airways.

NEP and ACE are co-expressed on the postcapillary venular endothelium, the elective site of mediator-induced plasma extravasation and leukocyte adhesion. Inhibition of NEP or ACE potentiates the increase in vascular permeability and the adhesion of neutrophils to the vascular endothelium produced by sensory nerve stimulation or by exogenous injection of SP. Simultaneous inhibition of both enzymes potentiates these effects more than a maximally effective dose of either inhibitor by itself. Differently from ACE, NEP is also expressed on the plasma membrane of neutrophils and seems to regulate their migration outside blood vessels. However, inhibition of this enzyme leads to different effects depending on the type of chemotactic substrate. In fact, phosphoramidon increases the number of neutrophils migrating after stimulation of capsaicin sensitive nerves, whereas it abolishes chemotaxis in response to the bacterial tripeptide formyl-Met-Leu-Phe.

16.4.2 Blood Flow

Blood flow in the airway microvascular bed may be a critical component of neurogenic inflammation. Changes in microvascular blood flow may affect the recruitment of inflammatory cells and mediators and the edematous swelling of the respiratory mucosa. On the other hand, the increase in blood flow may also have the protective function of washing away inflammatory mediators and exogenous irritants from inflamed tissues, thus limiting the extent and duration of acute inflammatory and hypersensitivity responses.

The endogenous release of neuropeptides from capsaicin-sensitive sensory afferents greatly increases blood flow in the microvascular bed of the rat extrapulmonary airways. Fivefold increase in airway blood flow is produced by a low dose of capsaicin (10$^{-7}$ mol/kg), which does not alter regional blood flow to other organs; thus, the resistance vessels regulating the blood flow to the extrapulmonary airways in the rat are more responsive to sensory neuropeptides than similar vessels in other organs. In addition, this dose of capsaicin does not increase airway vascular permeability; thus, the threshold nerve stimulation necessary to induce plasma exudation is much higher than that necessary to increase local blood flow. These observations
suggest that the intensity of noxious stimuli qualitatively modulates neurogenic inflammatory responses. Mild stimuli may be eliminated by increased blood flow, whereas stronger, persistent stimuli may require the activation of additional defense mechanisms, such as the recruitment of inflammatory cells and mediators through more permeable vessels. The fact that neuropeptides do not affect intrapulmonary bronchial blood flow, together with previous observations showing that antidromic vagal stimulation evokes plasma extravasation only in the proximal airways, indicate that neurogenic inflammation does not occur in the distal airways of adult lungs, probably due to sparse afferent innervation. In contrast, in young animals the highest density of sensory innervation is found in the distal airways, and this “inverted” distribution of afferent fibers may explain, at least in part, the different manifestations of airway inflammatory disease in childhood versus adulthood.

The vasodilator effect of capsaicin in the airways is reduced by pretreatment with a cocktail of autonomic inhibitors (atropine, guanethidine, and hexamethonium), suggesting that a vasodilator reflex is activated following sensory nerve stimulation, probably mediated by VIP released from parasympathetic efferents. Despite the fact that SP and CGRP immunoreactivities coexist in perivascular C-fibers and are co-released when these nerves are stimulated with capsaicin, only SP increases the blood flow in the airway microvascular bed, whereas CGRP does not produce significant changes. Studies with a selective receptor antagonist (CP-99,994) confirmed that neurogenic vasodilation, like the other vascular effects of sensory nerves in the airways, is due to the effect of SP on NK1 receptors.

In rats pretreated with captopril to inhibit ACE activity, the vasodilator effect of SP on airway microvessels is increased by approximately one order of magnitude. More important, ACE inhibition affects the duration of SP-induced vasodilation. In fact, the vasodilator effect of both SP and capsaicin is very transient, and no change in airway blood flow can be demonstrated 5 min after injection of either drug. In contrast, airway blood flow remains elevated 5 min after SP is injected in captopril-pretreated animals. Similar potentiation of airway blood flow is obtained using the selective NEP inhibitor, phosphoramidon. In contrast, the relaxant effect of SP in isolated pulmonary arteries is potentiated by selective ACE inhibition, but not by inhibition of NEP or other enzymes known to degrade vasoactive peptides, suggesting regional differences in the endothelial expression and functional importance of peptidases along the respiratory tract. On the basis of the dramatic effect of captopril on the vascular changes produced by SP, it can be speculated that potentiation of neurogenic inflammation may be one cause of the increased bronchial reactivity associated with the chronic therapeutic use of ACE inhibitors in humans.

### 16.4.3 Bronchomotor Tone

Tachykinins released from sensory nerves contract the airway smooth muscle. In isolated human bronchi, this effect is mimicked by selective NK2 agonists and blocked by selective NK2 antagonists, whereas NK1 agonists and antagonists are ineffective. Although the NK2 receptor can be activated by both SP and NKA, the latter is the most potent natural agonist of this receptor.
The contraction induced in isolated segments of ferret tracheal smooth muscle by exogenous tachykinins and by electrical nerve stimulation is potentiated by the NEP inhibitor leucine-thiorphan dose dependently. The effect of electrical stimulation is abolished by atropine and by tachykinin antagonism but is not affected by adrenergic or ganglion blockers; thus this effect is likely to be due to post-ganglionic stimulation of cholinergic nerves by tachykinins. In contrast, electrical nerve stimulation of guinea pig bronchi evokes early cholinergic contraction followed by long-lasting sensory nerve-dependent contraction that is mediated by NK1, NK2, and possible additional NK receptors. NEP inhibitors potentiate the contractile effect of capsaicin and electrical nerve stimulation in guinea pig bronchi, whereas inhibitors of ACE, aminopeptidases, acetylcholinesterase, and serine proteases are without effect.

These observations, together with other similar in vitro studies, indicate that endogenous tachykinins induce airway smooth muscle contraction and that this effect is modulated by NEP predominantly. However, the relative importance of different peptidases seems to be determined by geometric factors. The NEP inhibitor phosphoramidon given by aerosol or by intravenous injection potentiates the increase in total pulmonary resistance produced by exogenous SP. Aerosolized phosphoramidon also potentiates the atropine resistant response to vagus nerve stimulation. Captopril potentiates the effect of SP only when this peptide is given by intravenous injection or released by vagal stimulation, but does not have any effect on aerosolized SP. These findings probably reflect the different distribution of the two peptidases in the airway tissues, and confirm the predominance of ACE activity in the intravascular compartment and of NEP activity in the extravascular compartments.

### 16.4.4 Mucociliary Clearance

Tachykinins stimulate mucus secretion and ciliary beat frequency through activation of NK1 receptors. The first studies on the modulating role of NEP in neurogenic inflammation were in vitro studies of airway glands secretion. Inhibitors of NEP potentiate the effect of SP on mucus secretion in the ferret trachea, whereas inhibitors of other proteases (including the ACE inhibitors captopril and teprotide) have no effect. The fact that the N-terminal fragment SP1-9 generated by NEP does not induce gland secretion provides additional evidence that NEP cleavage generates inactive SP metabolites in this system. In addition, NEP inhibition potentiates the effect of bradykinin and SP on ciliary motility. The important regulatory influence exerted by NEP on different aspects of the airway mucociliary function and cough reflex suggest that this enzyme may play a key role in the defense of the airway mucosa against particulate irritants.

### 16.4.5 Cough

Although cough is one of the most conspicuous manifestations of lung diseases associated with inflammation (e.g., asthma, bronchitis, cystic fibrosis, viral infections), the mechanisms underlying this protective reflex are poorly understood. In
unanesthetized guinea pigs, inhalation of capsaicin and SP aerosols causes cough, and this effect is potentiated after inhalation of aerosols of NEP inhibitors, presumably by inhibiting the NEP present in the guinea-pig airway epithelium. A similar pathophysiologic mechanism can be proposed to explain the dry cough observed in humans as a well-known side effect of chronic therapy with ACE inhibitors.

16.5 NEUROGENIC INFLAMMATION IN THE PATHOPHYSIOLOGY OF AIRWAY DISEASES

16.5.1 RESPIRATORY INFECTIONS

Viral respiratory infections frequently cause exacerbations of asthma, particularly in children, and have been implicated in the pathogenesis of this disease. The following paragraphs review the effects of respiratory infections on the magnitude of the responses evoked by exogenous and endogenous tachykinins in the smooth muscle and microvascular bed of the airways.

The contractile response to SP of ferret tracheal segments increases by threefold following incubation in vitro with human influenza virus A1 Taiwan. Pretreatment with an NEP inhibitor increases the response to SP to the same final level in both infected and control tissues, whereas inhibitors of other peptidases (including ACE) are ineffective. Furthermore, NEP activity is decreased in infected tissues. In vivo, pathogen-free guinea pigs selectively infected with murine parainfluenza type I (Sendai) virus develop bronchial hyperreactivity to tachykinins associated with decreased airway NEP activity 4 days after inoculation. These findings suggest that these viral infections may potentiate the contractile response to tachykinins by decreasing the degradative activity of NEP.

The responses induced by tachykinins in the airway microcirculation are similarly affected by respiratory infections. The permeability of tracheal blood vessels to intravascular tracers was initially studied in rats with antibody titers showing natural exposure to three common murine pathogens: Sendai virus, rat coronavirus, and Mycoplasma pulmonis. Past exposure to these infectious agents is not associated with changes in baseline airway vascular permeability. However, the increase in tracheal vascular permeability evoked by nerve stimulation or by SP is potentiated markedly compared with pathogen-free controls. Similarly, in rats exposed to respiratory infections, nerve stimulation evokes a much larger increase in the number of neutrophils adhering to the venular endothelium, preparing to migrate into the perivascular tissues. Again, NEP inhibition increases the SP-induced change in vascular permeability in pathogen-free rats, but does not further amplify the already large increase in rats exposed to infections. NEP activity is decreased in the tracheal epithelium of infected rats compared with pathogen-free rats, but no difference in enzyme activity can be detected in the tracheal submucosa and in the esophagus. Exaggerated neurogenic plasma extravasation and leukocyte adhesion can be reproduced by selectively inoculating pathogen-free rats with Sendai virus or with Mycoplasma pulmonis. The long-lasting potentiation produced by the latter organism may be due in part to structural changes in the tracheal microvasculature (i.e., increased number of postcapillary venules).
Sendai virus infection potentiates the increase in airway blood flow induced by SP but not histamine. In contrast with the previous observations of the effect of infections on smooth muscle contraction and vascular permeability, after inhibition of the NEP activity alone SP-induced airway vasodilation in pathogen-free rats is still significantly reduced compared with infected rats, whereas inhibition of both NEP and ACE potentiates the effect of SP to the same final level in both infected and control animals. Thus, the effect of Sendai virus infection on airway resistance vessels may be due to a combined downregulation of the activities of both peptidases.

Collectively, these studies indicate that influenza and parainfluenza viruses potentiate neurogenic inflammatory and hypersensitivity responses, and propose the associated decrease in peptide-degrading enzyme activity as the cause for the increased responses. The decrease in NEP activity does not appear to be due to the release of a protease inhibitor produced during viral replication because the supernatant from tracheal rings infected with influenza virus does not affect the activity of purified NEP. Since viral infections are commonly associated with extensive damage of the respiratory epithelium, it is possible that the increased responses to endogenous and exogenous tachykinins are due in part to the physical loss of NEP-rich epithelium during viral replication. However, this mechanism alone cannot explain the increased response to intravenous SP, the potentiation produced by *Mycoplasma pulmonis* in the absence of significant epithelial damage, or the down-regulation of endothelial ACE activity. Therefore, additional mechanisms have to be involved in the interaction between infectious agents and peptidases.

### 16.5.2 Neuroimmune Interactions

Lower respiratory tract infections with RSV cause strong potentiation of neurogenic-mediated inflammation, as manifested by the exaggerated increase in microvascular permeability in response to endogenous and exogenous SP observed 5 days after inoculation of the virus. This potentiation of neurogenic inflammation is similar to that outlined above for other respiratory pathogens (influenza and parainfluenza viruses), however, RSV does not affect the enzymatic activity of NEP, as seen with the other viruses, and therefore its effect cannot be explained with decreased catabolism of the SP released from nerve fibers. Rather, our data suggest that the potentiation of neurogenic inflammation associated with the presence of RSV in the respiratory tract involves a different postsynaptic mechanism(s), independent from the activity of peptide-degrading enzymes.

SP binds with high affinity to the NK-1 tachykinin receptor subtype. RT-PCR analysis has revealed that the expression of the gene encoding this receptor is strongly upregulated in the lung tissues of rats inoculated 5 days earlier with RSV. At the same time, the density of binding sites for SP visualized by autoradiography is markedly increased in the bronchial mucosa of RSV-infected lungs. Selective antagonism of the NK-1 receptor abolishes the effect of RSV on airway neurogenic inflammation, confirming that this effect requires SP/NK-1 receptor interaction.

In adult rats, the potentiation of capsaicin-induced neurogenic inflammation during RSV-infection can be detected in the extrapulmonary, but not in the intrapulmonary airways. This observation suggests that the density of capsaicin-sensitive
C-type nerves is highest in the proximal airways and progressively decreases in more distal airways, confirming previous observations. However, intrapulmonary airways exposed to RSV respond to exogenous SP with a large increase in vascular permeability, suggesting that RSV-infected distal airways become hyperresponsive to intravascular SP even in the absence of specific innervation. This is consistent with other studies demonstrating a significant mismatch in the localization of SP-containing nerves and SP receptors in the brain and lungs of adult rats.

16.5.3 Age-Dependency

Stimulation of capsaicin-sensitive sensory nerves 5 days after inoculation causes a much larger increase of Evans blue-labeled albumin extravasation in the intrapulmonary airways of RSV-infected weanling rats compared with age-matched pathogen-free controls. In contrast, analysis of the extrapulmonary airways shows no difference in extravasation between infected and pathogen-free weanling rats. Therefore, the response to capsaicin of airway blood vessels in young rats is qualitatively different from adult rats. In separate experiments, we also found that the conversion to the adult-type pattern is completed by 48 to 50 days of age. The differences found between young and adult rats suggest age-dependent variability in the distribution of tachykinin-containing sensory nerves along the respiratory tract. We speculate that the different clinical manifestations of RSV disease in children (lower respiratory tract involvement with bronchiolitis) versus adults (usually upper airway symptoms) may at least in part result from age-related molecular differences in one or more inflammatory pathways, rather than be determined by simple anatomical factors (e.g., airway caliber).

In our weanling rat model, semi-quantitative RT-PCR analysis of the NK-1 receptor mRNA extracted from lung tissues reveals a consistently much stronger signal in RSV-infected weanling rats than in pathogen-free controls. Pretreatment with a monoclonal antibody against the F protein of RSV prevents upregulation of the NK-1 receptor. In contrast, VIP receptor expression is only slightly elevated in RSV-infected weanling rats compared with the pathogen-free controls. These data suggest that RSV differentially modulates the expression of specific neuropeptide receptors causing an imbalance between the proinflammatory (SP-dependent) and antiinflammatory (VIP-dependent) components of the NANC nervous system in the respiratory tract and favoring the development and maintenance of airway inflammation. In addition, because SP and VIP modulate several immune functions by exerting opposite influences (stimulatory for SP, inhibitory for VIP), the peptidergic imbalance caused by RSV may link the neurogenic and immuno-inflammatory mechanisms proposed by different authors to explain the pathophysiology of RSV disease and its sequelae.

16.5.4 Bronchomotor Tone

The high-affinity NKA receptor (NK-2 subtype) is expressed on smooth muscle cells and mediates NANC bronchoconstriction. RSV does not affect the expression of the NK-2 receptor subtype, as indicated by: a) the lack of change in the expression of
NK-2 receptor mRNA in infected lung tissues\textsuperscript{117}; and b) the sparse binding of radiolabeled ligand to the airway smooth muscle in infected bronchioles.\textsuperscript{113}

To confirm these findings, we have measured NKA-induced bronchoconstriction in our model of RSV bronchiolitis.\textsuperscript{118} No significant difference was found in NK-2 receptor mRNA from lung tissues or in NKA-induced bronchospasm between RSV-infected and pathogen-free rats.\textsuperscript{118} These data indicate that RSV does not affect NANC bronchoconstriction in the F-344 rat model. Based on this data, we speculate that bronchial obstruction in RSV bronchiolitis is primarily a function of inflammatory edema of the airway mucosa rather than constriction of the airway smooth muscle tone, which would also explain the frequent lack of clinical response to bronchodilators in infants with viral bronchiolitis.

16.5.5 Post-Viral Inflammation

Despite complete resolution of the infection, the potentiation of neurogenic-mediated inflammatory reactions is still present 30 days after inoculation of the virus, as manifested by the exaggerated increase of microvascular permeability in response to sensory nerve stimulation.\textsuperscript{119} This potentiation is not only qualitatively, but also quantitatively similar to the response measured during the acute phase of the infection. Similar long-term potentiation is observed in adult rats that had developed RSV infection during the weanling period. Surprisingly, the vascular response to exogenous SP is no longer potentiated 30 days after the infection. Although the expression of the NK-1 receptor remains slightly increased after resolution of the acute infection, this effect is no longer statistically significant, and thus it is unlikely to be the primary mechanism responsible for the post-infection changes in neurogenic inflammation. However, enzyme-linked immunoassay reveals a large increase of SP concentration in the lung tissues of rats during and after infection with RSV. Also, capsaicin-induced release of SP remains abnormal after the infection and is approximately twice that measured in pathogen-free controls injected with the same dose of capsaicin at 30 days postinoculation, suggesting structural and/or functional remodeling of the sensory innervation serving the airways. These findings are consistent with the hypothesis that the mechanism of post-infectious potentiation of neurogenic inflammation operates predominantly at the presynaptic level with minor involvement of the post-synaptic component active during the acute phase of the infection.

The experimental evidence supporting a presynaptic mechanism for the post-viral potentiation of neurogenic inflammation is threefold:

- Lack of significant vascular response at 30 days postinoculation of RSV to the same dose of SP that caused large exudation 5 days postinoculation
- Lack of persistent elevation of mRNA encoding the NK-1 receptor 30 days postinoculation
- Increased content and increased release of SP from capsaicin-sensitive sensory nerves in lung tissues 30 days postinoculation
16.5.6 NEUROIMMUNOMODULATION

Immunohistochemical and autoradiographic mapping of NK-1 receptor expression in RSV-infected airways shows clusters of cells staining deeply with an anti-NK-1 antibody and binding radiolabeled SP avidly, indicating that RSV induces overexpression of the NK-1 receptor on selected lymphocyte subpopulations within the BAL, thus “priming” these cells for the immunomodulatory effects of substance P. Based on this observation, it is likely that release of this peptide from the dense supply of afferent fibers innervating the mucosal lymphoid aggregates by airborne irritants inhaled during lower respiratory tract infections initiates a cascade-like sequence by recruiting the immunocytes primed by the virus. In fact, we have found that sensorineural stimulation during RSV infection leads to a markedly increased flux of lymphocytes and monocytes into the airways, with approximately 20-to-1 monocytes/lymphocytes ratio. This neurogenic-mediated recruitment of immunocytes is already measurable 1 day after nerve stimulation, peaks by day 5, and remains significant 10 days after a single stimulus, whereas in the absence of sensorineural activation, immunocyte recruitment by the infection alone is much more limited and transient. Thus, not only is the degree of the cellular response to the virus magnified by concurrent nerve irritation, but this response is also sustained and persists after nonstimulated airways have returned to normal. The simultaneous reduction of both CD4+ and CD8+ cells recovered from infected lung tissues suggests that the lymphocytes flowing into the airways originate from the mucosal immune system.

Neurogenic-mediated recruitment of immunocytes into infected airways is abolished by selective pharmacological antagonism of the NK-1 receptor, indicating that substance P binding to its high-affinity receptor is primarily responsible for this effect. The influence of substance P on immunocyte recruitment during RSV infection appears to be not only quantitative, but also qualitative as suggested by the disproportionate number of CD4+ cells versus CD8+ cells found in the BAL. However, the effect of sensory nerves on both CD4+ and CD8+ cells appears to resolve rapidly after stimulation with capsaicin, and therefore the late surge in lymphocyte counts measured 5 and 10 days later could not be accounted for in the flow cytometry data. There does not appear to be a CD3+ but CD4−, CD8− population that we could identify to account for the difference seen, and we were also able to determine that the cells in question are not of B cell lineage using antibodies against B cell-specific markers. It should be noted, however, that we set a very stringent lymphocyte gate on our samples based on resting spleen cells in an effort to overcome the background signal and obtain a true count of CD4+ and CD8+ cells, and the limited gate may have excluded activated lymphocytes from our flow cytometry analysis at days 5 and 10. Therefore, the differences noted between differential cell counts and flow cytometry data are probably related to the presence of activated lymphocytes and/or natural killer (NK) cells.

Both lymphocytes and monocytes recruited in the infected airways may amplify and propagate the chemotactic and modulatory functions of sensory nerves in an autocrine and/or paracrine fashion, as they are capable of producing and releasing substance P. Also, the subsequent activation of these cells with release of
cytokines and chemokines is likely to be responsible for the second phase, characterized by the build-up in the airways of a large population of activated lymphocytes and monocytes. The neurogenic-mediated recruitment of monocytes is of special interest, as these cells can promote airway inflammation and modulate immune responses via the production of cytokines like tumor necrosis factor alpha (TNFα),124,125 and interleukins 1 (IL-1),126 IL-6,124 IL-8,124 and IL-10.127 In addition, RSV-infected monocytes may amplify and spread the infection by delivering a greater load of viral particles to the host tissue than the initial load infecting the monocyte itself.128

16.5.7 Mast Cells

Histopathological analysis of lung tissues stained with an antibody against tryptase shows numerous mast cells in the sections from RSV-infected rats, with an average sevenfold increase compared with the lungs of pathogen-free controls.129 Most of these mast cells are in close spatial associations with nerve fibers (Figure 16.3), suggesting functional mast cell-nerve interactions similar to those previously reported in other organ systems,130 particularly the skin, central nervous system, and gastrointestinal tract.131

Among the mast cell-derived inflammatory mediators, leukotrienes appear to play a particularly important role in virus-infected airways.129 However, time course analysis of infected lung tissues suggests that the effect of RSV on 5-LO gene expression and leukotriene synthesis is transient, being maximal at 3 days postinoculation and already resolved at 5 days. Also, a significant increase in transcripts encoding the cys-LT1 receptor can be measured by RT-PCR from RSV-infected lung tissues (Figure 16.4). The exaggerated neurogenic inflammation in the intrapulmonary airways of
young rats infected with RSV involves the concomitant release of cysLTs and activation of the cys-LT1 receptor, as manifested by the potent inhibitory effect of the receptor antagonist montelukast on capsaicin-induced plasma leakage.\textsuperscript{129} Montelukast also has a much smaller, but still significant, inhibitory effect in the intrapulmonary airways of RSV-infected adult rats, whereas no effect is found in the extrapulmonary airways of either young or adult rats. The increased susceptibility of the intrapulmonary airways of RSV-infected young rats to the inflammatory effects of sensory nerves may be dependent, at least in part, on higher density of nerve-mast cells synapses compared with older rats.

Collectively, our observations suggest that, following the early phase of the viral infection, leukotriene production/release returns rapidly to baseline levels, but can be reactivated by neurogenic stimulation of the numerous mast cells that had differentiated along the airways during the infection. The consequent release of cysLTs can in turn amplify the release of tachykinins from sensory nerves\textsuperscript{132} forming local neuron-mast cell feedback loops. In addition, since it has been shown that also non-neuronal cells such as monocytes and macrophages express SP and its receptors and release this peptide in response to capsaicin stimulation,\textsuperscript{123} it is possible that cells involved in the immune and inflammatory response to the infection contribute to this mechanism of mast cell activation. Finally, RSV may induce qualitative/quantitative changes in mast cell-nerve synapses, such as upregulation of NK-1 receptor expression on mast cell membranes analogous to epithelial cells,\textsuperscript{113} vascular endothelial cells,\textsuperscript{113} and T lymphocytes\textsuperscript{133} in infected airways.

### 16.5.8 Neurotrophins

Recent studies have shown that the expression of nerve growth factor (NGF) and its receptors trkA and p75 in lung tissues decreases progressively with age.\textsuperscript{134}
Weanling rats have approximately a twofold higher level of NGF mRNA and protein than adult rats, and the adult expression pattern for these molecules is reached by 8 weeks of age. The expression of the high-affinity receptor trkA parallels that of its ligand, whereas the decline in the expression of the low-affinity p75 receptor is steeper and its mRNA level is more than fourfold lower in adult rats than in weanling rats. These findings are consistent with previous reports of minimal neurotrophin receptor expression in adult lung tissues and show a different profile of developmental maturation in the lungs compared with other non-neuronal tissues, e.g., the thymus where the expression of neurotrophin receptors peaks at 12 weeks of age in rats. This genetic blueprint can be modified dramatically by early-life viral infections.

In fact, a strong increase of NGF and neurotrophin receptors expression can be found in lungs infected with RSV 5 days after intranasal inoculation of the virus. Furthermore, the characteristic changes in NK-1 receptor expression in rats infected with RSV responsible for the potentiation of neurogenic inflammation are mimicked by the administration of exogenous NGF in the absence of infection, and blockade of NGF activity with a selective antibody inhibits both NK-1 receptor upregulation and the potentiation of neurogenic plasma extravasation in infected lungs, suggesting that NGF plays an important role in the mechanism of airway inflammation during RSV infection. This new observation can explain the report of NGF-induced airway hyperresponsiveness in guinea pigs, which is completely blocked by selective pharmacologic antagonism of NK-1 receptors. UV inactivation of the viral nucleic acid hinders the effect of RSV on the expression of NGF and its receptors in the lungs, suggesting that the changes observed in the neurotrophin pathways are linked to active viral replication and to expression of the viral genome in the respiratory epithelium.

Although the relative magnitude of the changes caused by RSV in the neurotrophin system is comparable in weanling and adult rats, absolute mRNA levels encoding NGF and its receptors are much higher in RSV-infected weanling rats due to the different baseline. Furthermore, the increase in NGF protein concentration and the inhibitory effect of anti-NGF on neurogenic plasma extravasation is greater in the lungs of weanling rats, suggesting that early-life RSV infections have a more profound influence on neurotrophin systems and lung development. The higher susceptibility of weanling rats appears to derive from a higher degree of plasticity of the peripheral nervous system of young animals.

Because NGF is released from airway epithelial cells, increases the production and release of SP and other tachykinins from adult sensory neurons, and induces sensory hyperinnervation in the airways of transgenic mice, it represents the ideal link between virus-infected respiratory epithelium and the dense subepithelial network of unmyelinated sensory fibers. Overexpression of NGF and its low- and high-affinity receptors during RSV infection can generate long-term consequences via: a) upregulation of the preprotachykinin A (PPT-A) gene encoding the precursors of SP and NKA; and b) upregulation of the genes encoding vanilloid-type transient receptor potential (TRPV) channels, which increases the responsiveness of sensory fibers and the release of proinflammatory peptides upon stimulation by airborne irritants.
Measurements of microvascular permeability in the intrapulmonary airways have confirmed that neurogenic inflammation is more prominent in the lungs of RSV-infected weanling rats and it is abolished by NGF blockade, whereas in the lungs of RSV-infected adult rats the magnitude of the neurogenic plasma extravasation and the inhibitory effect of NGF blockade are relatively small. These findings are consistent with the hypothesis that NGF plays an important role in the mechanism of neurogenic inflammation during RSV infection and that the magnitude of its effect is age-dependent, thus justifying the different distribution of sensory afferents across the respiratory tract at different ages.

16.5.9 THE RSV-ASTHMA LINK

RSV is the most important respiratory pathogen in infancy and can cause serious lower respiratory tract infections, particularly in prematurely born infants and children with underlying cardiopulmonary conditions. Epidemiological studies have indicated that RSV infection in infancy is associated with recurrent wheezing later in life, giving rise to the theory that there is a link between RSV and asthma. However, the pathogenetic mechanisms of RSV-induced airway inflammation and hyperreactivity remain largely unknown and no effective therapeutic option is currently available to manage the acute and chronic clinical manifestations of this infection.

It has been suggested that RSV infection may enhance the development of allergic inflammatory responses when a child is exposed to allergens after an episode of RSV bronchiolitis. Such a hypothesis, based on immune dysregulation, is supported by the observation that the expression of high levels of RSV-specific IgE occurs in some patients during the acute and convalescent stages of severe RSV infection, and this correlates with subsequent recurrent wheezing in early childhood. There is also evidence that an imbalance between immunologic pathways mediated by different subpopulations of helper T lymphocytes (T_{H}1 and T_{H}2) may be involved in the development of the atopic phenotype. In this form of immune dysregulation, RSV may create a persistent atopic state by skewing immunity towards T_{H}2-type responses. A third hypothesis, which has been demonstrated in animal models, is that abnormal neural control of the airways or neuroimmune interactions that generate a state of hyperreactivity in the airways may be operative.

We have shown that RSV increases markedly the expression of the NK1 tachykinin receptor in the respiratory epithelium and vascular endothelium in the lungs of adult rats, whereas the expression of the NK2 tachykinin receptor and of the VIP receptors is affected minimally. The physiological manifestation of this receptor imbalance is represented by exaggerated neurogenic-mediated inflammation in the airways during the acute viral infection. After resolution of the infection, NK-1 receptor expression progressively returns to baseline, but airway neurogenic inflammation remains abnormal due to remodeling of the sensory innervation, which involves amplification of peptide synthesis and release. RSV infections occurring in the early post-natal period have a profound impact on development of neuropeptide-mediated responses, which remain potentiated into adulthood. Also, neurogenic inflammation in infected young rats is prominent in the intrapulmonary airways,
whereas it is observed almost exclusively in the extrapulmonary airways during adulthood, reflecting age-dependent differences in the pattern of sensorineural innervation across the respiratory tract.

Extending these studies, we have identified additional important modes of interaction between sensory nerves and cellular effectors playing critical roles in inflammatory and allergic disorders, particularly lymphocytes, monocytes, and mast cells, and we have shown that all these neuroimmune interactions are amplified by RSV infection. Finally, we have found evidence that NGF is essential for the integration of the different neuroimmune interactions and represents the ideal link between virus-infected respiratory epithelium and the dense subepithelial network of unmyelinated sensory fibers. Figure 16.5 illustrates the general model of neuroimmune interactions in RSV-infected airways emerging from our studies.

This model implies that in the airways as in other organ systems (e.g., gastrointestinal tract, skin) there is close anatomical juxtaposition and functional interaction of sensory nerves with mucosal lymphoid tissue and mast cells. NGF has a profound impact on the distribution and function of sensory nerves, as well as on the regulation of multiple immuno-inflammatory pathways and infections like RSV bronchiolitis can alter both sensorineural and immuno-inflammatory pathways in the respiratory tract, with a final effect exquisitely dependent on chronological age and developmental window.

16.6 THERAPEUTIC STRATEGIES

16.6.1 Glucocorticoids

Several investigators have demonstrated that glucocorticoids induce ACE activity in cultured alveolar macrophages,\textsuperscript{144} in endothelial cells, and in the rat lung \textit{ex vivo}.\textsuperscript{145} In human monocytes, dexamethasone has been shown to induce the activity of ACE, but not that of other ectoenzymes,\textsuperscript{146} suggesting that glucocorticoid effect may be
rather selective. Similarly, the glucocorticoid budesonide induces a gradual increase of NEP gene expression in transformed human airway epithelial cells. The increase in ACE and NEP activities has a long latency and peaks after 5 to 6 days of incubation. The effects of glucocorticoids on other components of the neurogenic inflammatory pathway have different time courses; glucocorticoid inhibition of NK-1 receptor mRNA transcription peaks within a few hours, whereas the increase in the SP content of dorsal root ganglia following adrenalectomy can be detected at 10 days, but not at 5 days.

In vivo, dexamethasone inhibits in a dose- and time-dependent fashion the increase of vascular permeability produced in the rat trachea by endogenous and exogenous tachykinins. Marked inhibition can be obtained with doses of dexamethasone within the clinical therapeutic range. The inhibitory effect of dexamethasone has a latency of several hours and reaches its maximum after a 5-day course of dexamethasone injections, thus resembling the time dependency of glucocorticoid-induced upregulation of ACE and NEP activities in vitro. This time dependency suggests that the effect of glucocorticoids on peptidase activity is predominant over the effects on NK-1 receptors and neuropeptide content. The administration of dexamethasone can prevent the potentiation of neurogenic plasma extravasation induced in the trachea by Sendai virus infection. Furthermore, the inflammatory infiltrates and the epithelial damage associated with this acute viral infection are virtually absent after treatment with dexamethasone.

The simultaneous inhibition of NEP and ACE completely reverses the protective effect of dexamethasone against neurogenic inflammation. The dose of dexamethasone that inhibits by more than 50% the SP-induced extravasation (0.5 mg/kg per day for 2 days) has no effect on the plasma extravasation induced by an intravenous injection of a nonpeptide mediator, the platelet-activating factor (PAF). Furthermore, PAF-induced extravasation is not affected by the combined inhibition of NEP and ACE. These observations suggest that low-dose dexamethasone protects tracheal postcapillary venules by increasing tachykinin degradation without affecting the responsiveness of the venular endothelium to nonpeptide inflammatory mediators. However, the inhibitory effect of glucocorticoids against sensory nerve-mediated extravasation may also affect a variety of other inflammatory mediators indirectly. For example, it is known that histamine and bradykinin cause antidromic stimulation of sensory nerves in the lung, thus releasing SP and other neuropeptides, and that the elimination of these nerves reduces histamine’s effect on airway vascular permeability. Thus glucocorticoids may inhibit plasma extravasation produced by nonpeptide inflammatory mediators by inhibiting the effect of secondarily released tachykinins.

### 16.6.2 Leukotriene Modifiers

Recent studies outlined above have shown that acute RSV infection is associated with a marked increase in the number of mast cells in the airway mucosa and transiently increased expression of the 5-LO gene with production of cysteinyl leukotrienes, probably deriving from the expanded mast cell population. Interestingly, the mast cells in the infected mucosa appeared to form clusters around nerve
fibers, suggesting functional interactions through the formation of local neuron-mast cell feed-back loops. In fact, the leukotriene receptor antagonist montelukast potently inhibited neurogenic-mediated inflammation in the intrapulmonary airways of infected weanling rats, and to a much lesser degree of adult rats.21

Consistent with our findings in animal models, therapy with a leukotriene receptors antagonist was effective in reducing respiratory symptoms in RSV-infected children up to 3 years of age. An important similarity between Bisgaard’s data and ours is that a post-hoc analysis revealed a stronger effect of antileukotriene therapy in younger children (9 months of age). An ongoing randomized controlled trial will probably clarify if there is a group of patients who could particularly benefit by an early treatment with leukotriene receptor antagonist.

The great clinical relevance of these studies derives from the fact that at present there is no evidence-based therapy against acute viral bronchiolitis or chronic post-viral wheeze. The usefulness of bronchodilators is controversial,23 and steroids are ineffective on the acute and long-term clinical outcome, whether administered systemically24–26 or topically.27–29 Therefore, confirmation of the therapeutic efficacy of leukotriene and identification of patients overproducing leukotrienes may provide a new, targeted therapeutic approach to this common disease. Also, since RSV infection also influences the risk of subsequent asthma,18–20 the follow-up of these patients will elucidate whether increased leukotriene synthesis during the acute infection is predictive of the development of post-bronchiolitis complications, particularly childhood asthma.

16.6.3 β2 ADRENERGIC AGONISTS

Short-acting β2 adrenergic agonists have been for decades first line drugs in the management of acute bronchospasm, and albuterol is the most used of these drugs. This is a racemic mixture containing a 50:50 ratio of two isomers, designated as S (sinister) for the left-handed orientation and R (rectus) for the right-handed orientation. These stereoisomers are identical in chemical and physical properties but have distinct pharmacologic characteristics because the β2-adrenoceptor binds only the (R)-isomer with high affinity (IC50 of 1 μM), whereas the receptor binding affinity of (S)-albuterol is approximately 150-fold lower. Thus, essentially all of the β2-receptor binding and therapeutic effect of racemic albuterol derives from the (R)-isomer. Unfortunately, most studies exploring the efficacy of these drugs in viral bronchiolitis have failed in demonstrating clinical benefit.

Many clinical benefits of β2-agonists may depend not on the bronchodilatory properties of these drugs, but rather on their anti-inflammatory effects. Studies in our animal models suggest that (R)-albuterol has a marked inhibitory effect on neurogenic inflammation during acute lower respiratory tract infections. Interestingly, the potentiation of capsaicin-induced neurogenic inflammation in the extrapulmonary airways was totally abolished by the administration of 1.25 mg (R)-albuterol, whereas an equivalent dose of racemic albuterol was only able to reduce the effect of capsaicin by half. No inhibitory effect on vascular permeability was seen with low-dose (S)-albuterol (0.31 mg), whereas we found significant inhibition at high doses, suggesting that (S)-albuterol might act as a partial receptor agonist.
S-albuterol is considered to be devoid of clinical benefits, and on the contrary it opposes the bronchodilator activity of (R)-albuterol,\textsuperscript{153} may be pro-inflammatory,\textsuperscript{154} and exacerbates airway reactivity to spasmogens. The presence of (S)-albuterol may explain why racemic albuterol is not as potent as (R)-albuterol. The mechanism of the anti-inflammatory effect of (R)-albuterol is probably due to a direct effect on the specialized endothelium of the postcapillary venules, decreasing the formation of the endothelial gaps necessary for the leakage of plasma macromolecules.\textsuperscript{155}

This anti-inflammatory effect is generally not present when β₂-agonists are used parenterally, whereby a high concentration in the airways is avoided\textsuperscript{156} and may not be evident clinically with the currently recommended dosages. In addition, this effect is not likely to wane with frequent or repeated use, as there is no evidence of tolerance to the anti-leakage effect in rats treated with β₂-agonists.\textsuperscript{157}

16.6.4 TACHYKININ ANTAGONISTS

Several studies have shown that atopic individuals have increased levels of immunoreactive SP in bronchoalveolar and nasal lavage fluids compared with nonallergic controls, both at baseline and following allergen provocation.\textsuperscript{158} In addition, there is evidence that NK-1 receptor gene expression may be amplified.\textsuperscript{159} Another study found increased supply of SP-immunoreactive fibers in the airway mucosa of asthmatics compared with nonasthmatics,\textsuperscript{160} although this observation was not confirmed by subsequent studies.\textsuperscript{161,162} Inhaled NKA causes bronchoconstriction in asthmatics\textsuperscript{163} and tachykinins enhance mucus secretion from isolated human bronchi.\textsuperscript{164} Collectively, these observations support the hypothesis that tachykinins released from sensory C-fibers by inhaled irritants may contribute to the pathophysiology of airway inflammation in diseases such as asthma and chronic bronchitis.

Of course, this hypothesis needs to be confirmed by clinical studies exploring the effect of selective antagonists of tachykinin receptors. In the first clinical trial involving an NK receptor antagonist, a dual NK-1/NK-2 antagonist was shown to block the decrease in specific airway conductance induced by inhaled aerosolized bradykinin in asthmatic patients.\textsuperscript{165} The findings of this clinical trial suggested that neurogenic mechanisms play a relevant role in human airway inflammation and encouraged further clinical evaluation of tachykinin antagonists. On the other hand, preliminary studies with the selective NK-1 receptor antagonist CP-99,994 have failed to show significant protection against hypertonic saline-induced bronchoconstriction and cough in asthmatic subjects.\textsuperscript{166}

A possible explanation for these discordant findings is that selective blockade of a single NK receptor subtype may be by-passed by over-activation of the other subtypes, which may also assume the roles of the blocked receptor. This biological redundancy is well established in other inflammatory systems (e.g., cytokines), and it is likely that clinically effective tachykinin antagonists will have to resemble in part the lack of specificity typical of glucocorticoids. Another important issue is that the pharmacokinetics of the molecules tested may have been inadequate to provide significant results; as an example, the half-life of CP-99,994 is quite short, and
therefore unlikely to exert a sustained anti-inflammatory effect during a broncho-
provocation experiment. But maybe the most important flaw of the clinical studies
done so far resides in the choice of the test population, e.g., adult patients with
atopic asthma. From our most recent studies, emerges consistently the message that
the structure and function of the sensory innervation of the respiratory tract is
exquisitely age-dependent and that neurogenic-mediated inflammatory mechanisms
in the airways are much more active and important earlier rather than later in life,
when the predominant asthma phenotype is the nonatopic transient wheezing trig-
gerated by viral infections. Thus, future clinical trials should focus on pediatric patients
and infectious wheezing to understand whether this information obtained in exper-
imental cellular and animal models can be transferred to the clinical arena.

16.7 CONCLUSIONS

Tachykinins are peptide neurotransmitters released primarily from vagal sensory
afferents that cause powerful inflammatory effects in the airways of several species,
including humans. These effects are mediated by three different G-protein-coupled
NK receptors and are modulated by membrane-bound peptidases, particularly NEP
and ACE. The tachykinin peptides, their receptors and their peptidases have extensive
localization in the respiratory tract, where they have been shown to evoke a constel-
lation of biological responses termed “neurogenic inflammation.”

Exaggerated neurogenic inflammation results from overexpression of NK recep-
tors on target tissues or by changes in the peptide content or activation threshold of
sensory nerve fibers. Also, decreased peptidase activity as a result of biological or
chemical insults to the airway mucosa may result in exaggerated and uncontrolled
activation of otherwise physiological defense mechanisms, leading to hypersensitiv-
ity reactions and tissue damage. As neurogenic inflammatory mechanisms are oper-
ational in human airways and other organ systems, selective pharmacological inter-
ventions modulating this pathway may provide useful therapy in numerous human
diseases in which neuropeptides play a pathogenetic role.

REFERENCES

1. Cascieri, M. A., R. R. Huang, T. M. Fong, A. H. Cheung, S. Sadowski, E. Ber, and
C. D. Strader. 1992. Determination of the amino acid residues in substance P con-
ferring selectivity and specificity for the neurokinin receptors. Mol. Pharmacol.
41:1096–1099.
kinins are produced and secreted upon post-translational processing of three substance
peptide isolated from rabbit intestine that is derived from γ-preprotachykinin.


Advances in Vagal Afferent Neurobiology


17 Vagal Afferents and Visceral Pain

Wilfrid Jänig

CONTENTS

17.1 Introduction ..................................................................................................465
17.2 Vagal Afferents, Spinal Visceral Afferents, Visceral Pain, and Nociception ..................................................................................................467
   17.2.1 Pelvic and Abdominal Organs .........................................................467
   17.2.2 Thoracic Visceral Organs.................................................................468
17.3 Abdominal Vagal Afferents, Protection of the Body, and Illness Responses .....................................................................................................469
17.4 Role of Vagal Afferents in Nociceptive-Neuroendocrine Control of Experimental Inflammation ........................................................................472
17.5 Role of Vagal Afferents in Cutaneous Mechanical Hyperalgesic Behavior .......................................................................................................476
   17.5.1 Effect of Subdiaphragmatic Vagotomy on Mechanical Hyperalgesic Behavior ...................................................................................476
   17.5.2 Effect of Defunctionalization of the Sympatho-Adrenal System ...480
   17.5.3 Effect of Denervation of Adrenal Medullae 14 Days Following Subdiaphragmatic Vagotomy ...........................................................480
17.6 Vagal Afferents and Hyperalgesia: An Interpretation .................................484
17.7 Summary ......................................................................................................487
References ..............................................................................................................488

17.1 INTRODUCTION

Visceral organs in the thoracic, abdominal, and pelvic cavities are innervated by two sets of extrinsic primary afferent neurons: spinal visceral afferent neurons and vagal visceral afferent neurons (the latter also including afferent neurons from arterial-baroreceptors and chemoreceptors that project through the glossopharyngeal nerve). Spinal visceral afferent neurons have their cell bodies in the dorsal root ganglia and vagal afferent neurons in the nodose ganglion (a few in the jugular ganglion and some arterial baroreceptor and chemoreceptor afferents in the petrosal ganglion). Visceral afferent neurons are involved in specific organ regulations, multiple organ reflexes, neuroendocrine regulations, and visceral sensations (including visceral
pain), shaping emotional feelings, and other functions. They monitor the inner state of the body and serve to maintain homeostasis and to adapt the internal milieu and the regulation of the organs to the behavior of the organism. They are anatomically and functionally closely associated with the autonomic nervous system. However, they should not be labeled “sympathetic,” “parasympathetic,” or “autonomic” afferent neurons. This labeling is misleading since it implies that the visceral afferent neurons have functions that uniquely pertain to the sympathetic or parasympathetic autonomic nervous system, the exception being that afferent neurons of the enteric nervous system are per definition enteric afferent neurons. No functional, morphological, histochemical, or other criteria do exist to associate any type of visceral afferent neuron with only one autonomic system.

Usually vagal afferent neurons are described and characterized by the adequate physiological stimuli exciting them and by the main reflexes associated with the cardiovascular system, respiratory system, gastrointestinal tract (GIT), or other regulatory systems (see contributions to this volume). However, there are several indications showing that vagal afferents innervating visceral organs in the thoracic and abdominal cavity are involved in the control of nociception and pain, in neuroendocrine control of nociceptors, in the control of inflammation, and in the control of general protective body reactions including illness responses. Thus, vagal afferent neurons signal events from visceral organs, in particular the GIT, to the central nervous system that trigger protective body reactions not only in the viscera but also in the superficial and deep somatic body tissues.

This chapter will discuss experiments, showing that vagal afferents innervating viscera may have remarkable functions that are related to nociception, pain, and body protection. These functions are inferred from various types of experimental investigations and from some clinical observations. The exact nature of these vagal afferents, i.e., their functional properties, has not been investigated so far using neurophysiological methods. In fact, it is unclear whether a specific group or specific groups of vagal afferents are involved in the protective body reactions or whether vagal afferents responding to mechanical and intraluminal chemical stimulation and being involved in organ regulation are also responsible for the protective body reactions. The chapter will particularly concentrate on the abdominal vagal afferents. The abdominal vagus nerves contain 80 to 85% visceral afferent fibers and 15 to 20% preganglionic parasympathetic fibers. Most vagal afferents innervate the GIT and associated organs (e.g., liver, pancreas). They encode in their activity mechanical and chemical events of the GIT (see Chapter 12). They project viscerotopically to the nucleus tractus solitarii. The second-order neurons in the nucleus tractus solitarii project to various sites in the lower brain stem, upper brain stem, hypothalamus and amygdala, establishing well-organized neural pathways that are the basis for distinct regulations of GIT functions as well as of the functions to be discussed in this chapter. The central pathways underlying the representation of conscious sensations related to the gastrointestinal, cardiovascular, and respiratory vagal afferents in brain stem (nucleus tractus solitarii, parabrachial nuclei), thalamus and insular cortex have been discussed by Saper. This chapter:
Vagal Afferents and Visceral Pain

• Summarizes data related to the role of vagal afferents in generating pain
• Summarizes data about the role of vagal afferents in body protection and sickness behavior (illness responses)
• Describes animal experiments showing that activity in vagal afferents is important in the central control of inflammation involving neuroendocrine systems
• Describes animal experiments showing that activity in vagal afferents is important in the central control of mechanical hyperalgesic reactions (sensitization of nociceptors) involving the sympatho-adrenal system
• Discusses the mechanism underlying the modulation of inflammation and hyperalgesia by activity in vagal afferents

17.2 VAGAL AFFERENTS, SPINAL VISCERAL AFFERENTS, VISCERAL PAIN, AND NOCICEPTION

17.2.1 PELVIC AND ABDOMINAL ORGANS

Visceral pain elicited from pelvic and most abdominal organs is triggered by stimulation of spinal visceral afferent neurons and not by stimulation of vagal afferent neurons. However, the situation is not entirely clear for the gastroduodenal section of the GIT. Whether patients with complete interruption of spinal ascending impulse transmission at the thoracic level T1 or at a more rostral segmental level can experience pain from the gastroduodenal section (e.g., during gastritis, a peptic ulcer or distension of the stomach) has never been systematically investigated. Patients with complete lesion of the cervical spinal cord experience abdominal hunger, dread, and nausea. Furthermore, these patients may experience vague sensations of fullness after a hot meal. But acid reflux or an obstructed or distended viscus usually does not generate discomfort and pain. Occasional observations in these patients show that gastric distension may generate pain and discomfort (Dietz, personal communication) and that an acute perforation of a duodenal ulcer may be accompanied by violent pain in the right or left shoulder (page 284 in Guttmann) indicating that vagal afferents may be involved.

It is generally accepted that stimulation of vagal afferents innervating the stomach elicits emesis, bloating, and nausea, all three being protective reactions. Experiments on rats show that influx of acid into and other chemical insults of the gastroduodenal mucosa lead to a host of locally and centrally organized protective reactions that are mediated by spinal visceral and vagal afferent neurons. Activation of vagal afferents by these chemical stimuli leads to activation (expression of the marker protein Fos after activation of the immediate early gene c-fos) of neurons in the nucleus tractus solitarii, area postrema, lateral parabrachial nucleus, subceruleus nucleus, thalamic and hypothalamic paraventricular nuclei, supraoptic nucleus, and central amygdala, but not in the insular cortex (the major central representation area of the stomach). Furthermore, neurons in the spinal dorsal horn do not seem to be activated. Holzer has put forward the following idea about the role of vagal afferents in chemonociception of the gastroduodenal mucosa and about the differential (central and peripheral “efferent”) functions of spinal visceral afferents:
• Vagal afferents involved in chemosensory mediation mediate autonomic, endocrine, and behavioral protective reactions.
• Vagal afferents are not involved in pain perception, but in the emotional aspect of pain and, therefore, indirectly in upper abdominal hyperalgesia.
• Spinal visceral afferents are involved in pain perception, other sensations, and nociceptive and other reflexes elicited by mechanical and chemical stimulation.
• Some spinal peptidergic visceral afferents innervating the gastro-duodenal section of the GIT are only involved in peripheral efferent protective functions by releasing calcitonin gene-related peptide (CGRP) and substance P (SP; vasodilation, venular plasma extravasation, mucus secretion, bicarbonate secretion, activation of mast cells and leukocytes).  
• Some spinal visceral afferent neurons have dual (central and efferent) functions.

These fascinating ideas need verification by further experimentation. For example, it is unclear in which way activity in spinal visceral afferents and vagal afferents are centrally integrated in vivo so as to elicit the protective reflexes, protective behavior, and pain sensations including visceral hyperalgesia. Furthermore, it is unclear in which way activity in vagal afferents is responsible for the emotional aspects of visceral pain, but spinal visceral afferents for the conscious perception of visceral pain. Finally, it has to be shown that spinal visceral afferents may have only peripheral (efferent) functions. Some spinal visceral afferent neurons neither project to the spinal cord nor into the dorsal root, but into the ventral root (ending here blindly) and to the viscera.

17.2.2 Thoracic Visceral Organs

The situation is at least as complex for the thoracic visceral organs. Pain elicited from the proximal esophagus and proximal trachea is probably mediated by vagal afferents innervating the mucosa of these organs. These afferents are peptidergic (i.e., contain CGRP and/or substance P); their cell bodies are probably located in the jugular ganglion (see Reference 14 [cells in the nodose ganglion are almost exclusively peptide-negative, whereas neurons in the jugular ganglion may contain peptides]). Activation of these afferents generates neurogenic inflammation in the mucosa (venular plasma extravasation and vasodilation). Pain elicited from the most distal sections of esophagus and trachea, as well as from the bronchi, may be elicited by stimulation of spinal visceral afferent neurons and not of vagal afferents. However, this situation is unclear and needs further experimentation.

Cardiac pain (e.g., during ischemic heart disease) is generally considered to be mediated by spinal visceral afferents having their cell bodies in the dorsal root ganglia C8 to T9 (mainly T2 to T6). However, attempts to relieve pain associated with cardiac angina by surgical manipulations (cervico-thoracic sympathectomy, dorsal rhizotomy, injection of alcohol into the sympathetic chain) consistently showed that only 50 to 60% of patients report complete relief from angina following these interventions, while the remaining patients report either partial relief or no
relief at all. With the caveat that some failures to relieve pain were attributed to incomplete spinal denervation of the heart, it is concluded that vagal afferents innervating particularly the inferior-posterior part of the heart may mediate cardiac pain too (for review and references see Meller and Gebhart\textsuperscript{17}).

This conclusion is supported by neurophysiological investigations in monkeys and rats. The investigations show that some spino-thalamic tract (STT) neurons in the superficial dorsal horn and deeper laminae of the cervical segments C1 to C2 (C3) can be synaptically activated by electrical stimulation of cardiopulmonary spinal and vagal afferents or by injection of algogenic chemicals in the pericardial sac via both afferent pathways. The activation of the STT neurons by vagal afferents is relayed through the nucleus tractus solitarii. These STT neurons can also synaptically be activated by mechanical stimulation of the somatic receptive fields in the corresponding segments from the head, jaw, neck and shoulder (dermatomes, myotomes). These results are fully in line with clinical observations showing that cardiac pain may be referred to neck, shoulder and jaw\textsuperscript{17–19}. The same high cervical spinal segments contain neurons with similar convergent synaptic inputs from vagal, spinal visceral, and somatic afferents that project to more caudal spinal thoracic, lumbar, and sacral segments. These neurons are involved in the inhibitory control of nociceptive impulse transmission (for discussion and literature see References 20 and 21).

17.3 ABDOMINAL VAGAL AFFERENTS, PROTECTION OF THE BODY, AND ILLNESS RESPONSES

The small intestine and liver are very vulnerable portal of entries into the body. Both have potent local defense systems and serve as internal defense lines of the body. The small intestine contains a powerful immune system (the gut associated lymphoid tissue [GALT]\textsuperscript{23}). It is innervated by vagal afferents that project through the celiac branches of the abdominal vagus nerve. Specific modulation of activity in these afferents in conjunction with the reaction of the GALT may act as an early warning system to the rest of the body by transmitting important information to the brain about toxic events and agents in the small intestine that are dangerous for the organism\textsuperscript{14}. Therefore, vagal afferents in the celiac branches of the abdominal vagus nerves that innervate the small intestine (in addition to the distal duodenum and the proximal colon) and vagal afferents in the hepatic branch that innervate the liver (in addition to the proximal duodenum, pancreas, and pylorus) are of particular interest in this chapter.

Recent experimental investigations support the idea that abdominal vagal afferents may be important for protective functions of the GIT and the body:

1. Vagal afferents in the celiac branches of the abdominal vagus nerve monitor chemical and mechanical events that occur in the intestine under physiological and pathophysiological conditions (for a review, see Reference 24; also see Chapter 12), i.e., are related to meals, ingestion of toxic substances, inflammation, obstruction, etc. Neurophysiological recordings have shown that these afferents respond to distension or contraction of the small intestine and to intraluminal chemical stimulation. Responses
of vagal afferents to maltose, glucose, and intraluminal osmotic stimuli are mediated by enterochromaffin cells releasing 5-HT and by 5-HT\textsubscript{3} receptors on the terminals of the vagal afferents.\textsuperscript{25} Responses of vagal afferents to protein products of long-chain lipids are mediated by enteroendocrine cells releasing CCK and the CCK\textsubscript{A} receptor on the terminals of the vagal afferent neurons. These afferents do not seem to be mechanosensitive.\textsuperscript{26,27} Vagal afferent neurons may be associated with the gut associated lymphoid tissue (GALT) and excited by inflammatory processes and toxic processes. This excitation probably is also mediated by enterochromaffin cells releasing serotonin (5-HT), by enteroendocrine cells releasing cholecystokinin (CCK), and by mast cells releasing histamine and other compounds.\textsuperscript{28,29} The functional specificity of these afferents with respect to the different types of intraluminal stimuli is unknown. The vagal afferents are important for preabsorptive detection of energy-yielding molecules and probably for other properties of nutrient solutions that may be toxic and deleterious for the GIT and for the body.\textsuperscript{30,31} Additionally some vagal afferents that innervate the small intestine and the liver respond to cytokines (e.g. IL-1\textbeta); these afferents may encode events that are related to the immune system of the GIT and liver.\textsuperscript{32}

2. Electrical stimulation of abdominal vagal afferents exerts inhibition or facilitation of central nociceptive impulse transmission in the spinal dorsal horn and depresses nociceptive behavior depending on as to whether unmyelinated or myelinated vagal afferents are excited.\textsuperscript{33,34} Electrical stimulation of cervical vagal afferents in monkeys suppresses transmission of impulse activity in spinothalamic relay neurons with nociceptive function at all levels of the spinal cord. Electrical stimulation of subdiaphragmatic vagal afferents has no effect on spinothalamic relay neurons in this species. It is concluded that particular cardiopulmonary vagal afferents are involved in this inhibitory control. The central pathways mediating the inhibitory effect are neurons in the subceruleus-parabrachial complex (noradrenergic) and neurons in the nucleus raphe magnus of the rostroventromedial medulla (serotonergic) that project to the spinal cord.\textsuperscript{35} The central pathways mediating the facilitatory effect are mediated by suprapontine pathways.\textsuperscript{33} The functional types of vagal afferents being involved in inhibitory and facilitatory control of nociceptive impulse transmission are unknown.

3. When injected intraperitoneally in rats, illness-inducing agents, such as the bacterial cell wall endotoxin lipopolysaccharide (LPS), produce behavioral hyperalgesia. This is mediated by activity in subdiaphragmatic vagal afferents, specifically afferents running in the hepatic branch. It is suggested that LPS activates hepatic macrophages (Kupffer cells) which release interleukin-1\textbeta (IL-1\textbeta) and tumor necrosis factor \alpha (TNF-\alpha). This in turn activates vagal afferents from the liver. IL-1\textbeta and TNF-\alpha injected intraperitoneally themselves generate behavioral hyperalgesia that is abolished by vagotomy.\textsuperscript{36–41} These results suggest that vagal afferents, probably innervating the liver, are activated by proinflammatory cytokines (IL-1\textbeta, TNF\alpha) released by activated macrophages (Kupffer cells), dendritic cells
and leukocytes and signal these events to the brain resulting in pain behavior (i.e., an enhanced thermal nociceptive tail-flick reflex). Watkins and coworkers suggest that the proinflammatory cytokines either activate the vagal afferents directly or bind specifically to glomus cells in the abdominal paraganglia that are innervated by vagal afferents: the activated vagal afferents signal the peripheral events to the brain, leading to hyperalgesia.

4. The first (fast) phase of the fever response that is generated in the rat and guinea pig by intravenous injection of the endotoxin LPS is mediated by vagal afferents possibly innervating the liver.\textsuperscript{42,43} The fast component of fever generated by LPS is abolished after section of the hepatic branch, but not after section of the gastric and/or celiac branches of the abdominal vagus nerves.\textsuperscript{44} Activation of vagal afferents by LPS leads to activation of neurons in the nucleus tractus solitarii and subsequently of noradrenergic neurons in the A1 and A2 areas of the brain stem that project to the hypothalamus.

5. Pain behavior mediated by vagal afferents, that are activated by intraperitoneal injection of LPS and of the proinflammatory cytokines, is part of a general sickness behavior characterized by various protective illness responses (e.g., immobility, decreased social interaction, decrease in food intake, formation of taste aversion to novel foods, decrease of digestion, loss of weight [anorexia], fever, increase of sleep, change in endocrine functions [activation of the hypothalamo-pituitary-adrenal axis], malaise, etc.), and is correlated with marked alterations of brain functions. For example, food aversion and anorexia are generated in rats by TNF-\(\alpha\) (injected intraperitoneally) and by subcutaneous implantation of Leydig LTW(m) tumor cells. Both illness responses are abolished or attenuated by subdiaphragmatic vagotomy.\textsuperscript{45,46} It has been shown that endotoxin or IL-1\(\beta\) or TNF-\(\alpha\) injected intraperitoneally activates nodose ganglion cells\textsuperscript{47} and various brain areas in rodents (as indicated by the marker Fos in neurons of the nucleus tractus solitarii, parabrachial nuclei and hypothalamic supraoptic and paraventricular nuclei) as well as induction of IL-1\(\beta\) mRNA in the pituitary gland, hypothalamus and hippocampus. These changes do not occur or are significantly attenuated in subdiaphragmatically vagotomized animals\textsuperscript{48–50} (for critical discussion see References 51 through 53).

6. Watkins, Maier, and coworkers have developed the general idea that vagal abdominal afferents projecting through the hepatic branch of the abdominal vagus nerves form an important neural interface between immune system and brain. Activation of these afferents by signals from the immune system (proinflammatory cytokines IL-1\(\beta\), TNF-\(\alpha\), IL-6) trigger via different centers in brain stem and hypothalamus illness responses, one component being pain and hyperalgesia. The underlying mechanisms of these illness responses have been discussed.\textsuperscript{54–57} The physiology of the vagal afferents involved in the communication between the immune system, that operates as a diffuse sensory system to detect chemical constituents associated with dangerous microorganisms and their toxins, and the brain have to be worked out.
17.4 ROLE OF VAGAL AFFERENTS IN NOCICEPTIVE-NEUROENDOCRINE CONTROL OF EXPERIMENTAL INFLAMMATION

Under resting conditions, the synovia exhibits a baseline plasma extravasation. Bradykinin infused into the rat knee joint cavity increases plasma extravasation by about five times the baseline plasma extravasation (for details of knee joint perfusion, bradykinin concentrations and measurements of plasma extravasation see Figure 17.1 and legend of Figure 17.1, as well as literature Reference 58). This increase in plasma extravasation lasts throughout the bradykinin infusion with a decay by about 10% in 90 minutes (crosses in Figure 17.2A,B).*  

Stimulation of cutaneous nociceptors in the hindpaw (i.e., by injection of capsaicin at doses of 3 to 30 µg into the plantar skin of the contralateral hindlimb) decreases bradykinin-induced plasma extravasation only weakly but significantly at 30 µg (circles in Figure 17.2A).** Stimulation of nociceptors in the forepaw by intra-palmar injection of capsaicin decreases bradykinin-induced plasma extravasation significantly at doses of 3 to 30 µg. This depression is significantly stronger than that elicited from the hindpaw (circles in Figure 17.2B). After denervation of the adrenal medullae, by bilateral severing of the preganglionic axons innervating the adrenal medullae, intra-plantar injection of capsaicin no longer significantly inhibits bradykinin-induced plasma extravasation at 30 µg (open triangles in Figure 17.2A; not shown for the forepaw). Stimulation of spinal visceral afferents by intraperitoneal or intravesical injection of capsaicin also powerfully inhibits bradykinin-induced plasma extravasation.64,65  

After acute subdiaphragmatic vagotomy, depression of bradykinin-induced plasma extravasation generated by intraplantar injection of capsaicin is strongly enhanced. This inhibition is now already significant at 3 µg capsaicin injected in the hindpaw and plasma extravasation decreases to 20 to 30% of the maximal bradykinin-induced plasma extravasation at the dose of 30 µg (closed circles in Figure 17.2A). For the forepaw stimulation the potentiating effect of vagotomy is smaller than for the hindpaw stimulation being most notable at 30 µg (closed circles in Figure 17.2B). In vagotomized animals with denervated adrenal medullae, stimulation of nociceptors by capsaicin injected in the hindpaw or forepaw does not significantly depress bradykinin-induced plasma extravasation (closed triangles Figure 17.2A and Figure 17.2B).

* Bradykinin-induced plasma extravasation of the knee joint synovia that occurs at the venular side of the vascular bed is reduced to about 30% after surgical sympathectomy (removal of the postganglionic neurons) but not after decentralization of the postganglionic neurons (cutting the preganglionic axons). Thus this synovial plasma extravasation is dependent on the innervation of the synovia by sympathetic postganglionic axons but not on activity (action potentials) in these axons and not on release of noradrenaline. The varicosities of the sympathetic postganglionic axons mediate directly or indirectly the effect of bradykinin.58,60 Details about the underlying mechanism of this novel function of postganglionic sympathetic terminals have not been explored so far.

** Intra-plantar injection of capsaicin, in doses of 3 to 30 µg, into the denervated plantar skin (sciatic and saphenous nerves cut) has no effect on bradykinin-induced plasma extravasation. Furthermore, intra-plantar injection of capsaicin has no effect on plasma extravasation generated by platelet-activating factor (10⁻⁷ M) in the rat knee joint.63
The following conclusions are drawn from these experiments:

1. Noxious stimulation of skin by intradermal capsaicin and noxious stimulation of spinal visceral afferents by intraperitoneal injection of capsaicin leads to depression of synovial plasma extravasation. This depression is mediated by the sympatho-adrenal system (adrenal medullae). The signal to the synovia is most likely epinephrine. The mechanisms in the synovia by which epinephrine generates a depression of synovial plasma extravasation is unknown; but it is unlikely to be generated by decrease in blood flow since the synovial plasma extravasation produced by platelet activating factor is not decreased by noxious stimulation!*

* Continuous transcutaneous electrical stimulation of cutaneous afferent nerve fibers in the plantar skin at C-fiber strength (but not at A-fiber strength) and at low stimulation frequencies (≤4 Hz) also leads to depression of bradykinin-induced plasma extravasation. However, this depression is mediated by the hypothalamo-pituitary-adrenal (HPA) axis. In addition, this depression is enhanced after subdiaphragmatic vagotomy. These experiments principally show that inflammatory processes are under the control of both neuroendocrine systems. The differential activation of both neuroendocrine systems, the HPA axis or the sympatho-adrenal system, generated by the two modes of afferent stimulation is interesting but puzzling. The mechanisms underlying this differential activation are unknown and are presently explored.
2. The central pathways activated by the noxious input and leading to the reflex activation of the preganglionic neurons innervating the adrenal medullae is a spino-bulbo-spinal pathway and probably a spinal pathway (see circuit in bold and grey-shaded neurons in Figure 17.3). Such spinal and spino-bulbo-spinal somato-sympathetic reflex pathways to sympathetic preganglionic neurons innervating the adrenal medullae, which are activated by noxious stimulation, have been shown to exist in experiments in which activity in the adrenal nerve (which contains the preganglionic axons innervating the chromaffin cells of the adrenal medulla) and release of catecholamines from the adrenal medullae have been measured. For example, stimulation of cutaneous nociceptive afferents activates the preganglionic neurons to the adrenal medullae and leads to release of catecholamines from the adrenal medullae in rats with intact spinal cord and in spinalized rats (References 68 through 70; for review see References 71 and 72). From the studies reported by Levin’s group\(^{63,73}\) for the hindpaw-induced reflexes, a spinal pathway from the lumbar segments L\(_4\) and L\(_5\) (which receives the afferent input from the plantar skin of the hindpaw) to the preganglionic neurons in the segments T\(_4\) to T\(_{12}\) which innervate the adrenal medullae is postulated.\(^{74}\)

3. The nociceptive-neuroendocrine reflex pathways are normally inhibited by activity in vagal afferents. Experiments have shown that section of the celiac branches has the same effect as subdiaphragmatic vagotomy, but section of the gastric or hepatic branches has no effect.\(^{65}\) Thus, the vagal afferents involved project through the celiac branches of the abdominal vagus nerves that innervate the small intestine, distal duodenum and proximal colon.

4. Acute gastrectomy has no effect on the nociceptive-neuroendocrine reflex controlling synovial plasma extravasation, whereas resection of the duodenum has the same effect as sectioning the celiac vagal branches. Finally, fasting of rats produced the same effect as section of the celiac branches. The effect of fasting was reversed by distension of the duodenum.\(^{75}\)

5. After section of the dorsolateral funiculus of the spinal cord contralateral to the afferent nociceptive input (Figure 17.4A left), the depression of bradykinin-induced plasma extravasation is almost totally abolished in rats with intact vagus nerves, as well as in vagotomized rats (open triangles and open squares in Figure 17.4C), showing that the reflex activation of the preganglionic neurons requires a spino-bulbo-spinal positive feedback circuit (outlined in bold in Figure 17.3) and that the ascending limb of the postulated spino-bulbo-spinal reflex pathway projects through the dorsolateral funiculus of the spinal cord contralateral to the afferent nociceptive input from the hindlimb.

6. After section of the dorsolateral funiculus ipsilateral to the afferent nociceptive input in vagus-intact rats (Figure 17.4A right), the depression of bradykinin-induced plasma extravasation is quantitatively the same as in vagotomized rats (compare closed triangles with closed circles in Figure 17.4B). In this type of experiment the ipsilateral dorsolateral funiculus
FIGURE 17.2 Inhibition of bradykinin-induced plasma extravasation by stimulation of nociceptors by capsaicin (CAP) is mediated by the sympathoadrenal axis and enhanced after vagotomy. Effect of intradermal injection of CAP (3 to 30 µg) into the plantar skin of hindpaw (A) or into the palmar skin of the forepaw (B) on bradykinin-induced plasma extravasation in the knee joint in sham-vagotomized rats (open circles), in vagotomized rats (closed circles), in rats with intact vagus nerves and denervated adrenal medullae (open triangles), and in vagotomized rats with denervated adrenal medullae (closed triangles). Crosses (in A,B): No stimulation of nociceptors by capsaicin (control). Ordinate scale, degree of plasma extravasation in the rat knee joint normalized with respect to maximum (before stimulation of nociceptors by capsaicin). For details see legend of Figure 17.1. Mean ± S.E.M.; N = 8 knees animals with interventions; N = 16 knees in control. (Modified from Miao et al., 2000.63)
was sectioned at the spinal segmental lumbar level L2/L3 (Figure 17.3). This shows that the inhibition maintained by the activity in vagal afferents is mediated by a descending pathway ipsilateral to the afferent nociceptive input and occurs at the spinal level, possibly in the dorsal horn at the segmental level of the activated nociceptive afferent input (Figure 17.3).

7. Various other types of experiments were conducted (hemisection of the spinal cord, section of the dorsolateral funiculi at various segmental levels, section of the dorsal funiculi73) to support the key conclusions as graphically illustrated in Figure 17.3.

8. The central nociceptive-neuroendocrine reflex pathways controlling inflammation and their inhibitory modulation by activity in vagal afferents are under the control of the periaqueductal gray (PAG). However, PAG-induced inhibition and vagus-induced inhibition are not relayed through the same though overlapping central pathways.76

The hypothesis (as outlined in Figure 17.3) explaining the results of the spinal lesion experiments, the evidence supporting the existence of the spino-bulbo-spinal reflex loop, the evidence supporting the descending inhibitory pathway linked to vagal afferents and the evidence supporting the lateralization of ascending excitatory and descending inhibitory pathways have been extensively discussed.73 The functional characteristics of the vagal afferents that are involved are unknown (see discussion at the end of the next section).

17.5 ROLE OF VAGAL AFFERENTS IN CUTANEOUS MECHANICAL HYPERALGESIC BEHAVIOR

17.5.1 EFFECT OF SUBDIAPHRAGMATIC VAGOTOMY ON MECHANICAL HYPERALGESIC BEHAVIOR

Withdrawal threshold to stimulation of the rat hindpaw with a linearly increasing mechanical stimulus applied to the dorsum of the paw decreases dose-dependently after intradermal injection of bradykinin (open circles and closed squares in Figure 17.5). Following a single injection of bradykinin this decrease lasts for more than one hour for mechanical stimulation.77 This type of mechanical hyperalgesic behavior is mediated by the B2 bradykinin-receptor78 and is not present when bradykinin is injected subcutaneously.79* Bradykinin-induced hyperalgesic

* The decrease in paw-withdrawal threshold provided by bradykinin is significantly reduced after surgical sympathectomy. This shows that the sympathetic innervation of the skin is involved in the sensitization of nociceptors for mechanical stimulation.80 Interestingly, decentralization of the lumbar paravertebral sympathetic ganglia (denervating the postganglionic neurons by cutting the preganglionic sympathetic axons) does not abolish bradykinin-induced mechanical hyperalgesic behavior. This indicates that the sensitizing effect of bradykinin is not dependent on activity in the sympathetic neurons innervating skin and therefore not on release of norepinephrine. It is believed that bradykinin stimulates the release of prostaglandin from the sympathetic terminals. However, this has to be shown to occur.81
behavior is blocked by the cyclooxygenase inhibitor indomethacin and therefore mediated by a prostaglandin (probably PGE$_2$) that sensitizes nociceptors for mechanical stimulation.*

* Evidence for sensitization of cutaneous nociceptors to mechanical stimulation by bradykinin is poor or absent.$^{82}$ Some sensitization to mechanical stimulation by bradykinin has been demonstrated for afferents from the knee joint$^{83}$ and from skeletal muscle.$^{84}$ These discrepancies may be due to technical experimental difficulties.
FIGURE 17.4 Effect of section of the ipsilateral dorsolateral funiculus (DLF) at the segmental level of L2/L3 (B) or of the contralateral DLF at the segmental level of T8/T9 (C) on depression of bradykinin-induced plasma extravasation generated by intradermal injection of capsaicin (3, 10, and 30 µg) in the plantar skin of the hindpaw in sham-vagotomized and vagotomized rats. (A) Diagrams of transverse spinal sections. (B) Ipsilateral DLF section (closed inverted triangles). Closed circles: acute vagotomy. Open circles: sham vagotomy. (C) Contralateral DLF section without vagotomy (inverted open triangles) and with vagotomy (open squares). Crosses (in B, C): No stimulation of nociceptors by capsaicin (control). Ordinate scale, degree of plasma extravasation in the rat knee joint normalized with respect to maximum. For details see legend of Figure 17.1. Mean ± S.E.M.; N = 8 knees; N = 16 knees in control. (Modified from Miao et al., 2001.73)
If inhibition maintained by activity in vagal afferents acts continuously on the central nociceptive pathway one would expect, on the basis of studies reported by Gebhart, Randich and coworkers,¹³,¹⁴ that subdiaphragmatic vagotomy might enhance the mechanical hyperalgesic behavior, irrespective of the way the nociceptive afferents have been sensitized (e.g., by bradykinin or another hyperalgesic agent), and lower baseline threshold to mechanical stimulation.

Baseline paw-withdrawal threshold in normal (109 ± 2.1 g, mean ± SEM) and sham vagotomized (107 ± 2.8 g) rats is significantly decreased 7 days after subdiaphragmatic vagotomy (89 ± 1.7 g; triangles in Figure 17.5). Intradermal injection of bradykinin produces a dose-dependent decrease in mechanical nociceptive threshold (i.e., generates a mechanical hyperalgesic behavior) in normal rats and in rats 7 days after subdiaphragmatic sham-vagotomy (open circles and squares in Figure 17.5). Bradykinin-induced hyperalgesia is significantly enhanced 7 days after subdiaphragmatic vagotomy (triangles in Figure 17.5). There are three important characteristics of the effect of vagotomy on mechanical baseline threshold and on bradykinin-induced decrease of paw-withdrawal threshold to mechanical stimulation:
1. The dramatic enhancement of bradykinin-induced mechanical hyperalgesic behavior occurs also when only the celiac vagal branches are interrupted, but not when the gastric and/or hepatic branches of the abdominal vagus nerves are interrupted. Thus, the vagal afferents involved project through the celiac branches of the abdominal vagus nerves, which innervate the small intestine and proximal part of the large intestine, and not through the hepatic or gastric branches. Surprisingly, the baseline paw-withdrawal threshold to mechanical stimulation does not decrease when only the celiac vagal branches are interrupted.

2. Both vagotomy-induced changes (decrease in baseline paw-withdrawal threshold, bradykinin-induced hyperalgesic behavior) take about two weeks to reach maximum and remain stable over five weeks (Figure 17.7).

3. Subdiaphragmatic vagotomy does not have a significant effect on cutaneous mechanical hyperalgesic behavior produced by intradermal injection of prostaglandin E₂ (which is supposed to act directly to sensitize nociceptors).

Thus, the effect of vagotomy is not a general effect of all abdominal vagal afferents and cannot readily be explained by an immediate removal of inhibition from the central nociceptive system (e.g., acting in the dorsal horn) as predicted by the experiments of Foreman, Gebhart, Randich and coworkers (see above).

17.5.2 EFFECT OF DEFUNCTIONALIZATION OF THE SYMPATHO-ADRENAL SYSTEM

Bilateral removal or denervation of the adrenal medullae (cutting the sympathetic preganglionic axons) generates both a small increase in baseline paw-withdrawal threshold and paw-withdrawal threshold to intradermal injection of bradykinin compared to the controls (Figure 17.6). Under this condition of defunctionalized adrenal medullae, subdiaphragmatic vagotomy is followed by only a small decrease in paw-withdrawal threshold (compare open and closed squares in Figure 17.6) but not by the large changes seen in animals with functioning adrenal medullae. These small changes are significant, with the exception of the change of baseline threshold in animals with denervated adrenal medullae. They can fully be explained by removal of central inhibition of nociceptive impulse transmission occurring probably in the dorsal horn (see above).

17.5.3 EFFECT OF DENERVATION OF ADRENAL MEDULLAE 14 DAYS FOLLOWING SUBDIAPHRAGMATIC VAGOTOMY

If the decrease of baseline mechanical paw-withdrawal threshold and enhanced decrease of paw-withdrawal threshold to mechanical stimulation, generated by intradermal injection of bradykinin, are related to epinephrine released from the adrenal medullae, which is dependent on activity in sympathetic preganglionic axons innervating the adrenal medullae, one would expect that these changes are reversed when
FIGURE 17.6 Role of adrenal medulla in bradykinin-induced behavioral mechanical hyperalgesia and its enhancement after subdiaphragmatic vagotomy (SDV). (A) Baseline and decrease of paw-withdrawal threshold to mechanical stimulation of the dorsum of the rat hindpaw, induced by bradykinin (bradykinin-induced behavioral mechanical hyperalgesia) in sham vagotomized rats (closed circles, n = 18), in rats whose adrenal medullae are removed (Adrenal Medx, open squares, n = 12), and in rats with removed adrenal medullae and which are also vagotomized (closed squares, n = 12). Experiments conducted 5 weeks after removal of the adrenal medullae and 7 days after additional vagotomy. (B) Data from experiments in which the adrenal medullae are denervated: rats with denervated adrenal medullae (AM denerv, open squares, n = 6), vagotomized rats with denervated adrenal medullae (AM denerv plus SDV, closed squares; n = 10). Experiments were conducted 7 days after surgery. Paw-withdrawal thresholds of vagotomized rats in which the adrenal medullae are removed or denervated are significantly higher than those of rats which are only vagotomized (see open triangles in Figure 17.5; P<0.05). Paw-withdrawal thresholds of rats in which the adrenal medullae are removed or denervated are significantly higher than those of rats which were additionally vagotomized (P<0.05; compare open with closed squares). Data on sham-vagotomized rats in A and B are the same as in Figure 17.5. (Modified from Khasar et al., 1998.85)
FIGURE 17.7 Long-term enhancement of bradykinin-induced behavioral mechanical hyperalgesia after vagotomy (SDV, subdiaphragmatic vagotomy) and its disappearance after denervation of the adrenal medullae. (continued)
the adrenal medullae are denervated, that epinephrine administered chronically sim-
ulates the effects and that a chronic β-adrenoceptor-blockade prevents or attenuates
the effect of vagotomy.

Groups of rats were repeatedly tested over 5 weeks for their mechanical paw-
withdrawal threshold to 1 ng bradykinin injected intracutaneously (a dose that does
not decrease the threshold to mechanical stimulation in normal rats with intact vagus
nerves) (see Figure 17.5 and Figure 17.6): (1) Rats in which the vagus nerves were
severed subdiaphragmatically followed 14 days later by denervation of the adrenal
medullae. (2) Rats which were vagotomized only and repeatedly tested. (3) Control
rats which were repeatedly tested without any surgical intervention. Figure 17.7
demonstrates the results of these experiments for the baseline paw-withdrawal
threshold (A), for the decrease in paw-withdrawal threshold to intradermal injection
of 1 ng bradykinin alone (B), and for both effects together (C). After vagotomy, the
paw-withdrawal threshold slowly decreases reaching its lowest values after 7 to
14 days. The reversal of the vagotomy effect after denervation of the adrenal medul-
lae has also a slow time course similar to the time course of the decrease in baseline
paw-withdrawal threshold following vagotomy (closed triangles in Figure 17.7) over
a five-week period. Repeated testing of sham-vagotomized control rats over the same
period of time does not reveal a decrease in paw-withdrawal threshold produced by
1 ng bradykinin (closed circles in Figure 17.7). The paw-withdrawal thresholds, 14
and 21 days after denervation of the adrenal medullae, are significantly higher than
those measured in the animals that are only vagotomized (compare closed triangles
with open triangles in Figure 17.7). The paw-withdrawal thresholds in response to
1 ng bradykinin at 14 and 21 days after denervation of the adrenal medullae in
vagotomized animals are not significantly different from those in sham-vagotomized
animals that had repeatedly been tested over a period of 5 weeks after surgery
(compare closed triangles with closed circles in Figure 17.7).

Chronic administration of epinephrine (10.8 µg/h; using a subcutaneously
implanted microosmotic pump) generates the same effect as vagotomy: The brady-
kinin-induced paw-withdrawal threshold to mechanical stimulation significantly

**FIGURE 17.7 (CONTINUED)** Baseline paw-withdrawal threshold (A), difference between
baseline paw-withdrawal threshold and paw-withdrawal threshold in response to 1 ng BK
injected intradermally (B), and total change of paw-withdrawal threshold in response to
intradermal injection of 1 ng bradykinin (C) in rats before and 7 to 35 days after vagotomy
(open triangles, n = 6), before and 7 to 35 days after sham-vagotomy (closed circles, n = 8), and
in rats which are first vagotomized and whose adrenal medullae (AM) are denervated 14 days
after vagotomy and measurements taken up to 35 days after initial surgery. The latter group
of animals consists of two subgroups: rats which are tested after vagotomy and after additional
denervation of the adrenal medullae (closed normal triangles, n = 6) and rats which are only
tested after additional denervation of the adrenal medulla (closed inverted triangles, n = 4).
Ordinate scale is threshold in grams. Data of the sham-vagotomy and the vagotomy group of
rats are significantly different 7 days after vagotomy (P<0.01). Data of vagotomized rats with
denervated AM and rats that are only vagotomized are significantly different on days 28 and
35 (P<0.01). Data between sham-vagotomized rats and vagotomized rats in which the adrenal
medullae are denervated are not significantly different on days 28 and 35 (P>0.05). (Modified
from Khasar et al., 1998. 85)
Advances in Vagal Afferent Neurobiology
decreases. This decrease is delayed and reaches its peak effect 14 days after start of epinephrine infusion. After chronic infusion of the $\beta_2$-adrenoceptor blocker ICI 118,551, the decrease of bradykinin-induced paw-withdrawal threshold following vagotomy is significantly attenuated. The plasma levels of epinephrine following vagotomy significantly increase 3, 7 and 14 days after subdiaphragmatic vagotomy compared to sham-vagotomized animals.86

17.6 VAGAL AFFERENTS AND HYPERALGESIA: AN INTERPRETATION

These results suggest that two mechanisms contribute to the decrease of baseline paw-withdrawal threshold to mechanical stimulation and to enhancement of the decrease of paw-withdrawal threshold generated by intracutaneous injection of bradykinin following vagotomy:

1. Ongoing central inhibition of nociceptive impulse transmission (occurring probably in the dorsal horn) that is normally maintained by spontaneous activity in vagal afferents is removed after vagotomy (Figure 17.8), resulting in a small but significant enhancement of nociceptive behavior to mechanical stimulation (see Figure 17.6). This enhancement is in accordance with the idea that nociception and pain are centrally under inhibitory control from the visceral domain maintained by activity in vagal afferents.20,33–35

2. Vagotomy triggers the activation of sympathetic preganglionic neurons innervating the adrenal medullae (Figure 17.8), probably by removing central inhibition acting at this sympathetic pathway, thus leading to an increased release of epinephrine from the adrenal medullae and an increased epinephrine level in the plasma. Interruption of these sympathetic preganglionic axons innervating the adrenal glands (Figure 17.8), stops the release of epinephrine, and therefore prevents or reverses the decrease of baseline mechanical paw-withdrawal threshold and the enhancement of bradykinin-induced decrease of paw-withdrawal threshold to mechanical stimulation. This novel finding implies that the sensitivity of nociceptors to mechanical stimulation is potentially under control of the sympato-adrenal system and that nociceptor sensitivity can be regulated from remote body domains and by the brain via this neuroendocrine pathway.

The second mechanism is novel and has several implications. In connection with the effect of vagotomy several interesting questions and problems are raised:

• The vagal afferents that are involved in modulation of hyperalgesic behavior and in modulation of inflammation project through the celiac branches of the abdominal vagus nerves and supply small and large intestines, but probably not liver and stomach. These vagal afferents are capsaicin-sensitive
Vagal Afferents and Visceral Pain

whereas most of those vagal afferents that innervate stomach or liver are not.14,87 Do these vagal afferents monitor toxic and other events at the inner defense line of the body (the “gut associated lymphoid tissue,” GALT)? What are the physiological stimuli to activate these vagal afferents? Is it possible to increase mechanical paw-withdrawal threshold (baseline, to intradermally injected bradykinin) by physiological stimulation of these vagal afferents related to toxic events?

• The changes following vagotomy (decreased mechanical baseline threshold and enhanced bradykinin-induced mechanical hyperalgesic behavior81,85) are generated by the interruption of vagal afferents. Therefore, the vagal afferents involved must be tonically active88,89 (see Chapter 12). This conclusion is fully supported by experiments in which the influence of activity in vagal afferents on the nociceptive-neuroendocrine negative feedback pathway controlling neurogenic inflammation of the synovia of the rat knee joint has been studied (see above).

• Maier, Watkins and coworkers have shown, using the thermal tail-flick reflex, that behavioral hyperalgesia in rats produced by intraperitoneal

FIGURE 17.8 Schematic diagram showing the proposed neural circuits in spinal cord and brain stem which modulate nociceptor sensitivity via the sympatho-adrenal system (adrenal medulla). Sensitivity of cutaneous nociceptors for mechanical stimulation is modulated by a signal (probably epinephrine) from the adrenal medulla. Activation of the adrenal medullae increases the sensitivity of the nociceptors. Activity in preganglionic neurons innervating the adrenal medullae depends on activity in vagal afferents from the small intestine, which has an inhibitory influence on the central pathways to these preganglionic neurons. Thus, interruption of the vagal afferents leads to activation of the adrenal medullae. It is hypothesized that these neuronal (reflex) circuits in the brain stem are under the control of upper brain stem, hypothalamus, and forebrain. Dotted thin lines: Axons of sympathetic premotor neurons in the brain stem which project through the dorsolateral funiculi of the spinal cord to the preganglionic neurons of the adrenal medulla. For details see text. +, excitation; −, inhibition. (Modified from Khasar et al., 1998.85)
Advances in Vagal Afferent Neurobiology

injection of the illness-inducing bacterial cell wall endotoxin lipopolysaccharide (LPS) is mediated by activity in subdiaphragmatic vagal afferents projecting through the hepatic vagal branch. The results of these experiments imply that *stimulation* of vagal afferents generates thermal hyperalgesic behavior and are apparently at variance with the results reported here, showing that *decrease* of activity in vagal afferents leads to hyperalgesic behavior. However, it must be kept in mind that: (1) two different types of nociceptive behavior (mechanical, thermal) have been tested; (2) two different groups of vagal afferents (innervating small/large intestine and liver, respectively) are involved; (3) induction and depression of the two hyperalgesic behaviors occur at different time courses; (4) different (central and peripheral) mechanisms are probably involved in the modulation of both nociceptive behaviors from the viscera.

- The mechanism of the slow time-course of the changes of paw-withdrawal threshold is not clear at the moment. Epinephrine has obviously to act over a long period of time to induce changes in the micromilieu of the nociceptors which in turn leads to their sensitization. It does most likely act *not directly* on the nociceptors but on other cells (e.g., macrophages, mast cells, keratinocytes), which then release substances that generate the sensitization, this in particular since prostaglandin E₂-induced mechanical hyperalgesic behavior is not changed after vagotomy.

- The change of sensitivity of a population of cutaneous nociceptors generated by epinephrine, which is regulated by the brain would be a novel mechanism of sensitization. This novel mechanism of sensitization of the nociceptor population by epinephrine released by the sympathoadrenal system would be different from mechanisms that lead to activation and/or sensitization of nociceptors by sympathetic-afferent coupling under pathophysiological conditions.

- Which central pathways are involved leading to activation of preganglionic sympathetic neurons that innervate the adrenal medullae after subdiaphragmatic vagotomy? Are only sympathetic neurons that innervate the adrenal medullae activated after vagotomy or also other functional types of sympathetic neuron? Experimental investigations performed on rats show that sympathetic preganglionic neurons innervating cells of the adrenal medullae that release epinephrine are connected to distinct neuronal circuits in the neuraxis that are different from those connected to preganglionic neurons innervating cells of the adrenal medullae that release norepinephrine and from those connected to preganglionic neurons innervating postganglionic neurons supplying resistance vessels in skeletal muscle or viscera or functionally other types of sympathetic preganglionic neurons.

- Do the same changes, related to the abdominal vagal afferents and the adrenal medullae, also occur in other behavioral pain models? For example, do the changes, probably induced by epinephrine in the cutaneous nociceptor population, also occur in deep somatic and visceral nociceptive
afferents? Finally, is it possible that these mechanisms operate in such ill-defined pain syndromes as irritable bowel syndrome, functional dyspepsia, fibromyalgia etc. 102–104

17.7 SUMMARY

Vagal afferent neurons innervating visceral organs are involved in generation or modulation of pain and nociception in various ways. This influence originating in visceral organs has to be understood in the frame of protection of body tissues when the body is endangered by invading organisms, antigens, toxins, and other events.

1. Vagal afferents innervating the heart may be involved in pain during angina referred to the upper cervical dermatomes and myotomes.
2. Vagal afferents innervating the stomach and being excited by acid are involved in nociceptive protective reactions, but probably not in conscious perception of pain.
3. Transmission of nociceptive impulses in the spinal dorsal horn is under inhibitory control generated by activity in cardiopulmonary and abdominal vagal afferents.
4. Vagal afferents innervating the liver (thus passing through the hepatic branch of the abdominal vagus nerves) are involved in illness responses generated by lipopolysaccharides (bacterial antigens) or proinflammatory cytokines (e.g., interleukin-1β or tumor necrosis factor α) into the peritoneal cavity. One component of the illness responses is hyperalgesic behavior.
5. Experimental knee joint inflammation is under inhibitory control of nociceptive-neuroendocrine (spinal and supraspinal) reflex pathways involving the sympatho-adrenal (SA) system and epinephrine released by this system. The nociceptive-neuroendocrine reflex pathways are modulated by activity in abdominal vagal afferents innervating the small intestine. The inhibitory modulation occurs at the spinal level.
6. Mechanical hyperalgesic behavior (bradykinin-induced decrease of paw-withdrawal threshold to mechanical stimulation) is enhanced by activation of the SA system and epinephrine. Activation of the SA system is enhanced after interruption of vagal afferents passing through the vagal celiac branches and innervating the small intestine.
7. These results imply that nociceptors can be sensitized by epinephrine released by the SA system and that this sensitization is modulated from the visceral organs via vagal afferents.
8. The physiological response properties of vagal afferents being involved in the reflex modulation of nociception, pain, hyperalgesia, inflammation and illness responses are unknown. It is likely that not one functionally specific type of vagal afferent neuron is involved but several different types.
REFERENCES


Electrical Stimulation of the Vagus Nerve for the Treatment of Epilepsy

Steven C. Schachter

CONTENTS

18.1 Introduction .................................................................................................495
18.2 Relevant Anatomy .......................................................................................496
18.3 Mechanism of Action ..................................................................................496
  18.3.1 Neurophysiological Studies .............................................................496
  18.3.2 Neuroimaging Studies ......................................................................497
18.4 Efficacy of VNS in Animal Models of Epilepsy ........................................498
18.5 The VNS Therapy System ..........................................................................498
18.6 VNS Efficacy Studies in Patients with Epilepsy .........................................500
18.7 Effects of VNS on Other Conditions that Often Occur in Patients with Epilepsy .......................................................................................................501
18.8 Safety and Tolerability of VNS in Patients with Epilepsy ..............................502
18.9 Clinical Use of Electrical Stimulation of the Vagus Nerve as a Treatment for Epilepsy .................................................................503
18.10 Summary .....................................................................................................504
References ..............................................................................................................504

18.1 INTRODUCTION

Epilepsy is a disorder of the brain characterized by recurrent seizures, which occur in 0.5 to 1% of the population. While an unprecedented number of new antiepileptic drugs (AEDs) were approved by the Food and Drug Administration (FDA) in the past 15 years for the treatment of seizures, up to one in three patients whose seizures emanate from a focal area of the brain (called partial-onset seizures) continue to have seizures despite all trials of AEDs (termed medically refractory or pharmacoresistant epilepsy). Treatment options other than drug therapy (AEDs) are limited. Some patients may benefit from surgical resection of the seizure-generating brain tissue (epilepsy surgery), while others, especially children, may improve with the
ketogenic diet, which is high in fats and low in carbohydrates. Unfortunately, some patients are not candidates for resective surgery, and seizures in adult epilepsy patients do not generally improve with the ketogenic diet.

In 1997, the VNS Therapy System® (Cyberonics, Inc., Houston, Texas) was approved by the FDA as adjunctive (i.e., in addition to AED) therapy for adults and adolescents over 12 years of age whose partial-onset seizures were refractory to AEDs. Electrical stimulation of the vagus nerve thus became the first FDA-approved nonpharmacological treatment for epileptic seizures.

This chapter reviews the possible mechanisms of action of vagus nerve stimulation (VNS), experiments in animals, and clinical results in patients with epilepsy.

18.2 RELEVANT ANATOMY

Detailed discussions of afferent vagal anatomy are available elsewhere in this volume. Several particular aspects are relevant to epilepsy and to the safety of VNS.

The cell bodies of the nodose ganglion relay sensory information of vagal origin to the nucleus of the solitary tract (NTS). One of the subsequent pathways of relevance to epilepsy ascends to the forebrain via the parabrachial nucleus, which is lateral to the locus coeruleus.4 The parabrachial nucleus also transmits visceral sensations to the ventroposterior parvocellular nucleus of the thalamus, which in turn projects to the insular cortex,5 an area frequently involved in partial-onset seizures. Likewise, other projections from the parabrachial nucleus and the NTS provide visceral sensation to two other regions often affected by partial seizures, the amygdala and basal forebrain. Mapping studies using c-fos have confirmed that VNS activates these nuclei and pathways.6 Other work has shown that the locus coeruleus mediates the effects of VNS against seizures, suggesting the importance of norepinephrine in the mechanism of action of VNS.7

Because the right vagus innervates the cardiac atria more so than the left vagus nerve, and the left vagus nerve provides the predominant innervation of the ventricles,8 electrical stimulation of the left vagus nerve would be predicted to be less likely to cause serious cardiac arrhythmias. This prediction is borne out by clinical experience, though right-sided VNS is equally as effective against seizures as left-sided stimulation in rat models of epilepsy.9

18.3 MECHANISM OF ACTION

18.3.1 NEUROPHYSIOLOGICAL STUDIES

Early experiments showed that repetitive electrical stimulation of the vagus nerve either synchronized or desynchronized brain electrical activity in anesthetized animals, as measured by the electroencephalogram (EEG), depending on stimulus frequency and current strength (i.e., which in turn determined the fiber types [myelinated, nonmyelinated] that were recruited).10–12 Because desynchronization is generally viewed as reducing the brain’s vulnerability to seizing, these studies suggested a possible anticonvulsant benefit of VNS and prompted studies in animal models of epilepsy.
More recent animal work extended the earlier observations by focusing on cortical neurons rather than the EEG. Low-intensity trains of VNS (100 µA, 30 Hz, 500 µs, 20 seconds on time) were found to hyperpolarize pyramidal neurons of the parietal association cortex in rats. Neurons that are hyperpolarized are inhibited; i.e., less likely to be excitable and initiate or participate in a seizure. Seizures generally result from a change in the balance of excitation and inhibition of neuronal networks such that there is excess excitatory tone compared to inhibitory tone. Interestingly, low-intensity stimulation, which predominantly activates myelinated vagal fibers, was more effective in inducing long-lasting inhibitory effects than higher stimulus intensities, which also activate nonmyelinated vagus fibers.

Studying the changes in EEG or in single neurons in association with VNS is technically more difficult in awake humans. A study utilizing transcranial magnetic stimulation in five patients treated with VNS for epilepsy showed evidence of a pronounced increase in cortical inhibition with stimulation and no effects on cortical excitability. In support of this study, Marrosu et al. found normalization of impaired neuronal inhibition in the brains of patients whose seizures improved with VNS.

### 18.3.2 Neuroimaging Studies

The effects of VNS on activation of the brain have been studied using positron-emission tomography (PET), single photon emission computed tomography (SPECT), and functional magnetic resonance imaging (fMRI) scanning. PET studies, which evaluate changes in regional blood flow, have been inconsistent: one study noted increased blood flow in the ipsilateral anterior thalamus and cingulate cortex, and another noted increased flow in the contralateral thalamus and temporal cortex and ipsilateral putamen and cerebellum. Henry et al. found relationships between the extent of bilateral thalamic changes in blood flow and reductions in seizure frequency.

Precise anatomic localization with PET is technically difficult. Ring and colleagues obtained SPECT scans, which also evaluate regional cerebral blood flow (rCBF) changes, with particular attention to thalamic and insular regions, in seven subjects treated for at least six months with VNS. They found decreased rCBF in the medial thalamic regions bilaterally. Vonck et al. similarly found thalamic hypoperfusion with VNS, and no correlation between the extent of rCBF changes and degree of seizure reduction.

Bohning et al. were the first to document the feasibility of recording VNS-synchronized fMRI. This imaging technique has more precise resolution than either PET or SPECT scans. They studied nine patients enrolled in a depression protocol, they found VNS-associated changes in bilateral orbitofrontal and parieto-occipital cortices, left temporal cortex, the hypothalamus, and the left amygdala. The same group has further demonstrated that the pulse width (in microseconds), one of the stimulation parameters, influences which regions are activated. Sucholeiki et al. obtained fMRIs in four patients treated with VNS for epilepsy. Four anatomical subregions were consistently activated in each subject — left superior temporal gyrus, inferior frontal gyrus (bilateral), medial portions of the superior frontal gyrus in the region of the supplementary motor cortex (bilateral), and posterior aspect of
Advances in Vagal Afferent Neurobiology

the middle frontal gyrus (bilateral). Narayanan and colleagues also evaluated patients with epilepsy and found the most robust activations induced by VNS were seen in the thalami (left greater than right) and insular cortices.\textsuperscript{24} Thalamic activation on fMRI correlated positively with clinical outcomes in one study.\textsuperscript{25}

18.4 Efficacy of VNS in Animal Models of Epilepsy

Following the demonstration that VNS had measurable effects on electrical brain function, investigators tested the efficacy of VNS in several animal models of epilepsy.\textsuperscript{26–29} In other studies, the ability for VNS to have a sustained anticonvulsant effect was evaluated,\textsuperscript{30,31} which is more relevant to human epilepsy.

18.5 The VNS Therapy System

The VNS Therapy system consists of a programmable pulse generator, a bipolar VNS lead, a programming wand with accompanying software, a tunneling tool, and handheld magnets.\textsuperscript{32,33}

The pulse generator is implanted in the patient’s upper left chest (Figure 18.1) and is powered by a lithium carbon monofluoride battery. The current version of the hermetically sealed titanium generator (Model 102) weighs 25 grams, and is approximately 52 mm in diameter and 6.9 mm deep.

The bipolar stimulating electrodes directly connect the generator to the left vagus nerve and thereby convey the electrical signal produced by the generator to the vagus nerve. The two connector pins at one end are plugged into the generator. At the other end are two separate helical silicone coils (Figure 18.2). Each helix has three turns; on the inside of the middle turn is a platinum ribbon coil that is welded to

\textbf{FIGURE 18.1 (A color version of this figure follows page 236.)} Schematic representation of the placement of the VNS Therapy generator and the lead connecting the generator to the left vagus nerve. (Courtesy of Cyberonics, Inc., Houston, TX.)
the lead wire. The helical shape of the coils enables the surgeon to place the coils around the nerve nontraumatically so that the middle coil with the platinum ribbon maintains mechanical contact with the nerve.

The programming wand uses radiofrequency signals to communicate with the generator under the control of computer software. In this manner, the physician can program the output current (typically 1 mA), signal frequency (typically 30 Hz), signal pulse width (typically 500 microsec), signal on time (typically 30 seconds), and signal off time (typically 5 minutes). In addition, magnet activated stimulus parameters — pulse width, output current, and on time — are also programmable (a supplied handheld magnet turns on stimulation when briefly held against the chest over the generator). The generator delivers intermittent stimulation until the battery wears out, which is predicted to take 6 to 11 years with the Model 102, depending on stimulation parameters used for an individual patient.

The implantation procedure is performed as same-day surgery at some centers. At other facilities, patients remain in the hospital until the day after the implantation. The procedure lasts approximately one to two hours and is typically performed under

FIGURE 18.2 (A color version of this figure follows page 236.) Stimulating electrodes attached to the vagus nerve. The top helical coil is the negative electrode. The middle coil is the positive electrode. The bottom coil tethers the other electrodes to nearby connective tissue. (Courtesy of Cyberonics, Inc., Houston, TX.)
general anesthesia to minimize the possibility that a seizure will disrupt the operation.35–37

Besides intermittent stimulation, on-demand stimulation may be brought on by the patient or a companion by placing the supplied magnet on the patient’s chest over the generator for several seconds. The stimulator settings employed for on-demand stimulation usually utilize a higher current and pulse width than those used for intermittent stimulation. Some patients have reported that on-demand stimulation interrupts a seizure or reduces its severity if administered at the onset of the seizure.38,39

18.6 VNS EFFICACY STUDIES IN PATIENTS WITH EPILEPSY

The first time a human with epilepsy was implanted with the vagal nerve stimulator was in 1988.40 The first pivotal trial of VNS was the E03 study41–44 and the second pivotal clinical study was the E05 study.45 In 1991, a compassionate-use trial enrolled 124 patients with all types of intractable seizures (the E04 study).46

The E03 and E05 studies were multicenter, blinded, randomized, active-control trials that compared two different VNS stimulation protocols for the treatment of pharmacoresistant partial-onset seizures: high stimulation (30 Hz, 30 seconds on, 5 minutes off, 500 microseconds pulse width) and low stimulation (1 Hz, 30 seconds on, 90 to 180 minutes off, 130 microseconds pulse width). The hypothesis was that the low-stimulation treatment was less effective than the high-stimulation treatment.

Patients enrolled in the E03 and E05 studies were at least 12 years of age, had at least six seizures per month, had a history of epilepsy for more than 20 years on average and were taking a mean of 2.1 AEDs at study entry. In the E05 study, 254 patients were entered, including 55 who were discontinued from baseline for failing protocol eligibility, and 199 patients were implanted.

In both studies, the primary measure of efficacy was the percentage change in seizure frequency during VNS treatment compared with the pre-implantation baseline. Changes in seizure frequencies in the high- and low-stimulation groups were then compared in each study. In the E03 study, the high-stimulation group had a mean reduction in seizure frequency of 24.5%, versus 6.1% for the low-stimulation group (P = 0.01). In the E05 study, the corresponding decreases were 28 and 15% for the high- and low-stimulation groups, respectively (P = 0.039). Thus both studies showed that high-stimulation was more effective than low-stimulation.

Other efficacy analyses confirmed the primary outcome measure. In the E03 study, 31% of patients receiving high-stimulation had at least 50% reduction in seizures compared to 13% of patients in the low-stimulation group (P= 0.02). In the E05 study, 11% of patients in the high-stimulation group had at least a >75% reduction of seizures compared with baseline, versus 2% of patients in the low-stimulation group (P = 0.01).

The E03 and E05 studies evaluated seizure frequency over a period of three months. Considering that epilepsy is often a chronic condition, treatments should ideally remain effective indefinitely. The results of several long-term studies of VNS
suggest that this treatment does not lose effectiveness,\textsuperscript{43,47–49} including in patients aged 50 and older.\textsuperscript{50} However, because VNS treatment was given in an unblinded fashion during the long-term analysis and stimulation parameters, and AED dosages could be adjusted as clinically necessary, these results are not conclusive for long-term efficacy. Likewise, other long-term studies that have followed patients up to 12 years postimplantation show sustained seizure control over time, but are similarly limited by their open and often retrospective study designs, as well as flexibility in allowing changes in AEDs.\textsuperscript{51–54} The exception to the latter caveat is a retrospective study of 269 patients whose AEDs were kept constant for one year after VNS implantation that showed a 45\% reduction of seizure frequency at 3-months post-implantation compared with pre-implantation baseline and 58\% reduction at 12 months.\textsuperscript{55}

As noted earlier, VNS was approved by the FDA as adjunctive therapy for adults and adolescents over 12 years of age whose partial-onset seizures were refractory to AEDs. This indication was based on the patients enrolled in the E03 and E05 studies.

Several open, uncontrolled studies suggest that adjunctive VNS may reduce generalized seizures that have been refractory to drug therapy. Generalized seizures, in contrast to partial-onset seizures, are generally believed to originate deep in the brain and affect both cerebral hemispheres synchronously.\textsuperscript{46,56–58}

In addition, VNS also appears to be a promising treatment for pharmacoresistant epilepsy in children,\textsuperscript{59} including those with a particularly devastating form of epilepsy called Lennox-Gastaut syndrome\textsuperscript{60–69} and in developmentally disabled or mentally retarded patients with epilepsy.\textsuperscript{70}

18.7 \textbf{EFFECTS OF VNS ON OTHER CONDITIONS THAT OFTEN OCCUR IN PATIENTS WITH EPILEPSY}

Patients with epilepsy are more likely than the general population to have memory complaints and clinically significant depression. In addition, AEDs often cause impaired cognition and daytime sleepiness.

Clark et al. studied word recognition memory in patients with epilepsy,\textsuperscript{71} which was prompted by their findings of improved memory storage in rats exposed to VNS.\textsuperscript{72} After reading paragraphs that contained highlighted words, patients then received either VNS or sham stimulation. Retention of verbal learning (word recognition) was significantly enhanced by VNS but not by sham stimulation. Another study evaluated the effects of 4.5 minutes high-intensity VNS (> 1 mA) on material-specific memory and decision times in patients with medically refractory epilepsy.\textsuperscript{73} The results indicated reversible worsening of figural but not verbal memory. Controlled trials of VNS on memory function in epileptic patients with memory complaints have not been published.

Two studies evaluated the mood effects of VNS in patients with pharmacoresistant partial-onset seizures.\textsuperscript{74,75} Improvement in mood was found, and this interestingly did not correlate with improved seizure control. Other studies have documented improvements in mood and reductions of tenseness and dysphoria.\textsuperscript{76–78}
Dodrill and Morris\(^7^9\) evaluated cognition in 160 patients enrolled in the E05 study. Encouragingly, there was no statistically significant worsening of cognition with high-stimulation treatment groups, which might be expected with AEDs. Similarly, another study evaluated cognition in 36 adult patients before and at least 6 months after implantation and found no evidence of cognitive worsening.\(^8^0\) Martin et al. found that decision making, as reflected in a gambling task, was enhanced by VNS in a blinded, controlled study of patients with epilepsy, suggesting to the authors that the vagus nerve is a conduit for afferent somatic signals that can influence decision making.\(^8^1\)

In support of anecdotal clinical observations, studies suggest that VNS may improve daytime alertness and vigilance,\(^8^2,8^3\) as well as sleep quality in some epileptic patients with reduced daytime sleepiness,\(^8^4\) while having the opposite effect in other patients.\(^8^5\)

18.8 SAFETY AND TOLERABILITY OF VNS IN PATIENTS WITH EPILEPSY

While high-frequency stimulation may theoretically be associated with nerve damage,\(^3^3\) there is no evidence that the stimulation settings used clinically to treat epilepsy damage the vagus nerve.\(^3^5,8^6\)

Side effects of VNS are noted only while stimulation is actually occurring. Notably absent with VNS stimulation therapy are the typical side effects of AEDs, such as sedation, lack of coordination, and double vision, which can be persistent.

Randomized, double-blind studies provide a more reliable assessment of the safety and tolerability of a new treatment than open, nonrandomized studies. In the E03 study (described earlier), the side effects that occurred in at least 5% of patients in the high-stimulation group during treatment were hoarseness (37%), throat pain (11%), coughing (7%), shortness of breath (6%), tingling (6%), and muscle pain (6%). Hoarseness was the only side effect that occurred significantly more often with high stimulation than with low stimulation. Likewise, in the E05 study, the only two side effects that occurred significantly more often in the high-stimulation group than in the low-stimulation group were shortness of breath and voice alteration. No significant changes in heart rhythm (as measured by Holter monitoring) or pulmonary function were found. No deaths occurred during either study.

Long-term safety and tolerability studies of VNS generally show sustained safety and improved tolerability over time. For example, among 444 patients who continued VNS after participating in a clinical study, the most commonly reported side effects at the end of the first year postimplantation were voice alteration (29%) and tingling (12%); at the end of 2 years, voice alteration (19%) and cough (6%); and at 3 years, shortness of breath (3%).\(^8^7\)

The overall impact of seizure treatment can be assessed using quality-of-life (QOL) batteries. Studies of VNS treatment generally, but not always,\(^8^8\) show improved QOL scores beyond reduction in seizures.\(^8^9–9^1\)

Transient asystole lasting up to 20 seconds has been reported in association with the lead test during implantation in approximately 0.1% of cases.\(^9^2–9^4\) Complete heart
Electrical Stimulation of the Vagus Nerve for the Treatment of Epilepsy

block due to complete atrioventricular nodal block was documented in one series.95 The lead test is performed intraoperatively and assesses stimulator functioning and system integrity by turning on the generator briefly at 1.0 mA, 500 microseconds and 20 Hz. There have been no reported serious consequences in any patients.

Other studies have found no consistent clinically relevant effects of VNS on cardio-respiratory function.96,97 Galli and colleagues analyzed RR variability in 24-hour EKG recordings at baseline, 1 month and 36 months after initiation of VNS in 7 patients with epilepsy.98 They found a reduction of the high-frequency component of the spectrum during the night, and a flattening of sympathovagal circadian changes that was not clinically significant.

Other isolated, transient side effects attributed to VNS are the subject of case reports or small series.99–106 Children with severe mental and motor retardation who are dependent on assisted feeding may be at increased risk for aspiration while being fed during vagus stimulation,107,108 which is presumed to result from stimulation-induced swallowing impairment. Infections of the subcutaneous pocket into which the VNS generator is placed occur infrequently, but when present usually require explantation.109,110

The manufacturer of the VNS therapy system has cautioned patients treated with VNS not to undergo short-wave diathermy, microwave diathermy, or therapeutic ultrasound diathermy because of the theoretical possibility that the generator or lead could heat up and then cause thermal tissue damage. There are no documented cases of this complication in VNS-treated patients.

When the battery in the VNS generator is depleted, seizure frequency may increase in some patients.111 Fortunately, the end of usable battery service can be predicted with the current VNS model, allowing for elective generator replacement without an undue risk of seizure worsening.

18.9 CLINICAL USE OF ELECTRICAL STIMULATION OF THE VAGUS NERVE AS A TREATMENT FOR EPILEPSY

According to the manufacturer’s website (www.cyberonics.com), more than 28,000 patients have been treated with the VNS therapy system worldwide as of January 2005. Because VNS is the first nonpharmacological therapy approved for epilepsy, and owing to its initial high cost, which may be offset by later cost savings, clinicians have actively debated its role in the treatment of epilepsy.112–114 Today, VNS is generally considered as a possible treatment option for patients with medically refractory partial-onset seizures who are either opposed to intracranial surgery, are not candidates,54 or whose seizures were not substantially improved by prior intracranial epilepsy surgery.115,116 Typical stimulation parameters used in clinical practice are shown in Table 18.1.

The era of proven nonpharmacological treatment of seizures began with the introduction of VNS.117,118 Further controlled studies are needed to understand its role in patients with generalized seizures, to explore strategies for determining optimum stimulation settings,119,120 and to determine whether patients who benefit
Advances in Vagal Afferent Neurobiology

from VNS can be identified pre-operatively, thereby avoiding the potential surgical complications and costs of implantation in patients who have no clinical response to VNS.\textsuperscript{121}

\textbf{18.10 SUMMARY}

Electrical stimulation of the vagus nerve is effective, safe, and well tolerated in patients with long-standing, refractory partial-onset seizures.\textsuperscript{122} Evidence of benefit for other seizure types and in children with epilepsy is promising. There has been no indication of reduction of effectiveness in long-term, open studies.

Side effects occur during stimulation in the minority of patients, are usually mild to moderate in severity, and diminish with time or reduction in stimulation intensity. Caution should be exercised when considering VNS for patients with sleep apnea\textsuperscript{123} and cardiac conduction disorders. Surgical complications are infrequent,\textsuperscript{124} especially with enhancements in surgical techniques and postoperative care.

\textbf{REFERENCES}


Spanaki, M.V. et al., Vagus nerve stimulation therapy: 5-year or greater outcome at a university-based epilepsy center, *Seizure* 13 (8), 587–190, 2004.


Rafael, H. and Moromizato, P., Vagus nerve stimulation (VNS) may be useful in treating patients with symptomatic generalized epilepsy, *Epilepsia* 39 (9), 1018, 1998.


Advances in Vagal Afferent Neurobiology


96. Frei, M.G. and Osorio, I., Left vagus nerve stimulation with the Neurocybernetic Prosthesis has complex effects on heart rate and on its variability in humans, *Epilepsia* 42 (8), 1007–1016, 2001.
Index

A

A δ afferents, 289, 316
Abdominal organs, 467–468
Abdominal visceral afferents, 469–471
ACE, see Angiotensin converting enzyme
Acetylcholine (ACh), 40, 176, 317, 419
ACh, see Acetylcholine
Achaete-scute complex, 13
Acid sensitive ion channels (ASICs), 174, 176, 317, 419
Adenosine, 230, 298–301
Adenosine triphosphate (ATP), 175, 290
Adenylyl cyclase, 295
Adrenal medullae, 480–484
Adrenergic agonists, 452–453
Adrenergic receptors, 259–260, 417
AEDs, see Antiepileptic drugs
Aδ fibers, 290, 296, 297, 301, 358
Agatoxin, 83
AHR, see Airway hyperresponsiveness
Airway function; see also Airway-related vagal preganglionic neurons;
Bronchopulmonary vagal afferent nerves; C-fibers; Cough
autonomic reflexes, 412–420
blood flow, 438–439
bronchial vasculature regulation, 419–420
bronchoconstriction, 266–267, 287, 290, 296, 297
bronchomotor tone, 439–440, 443–444
CNS control of, 248–250, 264–265
diseases/pathophysiology, 249, 265, 291–293, 441–450
hyperresponsiveness (AHR), 265–266, 291, 299, 302
hypersensitivity, 294–302
inflammation (see inflammation)
mucociliary clearance, 440
mucosal edema, 287
neurogenic inflammation, 431–464
nociceptors, 405–406, 410–412, 419–420
norepinephrine-related responses, 260–261
pollutants/irritants, 265–266
serotonin-related responses, 264
smooth muscle, 249, 261, 265, 387, 412, 416–417
tachykinin effects, 437–441
tracheobronchial ganglia, 248, 249
vagal lung afferents, 279–313
Airway hyperresponsiveness (AHR), 265–266, 291, 299, 302
Airway mucosal edema, 287
Airway parasympathetic ganglia, 413
Airway/pulmonary reflexes, 286, 403–420
cough, 404–412
monitoring, 413–416
nociceptors, 405–406, 410–412
RARs and SARs, 404–405, 410–412, 417
vagally mediated autonomic reflexes, 412–420
vagally mediated respiratory reflexes, 406–412
Airway-related vagal preganglionic neurons (AVPNs), 247–275
adrenergic receptors, 259–260
autonomic reflexes, 412–420
catecholaminergic innervation, 255–261
central monoaminergic control, 255–267
CNS control system, 248–250, 264–265
CNS innervation, 254–255
inhibitory inputs, 265–267
microdialysis/HPLC, 259
neuroanatomical studies, 257–258
physiological responses, 260–261
serotonergic innervation, 261–264
structural characteristics, 250–255
ultrastructural characteristics, 252–254
Alcoholics, 388
Allergic inflammation, 292–293
α-Dendrotoxin, 84–86
Alveolar hypercapnia, 299, 300
Amiloride, 283
4-Aminopyridine (4-AP)-sensitive potassium channels, 282
AMPA receptors, 196, 201–203, 240, 329
Amplification, 169–170
Amygdale, 322
Anandamide, 181
Anaphylaxis, 296, 335
Angiotensin converting enzyme (ACE), 433–434, 436–442, 450–451
Angiotensin II, 230
Antiepileptic drugs (AEDs), 495–496
Advances in Vagal Afferent Neurobiology

4-AP, see 4-Aminopyridine-sensitive potassium channels
AP, see Area postrema
AP2α, 17
Apnea, 265, 281, 286, 411
Area postrema (AP), 201, 256, 316
Arterial chemoreceptors, 33–34
Arterial pressure, 234
Ash1, 13
ASICs, see Acid sensitive ion channels
Asthma, 293, 296, 299, 440–450
Atonal, 13
ATP, see Adenosine triphosphate
Autacoids, 286, 298
Autonomic reflexes, 412–420
  airway smooth muscle tone regulation, 416–417
  bronchial vasculature regulation, 419–420
  glandular secretion regulation, 417–419
  monitoring, 413–416
  morphology of airway autonomic innervation, 412–413
AVPNs, see Airway-related vagal preganglionic neurons
Axonal caliber, 46
Axonal transport (of neurotrophins), 48–50, 57–59

B
Baclofen, 181, 323
Baroreceptors, 28, 33–34, 111–112
  in diabetic patients, 54
  inputs, 230–231
  NO modulation, 224–228, 233, 235
BDNF, see Brain-derived neurotrophic factor
Bezold-Jarisch reflex, see Bradycardia
Biotin conjugated dextran amines, 131–134
BK, see Bradykinin
Blood brain barrier, 194, 201
Blood pressure, 234
BMPs, see Bone morphogenetic proteins
Bone morphogenetic proteins (BMPs), 16–17, 18
  BMP2, 20
  BMP7, 14, 15
Bowel, 316–318; see also Gastrointestinal tract
Bradycardia, 225, 286, 411
Bradykinin (BK), 113–114, 159, 286, 290, 295–297
  activation of nociceptors, 412, 417
  B2 receptor, 177
Brain-derived neurotrophic factor (BDNF), 323
  axonal transport of, 49–50
  in vagal afferent development, 29, 31–34, 38–39, 46, 51
Brainstem, see Monoaminergic modulation in brainstem; Nucleus tractus solitarii
Brainstem nuclei, 322
Bronchial vasculature regulation, 419–420
Bronchitis, 440
Bronchoconstriction, 266–267, 287, 290, 296, 297
Bronchodilation, 417
Bronchomotor tone, 439–440, 443–444
Bronchopulmonary function, see Airway function
Bronchopulmonary vagal afferent nerves, 279–313
  activation of airway/pulmonary reflexes, 403–420
  acute airway injury/inflammation, 291–292
  afferent phenotypes, 289–290
  chronic airway inflammation, 292–293
  classification, 280–290
  inflammatory mediators, 294–302
  laryngeal afferents, 280–281
  other lung afferents, 289
  pathophysiological conditions, 290–293
  RARs, 283–285, 290, 293, 296, 298, 404–405
  SARs, 281–282, 289, 290, 298, 404–405
Bronchospasm, 417–419
Butaprost, 295

C
C fibers, see C-fibers
Caffeine, 38
Calcitonin gene-related peptide (CGRP), 29–31, 40, 42–45
  allergic inflammation, 292
  bronchopulmonary C-fibers, 286–287
  neurogenic inflammation, 434–435, 439
  respiratory reflexes, 406
Calcium (Ca2+), 38–39
  binding proteins, 220
  currents, 89–92
  ion channels, 38–39, 82–84, 298
CALLA, see Common acute lymphocytic leukemia antigen
Cannabinoid (CB) receptors, 181–182
CAPON, 212, 229
Capsaicin, 286, 288–290, 302, 329–330
  activation of nociceptors, 410, 412, 417
  alveolar hypercapnia, 299–300
Capsazepine, 297–298

512
Carbocyanine dyes, 128–130, 132, 139
   immuno/counterstaining compatibility, 141
   multiple injections, 141
   permanence of labeling, 142
Carbon dioxide (CO₂)
   concentration, 281, 298
   partial pressure, 282
Cardiac mechanoreceptors, 359
Cardiac vagal afferent nerves, 351–375
   chemosensitive, 360–362
   chemotransduction, 362–363
   diabetes mellitus, 367–368
   electrophysiological characteristics, 353–363
   heart failure, 365–366
   hypertension, 366–367
   mechanosensitive, 353–359
   mechanotransduction, 359
   morphological characteristics, 352–353
   myocardial ischemia and reperfusion, 363–365
   patch-clamp recordings, 354–355
   pathophysiological states, 363–368
   sensory function classification, 353
   single-fiber recordings, 353–354
Cardiovascular function, 224–228
Cardiovascular reflexes, 221, 224–228, 420
Carotid body, 33–34, 36
Carotid sinus, 33–34, 230–231
Cash1, 13
Catecholamines, 40, 255–261, 318
CB, see Cannabinoid (CB) receptors
CCK, see Cholecystokinin
Central subnucleus of NTS (ceNTS), 221
CeNTS, see Central subnucleus of NTS
C-fibers, 112, 174–175, 201, 294–302, 358
   abdominal vagus, 316
   neurogenic inflammation, 432–437
   nociceptors, 405–406, 432
   nodose neurons, 175, 290
   respiratory reflexes, 406
C-fos, 322, 335
cGMP, see Cyclic guanine monophosphate
CGRP, see Calcitonin gene-related peptide
Channels, see Ion channels
Charybdotoxin (ChTX), 87, 88
CH₄T, see Choline acetyltransferase
Chemical transduction, see Chemotransduction
Chemoreceptors, mucosal, 331
Chemosensitivity, 109, 360–362, 391
Chemotransduction, 167–189, 362–363
   activation, 168–169
   amplification, 169–170
   chemotransducers, 171–182
   ionotropic receptors, 171–175
   modulation, 171
   stages of, 168–171
   transduction, 168
Chloride current, 113–114
Cholecystokinin (CCK), 40, 43, 159, 470
   behavioral aspects, 331–335
   receptors, 177–178, 393
Choline acetyltransferase (ChAT), 251–252
ChTX, see Charybdotoxin
Cigarette smoke, see Smoke/smoking
Ciliary neurotrophic factor (CNTF), 37–38
c-Jun, 45
Clara cells, 302
CNQX, 159
CNTF, see Ciliary neurotrophic factor
Cold receptors, 280
Colon, 392
Common acute lymphocytic leukemia antigen (CALLA), 433–434
Congestive heart failure, 28
Conotoxin, 83
Cough, 281, 291, 292, 297, 406–412
   mechanoreceptors, 411–412
   neurogenic inflammation, 440–441
   nociceptors, 412
   reflex, 289
   vagal afferent control, 406–409
CP, see Cricopharyngeus (CP) muscle
CP55940, 181
CPA, 301
Cranial ectodermal placodes, see Placodes
c-ret, 51
Cricopharyngeus (CP) muscles, 381, 382, 384
Cricothyroid muscles, 384
Currents, ionic, 87–94
Cutaneous mechanical hyperalgesic behavior, 476–480
Cyclic guanine monophosphate (cGMP), 213
Cystic fibrosis, 440
Cytokines, 335–336, 470–471

D
Dach, 9–10
DBH, see Dopamine β-hydroxylase
δ neurons, 200
α-Dendrotoxin (α-DTX), 85–86
Dexamethasone, 451
Dextran, 130–133
Advances in Vagal Afferent Neurobiology

Dextran amines, 130–134, 139–140
  immuno/counterstaining compatibility, 140–141
  multiple injections, 141
DGRR, see Duodeno-gastric reflex relaxation
Diabetes, 53–59
  effect on neuropeptides, 44
  mellitus, 28, 367–368
  streptozotocin (ZTC)-induced, 54–59
  TH-immunoreactive neurons, 42
  vagal nerve neurotrophins, 52–59
Dihydroxyphenylalanine (DOPA), 255
Di-I, 128–130, 132, 141
Dlx, 9, 15, 16
DM PX, 301
DMVN, see Dorsal motor vagal nucleus
DOPA, see Dihydroxyphenylalanine
Dopamine β-hydroxylase (DBH), 14, 220
Doppler probe monitors, 415–416
Dorsal motor vagal nucleus (DMVN), 250, 252, 316, 380, 389
Dorsal raphe neurons, 180
Dorsal root ganglia, 21, 180, 302
DPCPX, 301
Drive receptors, 280
α-DTX, see α-Dendrotoxin
Duodeno-gastric reflex relaxation (DGRR), 392–393
Dyspepsia, 320, 322
Dysphagia, 386
Dyspnea, 291, 292, 299

E
Ectodermal placodes, see Placodes
ED, see Esophagus, esophageal distension
Edema, 287
EGCR, see Esophagus, esophagoglottal closure reflex
EGR, see Esophagus, esophageo-gastric reflex relaxation
Eicosanoid receptors, 180
ELESR, see Esophagus, esophago-LES reflex relaxation
Embryology, 1–26
  jugular neurons, 16–22
  neural crest, 7–8, 15, 16–19
  nodose neurons, 8–16
  vagal sensory neurons, 1–26
ENaCs, see Epithelial Sodium Channels
ENK, see Enkephalin
Enkephalin (ENK), 40
ENS, see Enteric nervous system
Enteric nervous system (ENS), 317, 324
Enterochromaffin (EC) cells, 331–332
Epibranchial placodes, 12–14, 316
Epigenetic influences
  development/survival of neurons, 29–39
  maintenance of mature neurons, 40–59
Epilepsy, 495–510
Epithelial Sodium Channels (ENaCs), 283
Esophagus, 316, 318, 320–321; see also Gastrointestinal tract
  esophageal and supra-esophageal reflexes, 380–387
  esophageal distension (ED), 381, 384, 389
  esophageo-gastric reflex relaxation (EGR), 389
  esophagoglottal closure reflex (EGC), 384–385
  esophago-LES reflex relaxation (ELES), 388
  esophago-UES contractile reflex (EUC), 381
  gastroesophageal reflex (GER), 384
  pharyngo-esophageal inhibitory reflex (PEI), 386–387
  sphincters, 380–381
EUCR, see Esophagus, esophago-UES contractile reflex
Exocytosis, 197
External cuneate nuclei, 224
Extravasation, 437–438, 472
Eya family, 9–10, 15

F
Facial nerve, 223, 256
FGF, see Fibroblast growth factor
Fibroblast growth factor (FGF), 17
Flecainide, 282
Fluoro-Gold, 130–131, 137
Fluoro-Ruby, 130, 141
Fos, see c-Fos
FoxD3, 17
FoxI, 10, 15–16

G
GABA, 201, 318, 323
  NO modulation, 213, 230
  receptors, 102, 181, 323
Galantin, 40, 323
GALT, see Gut-associated lymphoid tissue
Gastric inflammation, 114–115
Gastric reflexes, 389, 390–393
  esophageo-gastric reflex relaxation (EGR), 389
gastro-colonic reflex (GCR), 392
Gastro-duodenal reflex (GDR), 391–392
Gastro-gastric reflex, 389
Gastro-LES reflex relaxation (GLRR), 390
Gastro-pyloric reflex relaxation (GPRR), 391
Laryngo-gastric reflex relaxation (LGRR), 389
Pharyngo-gastric reflex relaxation (PGRR), 389
Transient LES relaxation (TLESR), 390
Gastroesophageal reflex (GER), 384
Gastrointestinal tract reflexes, 379–401 esophageal and supra-esophageal, 380–389 gastric, 389, 390–393 intestinal, 393–394 GCR, see Gastric reflexes, gastro-colonic reflex GDNF, see Glial cell line-derived neurotrophic factor GDR, see Gastric reflexes, gastro-duodenal reflex Geniculate ganglion, 7 Geniculate placode, 7, 11–12 GER, see Gastroesophageal reflex GFR alpha 3, 51 Gigantocellular reticular nucleus (GiV), 256, 261 GiV, see Gigantocellular reticular nucleus Glial cell line-derived neurotrophic factor (GDNF), 36–37, 51, 323 Glial cells, 39 Glossopharyngeal afferent neurons, 28, 42, 223 Glossopharyngeal nerves (GPN), 223, 382, 385, 387 Glottis closure reflexes, 384–386 GLRR, see Gastric reflexes, gastro-LES reflex relaxation Glucocorticoids, 450–451 Glutamate, 40, 195–197, 201–202, 317 nNOS co-localization, 219–220 NO modulation, 213, 228–230 receptors, 196–197, 201–203, 323, 329 Golgi tendon organs, 319 GPN, see Glossopharyngeal nerves G-protein, 181
GPRR, see Gastric reflexes, gastro-pyloric reflex relaxation Guanylyl cyclase, see Soluble guanylyl cyclase Guillain-Barre syndrome, 28 Gut-associated lymphoid tissue (GALT), 469–470
H
IA, see Interarytenoid (IA) muscles
ICC, see Interstitial cells of Cajal
IGLEs, see Intraganglionic laminar endings
IL-1β, see Interleukins, IL-1β
IL-6, see Interleukins, IL-6
IL-11, see Interleukins, IL-11
IMA, see Intramuscular arrays
Immune system, 335–336, 442–443, 445, 450
Infections, respiratory, 441–442
Inflammation, 335–336
airway, 115, 249, 265, 266, 291–302
allergic, 292–293
experimental, 472–476
gastric, 114–115
inflammation-induced hypersensitivity, 294–302
inflammatory cell chemotaxis, 287
mediators, 294–302, 335–336
modulation of voltage-gated ion channels, 326–327
neurogenic, 431–464
Input signal, 195
Interarytenoid (IA) muscles, 384
Interleukins, 335–336
IL-1β, 336, 470–471
IL-6, 37–38
IL-11, 37
Interstitial cells of Cajal (ICC), 318
Intestine
anaphylaxis, 335
Gastrointestinal tract
reflexes, 392–393
Intraganglionic laminar endings (IGLEs), 149–161, 317, 319, 320
Intramuscular arrays (IMA), 317, 318–319
Ion channels
acid sensitive, 174
calcium, 38–39, 82–84, 298
HCN, 94–95
inflammatory modulation, 326–327
ionic currents in channel subtypes, 87–94
ligand-gated, 287–288, 323–331
plasticity, 38–39
potassium, 84–87, 94, 203, 235–236, 282, 298
sodium, 79–82, 283, 324–325
TRP family, 171–174, 288
voltage-gated, 77–99, 288, 323–327
Ionic currents, 87–94
Ionotropic receptors, 171–176
acid sensitive ion channels (ASICs), 174
excitatory amino acid receptors, 329
5-HT receptor, 174–175
ionotropic purine (P2X) receptors, 175
nicotinic acetylcholine (nAch) receptor, 176
TRP family, 171–174
Irritant receptors, see Rapidly adapting pulmonary stretch receptors
Ischemia, 363–365
ITTXR, 89–92
ITTXS, 89–92
J
J receptors, see Bronchopulmonary C-fibers
Jugular ganglion, 4–5, 8, 289–290, 316–318, 380
Jugular neurons, 20, 21–22
Juxta-pulmonary capillary receptors, see Bronchopulmonary C-fibers
K
Kurotoxin, 83
L
Labeling, see Tracers
Lactic acid, 297
Larynx, 380
laryngeal afferents, 280–281
laryngo-gastric reflex relaxation (LGRR), 389
laryngo-LES reflex relaxation (LLESR), 388
laryngo-UES contractile reflex (LUCR), 384
reflexes, 384, 388–389
Lateral cricoarytenoid (LCA) muscles, 384
Lateral line ganglia, 6
LCA, see Lateral cricoarytenoid (LCA) muscles
LES, see Lower esophageal sphincter
Leukemia inhibitory factor (LIF), 37–38
Leukotrienes, 417, 451–452
LGRR, see Larynx, laryngo-gastric reflex relaxation
LIF, see Leukemia inhibitory factor
Lipopolysaccharide (LPS), 336, 470–471
LLESR, see Larynx, laryngo-LES reflex relaxation
Locus coeruleus, 180, 256, 257
LOS, 322
Lower esophageal sphincter (LES), 388, 390
esophago-LES reflex relaxation (ELESR), 388
gastro-LES reflex relaxation (GLRR), 390
laryngo-LES reflex relaxation (LLESR), 389
pharyngo-LES reflex relaxation (PLESR), 388
transient LES relaxation (TLESR), 390
LPS, see Lipopolysaccharide
LUCR, see Larynx, laryngo-UES contractile reflex

Lungs
- Bronchopulmonary vagal afferent nerves
- Pulmonary/airway reflexes, 283, 286, 403–420
- Pulmonary C-fibers, 249, 264, 285–286, 288, 291
- Vagal lung afferents, 279–313

M

*Mash*, 13
*Mast cells*, 335–336, 446–447, 451–452
*Math3*, 13

Mechanoreceptors, 317, 409–410
- Cardiac, 359
- Chemical modulation of, 323
- Cough, 411–412
- Mechanical activation of, 152–157
- Muscle, 318–323
- Receptive fields of, 156–157

Mechanotransduction, 147–166, 359
- Endogenous chemicals, 159
- Evidence against chemical mediation, 157–158
- Mechanical activation of receptors, 152–157
- Molecular basis, 160–161
- Receptor morphology, 149–151

Medial vestibular nuclei, 224

Medulla oblongata, 248, 254–255

Melanocytes, 19

Memantine, 159

Metabotropic receptors, 176–189
- Bradykinin B1 receptor, 177
- Cannabinoid (CB) receptors, 181–182
- Cholecystokinin (CCK) receptors, 177–178
- Eicosanoid receptors, 180
- GABA4 receptors, 181
- Histamine H1 receptors, 178–180
- Neurokinin (NK) receptors, 178
- Opioid receptors, 181
- ORL-1 (nociceptin) receptor, 180–181

Modulation, 171

Monoaminergic modulation in brainstem, 247–275

MRS 1191, 301

Mucociliary clearance, 440

Mucosal chemoreceptors, 331

Mucosal edema, 287

Muscle mechanoreceptors, 318–323
- Chemical modulation of mechanosensitivity, 323
- Differences in mechanosensitivity, 319–320

sensitization, 322–323

threshold, 320–322

*Mycoplasma pulmonis*, 441–442

Myenteric ganglia, 319

Myenteric plexus, 318

Myocardial ischemia, 363–365

N

*nAch*, see Nicotinic acetylcholine (nAch) receptor

*NANC*, see Noncholinergic-noradrenergic nervous system

Natural killer (NK) cells, 445

Nausea, 336–337

*NEP*, see Neutral endopeptidase

Nerve growth factor (NGF), 322
- Axonal transport, 49–50
- Diabetes, 54–59
- Inflammation mediator, 301–302, 447–450
- RSV infection, 293
- Vagal afferent development/survival, 29–31, 46

Neural crest, 7–8, 11
- Central connections, 15
- Induction, 16–17, 21
- Jugular/nodose ganglia, 316
- Lineage, 17–19
- Sensory neurogenesis, 16, 19–20

Neural plate, 8, 9

Neural tracing, see Tracers

*NeuroD*, 13

Neurogenesis, 12–14, 19–20

Neurogenic inflammation
- 431–464; Inflammation
- Age-dependency, 443
- Blood flow, 438–439
- Bronchomotor tone, 439–440, 443–444
- Cough, 440–441
- Mast cells, 446–447
- Mucociliary clearance, 440
- Neuroimmunomodulation, 445–446
- Neurotrophins, 447–449
- Pathophysiology of airway diseases, 441–450
- Plasma extravasation/neutrophil adherence, 437–438
- Post-viral, 444
- Tachykinin effects in the airway, 437–441
- Tachykinin peptides, receptors, and peptidase, 432–437
- Therapeutic strategies, 450–454

*Neurogenins (ngns)*, 13, 14, 16, 20, 22

Neuroimmunomodulation, 442–443, 445–446, 450
Nitric oxide synthetase (NOS), 40–246
Nitric oxide (NO), 40, 83
Nicotinic acetylcholine (nAch) receptor, 176
Nicotine, 286; Smoke/smoking
NGF, NF
Neutrophil adherence, 437–438
Neutral endopeptidase (NEP), 293, 433–434
Neurotrophins
Neurotrophic factors
Neurotransmitters, 40–45, 50–51, 317–318;
Neuropeptides, neuropeptide K
Neurokinins (NKs)
A (NKA), 40, 406, 432–437
B (NKB), 432–437
receptors, 178, 198, 433–438, 442–448
Neuropeptides
42–44, 318, 432–437; specific neuropeptides
neuropeptide γ (NPγ), 432–437
neuropeptide K (NPK), 432–437
neuropeptide Y (NPY), 40, 44, 391, 419
Neurotransmitters, 40–45, 50–51, 317–318;
specific neurotransmitters and neuropeptides;
Neurotrophins
Neurotrophic factors
Neurotransmitters
axonal transport, 48–50, 57–59
BDNF (see brain-derived neurotrophic factor)
CNTF, 37–38
GDNF, 36–37, 51, 323
mRNAs, 52–53
neurogenic inflammation, 447–449
neurotrophin-3 (NT-3), 29, 34–36, 46, 48–51
neurotrophin-4/5 (NT-4), 29, 31–34, 36,
48–50
NGF (see nerve growth factor)
p65; p75
receptors, 29, 46–48, 52–59
vagal afferent development, 29–36
vagal afferent maintenance, 45–51
vagus nerve damage and disease, 52–59
Neutral endopeptidase (NEP), 293, 433–434,
436–442, 451
Neutrophil adherence, 437–438
NFkB, 38
NGF, see Nerve growth factor
Nicotine, 286; Smoke/smoking
Nicotinic acetylcholine (nAch) receptor, 176
Nifedipine, 38–39, 83
Nitric oxide (NO), 40–41, 209–246
cardiovascular response modulation, 224–228
excitatory/inhibitory synapses, 232–233
gene transfer studies, 234–235
neuro-modulation by, 210–214
NTS localization, 214–221, 231–232
smooth muscle tone regulation, 417
targets in brain, 213
transmitter interactions, 228–230
vasodilation, 419
Nitric oxide synthetase (NOS), 40–41, 210, 221
activation, 212–213
eNOS, 212–213, 216–219
gene transfer studies, 234–235
inhibitors, 225, 233–234
isoforms, 211–212, 231–232, 234–235
nNOS, 212, 216–224
NTS localization, 222–224
targets in brain, 213
transport, 222
Nitroxyergic modulation, 209–246
NK, see Natural killer (NK) cells
NKs, see Neurokinins
NMDA receptors, 196–197, 202–203, 329
nNOS co-localization, 220, 221
NO regulation, 212, 228–230
NO, see Nitric oxide
Nociception, 267–269, 472–476
airway nociceptors, 405–406, 410–412,
419–420
C-fibers, 201
C neurons, 295, 432
nociceptin receptor, 180–181
stimulants, 417
vagal vs. spinal nociceptors, 320
Nodose ganglion, 4–5, 7, 289–290
gastrointestinal sensory neurons, 316–318
laryngeal afferent cell bodies, 280, 290
neurotrophic factors in development, 36–39
neurotrophin receptors, 46–48, 57
neurotrophins in development, 29–36
plasticity in development, 38–39
Nodose neurons
activity and ion channel-dependent plasticity,
38–39
BDNF in development, 31–34, 38–39, 51
CCKα receptors, 334–335
C-fibers, 175, 290
CNTF in development, 37–38
embryology, 8–16
GDNF in development, 36–37
neurotransmitters, 39, 41–45
NGF and neuron survival, 29–31
nonneuronal cell trophic actions, 39
NT-3 and TrkB in development, 34–36, 47–48,
50
NT-4 and TrkB in development, 31–34, 47–48
tracers for, 142
TrkA in development, 47–48, 50
Type A, 91, 102
Nodose placode, 7–8, 10, 11–12, 15
Noncholinergic-noradrenergic nervous system
(NANC), 435
Nonneuronal cell trophic actions, 39
Noradrenaline, 255, 419
NOS, see Nitric oxide synthetase
Notch signaling, 12–13, 21
NPγ, see Neuropeptides, neuropeptide γ
NPK, see Neuropeptides, neuropeptide K
NPY, see Neuropeptides, neuropeptide Y
Nscl1, 13
NT-3, see Neurotrophins, neurotrophin-3
NT-4, see Neurotrophins, neurotrophin-4/5
NTS, see Nucleus tractus solitarii
Nucleus ambiguus, see Rostral nucleus ambiguus
Nucleus raphe obscurus (ROb), 256
Nucleus raphe pallidus (RPa), 256
Nucleus tractus solitarii (NTS), 193–208, 316
AMPAR receptors, 196, 201–203, 220
cardiovascular function modulation, 224–228
central sensitization, 293
central subnucleus (ceNTS), 221
eNOS, 212–213, 216–219
– 219
gastric vagal afferents, 380
glutamate, 195–197, 201–202
input signal, 195
intrinsic neuronal properties, 199–200
neurotransmitters, 317–318
nitrergic neuron localization, 219–221
– 221
nitroxergic modulation, 209–246
NMDA receptors, 196–197, 202–203
nNOS, 212, 216–224
NO interactions, 228–230
NO localization, 214–221, 231–232
NOS axons and fibers, 222–224
postsynaptic modulation, 199
presynaptic depression, 197–198, 202
reflexes, 380
second-order neurons, 195, 250, 316–317,
338, 380
synaptic plasticity, 200–201
synaptic transmission components, 194–200,
201–203
viscerotopic afferents, 230–231

O

Omega-agatoxin, 83
Omega-conotoxin, 83
Oncostatin M (OSM), 37–38
Opioids, 181, 323
ORL-1 (nociceptin) receptor, 180–181
OSM, see Oncostatin M
Ouabain, 282
Ozone, 291, 293

P

P2X, see Purine (P2X) receptors
p65, 38
p75, 29, 46–47, 51, 53, 447–448
PACAP, see Pituitary adenylate cyclase-activating polypeptide
Parainfluenza virus, 293, 441
Parasympathetic ganglia, 413
Parasympathetic nervous system, 416–420
Paratrigeminal nuclei, 224
Paraventricular nucleus, 230
PARs, 335
Pars alpha, 261
Pax family, 9–10, 12, 15
PBG, see Phenylbiguanide
PDZ domain, 212, 229
PEIR, see Pharynx, pharyngo-esophageal inhibitory reflex
Pelvic organs, 467–468
Peptidases, 432–437
Peptide histidine-isoleucine (PHI), 435
Peptide transmitters, see Neuropeptides
Peroxynitrite, 214
Petrosal ganglion, 7
– 7
neurotrophic factors in development, 36–39
neurotrophin receptors, 46–48
neurotrophins in development, 31–36
plasticity in development, 38–39
Petrosal neurons
activity and ion channel-dependent plasticity,
39
BDNF in development, 31–34
GDNF in development, 36–37
neurotransmitters, 39, 41–45
NT-3 and TrkC in development, 34–36, 47–48,
50
NT-4 and TrkB in development, 31–34, 47–48
TrkA in development, 47–48, 50
Petrosal placode, 7, 11–12
PGCR, see Pharynx, pharyngo-glottal closure reflex
PGE_{2}, see Prostaglandin
PGRR, see Pharynx, pharyngo-gastric reflex relaxation
Pharynx, 380
pharyngeal swallows (PS), 387–388
pharyngo-esophageal inhibitory reflex (PEIR), 386–387
pharyngo-gastric reflex relaxation (PGRR), 389
pharyngo-glottal closure reflex (PGCR), 385–386
pharyngo-LES reflex relaxation (PLESR), 388
pharyngo-UES contractile reflex (PUCR), 382–383
reflexes, 386–388
Phenyldiguanide (PBG), 289, 300, 361, 410–411
Phenyldiguanide, 289
Phenylethanolamine N-methyl transferase (PNMT), 220, 255
PHI, see Peptide histidine-isoleucine
Phospholipase C (PLC), 302
Phosphoramidon, 439
Phox2a, 13, 14, 15
Phox2b, 14, 15
Phrenic nerves, 265, 409
Pituitary adenylate cyclase-activating polypeptide (PACAP), 45, 417
PKA, see Protein kinase A
PKC, see Protein kinase C
Placodes, 6–15
development, 8–16
epibranchial, 12–14, 316
induction, 11–12
multi-placodal primordia, 10
pan-placodal primordium, 9, 15
Plasma extravasation, 437–438
Plasticity, 38–39, 200–201
PLC, see Phospholipase C
PLESR, see Pharynx, pharyngo-LES reflex relaxation
PNMT, see Phenylethanolamine N-methyl transferase
Pollutants/irritants, 265–266
Potassium currents, 92–93
Potassium ion channels, 84–87, 203, 298
voltage dependent calcium-activated, 94, 326
voltage-gated, 325–326
Pressure receptors, 280
Presynaptic depression, 197–198
Proneural genes, 12–13
Prostaglandin, 286, 294–295, 417
Protein kinase A (PKA), 295
Protein kinase C (PKC), 296
PS, see Pharynx, pharyngeal swallows
PSD95, 212
PUCR, see Pharynx, pharyngo-UES contractile reflex
Pulmonary C-fibers, 249, 264, 285–286, 288, 291
Pulmonary reflexes, 283, 286, 403–420
Purine (P2X) receptors, 175, 198, 221, 288, 290, 328–329
Pyloric sphincter, 391
Raphe nuclei, 223
Raphe obscurus, 261
Rapidly adapting pulmonary stretch receptors (RARS), 283–285, 290, 293, 296, 298, 404–405
Receptors, see specific receptors
Recurrent laryngeal nerve (RLN), 289, 382, 384, 385
Reflexes, 380–393
airway/pulmonary, 283, 286, 403–420
autonomic, 412–420
cardiovascular, 221, 224–228, 420
cough, 289, 406–412
esophageal and supra-esophageal, 380–389
gastric, 389, 390–393
gastrointestinal, 317, 380–393
intestinal, 392–393
laryngeal, 384, 388–389
monitoring, 413–416
pharyngeal, 386–388
respiratory, 406–412
Reperfusion, 363–365
Respiration, see Airway function
Respiratory infections, 441–442
Respiratory reflexes, 406–412
Respiratory syncytial virus (RSV), 293, 442–449
Respiratory viruses, 293
Reticular areas, 224
RLN, see Recurrent laryngeal nerve
rNa, see Rostral nucleus ambiguus
ROb, see Nucleus raphe obscurus
Rostral nucleus ambiguus (rNA), 250, 251, 252, 256, 261–262, 380
RPa, see Nucleus raphe pallidus
RSV, see Respiratory syncytial virus
S
SARS, see Slowly adapting pulmonary stretch receptors
Satiety, 333–335
Secretin, 332
Sendai virus, 293, 441
Sensory neurons, see Vagal sensory neurons
Serotonin (5-HT3), 40, 336–337, 470
central autonomic regulation, 261
GI tract, 327–328, 331–332, 336–337
receptors, 174–175, 288, 289, 295, 361
serotonergic neurons, 261–264
sGC, see Soluble guanylyl cyclase
Six family, 9–10, 15
Sleep-wake cycle, 266
SLN, see Superior laryngeal nerve
Slowly adapting pulmonary stretch receptors (SARS), 281–282, 289, 290, 298, 404–405
Slug, 17
Small bowel, 316-318; Gastrointestinal tract; Intestine
Smoke/smoking, 293, 382, 386, 388, 437–438
Smooth muscle, 249, 261, 265, 387, 412, 416–417
SNARE complex, 159
Sodium currents, 89–92, 288, 295, 323–324, 324–326
Sodium ion channels, 79–82
Epithelial Sodium Channels (ENaCs), 283 voltage-gated, 324–325
Smooth muscle, 249, 261, 265, 387, 412, 416–417
SOM, see Somatostatin
Somatostatin (SOM), 40, 220
Sphincters, 380–381, 380–382; specific sphincters
Spinal visceral afferents, 467–469
Spino-thalamic tract (STT), 469
Stomach
316–317, 319–321, 380; Gastrointestinal tract esophago-gastric reflex relaxation (EGRR), 389
gastric reflexes, 390–392
gastro-LES reflex relaxation (GLRR), 390
laryngo-gastric reflex relaxation (LGR), 389
pharyngo-gastric reflex relaxation (PGR), 389
Sulfur dioxide, 293
Superior laryngeal nerve (SLN), 384, 387, 389
Supra-esophageal reflexes, 380–387
Sympathoadrenal system, 480
Synaptic plasticity in NTS, 200–201
Synaptic transmission, 194–203
AMPA receptors, 196, 201–203
central sensitization, 293
glutamate, 195–197, 201–202
input signal, 195
modulation in CNS, 213–214
NMDA receptors, 196–197, 202–203
postsynaptic modulation, 199
presynaptic depression, 197–198, 202
second-order neuron, 195
T
Tachykinins, 286–287, 291–293, 302, 318
antagonists, 453–454
effects in the airway, 437–441
neurogenic inflammation, 432–437
specific peptides, 432
TDI, 293
TEA, see Tetraethylammonium
Tension receptors, 147–166, 317
in-series, 318, 319
morphological structure, 149–151
muscle mechanoreceptors, 318–323
tension-mucosal receptors, 148
Tetraethylammonium (TEA), 85–86
Tetrahydrobiopterin, 211
Tetramethylrhodamine (TMR), 130–134, 139
and biotin (TMR-B), 131–134, 135–136
immuno/counterstaining compatibility, 141
permanence of labeling, 142
resolution with, 140
Tetrodotoxin (TTX), 43, 89–92
TTX-resistant sodium current, 288, 295, 324–326
TTX-sensitive sodium ion channels, 79–82, 91, 324
TH, see Tyrosine hydroxylase
Thalamus, 322
Thoracic visceral organs, 468–469
TLESR, see Transient LES relaxation
TMR, see Tetramethylrhodamine
TNFα, see Tumor necrosis factor α
Tracers, 123–145
data and validations, 134–138
immunohistochemistry and counterstaining, 140–142
multiple injections, 141
permanence of labeling, 141–142
resolution, 140
selection of, 138–142
specific tracers, 125–136
transport time, 142
Tracheobronchial ganglia, 248
Tractus solitarius, 223
Transduction, see Chemotransduction
Transient LES relaxation (TLESR), 390
Transient receptor potential (TRP), 78, 171–174, 329–330
family of channels, 171–174, 178
vanilloid type 1 receptor (TRPV1), 287–288, 295, 296, 297–298, 318, 448
Transmitters, see Neurotransmitters
Trigeminal ganglion, 6, 7–8
Trigeminal nuclei, 224
Advances in Vagal Afferent Neurobiology

Trigeminal placode, 6, 7
Trk receptors, 322–323
NGF action, 51, 302
vagal nerve damage and disease, 52–59
Trks
TrkB, 29, 31–34, 46–48, 50
TrkC, 34–36, 47–48, 50
vagal nerve damage and disease, 52–59
Trophic factors, see Neurotrophic factors
TRP, see Transient receptor potential
TTX, see Tetrodotoxin
Tumor necrosis factor α (TNFα), 335, 446, 470–471
Tyrosine hydroxylase (TH), 31, 39, 41–42
AVPN localization, 257–258
DOPA production, 255
NGF and, 50–51
nNOS co-localization, 220
Tyrosine kinases, 29
Vagal afferent nerves
autonomic reflexes, 412–420
bronchopulmonary, 279–313
cardiac, 351–375
gastrointestinal tract, 315–350
GI reflexes, 379–401
NO formation, 222–223
NOS presence/absence, 222–223
respiratory reflexes and cough, 406–412
tracing, 123–145
Vagal afferent neurons
27–59, 101-120; Jugular neurons; Nodose neurons; Petrosal neurons
anatomy, 103–104
cell body electrophysiology, 101–120
δ neurons, 200
development and survival, 29–39
ion channels, 77–99
ionic currents, 87–94
maintenance, 40–59
neurotransmitters, 39, 41–45, 50–51
neurotrophic factors, 36–39, 51
neurotrophin receptors, 46–48, 52–59
neurotrophins, 29–35, 45–59
pathophysiological studies, 114–115
phenotypes, 124–125
physiological studies, 111–114
plasticity, 38–39
retrograde labeling, 105–107
target-identified, 107–116
threshold, 320–322
tracing, 123–145
Type A neurons, 91, 102
Type C neurons, 102
vagus nerve damage/disease, 52–59
Vagal lung afferents, 278–313
Vagal preganglionic neurons, see Airway-related vagal preganglionic neurons
Vagal sensory ganglia, 289–290
Vagal sensory neurons, 1–26
airway function, 248–250
airways, 113
differentiation, 21
electrophysiology, 101–120
embryology, 4–26
gastrointestinal tract, 316–318
neurogenesis, 19–20
somatic vs. visceral, 4–7
subtype identity, 14, 16, 19–21
survival, 14
voltage-gated ion channels, 77–99
Vagal tension receptors, see Tension receptors
Vagotony, 320, 391, 413, 476–480
Vagus nerve
abdominal, 316
cervical/thoracic, 381, 384
damage and disease, 52–59
dorsal motor nucleus, 250, 252, 316, 380, 389
embryology, 4–5
epilepsy, 495–510
neurotrophin content, 55–57
neurotrophin receptors, 57
recurrent laryngeal nerve, 289
subdiaphragmatic trunks, 316–318
Trk localization, 48
Vasoactive intestinal peptide (VIP), 40, 43–44, 417
AVPN expression, 252
neurogenic inflammation, 435, 439
NGF/NT-3 effects, 51
vasodilation regulation, 419
Vasoconstriction, 419–420
Vasodilation, 419–420
Verapamil, 38–39
Veratridine, 42, 282
Vestibuloacoustic (VIII) ganglion, 6
VGC, see Ion channels, voltage-gated
Vinblastine, 41–42, 43
VIP, see Vasoactive intestinal peptide
Viruses, 293, 440, 442–449
  abdominal vagal afferents, protection, and illness responses, 469–471
  pelvic and abdominal organs, 467–468
  thoracic organs, 468–469
VNS Therapy System®, 496, 498–504
Voltage-gated ion channels, see Ion channels, voltage-gated
Vomiting, 336–337

W
WGA-HRP, see Wheatgerm agglutinin-horseradish peroxidase
Wheatgerm agglutinin-horseradish peroxidase (WGA-HRP), 125–128, 139
  immuno/counterstaining compatibility, 141
  multiple injections, 141
  permanence of labeling, 142
  resolution, 140
  transport time, 142
Win 55212-2, 181
Wnt signaling, 17, 19–20, 22

Z
ZTC, see Streptozotocin