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Max Planck Institute of Psychiatry, Kraepelinstrasse 2, 80804 Munich, Federal Republic of Germany

*Corresponding author: E-mail: dodt@mpipsykl.mpg.de

Abstract

Infrared-guided laser stimulation is a new technique that allows precise and rapid stimulation of visualized neurons in brain slices. Infrared imaging of neurons with a new contrast system is combined with the photolytic release of caged neurotransmitters by an ultraviolet (UV) laser. Addition of caged neurotransmitters to the superfusion medium of neurons in brain slices allows local excitation in the micrometer range with a focused spot of UV light. In this way, the distribution of glutamate or γ-aminobutyric acid (GABA) receptors on neuronal dendrites can be mapped. Furthermore, this technique can be used to map the connectivity of neuronal networks through the controlled stimulation of neighboring neurons. Because the laser stimulation can be performed much faster than can paired recording, it is also possible to search for synaptic connections between distant neurons that have a low probability of connectivity.

Introduction

Many neurobiological questions could be readily addressed by a technique that allowed for precise and quick stimulation of visualized neurons in brain slices. Such a technique is now available: the visualization of neurons with infrared (IR) light in combination with the photolytic release of caged neurotransmitters. When a caged neurotransmitter (*1*, *2*) is added to the superfusion medium, neurons in brain slices can be excited by shining light on them. After setting up the equipment, this kind of stimulation is almost as simple as aiming a laser pointer at a picture of a neuron displayed on a screen. This very localized application allows one to scan dendrites for the distribution of neurotransmitter receptors. Furthermore, by stimulating neighboring neurons, the connectivity of neuronal networks can be investigated. Because the laser stimulation can be performed quickly, this technique is well suited to searching for synaptic connections between distant neurons that have a low probability of connectivity.

Experimental Setup

The experimental setup is designed to allow simultaneous visualization, patch-clamping, and stimulation of neurons in brain slices (Fig. 1A). After patch-clamping a neuron, laser stimulation of any part of the neuron or neuronal circuit must not disturb the recording electrodes. To accomplish this most easily when the field of view is very restricted at high magnification, we recommend mounting the microscope on a two-axis translation stage so that the microscope can be moved relative to the brain-slice chamber and the recorded neuron. The micromanipulator holding the patch pipette has to be motorized. The translation stage of the brain slice chamber, as well as the translation stage and focus drive of the microscope, should also be motorized for easiest manipulation. For patch-clamping, "healthy"-looking neurons should be identified in the slice at a lower magnification, and then the selected neuron approached with the patch pipette using a higher magnification. For this procedure, it is helpful to place an additional magnification changer in front of the video camera, allowing for an intermediate magnification of 1× or 4× the magnification of the objective.

Because caged compounds are rather expensive, a recirculation system for their use is helpful (*3*). Slices are submerged in a slice chamber, which is initially gravity-fed with standard Krebs-Ringer solution. Before the experiment is started, the slice chamber is connected to a vial in a closed loop with 0.3-mm inner diameter Teflon tubing. The vial, a 5-ml open-standing glass cylinder, contains the Krebs-Ringer solution, which is oxygenated continuously with carbogen (95% O_2 and 5% CO_2) introduced by fine Teflon tubing (0.5 mm inner diameter). Recirculation of a small volume (about 3 ml) of the Krebs-Ringer solution, is initiated: The Krebs-Ringer solution is aspirated through one Teflon tubing from the oxygenation vial by a peristaltic pump and introduced into the brain slice chamber. The same pump then aspirates the solution that superfused the slice through a second Teflon tubing and recirculates it to the oxygenation vial. The caged neurotransmitter is added from a concentrated stock to the oxygenation vial just before the recording is started.

Visualization of Neurons

Neurons in brain slices can be visualized by a combination of IR light, a contrast-generating system, and contrast enhancement by video technique. With standard microscopy, single neurons cannot be seen in thick brain slices because the neuronal network consists of a large number of neurons packed close together. These cells act as birefringent-phase objects that scatter light very effectively. Neurons that can be seen easily in dispersed cell cultures are obscured in brain slices by the scattered light produced by other cells in the layers above and below them. Any method for visualization of neurons in slices must therefore reduce this scattered light. Light scattering can be reduced by increasing the wavelength of illumination directly by the optics used for contrast generation, and indirectly by electronic contrast enhancement (*4*, *5*). The first reduction of light scattering is achieved by the use of near-IR radiation instead of visible light (*6*). Because of its longer wavelength (λ), IR radiation scatters less than visible light. This effect, well known to astrophysicists (*7*), can also be used to examine brain tissue (*8*). For IR videomicroscopy, a wavelength of 780 nm was found empirically to give the best results (*9*). Longer wavelengths give slightly better results, but the standard Newvicon tubes in video cameras are only sensitive to light up to a wavelength of 850 nm.

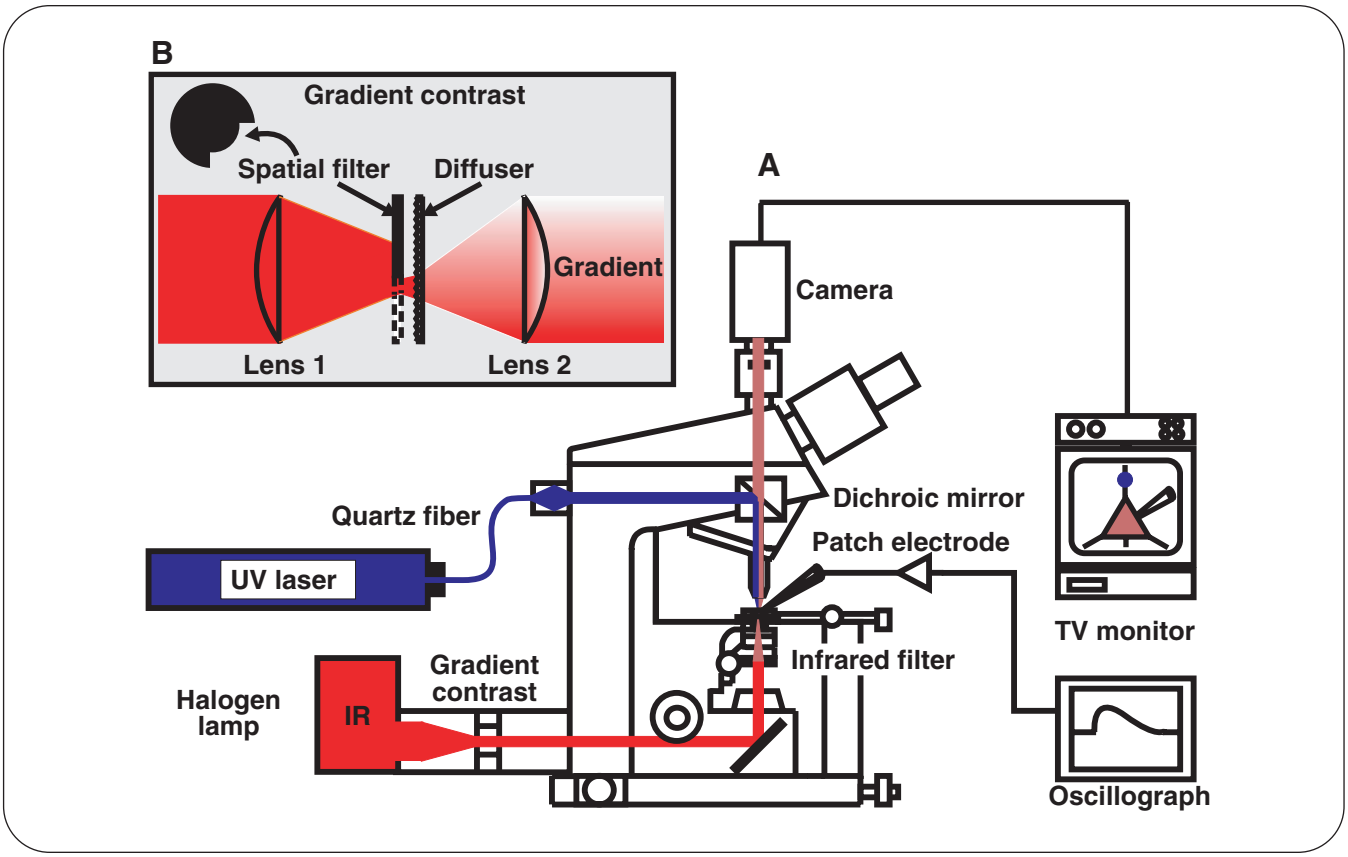

Fig. 1. (**A**) Experimental setup used for IR-guided laser stimulation (*17*). Neurons in the brain slice were visualized by illumination with IR light and the gradient contrast system. At the same time, light pulses from a UV laser were fed by a quartz fiber into the microscope and directed by a dichroic mirror onto the recorded neuron. Both the slice chamber and the microscope could be positioned in x and y dimensions by remote controls. The laser spot with an optical diameter of 1 μm formed by the objective (60×, 0.9 N.A.) in the specimen plane was made visible before the experiment with fluorescent paper, and its position was marked on the video monitor. By positioning the neuron to be stimulated on this point, the laser stimulation could be precisely guided by visual control. (**B**) Principle of gradient contrast. With a quarter annulus left open, a light stop is inserted as spatial filter in the condenser's aperture plane, which is then re-imaged by two lenses between the lamp house and the microscope. A diffuser that can be shifted relative to the light stop then generates an adjustable gradient of illumination across the aperture plane of the condenser. Because only a smoothed, hollow quarter-cone of light emerges from the condenser and transverses the brain slice, less stray light is generated than with standard full-cone illumination. This allows one to use a 1.4-N.A. oil immersion condenser, which gives superior resolution.

Standard halogen lamps serve adequately as light sources for IR radiation because their peak emission is in the near-IR range. The wavelength of illumination can be selected by placing a broadband interference filter ($\lambda = 780 \pm 50$ nm) in the filter holder of the microscope. The heat protection filter of the halogen lamp, which blocks IR light, can be removed and replaced by a filter on the field diaphragm in the foot of the microscope. This special filter (KG-1, Schott, Wetzlar, Germany) protects the brain slice against heat during visual inspection of the slice, when the interference filter is removed.

Unstained neurons in brain slices are phase objects. To render them visible, their phase gradients have to be converted into amplitude gradients by the optics. The optical system that provides images of neurons with the highest contrast is the gradient-contrast system (Fig. 1B) (*10*). In this system, the aperture plane of the condenser is re-imaged with a lens system between the rear of the microscope body and the lamp house to make it accessible for spatial filtering. A light-stop in the form of a quarter circle is positioned in the illumination beam path. At a small distance from the slit, a diffuser is introduced that generates a "gradient" of illumination across the condenser aperture plane. No spatial frequencies in the illuminating light are completely filtered out, so the image remains similar to the object. In addition, the curved form of the slit gives a gradient of illumination in two perpendicular directions, left to right and up to down. This can be very helpful for visualization of dendritic branches traversing in different directions. Because the light-stop blocks much of the illuminating light, only part of the normal, illuminating light cone is used and therefore less stray light is generated in the slice. The same principle is used with the slit-lamp by ophthalmologists. The contrast generated in this way is so high that gradient contrast alone allows visualization of neurons even in thick slices without a camera. Because no light-

consuming optical elements have to be placed in the beam path after the objective, gradient contrast can be combined with techniques such as fluorescence and photostimulation.

The visibility of neurons in brain slices can be greatly improved by electronic contrast enhancement. Contrast enhancement in real time is most easily achieved by the use of video technology, using cameras with Newvicon tubes (Hamamatsu). The "shading correction" performed by this camera is very useful because it electronically corrects image "shades" caused by optic distortions. Thus, the experimenter can usually obtain an evenly illuminated field of view.

Stimulating Neurons with UV Light

The setup (Fig. 1) allows simultaneous visualization of the neurons and of the focused UV light (spot) to be used to activate the caged neurotransmitter. By aiming the UV spot onto the neuron of interest, the experimenter is able to perform "IR-guided photostimulation." The light of a UV source is coupled into the epifluorescence port of the microscope through a quartz fiber and lens assembly and is reflected by a dichroic mirror onto the brain slice. As the light enters the objective from the back, the light-emitting end of the quartz fiber is demagnified, producing a small spot of UV light in the focus plane of the objective. By fine positioning of the quartz fiber, the UV spot can be directed precisely onto a point in the middle of the image plane. Before the sample is placed on the microscope, the UV spot is visualized by putting a small piece of white paper in the slice chamber. The position of the UV spot is then marked on the screen of the video monitor. After replacing the paper with a brain slice, the neuron to be stimulated is positioned on the mark on the video monitor during the experiment. The UV spot also becomes visible in the brain slice by autofluorescence of the tissue. For UV spots with diameters in the 10-µm range, a high-pressure mercury burner is sufficient as light source. For UV spots in the 1-µm range and for light flashes of short duration (in the 3-ms range), a UV laser has to be used to induce depolarizations of the neuron in the millivolt range. At present, a water-cooled argon-ion laser of up to 90 mW UV output is the most suitable laser.

Materials

Adenosine triphosphate, magnesium salt (Mg-ATP)

$CaCl_2$

Ethylene glycol bis (β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA)

Glucose

Hepes

$MgCl_2$

NaH_2PO_4

$NaHCO_3$

Potassium gluconate

Fluorescent paper (Linos, Göttingen)

Caged Neurotransmitters

γ-(CNB-caged) glutamate [L-glutamic acid, γ-(α-γ-[α-carboxy-2-nitrobenzyl (CNB)] ester, trifluoroacetic salt], [Catalog #G-7055, Molecular Probes (http://www.probes.com)]

O-(CNB-caged) GABA [γ-aminobutyric acid, (α-carboxy-2-nitrobenzyl (CNB)) ester, trifluoroacetic salt], [Catalog # A-7110, Molecular Probes (http://www.probes.com)]

Inhibitors

3-aminopropyl(diethoxymethyl)phosphinic acid (CGP-35348) (Sigma-Aldrich)

Picrotoxin (Sigma-Aldrich)

Tetrodotoxin (TTX) (Sigma-Aldrich)

Animals

2- to 3-week old male Sprague-Dawley rats

Equipment

The equipment that is used to perform this technique is specialized. We list below the specific equipment that we use, along with a brief explanation of the advantages of the listed products.

Carbogen (95% O_2 and 5% CO_2) tank with a pressure reduction valve. This is connected with polyethylene tubing (inner diameter 6 mm) to the tip of a 5-ml glass pipette standing in a 2-liter Erlenmeyer glass container to bubble the Krebs-Ringer solution continously.

Peristaltic pump [Type Reglo Analog MS-4/8, Ismatec (http://www.ismatec.com)]

Microscope and Video Camera

Axioskop 2FS microscope [Zeiss (http://www.zeiss.com)]

60× objective [Type LUMPLFL 60×/IR, Olympus (http://www.olympus.com)]

Note: The Olympus objective has good UV transmittance and is nearly parfocal for both IR and UV. It is completely compatible with the Zeiss microscope.

Magnification changer [Luigs and Neumann (http://www.luigs-neumann.com)]

Broadband interference filter ($\lambda = 780 \pm 50$ nm, Type KMZ 50-2; Schott, Wetzlar, Germany)

Gradient contrast system (Luigs and Neumann)

Video camera with Newvicon tubes [C2400-07, Hamamatsu (http://www.hamamatsu.de)]

UV Source

Enterprise II argon ion UV laser [Coherent (http://www.coherentinc.com)]

Note: This laser delivers 90 mW of multiline UV (351 to 364 nm). The laser requires water cooling; standard lab water connections are sufficient. The laser output power can be conveniently adjusted with a remote control in increments of 1 mW.

100-W high-pressure mercury lamp [Zeiss (http://www.zeiss.com)]

Note: This UV lamp can be used as an alternative to the UV laser. The Zeiss "Attoarc system" is very convenient and its output power can be regulated from 15 to 100%.

Infrared Radiation Source

Halogen lamp with KG-1 filter, heat protection filter removed (Schott, Wetzlar, Germany)

Note: The KG-1 filter protects the brain slice from heat when the heat protection filter is removed from the halogen lamp.

Electrophysiology and Micromanipulator Equipment

Optomechanical setup package [Luigs and Neumann (http://www.luigs-neumann.com)]

Note: The package comprises a two-axis translation stage, slice chamber, mechanical mounts and manipulators (240 standard setup), infrared gradient contrast, 4× magnification changer, and fiber optic coupling from laser or high pressure mercury lamp to microscope. Luigs and Neumann can also supply, on request, Zeiss or Olympus microscopes.
Recording amplifier [NPI electronic (http://www.npielectronic.com)]

Data acquisition system with PULSE software [HEKA (http://www.heka.com)]

Note: The SEC-10LX single-electrode voltage-clamp amplifier with low-noise recording head can be calibrated for the HEKA data acquisition system.

Brain Slice Preparation

Vibrating tissue slicer [FTB (http://ftb.vibracut.home.pages.de)]

Recipes

Recipe 1: Krebs-Ringer Superfusion Solution

NaCl	125 mM
KCl	2.5 mM
NaH_2PO_4	1.25 mM
$CaCl_2$	2 mM
$MgCl_2$	1 mM
$NaHCO_3$	25 mM
Glucose	25 mM

Saturate with 95% O_2 and 5% CO_2 by bubbling in carbogen for 30 min to achieve a pH of 7.4.

Recipe 2: Filling Solution for Patch-Clamp Pipettes

Potassium gluconate	130 mM
KCl	5 mM
EGTA	0.5 mM
Mg-ATP	2 mM
Hepes	10 mM
Glucose	5 mM

Adjust the pH to 7.2 with KOH.

Recipe 3: Caged Neurotransmitter Solutions

Prepare stock solutions of 30 mM (caged glutamate) or 60 mM (caged GABA) in Krebs-Ringer Superfusion Solution (Recipe 1). Store in 50-μl aliquots at –20°C wrapped in aluminum foil to protect from light.

Recipe 4: TTX Stock Solution

TTX	1 mM

Prepare in distilled water.

Recipe 5: Picrotoxin Stock Solution

Picrotoxin	5 mM

Prepare in distilled water.

Recipe 6: CGP35348 Stock Solution

CGP35348 50 mM

Prepare in distilled water.

Instructions

Preparation of the Brain Slices and Application of Caged Compounds

1. Anesthetize male Sprague-Dawley rats (2 to 3 weeks old) with ether.
2. Decapitate.
3. Remove the section of brain corresponding to the sensorimotor area of the cortex.
4. Cut 300-μm-thick parasagittal neocortical slices using a vibrating tissue slicer following standard procedures (*10*).
5. Submerge the slices in a slice chamber, which is gravity supplied with 1.5 ml of standard Krebs-Ringer Superfusion Solution (Recipe 1).
6. Connect the slice chamber to a small vial (large enough to hold 5 ml) with Teflon tubing with an inner diameter of 0.3 mm and connect to a peristaltic pump to create a closed recirculating system.
7. Fill the vial with 1.5 ml of Krebs-Ringer Superfusion Solution (Recipe 1) that is supplied continuously with carbogen (95% O_2 and 5% CO_2) (oygenation vial).
8. Prepare patch-pipettes from borosilicate glass capillaries using a horizontal pipette puller.
9. Fill recording pipettes with Filling Solution for Patch-Clamp Pipettes (Recipe 2).

   Note: DC resistance of the filled pipettes should be between 3 and 6 MΩ.
10. Record membrane currents from the slices under voltage clamp conditions using conventional whole cell-patch techniques (*10*) with a patch-clamp amplifier.
11. Add a small aliquot of highly concentrated Caged Neurotransmitter Solution (Recipe 3) to the oxygenation vial just after a stable patch-clamp recording has been obtained.

    Note: The final concentration of caged neurotransmitter needed should be empirically determined from a range of 250 μM to 1 mM.
12. Switch the solution supply to the recirculating system. The peristaltic pump recirculates 3 ml of Krebs-Ringer containing the caged neurotransmitter.

Positioning Neurons for Stimulation with UV Light

1. Visualize the UV spot on a small (1 cm × 1 cm) piece of white fluorescent paper in the slice chamber.
2. Mark the position of the spot on the screen of the video monitor.
3. Replace the paper with the brain slice.
4. Identify the neuron to be stimulated using the IR radiation and contrast-generating and contrast-enhancement systems described in "Visualization of Neurons."
5 Position the neuron to be stimulated in the same location as the mark on the video screen.
6. Activate the caged neurotransmitter with spots from the UV lamp or laser, ranging from 1 μm to 10 μm for 3 ms to 10 ms.

Sample Conditions for Monitoring Synaptic Plasticity with Caged Glutamate

Determining Pre- or Postsynaptic Source of Long-Term Depression (LTD)

Synaptic glutamate release can be mimicked by the photolytic glutamate release that occurs in a small focused UV laser spot. This technique offers a new approach to longstanding neurobiological questions, such as whether the locus of long-term potentiation (LTP) and long-term depression (LTD) are situated on the pre- or postsynaptic site. Unlike experiments that rely on normal synaptic transmission, one parameter (release from the presynaptic site) is held constant. Glutamate is released from its caged form by constant UV flashes (energy of the laser varies by less than 1%), and all presynaptic input is blocked by TTX. Therefore, any change in neuronal response has to be postsynaptic in origin.

1. Block all presynaptic input by bathing the brain slice in 1 μM TTX Solution (Recipe 4) for 10 min.
2. Position neurons of the neocortical lamina V region in the path of the UV laser using the IR radiation and contrast-generating and contrast-enhancement systems described in "Visualization of Neurons."
3. Add 0.5 mM γ-(CNB-caged) glutamate (Recipe 3) to the recirculating bath.
4. Release the caged glutamate with 3-ms UV laser flashes every 20 s for 10 min, and record the synaptic activity by patch-clamp techniques.

   Note: This establishes the baseline glutamate responses of the recorded neurons.
5. Release the caged glutamate with a train of 3-ms UV flashes at a frequency of 5 Hz for 1 min, and record the glutamate responses by patch clamp techniques.

   Note: This stimulation paradigm produced LTD in about 30% in all neurons tested for up to 1 hour of recording (Fig. 2A).

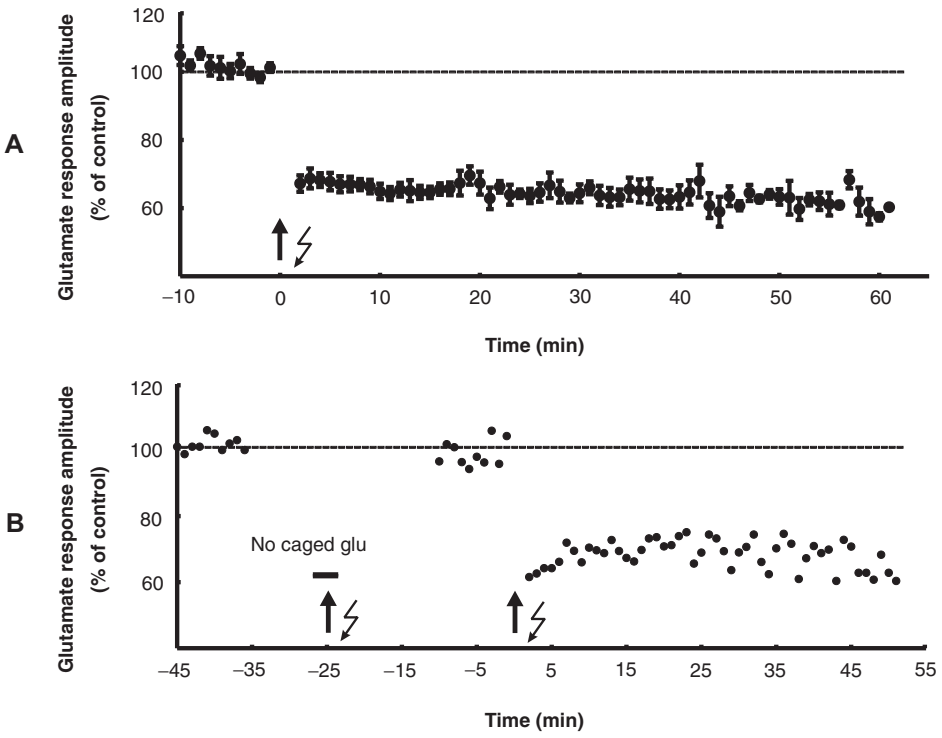

Fig. 2. Focal photolysis of caged glutamate induces LTD of glutamate receptors. (**A**) Tetanic release of glutamate from caged glutamate by a burst of UV-light pulses (3 ms long at 5 Hz for 1 min) causes LTD of membrane depolarizations mediated by glutamate (glutamate responses). Before and after the tetanus (arrow), glutamate was released by 3-ms light pulses every 20 s. The data shown are averages for every minute. Average of nine neurons (mean ± SEM). The 100% value in all figures represents the average of the last 5 min before tetanization. (**B**) LTD is not induced by the laser radiation itself. After establishing a baseline, caged glutamate was washed out and the 5 Hz light tetanus was applied. Afterwards, caged glutamate was washed in again. Glutamate responses were not affected by the light tetanus (single experiment shown). Reapplication of the light tetanus in the presence of caged glutamate induced robust LTD.

Determining the Spatial Specificity of LTD

The high spatial resolution of the UV laser stimulation can also be used to investigate the spatial extent of neurobiological phenomena related to neuronal plasticity. For this kind of experiment, it is of crucial importance to determine the accuracy of the laser stimulation. We determined this accuracy by laterally offsetting the laser point and measuring the induced neuronal depolarizations (Fig. 3A). Based on the measured effective glutamate release diameter, the spatial specificity of synaptic plasticity (LTD, in this case) can be determined.

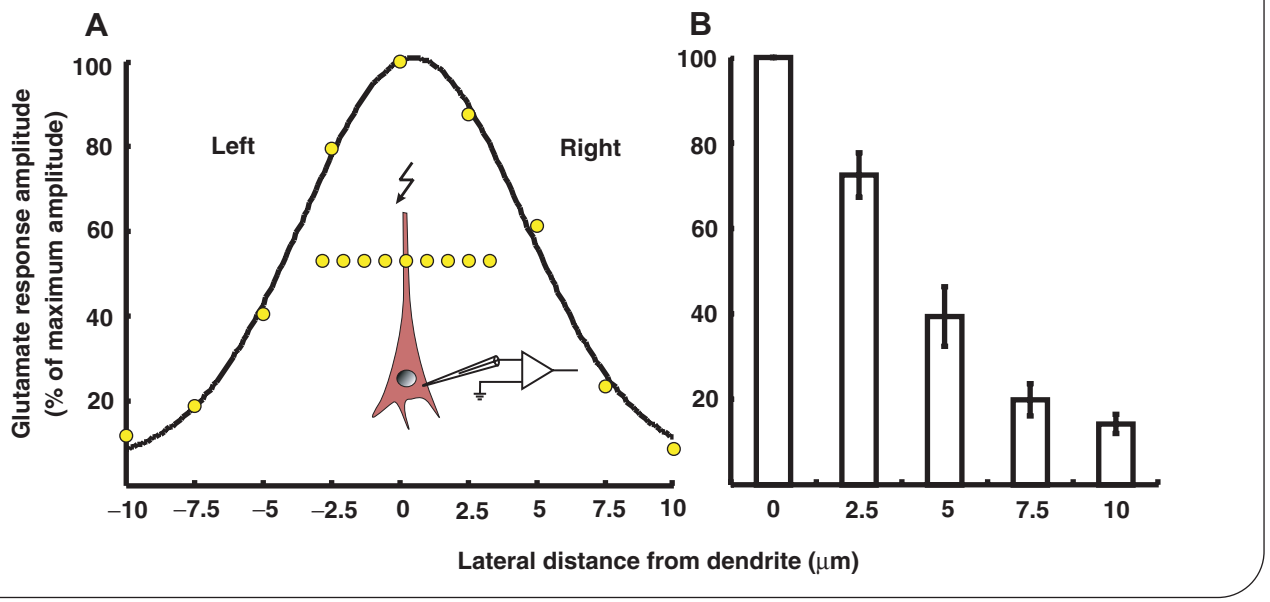

Fig. 3. LTD of glutamate receptors is spatially highly restricted. (**A**) Spatial specificity of the laser stimulation. The UV-stimulation point was moved laterally away from the dendrite in increments of 2.5 μm, and the decrease of the glutamate response amplitude was plotted as a function of this distance (single experiment shown). (**B**) Average of results of six neurons (from the experimental setup shown in Fig. 1 and Fig. 3A). Only the measurements of one side were considered, because often the Gaussian shape of the other side was distorted, probably due to an invisible secondary dendritic branch (*17*), full width half maximum, 4 ± 1 μm.

1. Position neurons of the neocortical lamina V region in the path of the UV laser using the IR radiation and contrast-generating and contrast-enhancement systems described in "Visualization of Neurons."
2. Add 0.5 mM γ-(CNB-caged) glutamate (Recipe 3) to the recirculating bath.
3. Offset the UV laser spot by 2.5 μm and measure the induced neuronal depolarizations.
4. Repeat the stimulation at distances of −10 to +10 μm to determine the accuracy of the laser stimulation.

   Note: We obtained a precision of laser stimulation of 4 ± 1 μm laterally [full width half maximum (FWHM), n = 6 neurons] and 18 ± 2 μm axially (n = 4 neurons) (Fig. 3, A and B). Thus, the effective "glutamate release site" can be regarded as a spot of about 10 μm in diameter.
5. Release the caged glutamate at several sites separated by 10 μm at a low frequency (3-ms UV laser flashes every 20 s for 10 min) and record the glutamate responses by patch clamp techniques to establish a baseline of activity.

   Note: We tested seven sites (Fig. 4).
6. At one of the centrally located sites, induce LTD with a train of 3-ms UV flashes at a frequency of 5 Hz for 1 min and record the glutamate responses by patch-clamp techniques.
7. Release the caged glutamate at the adjacent sites and record by patch clamp techniques to determine the effect of a locally induced LTD on adjacent synaptic activity.

   Note: We found significant reductions in glutamate response only at the site of LTD induction. The reductions of the glutamate responses measured 10 μm to the left and right of this point were statistically insignificant ($p > 0.05$). Thus, the LTD observed in these experiments had a spatial specificity of at least 10 μm, which is currently the limit of spatial resolution achievable.

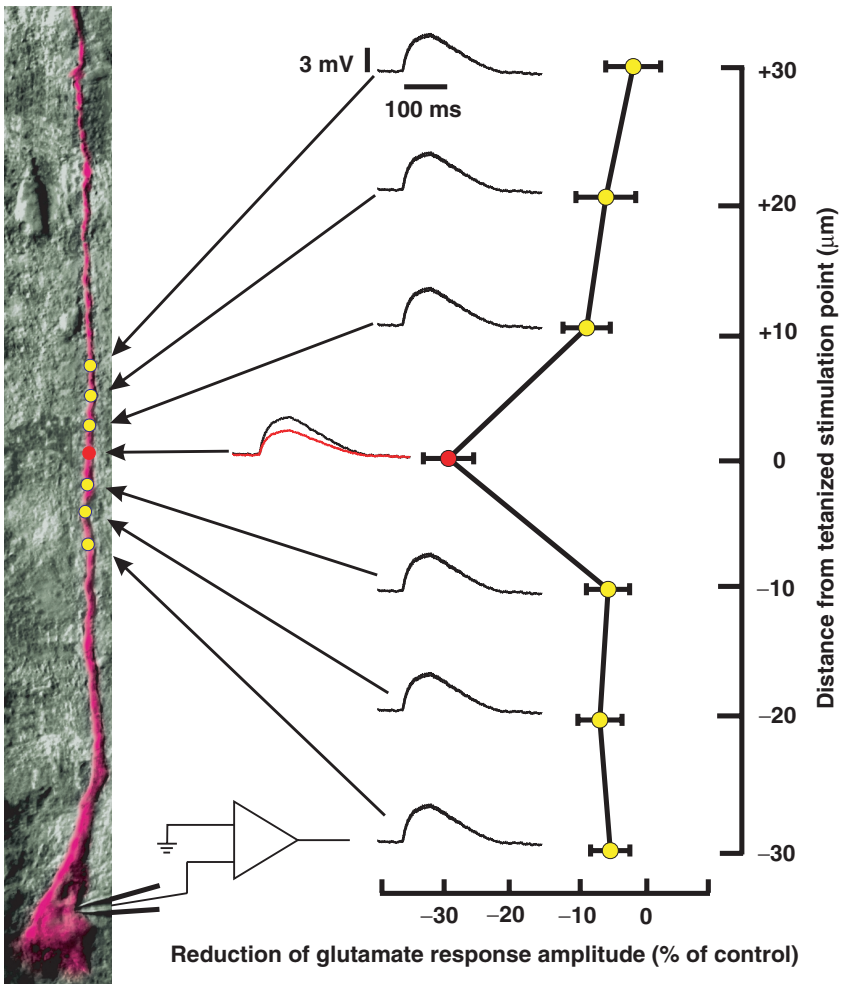

Fig. 4. Example of a pyramidal neuron of neocortical layer V overlaid with seven UV-stimulation points on the dendrite (scale bar, 10 μm). The reduction of the glutamate responses after 5 Hz stimulation to the central point only is indicated in red. LTD (29 ± 4 %) was induced only at this stimulation site, illustrated in the figure by the glutamate responses, scaled according to the reduction. At all other stimulation sites reductions were not significant compared with values before tetanic stimulation ($p > 0.05$, $n = 9$ neurons). Values were obtained by averaging five stimulations for each stimulation site on each neuron before, and 10 to 20 min after, the 5 Hz stimulation, and by calculating the relative reduction.

Sample Conditions for Mapping Receptor Distribution

Infrared-guided photostimulation of pyramidal neurons allows one to investigate the distribution of neurotransmitter receptors on the soma and dendrites. Specific receptor antagonists can also reveal the distribution of subtypes of receptors. For example, the addition of the specific *N*-methyl-D-aspartate (NMDA)-receptor antagonist D-APV revealed a higher density of NMDA receptors on the apical dendrite near the soma than on more remote sites on the dendrite (*10*). This kind of receptor density determination can be performed with a high-pressure mercury lamp as light source. Increasing the resolution of the method by using a laser (*11*) revealed hot spots of neuronal excitability (*12*). With the technique of IR-guided laser stimulation, it should be possible to map the distribution of all neurotransmitter receptors whose ligands are available in caged form. We describe the mapping of GABA receptor subtypes using laser stimulation and $GABA_A$ and $GABA_B$ receptor antagonists (*13*).

1. Position the neuron to be mapped into the path of the UV laser using the IR radiation and contrast-generating and contrast-enhancement systems described in "Visualization of Neurons."
2. Add 1 mM γ-(CNB caged) GABA to the recirculating bath.
3. Elicit GABA responses with 3-ms pulses of 10- to 40-mW UV laser energy along the dendrite up to a distance of 400 μm from the soma, and record the neuronal response using patch-clamp techniques.
4. Add 75 μM picrotoxin (Recipe 5) to the recirculating bath to specifically inhibit $GABA_A$ receptors, or 200 μM CGP35348 (Recipe 6) to specifically inhibit $GABA_B$ receptors.
5. Elicit GABA responses by 3-ms pulses of 10- to 40-mW UV laser energy along the dendrite up to a distance of 400 μm from the soma and record the neuronal response using patch-clamp techniques.

Related Techniques

To study neurotransmitter receptor distribution on the neuronal surface, the classical method of neurotransmitter application by microiontophoresis can be used (*10*). However, repeatedly moving a thick microiontophoretic pipette through a brain slice can dislodge the recorded neuron from the patch-clamp pipette. In addition, positioning the microiontophoretic pipette at close to the dendrite is only possible by touching the dendrite with the pipette. This increases the danger of desensitizing the glutamate receptors or damaging the neuron.

For the study of neuronal networks, a straightforward alternative to photostimulation is to patch-clamp a putative presynaptic neuron, then elicit an action potential in this neuron by current injection. This approach of paired recording is feasible using both sharp electrodes (*14*) and patch-clamp electrodes (*15*). Paired recording provides excellent temporal resolution but is very time-consuming, because every putative presynaptic neuron has to be patch-clamped. Thus, paired recording has been applied mainly to neighboring neurons and becomes nearly impossible at neuronal distances >500 μm.

Because neocortical neurons have very large dendritic trees with lengths that can be >1000 μm and high glutamate sensitivity throughout the dendrite, it is mandatory to visualize the soma of the stimulated neuron. This prevents stimulation of an invisible part of a dendrite mistaken as the soma. Approaches claiming to produce maps of neuronal networks by blind stimulation in an automated raster fashion cannot give information on the position of the somata of synaptically connected neurons.

Notes and Remarks

A possible improvement of the method of infrared-guided laser stimulation might be the photolysis of the caged glutamate by a "two-photon" process. Photolysis of double-caged glutamate (*16*) did not give higher spatial resolution than single-caged glutamate in our test experiments. Thus, the two-photon process has to be induced by nonlinear excitation using a "two-photon" laser. Until now, no commercially available caged compounds exist that can be photolyzed by two-photon excitation. This may change soon. However, after photolysis, even an infinitesimally small release volume of glutamate would always result in a cloud of diffusing transmitter. Experiments will have to first prove whether this approach will really bring a significant improvement.

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