# The living state: how cellular excitability is controlled by the thermodynamic state of the membrane

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# **Abstract**

The thermodynamic (TD) properties of biological membranes play a central role for living systems. It has been suggested, for instance, that nonlinear pulses such as action potentials (APs) can only exist if the membrane state is in vicinity of a TD transition.

Herein, two membrane properties – excitability and AP velocity – are investigated for a broad spectrum of conditions in living systems (temperature (T), 3D-pressure (p) and pH dependence). Based on these data we predict parameter ranges in which a transition of the membrane is located  $(15 - 35^{\circ}C)$  below growth temperature; 1 - 3 pH units below pH 7; at ~800 atm) and propose the corresponding phase diagrams. The latter explain: (i) changes of AP velocity with T, p and pH. (ii) The existence and origin of two qualitatively different forms of loss of nonlinear excitability ("nerve blockage", anesthesia). (iii) The type and quantity of parameter changes that trigger APs. Finally, a quantitative comparison of between the TD behavior of 2D-lipid model membranes with living systems is attempted. The typical shifts in transition temperature with pH and p of model membranes agree with values obtained from cell physiological measurements (excitability and propagation velocity). Taken together, these results suggest that it is not specific molecules that control the excitability of living systems but rather the TD properties of the quasi two-dimensional membrane interface. The approach as proposed herein can be extended to other quantities (surface potential, calcium concentration, etc.) and makes clearly falsifiable predictions, for example, that a transition exists within the specified parameter ranges in excitable cells.

# **Keywords**

action potential, lipid bilayer, cell membrane, phase diagram, anesthesia, excitability

# **Abbreviations**

AP – action potential

TD - thermodynamic

LE – liquid-expanded phase

LC - liquid-condensed phase

# 1. Introduction

This work deals with the central question: is the existence of a thermodynamic transition in the cell membrane the origin of nonlinear cellular excitability?

Membrane interfaces are generally responsive to environmental stimuli (mechanical, thermal, chemical, electrical, etc.). For some thermodynamic (TD) states of membranes, stimuli do not only lead to a linear response but – above a threshold – result in all-or-none pulses (Mussel and Schneider, 2019a; Shrivastava and Schneider, 2014). In biology, such pulses are referred to as action potentials (AP). APs have been detected in different cells of animals (Aidley, 1998), plants (Wayne, 1994), etc. In addition, nonlinear pulses with the same characteristics have been excited in phospholipid monolayers at the air-water interface (Shrivastava et al., 2015; Shrivastava and Schneider, 2014). The universal character of the phenomenon, *i.e.* the existence of pulses in different species and in simple model membranes indicates that nonlinear excitability *does not depend on specific molecules*. Therefore, it is of interest to ask which general properties a membrane must possess in order to support such pulses. Furthermore, it is of interest to identify the regime of states in which a membrane is excitable, in order to better understand the "living state" that has established itself in biological systems. It is the intent of the present work to approach this problem from a classical TD perspective following the work of Einstein (Einstein, 1910).

Theoretical predictions (Heimburg and Jackson, 2005; Kaufmann, 1989; Mussel and Schneider, 2019a, 2019b) and experimental evidence (Shrivastava et al., 2015; Shrivastava and Schneider, 2014) have indicated that nonlinear pulses can arise when a lipid membrane is perturbed close to an ordereddisordered transition. Heimburg and Jackson, in particular, proposed the existence of stable propagating solitary waves, when the membrane state is transiently forced into a transition regime (Heimburg and Jackson, 2005). Indeed, many synthetic phospholipid membranes, including those in which nonlinear pulses were observed (Shrivastava et al., 2015; Shrivastava and Schneider, 2014), exhibit transitions (e.g. (Lee, 1977)). There also exists evidence for transitions in biological membranes (platelets (Tablin et al., 1996), plant germ tips (Crowe et al., 1989), sperm cells (Crowe et al., 1989), bacteria (Heimburg and Jackson, 2005), spinal cord (Wang et al., 2018), etc.). Furthermore, optical studies (Georgescauld et al., 1979; Ueda et al., 1974) and membrane potential measurements (Inoue et al., 1973; Ueda et al., 1974) have reported typical signs of transitions in excitable cells. However, the basis of evidence is too small and scattered for systematic conclusions. This is unfortunate, because it impedes central progress in our understanding of the origin of cellular excitability, non-excitable states of cells (anesthesia, etc.), means to stimulate cells, etc. We have proposed that a transition in an excitable membrane can be located from the "physiological phenomenology" (Fillafer and Schneider, 2013). The approach shall be explained briefly: in essence,

we assume that physiological membrane functions (permeability (Mosgaard and Heimburg, 2013; Wunderlich et al., 2009), catalytic rate, excitability, pulse velocity (Griesbauer et al., 2012; Shrivastava and Schneider, 2014), etc.) are determined by the TD state of the membrane interface. We call this "the state-to-function relation". Thus, measurement of a function reflects the underlying state. It is one hallmark of a TD transition that drastic changes of the material properties occur and this should dramatically alter the physiological functions. The velocity of pulses in a membrane, for example, should undergo a characteristic decrease upon entering a transition. The underlying reason is that the TD susceptibilities (e.g. compressibility, heat capacity), are increased in the transition regime as compared to the neighboring phases (Albrecht et al., 1978; Steppich et al., 2010). At least in the linear case, the relation between pulse velocity and the isentropic compressibility  $\kappa_s$  is

$$c \approx 1/\sqrt{\rho \kappa_S}$$
 (1)

with  $\rho$  as the density of the material. Therefore, if the velocity of pulses starts to decrease, the system likely enters a transition regime. This correlation between compressibility of the system and pulse velocity has been confirmed for linear (Griesbauer et al., 2012) and interestingly also for nonlinear waves (Shrivastava and Schneider, 2014) in phospholipid monolayers.

It is the goal of the present work to analyze the thermodynamics of living, excitable systems in light of the relation between physical state and biological function (the *state-to-function* approach). In particular, we propose that the origin of nonlinear cellular excitability is a transition of the 2D-membrane interface. This assumption leads to intuitive and testable interpretations of the perturbations that stimulate cells and about the manipulations of the cell by which the nonlinear response can be suppressed (anesthesia, nerve block, etc.). We first describe the theoretical concepts and predictions of the approach (3.1) and subsequently test these predictions qualitatively (3.2 and 3.3) as well as quantitatively (3.4-3.6). We conclude with further predictions of phase transitions and/or nonlinear material property changes that should be observable near the resting state of living systems.

## 2. Materials and methods

*Materials.* All reagents used were purchased from Sigma-Aldrich (St. Louis, MO, USA) and were of analytical purity (≥ 99%).

Cell cultivation and storage. Chara australis was cultivated in glass aquariums filled with a layer of soil ( $\sim 2~cm$ ), quartz sand ( $\sim 1~cm$ ) and deionized water. The plants were grown under illumination from an aquarium light (14 W, Flora Sun Max Plant Growth, Zoo Med Laboratories Inc., San Luis Obispo, CA, USA) at a 14:10 light:dark cycle at room temperature ( $\sim 20~°C$ ). Prior to use, single internodal cells were stored for a minimum of 12 h in a solution containing 0.1 mM NaCl, 0.1 mM KCl and 0.1 mM CaCl<sub>2</sub>.

**Propagation velocity measurements.** A setup similar to that described in (Lühring, 2006) was used. It consists of a compartmentalized plexiglas chamber which can be controllably heated/cooled and which can hold cells up to 10 cm in length. Small extracellular sections (length  $\sim 5 \text{ mm}$ ) of the cell were electrically isolated against each other with vacuum grease (Dow Corning Corporation, Midland, MI, USA). The K<sup>+</sup>-anesthesia technique (Lühring, 2006) in combination with extracellular Ag/AgClwire electrodes was used for monitoring the membrane potential at two sites along the internode. The extracellular solutions contained 110 mM KCl in the outmost compartment and artificial pond water in all other compartments (APW; 1 mM KCl, 1 mM CaCl<sub>2</sub>, 190 mM D-sorbitol, and 5 mM buffer substance (MES for pH 4.5 – 7; TRIS for pH 7 – 10); pH was set to 7.0 with HCl/NaOH). The potential between the virtual intracellular electrode (KCl-compartment) and extracellular electrodes was recorded with voltage sensors (PS-2132; 100 Hz sample rate; PASCO scientific, Roseville, CA, USA). When a supra-threshold electrical stimulus was applied, an AP was excited and subsequently passed along downstream electrodes. The delay time of the pulse between two electrodes with a known spatial separation is inversely proportional to the pulse propagation velocity. Since the conduction velocity varied between cells and since the main interest was on identifying relative changes, the data were normalized to the velocity in the basal state  $(T = 20^{\circ}C; pH = 7.0)$ . To vary the temperature of the cell in a controlled manner, the measuring chamber was placed on a solid copper block which was perfused by a heat bath (RM6, Lauda GmbH, Lauda-Königshofen, Germany). The pH of the extracellular medium was varied by titration with HCl and NaOH respectively.

Excitation by cooling. The medium in one of the compartments  $(0.5 - 1 \, mL)$  was replaced with a micropipette by the same medium at a lower temperature. First, relatively large stepwise temperature drops were employed  $(\Delta T \sim -5^{\circ}C)$  until the threshold was encountered. Subsequently, the threshold was narrowed down by smaller steps. In between different trials, the cell was allowed to re-equilibrate at the basal temperature for  $\sim 10 \, min$ . Typically, a cell was exposed to 10 - 15 temperature steps.

## 3. Results and Discussion

#### 3.1. Theory. TD state controls cellular excitability

Simply put, the physical theory of nerve pulse propagation – as opposed to the electrical circuit theory by Hodgkin Huxley (HH) – rests on the idea that *energy-, entropy- and momentum conservation must not be violated in biology*. Since a membrane is decoupled from its surroundings (Griesbauer et al., 2009), a perturbation of the membrane will propagate. *This follows straight from fundamental physical principles*. Wilke was presumably one of the first to mention "adiabatic propagation" in the context of nerve pulses as early as 1912 (Wilke, 1912), but the key theoretical step came by Kaufmann in 1989 who put the idea on a thorough theoretical footing (Kaufmann, 1989). From there, Heimburg & Jackson

(HJ) developed the "soliton model" of nerve pulse propagation (Heimburg and Jackson, 2005). Apart from some details (e.g. we do not believe that the propagating pulse is a soliton (Fillafer et al., 2017; Shrivastava et al., 2018)) we agree with the framework set forth by HJ. The key idea is that action potentials (APs) are nonlinear sound pulses that propagate in the membrane. The origin of the nonlinear response is a TD transition in the membrane interface (Heimburg and Jackson, 2005; Kaufmann, 1989; Mussel and Schneider, 2019a, 2019b). When a membrane is compressed from the fluid towards the gel phase (liquid-expanded to liquid-condensed in terminology of lipid monolayers), the system passes through this transition and the characteristic maximum in susceptibilities is crossed (e.g. compressibility; see red curve in Fig. 1(a)). Furthermore, the framework predicts a relation between *propagation velocity c* and compressibility  $\kappa_S$  of the membrane (see (1) and for more details (Griesbauer et al., 2009; Kappler et al., 2017)). It should be mentioned that the isothermal state diagrams, which are used as a basis for the analysis herein, are not strictly correct for all types of disturbances of the membrane (slow up to adiabatic state changes). Importantly, however, the transition and its consequences will be present in all cases (Mussel and Schneider, 2019a).

To use this theory and to arrive at testable predictions for biology, we start with hypothetical phase diagrams that were inspired from lipid model membranes (Fig. 1).

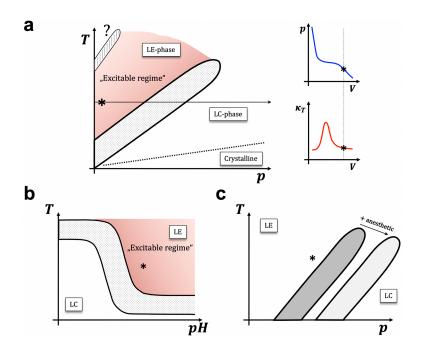


Figure 1. The relation between state and excitability as summarized in a putative phase diagram. (a) The cell membrane is excitable if the resting state (asterisk) is in the vicinity of an ordered-disordered transition (e.g. from liquid expanded (LE) to liquid condensed phase (LC) in liquid crystals; or from lamellar fluid to gel in lipid bilayers). This is illustrated by a p - V isotherm and the derived isothermal compressibility  $\kappa_T$  on the right (the arrow illustrates the respective slice through the phase plane). State changes move the system state (asterisk) through phase space and hence change the physical properties of the membrane. At low T and/or high p the

membrane "freezes" into a crystalline-like state. Note: There are indications that an additional transition is present at higher temperatures ("heat block"). (b) In the T - pH plane the curve progression of the phase boundary is sigmoidal. The underlying reason for this additional nonlinearity is that the headgroups of phospholipids have a pK (protonation transition). This results in a nonlinear change of the transition temperature  $T_m$  with pH. Thus, acidification at constant T and p can move the resting state into the crystalline-like phase. (c) According to one theory of anesthesia (melting point depression (Heimburg, 2018; Heimburg and Jackson, 2007; Ueda and Yoshida, 1999; Wang et al., 2018)), anesthetics leave the resting state in the disordered phase, but increase its distance to the transition.

It is the goal of this work to test, if these state diagrams correctly reflect the cellular phenomenology (excitability and pulse velocity).

#### 3.1.1. Predictions

According to the TD theory the resting state of an excitable cell should be in vicinity of a transition (see asterisk in Fig. 1). The location of the "living state" in phase space has been chosen as indicated, because ample evidence suggests that biological membranes reside on the fluid side of a transition (Crowe et al., 1989; Heimburg and Jackson, 2005; Tablin et al., 1996; Wang et al., 2018). In this regime a suitable stimulus/perturbation can force the cell membrane into the nonlinear regime (e.g. a sudden decrease of T or an increase of p). At the same time, slow changes of the cellular environment will lead to new resting states and thus to different excitability and pulse velocity. Since the (mean) compressibility of fluid lipid membranes decreases as temperature increases (Evans and Kwok, 1982; Steppich et al., 2010) we predict – according to (1) – an increase in AP propagation velocity c with temperature. In contrast, cooling down below  $T^*$  should have the opposite effect. Upon further cooling one crosses the transition regime into the gel or liquid-condensed state in lipid terminology. Any stimulus which previously "kicked" the system into this transition and thereby excited a pulse will now force the system state even further away from the nonlinear behavior. Effectively, the system has become *non-excitable*. We claim that such changes of state are the physical origin of several forms of loss of nonlinear excitability that have been observed in experiments ("cold block", "acid block", "3D-pressure block", etc.). Taken together, these arguments also provide a simple, testable explanation for an old pharmacological rule as expressed by one of the originators of the receptor concept, J.N. Langley: "[...] it is extremely common for a drug to stimulate first and then paralyze [...]" (Langley, 1906). Any substance (or other TD force) that is capable of taking the system towards a transition will be called excitatory (→ stimulation). If the system is continuously exposed to this substance (or TD force), however, a transition is effected and as a result the system will lose its non-linear response to this type of stimulus (→ nerve block, paralysis, desensitization, etc.).

In the framework of the "sound pulse"-description of APs (Heimburg and Jackson, 2005; Mussel and Schneider, 2019a; Shrivastava and Schneider, 2014), the phase diagram implies further testable

predictions. Cooling and pressurization, for instance, should have a similar impact on the state and thus excitability and velocity of APs (Fig. 1(a)). In consequence, it should be possible to compensate the cellular effects of pressurization by heating. Of course, this is only the tip of the iceberg, because the membrane state (and thus excitability and velocity) can also be changed by other thermodynamic forces such as  $\mu_{H^+}$ ,  $\mu_{Ca^{++}}$ , adsorption of molecules (*e.g.* anesthetics, toxins or proteins; note: the impact of membrane proteins on the state has been likened to an alloy (Sackmann, 1995)), transmembrane potential ( $\psi$ ), etc.). In fact, one should imagine a hypothetical equation of state  $p(T,pH,\mu_{lon},\mu_{protein},\psi)$ , which means that there is not only a relation between T and T (Fig. 1(a)), but also one between T and T and T (Fig. 1(b)) or between T and the concentration of anesthetics, etc. We proceed to some concrete predictions: based on the phase diagrams of anionic phospholipids, acidification is expected to reduce the velocity of APs, because it typically brings the system state closer to the transition (Fig. 1(b)). Furthermore, it should be possible to compensate this state and thus velocity change by an increase of T. Table 1 summarizes several conjectures that directly emerge from the *state-to-function* approach. The remainder of the present work is dedicated to test these predictions in living systems.

**Table 1.** Qualitative predictions based on the phase diagrams (Fig. 1). Increase and decrease of parameter is indicated by upward and downward arrows respectively.

	Velocity of AP	Loss of excitability	Triggering of AP
T	$c\downarrow$ with $T\downarrow$	$T\downarrow$	$T\downarrow$
p	$c\downarrow$ with $p\uparrow$	$m{p}\uparrow$	$m{p}\uparrow$
рН	$c\downarrow$ with $pH\downarrow$	pH ↓	$pH\downarrow$

We proceed as follows: The TD behavior of excitable living systems will be presented in chapters 3.2 - 3.4 Not only the roles of T, p and pH but also the relation to the melting-point-depression-theory of anesthesia will be discussed and quantitative agreement between our predictions and physiology will be presented. In 3.5 evidence for additional transitions in living systems will be provided and in 3.6 we discuss excitation of APs by different TD forces.

# 3.2. Thermodynamic behavior of action potentials

# 3.2.1. Loss of excitability upon cooling and heating

When an excitable plant cell (*Chara* internode) was cooled from the reference state, a progressive slowing down of APs was observed herein (Fig. 2) and by others (Beilby and Coster, 1976; Blatt, 1974).

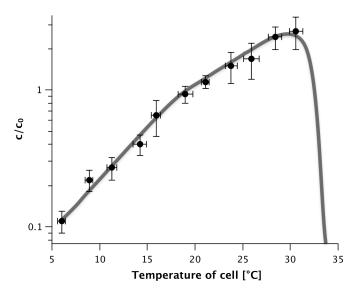


Figure 2. Temperature dependence of action potential velocity in *Chara*. Normalized velocity as a function of temperature. Each data point represents the average  $\pm$  StDev. of n=5-27 measurements in a total of N=10 cells. All data are normalized to the reference propagation velocity  $c_0$ , which ranged between 4-16 mm  $s^{-1}$  ( $T=20^{\circ}C$ ; pH=7.0).

Qualitatively similar results have been reported for axons (Chapman, 1967; Engelhardt, 1951; Franz and Iggo, 1968; Hodgkin and Katz, 1949; Kukita, 1982; Rosenberg, 1978) and muscular tubes (Fillafer and Schneider, 2013). At ~5°C, the pulse velocity was reduced to approximately a tenth of that at 20°C. Upon further cooling, APs often did not propagate along the entire length of the cell any more (*i.e.* the pulse only reached the proximal but not the distal region). This effect was not due to damage sustained to the distal parts, because (*i*) the distal part was still excitable by mechanical/electrical means and (*ii*) propagation over the full cell length was restored upon heating to ~10°C. Similar to *Chara*, squid axons are also excitable close to the freezing point of water (Hodgkin and Katz, 1949; Kukita and Yamagishi, 1981) (only some specimen of the latter exhibit a cold-block of excitability close to 0°C (Chapman, 1967)). In contrast, many nerves exhibit a reversible "cold block" of excitability above 0°C (Engelhardt, 1951; Franz and Iggo, 1968; Paintal, 1965; Rosenberg, 1978). The temperature of this block shifts during thermal acclimation (Engelhardt, 1951) and differs between species (Engelhardt, 1951) as well as myelinated and non-myelinated axons (Franz and Iggo, 1968).

Upon heating a *Chara* cell above its growth temperature the velocity increased monotonically up to a maximum at  $\sim 30 - 35^{\circ}C$  (Fig. 2). More importantly, a further increase in temperature led to cessation of excitability and cellular death (as evidenced by microscopy). This "heat block" of AP propagation

is remarkably universal and has been observed in squid giant axons (Chapman, 1967), *N. ischidiacus* from frog and cat (Engelhardt, 1951), dorsal column of tortoise (Rosenberg, 1978). In squid giant axons (Chapman, 1967; Hodgkin and Katz, 1949) and in blood vessel of blackworms (Fillafer and Schneider, 2013) the heat block was often reversible upon cooling. In the present experiments, this was not observed, presumably due to prolonged exposure (> 10 *min*) of the cell to the elevated temperatures.

Taken together, the phenomenology of the temperature dependence of *c* is very well conserved among plant (*Characean*) and animal cells (frog, cat, squid, etc.). The limits of the excitable regime are set by a cold and heat block and the velocity decreases in the vicinity of either block. This is in line with the predictions based on our phase diagram (Fig. 1 and Tab. 1). It is important to note that the cold block has been observed in many living systems that have been studied. In some experiments it was not detected (*e.g.* in *Chara* and some squid axons), presumably, because it is located below the freezing point of the aqueous medium, where experiments are not performed easily.

## 3.2.2. Loss of excitability upon acidification

Alkalization of the extracellular medium of a *Chara* cell up to  $pH \sim 10$  had negligible effects on the AP velocity (Fig. 3).

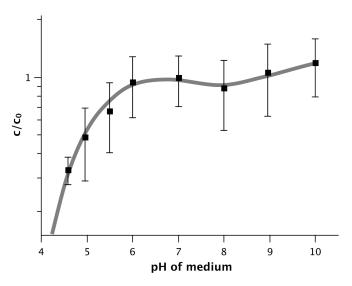


Figure 3. pH dependence of action potential velocity in *Chara*. Each data point represents the average  $\pm$  StDev. of n=12-17 measurements in a total of N=23 cells (note: pH 4.6 was an exception with only n=3 data points). All data are normalized to the reference propagation velocity  $c_0$  ( $T=20^{\circ}C$ ; pH=7.0).

In contrast, when the pH of the extracellular medium was lowered, a progressive slowing down of APs was observed. At  $pH \sim 5.0$  the pulse velocity was about half of that at pH 7. These results are in line with previous reports of a prolongation of APs with decreasing pH in Characean cells (note: a pulse

that propagates with a lower velocity will appear prolongated in time) (Beilby, 2007). A decrease in conduction velocity upon acidification has also been observed in cardiac muscle (Vaughan-Williams and Whyte, 1967) and diaphragm (Brody et al., 1991). In *Chara*, further acidification of the extracellular medium ( $pH \sim 4.0 - 4.5$ ) led to a pH-induced block of excitability (Tab. 1). Within the timescales of a typical experiment ( $10 - 30 \, min$ ) this block was reversible, *i.e.* when the extracellular pH was increased, excitability was reestablished. The pH-block has also been observed in other excitable cells, for example, upon acidification by  $\sim 1 \, \text{pH-unit}$  in heart cells (Harary and Farley, 1963) and squid giant axons (Tasaki et al., 1965). Again, the predictions from Tab. 1 (decrease of c with decreasing pH followed by block of excitability) are confirmed.

#### 3.2.3. Loss of excitability upon pressurization

To incorporate a third TD-parameter, we briefly review the effect of 3D-pressure on the propagation velocity and excitability of APs (for a more extensive review, see e.g. (Wann and Macdonald, 1980)). In one of the few works on the subject, Grundfest reported that an increase in hydrostatic pressure first leads to a slight increase of c followed by a slowing down of pulses  $(0.55 \cdot c_0)$  at  $\sim 800 \ atm$ ) (Grundfest, 1936). The decrease of c with an increase of c was confirmed in myelinated frog axons (Tasaki and Spyropoulos, 1957) and squid giant axons (decrease by c 15% at c340 c40 c41) (Spyropoulos, 1957). In the high pressure range (c550 – c61) c7100 c81 c72 at c7340 c735 c736. This c74 c757 c

#### 3.2.4. The action of anesthetics

In principle most of our arguments are compatible with a melting point depression theory of anesthesia set forth by Heimburg and others, which states that anesthetics are substances that interfere with nerve excitability via the location of the membrane transition (Fig. 1(c)) (Heimburg, 2018; Heimburg and Jackson, 2007; Ueda and Yoshida, 1999; Wang et al., 2018). It is a basic premise of this theory that that anesthetics preferentially dissolve in the fluid membrane phase and thereby induce a melting point depression. This means that the main transition is shifted to lower temperatures and thus away from the resting state (Fig. 1(c)). Several experimental findings in nerves agree with this assertion. If the *threshold* stimulus is administered, for instance, and subsequently the cell is exposed to anesthetics, the amplitude of the resulting pulse to this stimulus becomes progressively reduced until the stimulus-response curve is linear (Tasaki and Spyropoulos, 1957). This transition from a nonlinear to a linear response is in line with the expected removal of the resting state from the nonlinear behavior. However, it is possible to compensate this increased distance between resting state and depressed

melting point by stronger stimulation (Wang et al., 2018) (*i.e.* the nonlinear response of the system is not lost, just shifted). Furthermore, cooling, pressurization and acidification should counteract the effect of anesthetics, because these state changes are capable of moving the resting state towards the transition regime ((Heimburg and Jackson, 2007); *c.f.* Fig. 1). If the melting point depression theory is correct, loss of excitability by anesthetics is a different phenomenon as compared to the nerve blocks that have been discussed so far (cold block, acid block, etc.). The latter are a result of crossing the transition and thus cannot be compensated by an increase in stimulus strength (this will only drive the system further into the ordered phase). The physiological outcome of loss of nonlinear excitability can therefore be achieved by at least two different thermodynamic mechanisms.

# 3.2.5. Is nonlinear excitability limited to a phase?

In Fig. 1, we designated the liquid expanded (fluid) phase as the "excitable regime". This was based on the finding that the typical cell membrane at rest is in a fluid state. It was demonstrated, however, that experimental manipulations of a cell – most notably extensive cooling (Chang and Schmidt, 1960) - can lead to stable resting states that are characterized by a depolarized membrane potential level (Chang and Schmidt, 1960; Tasaki, 1959; Terakawa, 1981). It is tempting to speculate that in these experiments the cell was taken into the ordered phase. If the cellular resting state is indeed below the transition (c.f. Fig. 1(a)), one expects that a nonlinear response will now be generated by the exact opposite type of stimuli (heating, depressurization, etc.). The underlying reason is the inversion of the asymmetry between resting state and transition. In terms of the electrical stimulus, this prediction is indeed fulfilled: whereas cells exhibit a nonlinear response to outward membrane currents in their typical resting state, the cited studies reported an all-or-none behavior to inward currents (termed "hyperpolarizing response" or inverted APs in the literature (Chang and Schmidt, 1960; Tasaki, 1959)). These experiments indicate that the key to understanding the nonlinear response is the transition and its position in relation to the resting state in phase space. Therefore, it must be emphasized that the designation of the LE phase or fluid phase as the sole "excitable regime" in Fig. 1 is not strictly correct.

#### 3.3. Reversibility. TD cycles in a living system

It was one of our main assumptions that the cell membrane can be treated like a classical TD system in the sense that it is in a stable state and that reversible state changes are possible. This assumption will be proven wrong, for example, if the system does not return to its initial state upon performing a circular excursion in phase space. The assumption was addressed experimentally in *Chara* cells. When the pH of the extracellular medium was decreased by 2 units from the reference state (from pH 7 to 5) the conduction velocity was reduced to  $\frac{c_0}{2}$  (Fig. 5). It was possible to reverse this change by heating of

the cell to  $\sim$ 27°C. Upon returning the pH of the external medium to 7 the velocity was  $2 \cdot c_0$ . Subsequent cooling by 7°C to  $\sim$ 20°C led to restoration of the initial state and also of  $c_0$  in this cyclic process. This result indicated that a TD cycle in a living system is indeed possible on the timescales of the experiment ( $\sim$ 2 – 3 hours). It also underlines that different parameters can compensate each other, *i.e.* that a change in biological function (velocity) induced by one TD force (lowering the pH) can be compensated by the change of another force (e.g. an increase in T). This leads to a critical prediction: it must be possible to lift excitation blocks by the change of other parameters. According to the phase diagrams (Fig. 1), it should be possible, for example, to reverse a p – or pH–induced block by heating.

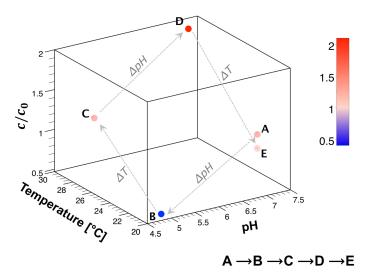


Figure 4. TD cycle in a living system. A decrease of the propagation velocity by acidification  $(A \to B)$  can be compensated by heating  $(B \to C)$ . The resting state is reversibly re-established by alkalization  $(C \to D)$  and subsequent cooling  $(D \to E)$ . Values were normalized to the velocity of the initial state  $(T \approx 21^{\circ}C; pH = 7.0)$ ). Each data point represents the average value of n = 6 - 7 measurements in a total of N = 3 cells.

# 3.4. Quantitative evidence for the state-to-function hypothesis in excitable systems

Chapter 3.2 and 3.3 provided the thermodynamic phenomenology of livings systems and demonstrated the agreement with the phase diagram that was borrowed from lipid membrane thermodynamics (Fig. 1). As stated above, the state-to-function hypothesis implies that it is the change in thermodynamic state of the 2D membrane (*not* the parameters and constituents that create it) that makes the difference in biological function. In particular, to be excitable, the membrane must reside near and above a transition in order to be able to access the nonlinearity. In addition to the qualitative predictions (Tab. 1), we now proceed to *quantitative comparisons*. The position of the transition will be given in relation to the cellular resting state. The induction of this transition by T, p, pH will be analyzed and

the underlying TD coefficients will be compared with those of lipid model membranes. These coefficients represent a quantitative scale, which can be used to correctly predict the cellular stimulation threshold for  $T_1p_1pH$ .

#### 3.4.1. Location of a transition and thermodynamic relations in living cells

TD transition below the growth temperature. Several independent investigators have demonstrated that cell membranes of bacteria, plants and animals reside on the fluid (disordered) side of an ordered-disordered transition (Crowe et al., 1989; Heimburg and Jackson, 2005; Tablin et al., 1996; Wang et al., 2018). These studies have indicated that the midpoint (e.g. maximum in  $c_p$ ) of a reversible transition is located  $\sim 10-30^{\circ}C$  below the growth temperature of the organism. The latter is in very good agreement with the location of the cold block as obtained from physiological phenomenology ( $\sim 15-35^{\circ}C$  below the growth/body temperature of the organism). This suggests that the physical origin of the "cold block" is indeed the crossing of a membrane transition.

Shift of membrane transition by p. The volume of phospholipid membranes is usually smaller in the ordered phase as compared to the disordered phase (Heimburg, 1998; Winter and Jeworrek, 2009). Accordingly, an increase in p stabilizes the ordered phase and thereby leads to a rise of melting temperature  $T_m$  on the order of  $1 - 3^{\circ}C/100$  atm (Tab. 2) (Cossins and Macdonald, 1989; Wann and Macdonald, 1980; Winter and Jeworrek, 2009). In cells, a very similar relation is found. There, an increase in pressure by +100 atm is equivalent to cooling by  $\sim 3^{\circ}C$  (quantity required for cold block divided by quantity for pressure block, Tab. 2). When pressurizing frog nerves, Grundfest found that an increase of p by ~800 atm leads to cessation of excitability (Grundfest, 1936). This is equivalent to cooling by  $\sim 24^{\circ}C$ , which is in remarkable agreement with the quantity required for cold block (cooling by  $15 - 35^{\circ}C$  below growth temperature). Therefore, the cold- and pressure-induced block are likely to have the same underlying cause: the shift of the system state across a transition regime. Shift of membrane transition by pH. Variations of pH around a membrane interface will change the degree of dissociation of the molecules and thereby will alter the charge state as well as the melting temperature  $T_m$  of the membrane. Acidification of typical cellular phospholipids (e.g. PS) is expected to lead to a reduction of membrane charge density and to an increase of  $T_m$  that is particularly pronounced if the pK of the interface is crossed (Fig. 1(b)).

**Table 2.** Parameter changes that lead to loss of excitability in cells and TD coefficients for cells (inferred) and phospholipid membranes (measured).

	Loss of excitability in cells	Coefficient in cells	Coefficient in phospholipid membranes
<b>\Lambda T</b>	15 – 35 °C a		

$$\begin{vmatrix} \Delta p \end{vmatrix} = 800 \ atm^b \qquad \qquad \begin{vmatrix} \frac{\Delta T}{\Delta p} \end{vmatrix} \sim \frac{2 - 4 \,^{\circ} C}{100 \ atm} \qquad \qquad \begin{vmatrix} \frac{\Delta T_m}{\Delta p} \end{vmatrix} \sim \frac{1 - 3 \,^{\circ} C}{100 \ atm}^d$$

$$|\Delta pH| \qquad 1 - 3 \ units^c \qquad \qquad \left| \frac{\Delta T}{\Delta pH} \right| \sim \frac{5 - 35 \,^{\circ} C}{1 \ unit} \qquad \qquad \left| \frac{\Delta T_m}{\Delta pH} \right| \sim \frac{2 - 20 \,^{\circ} C}{1 \ unit}^e$$

From this, it follows that acidification can induce a membrane transition at constant T and p (Träuble,

1977). The typical shift of the transition by pH for phospholipid membranes is  $\frac{\Delta T_m}{\Delta pH} \sim \frac{2-20^{\circ}C}{1 \text{ unit}}$  (Blume and Eibl, 1979; Heimburg and Jackson, 2007; Träuble, 1977), which is of the same order of magnitude as the quantity calculated for cells  $\left(\frac{\Delta T}{\Delta pH} \sim \frac{5-35^{\circ}C}{1\ unit}\right)$ . It should be noted that the latter value has to be taken with a grain of salt, because it linearizes the sigmoidal progression of the phase boundary (c.f. Fig. 1(b)). Based on the values for typical lipid membranes, a drop of pH by 1-3 units has the potential to raise the melting temperature by  $6-60^{\circ}C$ . This puts it well within reach that membrane protonation can raise the main transition to the growth temperature at constant T and p. The TD coefficients for lipid membranes and cells  $\left(\frac{\Delta T_m}{\Delta p}, \frac{\Delta T_m}{\Delta p H}\right)$ , which in principle represent the slopes of phase boundaries in the phase diagrams (Fig. 1), are based on the data of different authors and are summarized in Tab. 2. When calculating these coefficients, it was assumed that the distance of the resting state to the transition was the same in all of the collected literature results. The coefficients for cells closely resemble those of pure phospholipid membranes. This underscores the role of the twodimensional membrane state for the biological system. Taken together, these results strongly indicate that cooling, acidification and pressurization take excitable cell membranes towards (→ reduced velocity) and across a transition ( $\rightarrow$  loss of nonlinear excitability). This transition must be observable in excitable cell membranes within the specified parameter ranges.

#### 3.5. The heat block of excitability

From the phase diagram in Fig. 1 it is obvious that heating at constant pressure will remove the resting state from the main transition. As a consequence, compressibility will remain constant or will decrease (velocity is constant or increased respectively). Ultimately, heating should take the system above a critical temperature  $T_c$ . In this regime, p-A isotherms do not exhibit an inflection point anymore and thus the nonlinearity vanishes. In this part of phase space nonlinear excitability is not expected at least for stimulation of the system by an increase in p (c.f. Fig. 1). However, there exist indications that

<sup>&</sup>lt;sup>a</sup> (Engelhardt, 1951; Franz and Iggo, 1968; Paintal, 1965; Rosenberg, 1978); <sup>b</sup> (Grundfest, 1936); <sup>c</sup> Fig. 2 and (Harary and Farley, 1963; Tasaki et al., 1965); <sup>d</sup> (Cossins and Macdonald, 1989; Wann and Macdonald, 1980; Winter and Jeworrek, 2009); <sup>e</sup> (Blume and Eibl, 1979; Heimburg and Jackson, 2007; Träuble, 1977)

heating of an excitable cell does not merely take the system further into the disordered phase. It was demonstrated, for example, that the electrical potential and surface tension of a *Nitella* membrane change abruptly at  $\sim 35$  °C (Ueda et al., 1974). Moreover, the heat capacity profile of *Chara* membranes exhibits a second maximum at  $\sim 32$  °C (Beljanski et al., 1997), which is identical to the heat block temperature as found herein (Fig. 2). Fluorescence spectroscopic investigations in crab nerve membrane also indicated a distinct change in emission ratios at  $\sim 36$  °C (Georgescauld et al., 1979). In the vicinity of the heat block in squid axons the electrical membrane capacitance increases drastically (Palti and Adelman, 1969). Such deviations of the susceptibilities ( $c_P$ ,  $C_m$ ) are characteristic for transitions (Steppich et al., 2010). An increase in susceptibilities should also lead to a slowing down of APs and this was indeed observed in nerve preparations in proximity of the heat block (Chapman, 1967; Rosenberg, 1978). Taken together, these pieces of evidence suggest that an additional transition occurs in the biological membrane in the temperature range of the heat block (Fig. 1(a), indicated by question mark). It will be of interest to clarify the origin (protein denaturation, tendency for hexagonal phase formation, ...) and role of this transition for nonlinear excitability of the cell.

## 3.6. Means to stimulate cells ("reception by TD systems")

#### 3.6.1. Excitation close to a transition

So far, we considered state changes of the membrane interface in a quasi-stationary manner. The state of a system, however, can also be perturbed rapidly. Such a perturbation will lead to the TD forces being nonzero and this will drive propagation of the disturbance (Kaufmann, 1989). In the vicinity of a transition regime, nonlinear pulses can be induced (Heimburg and Jackson, 2005; Mussel and Schneider, 2019a; Shrivastava et al., 2015; Shrivastava and Schneider, 2014). From the TD perspective, this is the usual process for excitation (*i.e.* how an AP is triggered). In order to be a reasonable stimulus (*i.e.* to create a physiological response such as an AP) a perturbation in  $T, p, pH, \Psi, etc$ . has to be strong enough to locally induce a transition. Based on the phase diagrams, it is predicted that APs can be triggered by an increase in p, a decrease in p and by a decrease in pH because these parameter changes shift the system state into a transition (Fig. 1 and Tab. 2). The closer the resting state of the system is located to the transition, the more "receptive" the interface will appear.

#### 3.6.2. Triggering of APs by local cooling

The first prediction was tested experimentally in *Chara* cells. As expected, a sudden drop in temperature of the external medium by  $10 - 15^{\circ}C$  resulted in excitation (Fig. 5). The triggering of APs by local cooling has also been observed by others in a variety of excitable cells (Harvey, 1942;

Hill, 1935; Kobatake et al., 1971; Spyropoulos, 1961). It is worth noting that the same decrease in temperature did not trigger an AP if it was applied in a slow manner (*c.f.* Fig. 2). This underlines the difference between a rapid (adiabatic, Fig. 5) versus a quasi-stationary (isothermal, Fig. 2) state change.

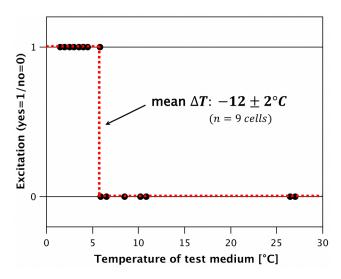


Figure 5. Triggering of an AP by sudden local cooling. The thermal excitation threshold for a cell was identified by local addition of cold medium. For this cell, which was kept at  $20^{\circ}C$ , the required temperature jump  $\Delta T$  was  $\sim -14^{\circ}C$ .

# 3.6.3. Excitation by pressurization, acidification and lateral pressure

In combination with the results in Fig. 5 the TD coefficients (Tab. 2) allow to make predictions about the excitation of APs by non-thermal means. Based on  $\frac{\Delta T_m}{\Delta p}$ , it can be estimated that a state change similar to a drop of temperature by  $10-15^{\circ}C$  (the thermal excitation threshold) can be effected, by pressurization by  $\sim 500-700$  atm. Indeed, it has been demonstrated that Characean cells (Harvey, 1942), squid axons (Tasaki and Spyropoulos, 1957) and other neurons (Grundfest, 1936; Wann et al., 1979) can be excited by application of hydrostatic pressure on the order of 350-550 atm. It has to be noted that in the latter experiments p was increased slowly to avoid heating of the aqueous bulk medium. Thus, the observed excitation is probably a consequence of slow transfer of the cellular resting state into the instability of the transition regime.

A similar argument as for p can be applied to excitation by protons. Based on  $\frac{\Delta T_m}{\Delta pH}$  (Tab. 1) it can be estimated that a decrease of pH by 2 – 3 units is required for excitation of a *Chara* cell. This is in fact the experimentally observed excitation threshold (Fillafer and Schneider, 2016). The triggering of APs by acidification is also a very common phenomenon in neurons (Frederickson et al., 1971; Krishtal and Pidoplichko, 1980; Varming, 1999) and it has been proposed that protons are the actual excitatory substance in some of the most important synapses, the cholinergic system (Fillafer and Schneider, 2016; Kaufmann, 1980).

Based on the relation between  $\tau$  and p, it can furthermore be predicted that a sudden increase in lateral membrane pressure by  $\sim 5-15 \, \frac{mN}{m}$  should lead to triggering of an AP (see Supporting Information).

## 4. Conclusions and outlook

Herein, we presented evidence that the origin of nonlinear cellular excitability is a TD transition in the membrane interface. This transition is located below the growth temperature of the cell and can be induced by cooling, acidification and pressurization. Furthermore, a phase diagram was proposed which allows for an intuitive understanding of several key points (Fig. 1):

- (i) the regime of excitable states in a living system,
- (ii) changes of AP velocity,
- (iii) nonlinear stimulation of a cell (TD parameter change that shifts the state into the transition regime) and
- (iv) loss of nonlinear excitability (due to crossing of a transition (cold-block, acid-block, pressure-block) or if the distance between resting state and transition is increased (e.g. by melting point depression (Heimburg, 2018; Heimburg and Jackson, 2007; Wang et al., 2018))).

We anticipate that it will be of particular interest to study the state diagrams of biological membranes to identify the mechanisms of evolutionary "fine-tuning" of certain sensitivities of the 2D-interface, *e.g.* to chemicals and light. It must be emphasized as well that the existence of nonlinear pulses is only one consequence of "living close to transitions". In a broader sense, transitions allow for a nonlinear response of all membrane functions (chemical reaction rates, adhesion, permeability, etc.). It follows that the biological system will have a higher response for certain environmental changes, namely the ones that move the system towards a transition. Furthermore, it is likely that transitions play an important role as a boundary for stability of the living system, because they involve drastic changes of the material properties (*e.g.* membrane crystallization). In this regard it is of particular interest that the transitions – and thus the limits of life – are not fixed but dynamic (thermal acclimation/adaptation of membranes (Engelhardt, 1951)). Finally, we consider it perhaps the most intriguing question of all: by which mechanism have cells acquired and retained such a peculiar resting state?

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# **Supporting information**

#### Predictions about membrane tension

The 3D pressure that is required to compress a 2D system scales as  $P_{3D} \sim P_{2D}/d$  (where d is the thickness of the interface and is in the range of  $\sim nm$ ). From geometrical considerations, one obtains the relation

$$p = \frac{2\tau}{d_0} \cdot \left(1 + \frac{\Delta d/d_0}{\Delta A/A_0}\right)^{-1} \tag{S1}$$

with  $^{\Delta d}/_{d_0}$  and  $^{\Delta A}/_{A_0}$  as the relative change in membrane thickness and area respectively (related to the Poisson ratio). At the main transition of dimyristoylphosphatidylcholine (DMPC),  $^{\Delta d}/_{d_0} \approx -0.16$  and  $^{\Delta A}/_{A_0} \approx 0.25$  (Heimburg, 1998). From this it can be estimated that an equivalent change of state induced by  $^\Delta p = 100~atm$  can be achieved by an increase in lateral pressure by  $^{\sim}1.8~\frac{mN}{m}$ . Therefore, it can be predicted that lateral pressurization of a biological membrane by  $^{\sim}15~\frac{mN}{m}$  should lead to a crossing of the transition regime and to a block of AP propagation. In contrast, a decrease in lateral pressure will remove the resting state from the transition regime.