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·Review·

Proton production, regulation and pathophysiological roles in the mammalian brain

Wei-Zheng Zeng[1,2], Tian-Le Xu[1]

[1]*Neuroscience Division, Department of Biochemistry and Molecular Cell Biology, Institute of Medical Sciences, Shanghai Jiao Tong University School of Medicine, Shanghai 200025, China*
[2]*Institute of Neuroscience and State Key Laboratory of Neuroscience, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China*

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Abstract: The recent demonstration of proton signaling in *C. elegans* muscle contraction suggests a novel mechanism for proton-based intercellular communication and has stimulated enthusiasm for exploring proton signaling in higher organisms. Emerging evidence indicates that protons are produced and regulated in localized space and time. Furthermore, identification of proton regulators and sensors in the brain leads to the speculation that proton production and regulation may be of major importance for both physiological and pathological functions ranging from nociception to learning and memory. Extracellular protons may play a role in signal transmission by not only acting on adjacent cells but also affecting the cell from which they were released. In this review, we summarize the upstream and downstream pathways of proton production and regulation in the mammalian brain, with special emphasis on the proton extruders and sensors that are critical in the homeostatic regulation of pH, and discuss their potential roles in proton signaling under normal and pathophysiological conditions.
Keywords: proton extruders; proton sensors; pH homeostasis; proton signaling; pH microdomains; local accumulation

1 Introduction

Protons are essential ions for fundamental biological processes such as pH homeostasis, synaptic transmission and the mitochondrial respiratory chain. Slight fluctuations in intracellular or extracellular pH can have marked effects on protein function, synaptic vesicle trafficking, and the electrical machinery of neuronal and glial cells. Moreover, the proton-driving force (ψ_{H+}) in organelles is crucial for the generation and conversion of energy. Theoretically, the ionization state of all protein side-groups can be influenced by ambient pH. However, only few of these changes have physiological relevance in the brain[1]. Elimination or inhibition of pH-sensitive proteins, such as Na^+/H^+ exchangers (NHEs)[2], monocarboxylate transporters (MCTs)[3], and acid-sensing ion channels (ASICs)[4], results in seizures, ataxia, impaired acid secretion or sour-sensing in mice, suggesting that protons are involved in signal transduction or the maintenance of cellular function. In particular, recent studies in *C. elegans* demonstrated that protons even directly serve as transmitters during muscle contraction[5,6].

Signal transduction occurs when an extracellular signaling molecule activates specific cell surface receptors, causing a second messenger to continue the signal into the cell and elicit a physiological response. While global changes in intracellular or extracellular pH are most likely

Corresponding author: Tian-Le Xu
Tel: +86-21-34696302; Fax: +86-21-53065329
E-mail: xu-happiness@shsmu.edu.cn
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harmful and unlikely to serve as signals, transient and localized pH changes may play such a role. To act as a signal transmitter, proton must be released in a regulated manner. Most proton transients in the brain are generated by two basic mechanisms: the *de novo* production of acid species, and net transmembrane fluxes of protons or their equivalents. The former arises mostly from energy metabolism while the latter results from the activation of membrane channels and pumps that transport protons. On the other hand, if protons mediate signals between cells, there must be proton sensors for signal transduction. The discoveries of proton effects on ASICs[7], voltage-gated Ca^{2+} channels (VGCCs)[8], ionotropic acetylcholine receptors[9], N-methyl-*D*-aspartate receptors (NMDARs)[10,11] and gamma-aminobutyric acid type A ($GABA_A$) receptors[12] unveiled their important roles in brain function under normal and pathophysiological conditions.

The present review mainly focuses on proton extruders and sensors in the brain that participate in proton production and regulation, and play potential roles in proton signaling. A hypothesis involving proton extruder-sensor coupling is attractive because it underscores the importance of a novel and unusual mechanism of extracellular communication under normal and pathophysiological conditions.

2 Proton-generating pathways and their pathophysiological roles in the brain

In mammalian cells, the production of protons is mainly accomplished by two mechanisms. The first is *de novo* production of acid species by energy metabolism. Many fundamental metabolic processes such as glycogenolysis, lactate utilization, and electron transport in mitochondria produce and consume protons. In these cases, proton production and consumption are well balanced to maintain intracellular pH homeostasis[1]. Neurological disorders such as ischemia, hypoxia, epileptic seizures and hypoglycemia result in an increased production or consumption of protons which consequently disrupts pH homeostasis[13]. Global changes in pH, such as metabolic acidosis[14], are generally harmful and have prolonged effects on downstream pathways. By contrast, protons that act as signaling molecules must be released and sensed locally.

The second mechanism of proton production is the net transmembrane efflux through proton-permeable ion channels or pumps. The activity of these channels or pumps is regulated to achieve local pH homeostasis and to fulfill the tasks of signal transduction[1]. This section focuses on the normal and pathophysiological significance of proton production pathways revealed by recent studies.

2.1 *De novo* proton-generating pathways and their pathophysiological roles Various metabolic reactions such as glycolysis in the cytoplasm and ATP production in mitochondria generate protons (Fig. 1). In general, protons are produced in reactions in which ATP is hydrolyzed or the oxidized form of nicotinamide-adenine dinucleotide (NAD^+) is reduced ($RH_2 + NAD^+ \rightarrow NADH + H^+ + R$)[13,15,16]. However, the majority of protons produced during metabolism are consumed in reactions related to energy metabolism. For example, protons are generated when ATP is hydrolyzed and consumed when it is re-formed. While the protons produced by metabolism contribute little to extracellular proton signaling, a burst of proton release, which can occur under specific conditions such as muscle contraction and activation of leukocytes by pathogens[17], may function beyond metabolism. During infection or inflammation in the mammalian brain, activated microglia and other neutrophils can also generate a proton burst through the NADPH oxidase complex[18]. The protons generated are then extruded to the extracellular space by Hv1 proton channels to kill the bacteria engulfed by phagocytes[18,19]. The voltage-gated proton channel Hv1 is discussed later.

An important reason to study proton production during metabolic reactions is to have a better understanding of brain ischemia and hypoxia. It is well known that under anaerobic conditions, excessive protons and lactate are produced in the cytoplasm by glycolysis. Under such circumstances, lactate concentrations in the tissue may increase to above 20 to 25 μmol/g, causing a decrease in pH to around 6.0. If the protons are not cleared or consumed immediately, acidosis occurs[13,20] which in turn causes irreversible damage to cell morphology and function.

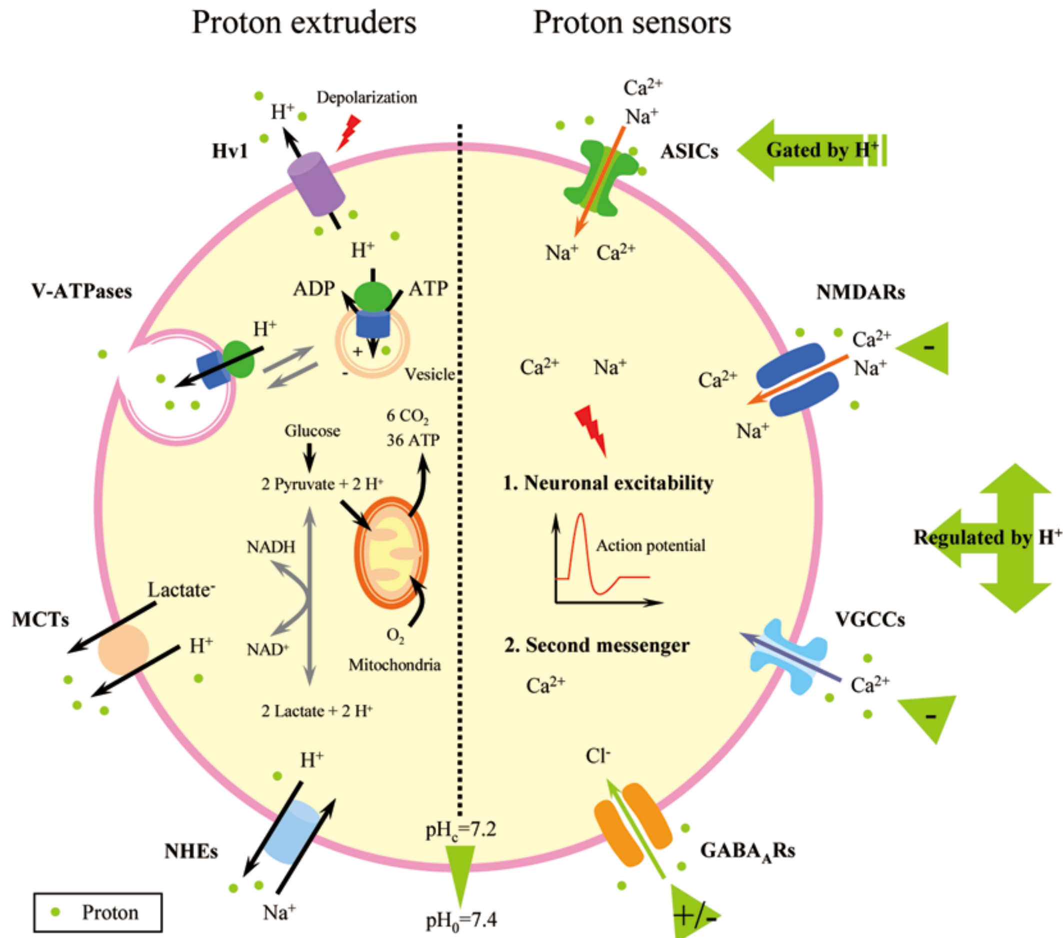

Fig. 1. Proton extruders and sensors in mammalian brain cells. Left: The cytoplasm tends to acidify due to the activity of various metabolic pathways, such as the anaerobic pathway in cytoplasm and the aerobic pathway in mitochondria. The predominant proton extruders include the plasma membrane NHEs, MCTs, and Hv1 channels. Although mainly acting in organelles, V-ATPases can also be incorporated into the plasma membrane during synaptic vesicle release. pH_c, cytosolic pH; pH_o, outside (extracellular) pH. Right: Main proton sensors in neurons. The functional outputs of proton sensors can alter neuronal excitability (1) or induce intracellular signal transduction (2). Hv1, hydrogen voltage-gated channel 1; V-ATPases, vacuolar type H^+-ATPases; MCTs, monocarboxylate transporters; NHEs, Na^+/H^+ exchangers; ASICs, acid-sensing ion channels; NMDARs, N-methyl-*D*-aspartate receptors; VGCCs, voltage-gated Ca^{2+} channels; $GABA_A$Rs, gamma-aminobutyric acid type A receptors.

Moreover, as discussed above, acidosis can affect synaptic transmission by modulating NMDARs[10,11], VGCCs[8] and $GABA_A$ receptors[21].

2.2 Controllable proton extrusion pathways and their pathophysiological roles In addition to metabolic pathways, protons can be generated by the action of proton-permeable channels or pumps. The major role of these proton extruders is to achieve pH homeostasis which is a vital function shared by all tissues[1,22]. However, accumulating evidence shows that localized proton gradients exist within[23-26] and even between[5,6] cells. Such pH microdomains are likely to have consequences for localized receptors, channels and enzymes, which may be involved in proton signaling. We will discuss some typical proton extruders below (Table 1, Fig. 1).

2.2.1 Vacuolar type H^+-ATPases (V-ATPases) Vacuolar type H^+-ATPases or V-ATPases play critical roles in pH homeostasis such as synaptic vesicle acidification, membrane trafficking and lysosomal protein degradation[27]. The maintenance of proton gradients in organelles such as

Table 1. Proton extruders in the brain

Proton extruders	Cell types	Membrane location	Transport modes
Na^+/H^+ exchangers	Neuron, astrocyte	Plasma membrane	Na^+ / H^+
Vacuolar type H^+-ATPases	Ubiquitous	Synaptic vesicles, endosome/ lysosome, presynaptic membrane	H^+
Hydrogen voltage-gated channel 1	Microglia	Plasma membrane	H^+
Monocarboxylate transporters (MCTs)	MCT1: ubiquitous; MCT2: neuron; MCT4: astrocyte	Plasma membrane	Monocarboxylate / H^+
Carbonic anhydrases (CAs)	Oligodendrocyte, astrocyte, neuron	Plasma membrane CA4, CA9, CA12, CA14	$CO_2+H_2O\xleftrightarrow{CA}H_2CO_3\longleftrightarrow HCO_3+H^+$

synaptic vesicles, endosomes and lysosomes is mainly accomplished by V-ATPases. V-ATPases are large complexes composed of 14 different subunits. The functional V-ATPases are composed of two domains named V_0 and V_1 which work together like a rotary machine. Some isoforms of V-ATPase subunits are specifically expressed in brain and are involved in many fundamental cellular events[27]. For example, the neuron-specific V-ATPase subunit V_0a1 interacts with presenilin 1 (PS1) and this interaction is important for lysosomal proteolysis[28]. Lysosomal acidification and autophagy are disrupted in neurons bearing the Alzheimer-related *PS1* mutation.

In addition to regulating pH homeostasis in intracellular organelles, V-ATPases are also found in the plasma membrane of a variety of cells, where they carry out specific functions such as renal acidification, bone resorption, sperm maturation and tumor metastasis[27]. Interestingly, Philippe and colleagues[29] reported that V-ATPases are expressed in nerve terminals where they are associated with presynaptic plasma membrane. It seems that the location of V-ATPases in neurons is highly variable depending on the destination of synaptic vesicles. The function of V-ATPases in presynaptic membrane is still unclear. Evidence from live cell imaging provided by David and colleagues[30] demonstrated that V-ATPases in the synaptic vesicle are inserted into the plasma membrane temporally during vesicular exocytosis elicited by action potential trains (12.5–100 Hz). By measuring the fluorescent signal of pH-sensitive yellow fluorescent protein expressed in mouse motor nerve terminals, they found cytosolic alkalization caused by V-ATPase activity. These results suggest that V-ATPases not only transport protons into organelles, but also extrude cytosolic protons after being exocytotically incorporated into the presynaptic plasma membrane (Fig. 1). Thus, the actions of V-ATPases in the plasma membrane[27,29,30] suggest a potential signaling role for generating localized protons. It would be interesting to explore the physiological consequences of these events.

2.2.2 Na^+/H^+ exchanger isoform 1 (NHE1) The Na^+/H^+ exchanger NHE1 is a secondary active transporter that mediates the equal exchange of extracellular Na^+ for intracellular protons across the plasma membrane. NHE1 is ubiquitously expressed on the plasma membrane of many types of cells and plays central roles in a variety of housekeeping functions such as cell volume regulation and intracellular Na^+ and pH homeostasis. The activation of NHE1 is often regulated by intracellular acidosis and many other extracellular cues such as growth factors, hormones, Ca^{2+} and osmotic cell shrinkage[31]. NHE1 deficiency leads to growth retardation, ataxia, and seizures[2] as well as abnormal cell morphology and adhesion[32]. It is reported that the asymmetrical expression and activation of NHE1 in cells generates a local intracellular pH gradient and thus induces or at least supports the rearrangement of the cytoskeleton by affecting cofilin, an actin-binding protein that increases the recycling of actin monomers[1].

While a great deal of effort has been focused on the involvement of NHE1 in cell migration and polarity[33-36], two studies have demonstrated that protons, released via *C. elegans* intestinal NHE PBO-4 (also known as NHX-7, the ortholog of mammalian NHE1), act on a proton-gated cation channel (comprised of PBO-5 and PBO-6 subunits) located in the adjacent muscle cells to induce contraction during the defecation cycle[5,6]. These studies provided solid evidence supporting the idea that localized protons can operate as direct signals. In the central nervous system, it is also possible that the NHE1 knock-out phenotypes are due to dysfunctional pH homeostasis or loss of proton-signaling function. Given the presence of many proton sensors such as ASICs, NMDARs and $GABA_A$ receptors in the brain, it still remains an open question whether localized proton signaling occurs between NHE1 and these proton sensors in the mammalian brain (Fig. 1).

2.2.3 MCTs Monocarboxylates such as lactate and pyruvate are important for cellular metabolism and metabolic communication between cells. Thus far, seven MCT isoforms (MCTs 1–4, 6, 8, and 10) have been functionally characterized[37]. The expression patterns of MCT isoforms vary among different tissues. For example, MCT1 is ubiquitously expressed, whereas MCT2 is found primarily in neurons and MCT4 in astrocytes[38]. MCT1–4 isoforms are described as proton-linked MCTs which co-transport a proton with a monocarboxylate (predominantly lactate) by catalysis[39]. In tissues undergoing anaerobic metabolism during oxygen deprivation, cytosolic lactate accumulation provides an outward gradient resulting in the excessive movement of protons to the extracellular space by MCTs. A study in muscle cells showed that during ischemia-induced acidosis, 40% of the protons and lactate generated are exported by MCTs[40].

The abundance of MCTs 1, 2 and 4 in the brain suggests a potential regulatory role of these proteins in brain function. An important energy source for the brain is glucose, which is transported across the blood-brain barrier. Astrocytes, but not neurons, store glycogen that can be rapidly converted to glucose, which then produces pyruvate and lactate. Astrocytes have always been described as supporting neurons by releasing transmitters and energy fuels. Emerging evidence suggests that astrocyte-neuron metabolic communication plays a critical role in long-term plasticity and memory formation[3]. It was found that lactate transport mediated by MCT1 and MCT4 in astrocytes plays an essential role in long-term memory formation. Disrupting MCT1 or MCT4 causes amnesia and long-term potentiation impairment, which is reversed by *L*-lactate but not glucose. Interestingly, disrupting neuron-specific MCT2 also leads to amnesia but is unaffected by either *L*-lactate or glucose, suggesting that lactate uptake by MCT2-expressing neurons is necessary for long-term memory. In addition, glycogenolysis in neurons is important for inducing the molecular changes required for memory formation, such as the induction of phospho-CREB, phospho-cofilin, and Arc[41].

It is notable that astrocytes not only present metabolic species, transmitters and cytokines to neurons, but also generate localized protons via the activity of MCTs (Fig. 1). Because of the link between astrocytes and neurons[42], it is possible that protons released from astrocytes may activate pH-sensitive surface receptors on neurons. Protons released from other cell types such as microglia and leukocytes are discussed below.

2.2.4 Hydrogen voltage-gated channel 1 (HVCN1) HVCN1, also called Hv1 or VSOP, is an intriguing target for the study of proton signaling. Two decades after the discovery of voltage-gated proton currents[43], the genes for HVCN1 were identified in humans and mice[44,45]. HVCN1 is described as highly proton-selective and activated by membrane depolarization with a sigmoid time-course of opening. The opening of HVCN1 is strongly dependent on the pH at both sides of the membrane. Until now, the most effective antagonist found for HVCN1 is Zn^{2+}. At a physiologically achievable pH range, the channel opens only when the proton gradient is outward, meaning it extrudes acid under most circumstances[46].

HVCN1 is widely expressed in immune cells including microglia[47], macrophages, monocytes[46], and granulocytes[46] as well as other cell types such as spermatozoa[48], respiratory epithelial cells[18,49] and neurons[43,50]. Cell lines

such as CHO and HEK293 also express HVCN1 endogenously. Because there is no measurable endogenous proton current in COS7 cells, they are best suited for the study of heterologously expressed HVCN1. The highest level of native expression of HVCN1 was found in human eosinophils where the current density of H^+ reaches 200 pA/pF[47]. HVCN1 has multiple functions in different cell types. For example, in neutrophils it mediates the positive charge compensation associated with oxidative bursts during phagocytosis[51,52], while in tracheal epithelium it mediates acid secretion[49]. In addition, activation of HVCN1 has been shown to create intracellular pH gradients in snail neurons[53,54]. Importantly, maintenance of the intracellular pH gradients by HVCN1 is critical for the pH-dependent activation of spermatozoa[48,55]. Spermatozoa in the quiescent state maintain a low cytosolic pH, <6.5. Once the sperm enters the female reproductive tract, HVCN1 is activated and extrudes protons to increase pH_i, triggering the capacity for fertilization.

Microglia, the resident macrophages in the brain, also express HVCN1. As phagocytes, activated microglia undergo a respiratory burst to release bactericidal superoxide anions to the outside. HVCN1 has important roles in regulating both the membrane potential and intracellular pH during this respiratory burst[47]. These features make microglia a proton source with high mobility during brain inflammation. Considering the tight interaction between microglia and neurons[42], it is worthwhile to determine whether the protons transiently released from microglia act on downstream sensors and lead to further signal transduction (Fig. 1).

3 Proton-sensing pathways and their pathophysiological roles in the brain

A complete signal transduction pathway of protons must have appropriate receptors to sense changes in local pH and activate downstream cascades. It is well known that protons modulate neurotransmission. For example, NMDARs are inhibited by acidic and potentiated by alkaline pH[10,11], whereas ionotropic $GABA_A$ receptors are differentially regulated by protons depending on the receptor subunit composition[12,56,57]. Other synaptic responses such as those mediated by glycine receptors are inhibited by tissue acidification[58,59] whereas AMPA or kainate receptor-mediated responses remain unchanged at all physiological pH levels[10,11,60]. In addition to modulating neurotransmission, protons have been indicated the principal endogenous ligand for a separate class of ligand-gated ion channels. Typical proton-gated ion channels, now called ASICs, were first described in sensory neurons[7,61]. Studies from *Drosophila* and the mammalian sour taste detection system also highlighted channels from the transient receptor potential (TRP) family, such as PKD2L1 and TRPA1, as sensors for acid or CO_2[62,63]. To fully illustrate proton-sensing pathways in the brain, we will discuss some typical receptors which are directly gated or modulated by protons (Table 2, Fig. 1).

3.1 ASICs Acid-sensing is associated with nociception and sour taste transduction. The pain caused by acid was first described to be mediated by H^+-gated cation channels present in sensory neurons[61]. The first gene of ASICs was cloned in 1997[7] and shown to belong to the ENaC/degenerin subfamily. To date, seven ASIC isoforms arising from four genes have been identified, ASIC1, ASIC2, ASIC3, and ASIC4 where 1a, 1b, 1b2, 2a, and 2b are splice variants. ASICs are trimeric protein complexes that can be made up of different combinations of subunits[64,65]. The main ion conducted by ASICs is the sodium ion, whereas the ASIC1a and human ASIC1b homomers[7,66], as well as the recently discovered ASIC2b/1a heteromer[67] are also permeable to Ca^{2+} (Fig. 2). The first crystal structure of chicken ASIC1a (cASIC1a) was solved in 2007[68,69], and the pore architecture and ion binding sites were solved in 2009[68,69]. It is anticipated that a great boost to ASICs research will come from unveiling their crystal structures.

ASICs are widely expressed throughout both the central and the peripheral nervous systems. ASIC1a, 2a and 2b are the major isoforms in the brain and spinal cord, whereas ASIC3 is present in dorsal root ganglia neurons. To date, several lines of evidence show that ASIC1a is involved in spine development[70], synaptic plasticity, spatial learning[4], and fear memory[71,72]. Under pathological conditions of

Table 2. Proton sensors in the brain

Proton sensors	Cell types	Tissue distribution	Gated by
Acid-sensing ion channels	Neuron	Central nervous system (CNS), dorsal root ganglion (DRG); Strongest in amygdala	Extracellular protons, 2-guanidine-4-methylquinazoline, MitTx
TASK K^+ channels	Neuron	Ubiquitous Strongest in pancreas and placenta	Halothane, isoflurane Inhibited by external acidification
NMDA receptors	Pyramidal neuron	CNS	Glutamate, NMDA, aspartate, glycine
$GABA_A$ receptors	Neuron	CNS	γ-aminobutyric acid
Voltage-gated Ca^{2+} channels	Neuron	Ubiquitous	Depolarization
Transient receptor potential type V 1	Neuron	DRG and trigeminal ganglion neurons	Capsaicin, heat, extracellular protons
Transient receptor potential type A 1	Neuron	Trigeminal ganglion neurons Nociceptive neurons	Intracellular protons and Ca^{2+}, mustard oil, cinnamaldehyde, cold
Transient receptor potential polycystic 2	Sensory neuron	Taste receptor	Extracellular protons

TASK: TWIK-related acid-sensitive K^+ channel. NMDA: N-methyl-*D*-aspartate. $GABA_A$ receptor: γ-aminobutyric acid type A receptor.

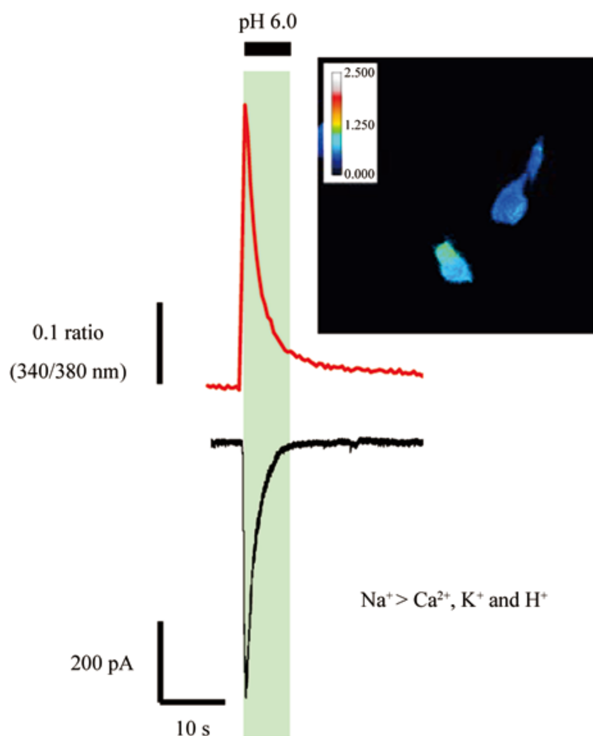

Fig. 2. Two downstream consequences occur following the reduction of extracellular pH in neurons. Upper: Rapidly dropping the extracellular pH to 6.0 induces intracellular Ca^{2+} elevation in cultured rat hippocampal neurons. Fura-2-AM was used as the Ca^{2+} indicator. Lower: Inward current is induced by rapidly dropping the extracellular pH to 6.0, and this is attributed to ASIC activation. Whole-cell recording was performed with holding voltage at -60 mV (Zeng *et al.*, unpublished data).

tissue acidosis, activation of ASIC1a channels contributes to axon degeneration[73], ischemic neuronal death[66,74,75], chronic pain[76] and termination of epileptic seizures[77].

ASICs are activated *in vitro* by a rapid fall of extracellular pH. Proposed as the H^+-gated ion channels in the nervous system, how are these channels activated *in vivo*? This question has been addressed in two aspects. One is to identify the physiological ligands. Wemmie and colleagues[78] demonstrated that ASIC1a in the amygdala is sufficient to detect CO_2 to elicit fear behavior. They also found that inhaled CO_2 reduces brain pH and evokes fear-related behavior in mice, whereas eliminating or inhibiting ASIC1a markedly impairs this activity. The study provides evidence that ASIC1a can be gated by physiological ligands such as CO_2 to elicit behavioral responses. However, the exact mechanism of endogenous activation of ASIC1a is still poorly understood. Based on the exciting discovery in *C. elegans*[5,6], one possibility would be to couple ASIC1a activation with endogenous proton sources such as NHEs, V-ATPases or carbonic anhydrases (CAs) (Fig. 1). On the other hand, much effort has been devoted to screening for the non-proton ligands of ASICs. Recently Xu and colleagues[79] identified a small molecule, 2-guanidine-4-methylquinazoline (GMQ), which evokes sustained ASIC3 currents, based on electrophysiological screening of a

library of 300 molecules. This exciting finding encourages further searches for endogenous non-proton ligands such as peptides and metabolic species which can directly gate ASICs under normal and pathophysiological conditions. Most recently, an elegant report from Julius and colleagues[80] showed that a Texas coral snake toxin, MitTx, directly activates ASICs to elicit robust pain-related behaviors in mice. This study further supports the existence of endogenous non-proton ligands of ASICs.

3.2 Acid-sensing receptors in sensory system Sour taste and carbonation sensing are critical sensory inputs for animals from insects to mammals. They provide animals with important information about the nature and the quality of food. In addition to ASICs, sour taste has been proposed to be mediated by several candidate receptors such as H^+-gated Ca^{2+} channels, hyperpolarization-activated channels (HCNs) and TWIK-related acid-sensitive K^+ channels (TASK1 and TASK3), most of which are broadly expressed in taste receptor cells (TRCs) and other tissues[81]. Recent studies have demonstrated that sour taste is mediated by distinct and independent populations of TRCs which express the TRP channel PKD2L1 (also known as TRPP2). Animals lacking PKD2L1-expressing cells are completely devoid of taste responses to sour stimuli[63]. A follow-up study showed that PKD2L1 is directly activated by exposure to solutions of low pH. The major component of the inward current activated by acid is mediated by protons[82].

Carbonation sensing is also conserved from *Drosophila* to humans. In the mammalian system, CO_2 elicits a pungent sensation and activates gustatory neurons. Because CO_2 works in both gas and dissolved forms (converted to H^+ and HCO_3^-), it evokes diverse responses in different tissues and cell types[83]. In fruit flies, gaseous CO_2 is detected by TRCs on the antenna whereas dissolved CO_2 is detected by those on the proboscis[84-87]. It is intriguing to work out how CO_2 activates these receptors. In mice, ingested CO_2 is sensed by TRCs on the tongue and soft palate, the same group of cells that sense sour. A notable finding by Zuker and colleagues is that taste responses to CO_2 can be functionally separated from acid detection using pharmacological and genetic approaches. Their study demonstrated that Car4, an extracellular glycosylphosphatidylinositol (GPI)-anchored carbonic anhydrase, acts as the main CO_2 sensor in the taste system[88]. CAs reversibly catalyze the conversion of CO_2 into bicarbonate and free protons. Given that Car4 is specifically expressed on the surface of sour-sensing neurons, it is ideally situated to provide localized protons to sour TRCs. Blood CO_2 is converted into H^+ and HCO_3^- and detected by chemosensors in the nervous system. TASK1 channels in the brainstem[89] and ASIC1a in the amygdala[78] have been demonstrated to be important for CO_2 sensing. In addition, TRPA1 expressed in trigeminal neurons mediate CO_2 responses. In this case, CO_2 diffuses into cells and produces intracellular acidification, which subsequently activates TRPA1 channels[62]. Moreover, ASIC functions are reported to be regulated by intracellular pH[90]. These results indicate that intracellular protons can function as modulators.

3.3 VGCCs Synaptic vesicles are always acidified by V-ATPases providing the electrochemical gradient for the uptake of neurotransmitters such as glutamate, GABA and acetylcholine[27]. As a consequence, intravesicular pH is ~1.5 units lower than cytoplasmic pH[91,92]. During fusion with the presynaptic membrane, both transmitters and protons are released to the synaptic cleft. Vesicular protons can therefore be an important modulator of synaptic function. Indeed, many channels localized on pre- or postsynaptic membranes are modulated by extracellular pH changes[10]. Because different brain regions may experience different levels of acidification at synapses and the pH buffering of solutions is typically not optimized to physiological conditions, so far, there is little experimental evidence showing that protons released from synaptic vesicles can modulate synaptic function.

It is well known that vertebrate photoreceptor activity is associated with pH changes in the retina. Studies on the VGCCs from mammalian photoreceptor synapses provided strong evidence that the pH changes alter synaptic function. VGCCs are a group of voltage-gated ion channels found in excitable cells. These channels are activated by depolarized membrane potentials and they have high Ca^{2+} permeability and selectivity, making them important signal

transducers linking membrane depolarization to intracellular signaling. Functional VGCCs are complexes made up of a core α1 subunit and auxiliary subunits such as β, α2, δ and γ. Based on several criteria including voltage sensitivity, single-channel conductance and pharmacology, VGCCs are divided into Cav1 (L-type Ca^{2+} channels), Cav2 (P/Q-, N-, and R-type Ca^{2+} channels) and Cav3 (T-type Ca^{2+} channels). Cav1 and Cav2 channels are widely expressed in the central and the peripheral nervous systems[93,94]. Synaptic Ca^{2+} currents are always mediated by more than one kind of VGCC. In mammalian photoreceptor synapses from the cone cells[8], bipolar cells[95] and horizontal cells[96], protons released into the cleft mediate negative feedback to presynaptic Ca^{2+} channel activity. Increasing the proton buffering capacity in the perfusion solution reduces the inhibitory effect, as does alkalization (Fig. 1). Moreover, studies demonstrated that protons modulate VGCCs in two ways. They interfere with ion fluxes and alter charged amino acids near the pore thus reducing conductance. Protons also neutralize negative surface charge, shifting the voltage-dependence of Ca^{2+} channels to more positive potentials[97,98].

3.4 $GABA_A$ receptors Another target of protons involved in regulating synaptic functions is the $GABA_A$ receptor. GABA receptors can be divided into two groups, $GABA_A$ receptors that are ionotropic channels from the Cys-loop family of ligand-gated ion channels, and $GABA_B$ receptors that are metabotropic receptors belonging to the G-protein-coupled receptor family. $GABA_A$ receptors are the major mediators of inhibitory neurotransmission in the brain and are gated by the endogenous ligand, GABA, which is released from GABAergic neurons. Activation of the $GABA_A$ receptor causes membrane hyperpolarization by allowing Cl^- influx in mature neurons. In some immature central neurons and mature peripheral neurons (e.g. primary afferent neurons), the intracellular concentration of Cl^- is maintained at a level that gives rise to an equilibrium potential more positive than the resting potential and activation of $GABA_A$ receptors causes Cl^- efflux, leading to depolarization. Numerous subunits of $GABA_A$ receptors have been identified and the receptor is a pentamer with the most common type in the brain being composed of two α, two β and one γ subunit (α2β2γ)[99-101]. The subunit composition defines agonist affinity, conductance, open probability and other properties of the $GABA_A$ receptor.

Various studies have indicated that protons can affect neuronal $GABA_A$ receptors differently depending on the receptor subunit composition, resulting in potentiation, inhibition, or no effect on GABA-induced responses[12,57,102-106] (Fig. 1). The modulation of $GABA_A$ receptors by protons has been demonstrated in both miniature inhibitory postsynaptic currents (mIPSCs) and whole-cell recordings using native or recombinant systems. However, evidence for $GABA_A$ receptor regulation by synaptic protons is still lacking. A recent study suggested that endogenous protons released from the presynaptic sites of rat cerebellar granule cells regulate $GABA_A$ signaling[21]. The authors changed the proton-buffering capacity simply by varying the concentrations of HEPES in the perfusion solution, a method that was validated in the study of endogenous proton modulation of presynaptic VGCCs[8,95,96]. Moreover, activity of the NHE appears to partly contribute to synaptic acidification in cultured rat cerebellar granule cells. Inhibition of NHE activity by amiloride or lithium alters mIPSCs in a manner consistent with alkalization. Interestingly, another amiloride-sensitive synaptic acidification mechanism has been reported to play an inhibitory role in VGCC activity[96]. These results provide evidence for endogenous synaptic acidification and its influence on synaptic function under pathophysiological conditions.

4 Conclusion

Protons play important roles in the brain. Here we reviewed a collection of proton extruders and sensors (Fig. 1) that are critical to both physiological and pathological brain functions. Proton extruders localize on the plasma membrane and generate H^+ transients upon activation of upstream signals. Proton sensors can be directly gated or modulated by protons and they elicit downstream consequences, for example, depolarizing or hyperpolarizing neurons (Figs. 1, 2). Unlike Ca^{2+}, which is mainly an intracellular second messenger, H^+ has been considered as an extracellular transmitter[6,107]. Although excellent mecha-

nisms exist in biological systems to maintain the overall balance between acids and bases, it is possible that brief fluctuations of the proton concentration in the microenvironment play roles in signal transduction[1]. According to current understanding, proton transmission occurs either between adjacent cells or within the same cell that expresses appropriate proton extruders and sensors. In the brain, the tight interactions between neurons themselves and between neurons and glia provide a potential environment for localized proton transmission[42]. However, the highly temporal and spatial dynamics of proton signaling makes it hard to capture local proton transmission experimentally *in vivo*. In future, functional studies using pH-sensitive dyes and/or fluorescent proteins for *in vivo* imaging will help to elucidate proton signaling and its pathophysiological roles in the brain.

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