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pH and proton-sensitive receptors in brain ischemia

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Abstract

Extracellular proton concentration is at 40 nM when pH is 7.4. In disease conditions such as brain ischemia, proton concentration can reach μM range. To respond to this increase in extracellular proton concentration, the mammalian brain expresses at least three classes of proton receptors. Acid-sensing ion channels (ASICs) are the main neuronal cationic proton receptor. The proton-activated chloride channel (PAC), which is also known as (aka) acid-sensitive outwardly rectifying anion channel (ASOR; TMEM206), mediates acid-induced chloride currents. Besides proton-activated channels, GPR4, GPR65 (aka TDAG8, T-cell death-associated gene 8), and GPR68 (aka OGR1, ovarian cancer G protein-coupled receptor 1) function as proton-sensitive G protein-coupled receptors (GPCRs). Though earlier studies on these GPCRs mainly focus on peripheral cells, we and others have recently provided evidence for their functional importance in brain injury. Specifically, GPR4 shows strong expression in brain endothelium, GPR65 is present in a fraction of microglia, while GPR68 exhibits predominant expression in brain neurons. Here, to get a better view of brain acid signaling and its contribution to ischemic injury, we will review the recent findings regarding the differential contribution of proton-sensitive GPCRs to cerebrovascular function, neuroinflammation, and neuronal injury following acidosis and brain ischemia.

Keywords

Acid signaling, acidosis, brain pH, ischemia, neuroinflammation, neuronal injury

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Introduction

It has long been known that the brain becomes acidic during and following ischemic stroke.[1–3] There have been extensive studies on the multiple mechanisms contributing to the regulation of brain pH homeostasis in health and disease. The data have revealed that carbonic anhydrases, sodium-proton exchanger, different proton, bicarbonate, and lactate transporters all contribute to pH regulation in the brain (for reviews, see[4–7]) On the other hand, it remains underappreciated regarding the complexity of brain acid signaling or its contribution to ischemia-induced cerebrovascular dysfunction. Protons can modulate the activities of multiple membrane proteins, e.g., inhibiting the NMDA receptors.[8,9] Moreover, proton can serve as ligands and signal directly through three classes of proton-sensitive receptors. These include the cationic acid-sensing ion channels (ASICs),[10] the proton-activated chloride channel (PAC)/acid-sensitive outward rectifying anion channel (ASOR),[11,12] and the family of proton-activated GPCRs, which include GPR4, GPR65 (aka T cell death associated gene-8, TDAG8), and GPR68 (aka ovarian cancer G-protein coupled receptor 1, OGR1).[13] Section 'An overview of brain pH regulation' of this review will summarize what the literature has documented regarding brain pH regulation and pH dynamics in brain ischemia. Next, we will discuss acid-induced signaling through proton-sensitive receptors in the brain, with a focus on recent findings of PAC/ASOR and proton-sensitive GPCRs.

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An overview of brain pH regulation

Within the brain parenchyma, the main cell types contributing to brain pH homeostasis include neurons, astrocytes, and microglia.[4,5] One universal buffer in these cells is the carbon dioxide (CO_2)-bicarbonate system. However, the conversion between carbonic acid and bicarbonate is a very slow reaction on its own. For the CO_2/HCO_3^- buffering system to work efficiently, the presence of functional carbonic anhydrase is a key.[6,7] Indeed, the brain expresses eleven of the thirteen functionally active carbonic anhydrases, with some exhibit preferential localization to specific domains (intracellular, membrane bond, or extracellular) or cell types. For example, carbonic anhydrase IV and XIV, which are membrane bond, exert their catalytic function on the extracellular side. For a detailed review on carbonic anhydrases in the brain, see reference.[7]

Besides the bicarbonate system, non-bicarbonate buffering also contributes to pH regulation in the brain. While most molecules can act as proton donor and receiver and thus contribute to buffering, lactate is one important molecule for brain pH during neural activity or in anaerobic metabolism.[6,14] This is in part because lactate levels exhibit over 10 fold change under anaerobic conditions such as ischemia.[15] The regulation of brain pH apparently depends on the crosstalk between neurons and astrocytes.[5,6,14] Under physiological conditions, neurons preferentially utilize aerobic respiration for its ATP production. Astrocytes, on the other hand, tend to go through aerobic glycolysis, which converts glucose to lactate even when there is sufficient oxygen supply.[6] Astrocytes also are the main cells for glucose uptake, especially in response to increased activities. These properties together enable the astrocytes to uptake glucose, convert it to lactate, and then shuttle it to neurons as the energy source for aerobic respiration.[6,14]

The monocarboxylate transporters (MCT2 in neurons; MCT1 and MCT4 in astrocytes) are responsible for transporting lactate in and out of astrocytes and neurons.[6] Multiple other transporters in neurons and astrocytes are important for pH regulation. These include the sodium-hydrogen exchangers, sodium bicarbonate cotransporter, sodium-driven chloride/bicarbonate exchanger, calcium/proton exchangers. For more information on these topics, see reviews.[5,6,14]

Brain pH dynamics in ischemia

Protons get buffered fast as essentially all biomolecules can act as proton donors/acceptors. Thus, accurate pH measurement, especially *in vivo*, is challenging. Typical approaches used in previous studies include direct measurement with pH microelectrode, fluorescent imaging with pH sensitive dyes or reporters, and functional pH imaging. It has long been known that ischemia leads to brain acidosis, both during ischemia and after reperfusion. The exact pH values measured differ between studies. In various studies, the resting or physiological extracellular brain pH is in the range of 7.2–7.4, while that of intracellular pH can be slightly lower.[16–19] Based on these data, in our discussion here, we consider extracellular "acidotic or acidosis" starts at ~pH 7.2.

pH changes during the ischemic phase

During ischemia, the stop of blood flow limits the supple of oxygen to brain tissue, and consequently brain cells transit from aerobic respiration to anaerobic glycolysis.[20] This conversion appears to occur within minutes of ischemia. As a result, neurons quickly deplete ATP, creatine, and phosphor-creatine but build up lactate and protons, and the intracellular pH (pH_i) becomes acidic.[3,15,20] The subsequent exporting of lactate could contribute in part to extracellular acidification. Other mechanisms which can contribute to interstitial pH reduction include the activation of NHE and the import of bicarbonate inside to counteract intracellular acidification.[4,5] In several reports, mostly using rodent models, a rapid pH reduction occurs within minutes of ischemia, to the range of 6.5–6.0.[17,21–23] At about 30 min after occlusion, brain pH is typically down to 6.2 or even below 6 in some cases. Hyperglycemia further worsens the degree of acidification while hypoglycemia alleviates it.[17,19,24–26] When there is no reperfusion, this level of pH reduction is maintained for up to 6 hr. In cats, acidosis persists to the second day, though the degree of acidosis becomes milder.[27] In human, at an average of 6 days after ischemic stroke, the brain exhibits a slight alkaline shift.[28]

pH changes following reperfusion

In transient ischemia, reperfusion quickly normalizes the metabolites and brings pH back to the normal range.[15] In another study, Maruiki et al performed 12 min complete ischemia in dogs and found that brain pH returned to pre-ischemia level within 30–60 min of reperfusion.[23] However, if the ischemic event lasts longer, the rise in oxidative stress accompanies disrupted metabolism at the reperfusion stage can lead to another phase of prolonged reduction in brain pH.[18,29,30] In one study, following 60 min transient middle cerebral artery occlusion (tMCAO), a transient rebound into the alkaline range occurs at 2-hr after reperfusion, then brain pH reaches ~6.5 at the 4-hr time point.[18] It is worth noting that while the

extracellular brain pH typically maintains in acidic phase for hours following reperfusion, intracellular pH can become slightly alkaline within an hour.[15] Figure 1(a) illustrates qualitatively the dynamics of pH changes in ischemia, either with or without reperfusion.

The proton-sensitive receptors

The first receptor identified to functionally mediate acid-sensing in the brain is the acid-sensing ion channel 1a (ASIC1a) .[31] It is now evident that the brain expresses most members in the three families of proton-sensitive receptors, which include the ASICs, the PAC/ASOR, and the proton-sensitive GPCRs. However, these receptors exhibit distinct patterns of expression and downstream signaling. In Table 1, we summarized the genomic location, main downstream signaling of these receptors, and some of the reagents which are relatively specific for the subtypes of the receptors. It is important to note that, especially for ASIC channels, there are additional reagents which are less selective among the subtypes. For more extensive reviews on ASIC modulation, including additional pharmacological reagents, see references.[32,33]

In previous studies, multiple groups have generated various kinds of mouse models for these receptors, including global knockout, conditional knockout, and reporter lines. Table 2 summarizes the mice which have been reported in the literature. Table 2 also presents a summary of the overall expression of these receptors in the central nervous system. In the brain, ASICs are mostly present in neurons.[32,34] ASIC1a, 2a, and 2 b are the major ASIC subunits expressed in brain neurons. The PAC/ASOR channel are more ubiquitously expressed in both neurons and non-neuronal cells.[11,12,35,36] For the GPCRs, GPR4, GPR65, and GPR68 exhibit preferential expression in endothelium, neuron, and microglia, respectively (see text below for more detailed discussion).

Besides differential expression, another important difference exist among these receptors is their pH sensitivity and kinetics. The ASICs, depending on the subunit composition, have a pH_{50} around 6.8–5.[37] The PAC requires much lower pH to get activated, with pH_{50} around 5.[11,12,35] Compared to the ASICs and PAC, the GPCRs exhibit higher pH sensitivity (Figure 1(b)). All three receptors start to get activated at about pH 7.4 or even higher, and typically reach maximal activation at pH 6.8–6.2[38–40]. One caveat of these studies, however, is that the majority of these studies on pH responses in GPCRs used ectopic expression systems. Nevertheless, it is apparent that the proton-sensitive GPCRs do not exhibit fast desensitization, making them an attractive mediator of acid signaling during persistent acidosis.

Acid-sensing ion channels (ASICs)

The ASICs are a family of proton-gated cation channels. There are four genes encoding ASICs: ASIC1-4.[10,32] ASIC1 and ASIC2 have two splice variants a and b. The ASIC subunits have two transmembrane domains with a huge extracellular domain. Three subunits form one functional ASIC channel.[41] ASIC1a, 1 b, 2a, and 3 can conduct acid-activated cation currents. ASIC1 and 3 respond to pH drop with a threshold range of 7.0-7.2 while ASIC2a are much less pH sensitive and starts to get activated at pH close to 5.5.[33,37] ASIC channels mostly conduct Na^+.

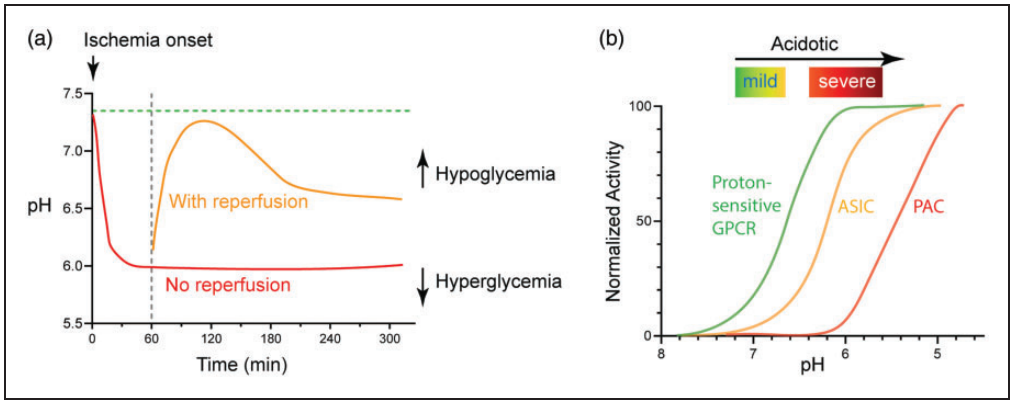

Figure 1. pH dynamics and the three classes of acid-sensitive receptors in the brain. (a) Illustration showing brain pH changes during brain ischemia and following reperfusion. The red line illustrates the change in permanent occlusion. The orange line illustrates the approximate change following reperfusion. These curves may shift upward or downward in hypoglycemic or hyperglycemic conditions, respectively. See text for details. (b) Diagram illustrating the pH sensitivity of the three classes of proton receptors. The curves are qualitative representation of the approximate/average pH response curves of a group of receptors within that family. See text for more explanation.

Table 1. Summary of signaling and pharmacological reagents for proton-sensitive channels and GPCRs.

Gene	Location and accession number	Ion selectivity or signaling	Agonist (EC_{50})	Antagonist (IC_{50})	Modulator	References for pharmacological reagents
ASIC1	Human: 12q13.12 NM_001095.4 (ASIC1a) HM991481 (ASIC1b) Mouse: chr 15 NM_009597.2 (ASIC1a) NM_001289791 (ASIC1b) Identity: ASIC1a 97.9% ASIC1b 93.8%	Na^+/Ca^{2+}	MitTx: ASIC1a (9.4 nM) ASIC1b (23 nM)	PcTx1 (1 nM) Mambalgin ASIC1a (55 nM) Hi1a Diminazene Amiloride A-317567	Big dynorphin & dynaorphin A (EC_{50} ~30 μM) Spermine	120–124 125,126
ASIC2	Human: 17q11.2-q12 NM_001094 (ASIC2a) NM_183377.2 (ASIC2b) Mouse: chr 11 NM_001034013 (ASIC2a) NM_007384 (ASIC2b) Identity: ASIC2a 99.2% ASIC2b 97.2%	Na^+		Diminazene Amiloride	Zn^{2+}	125, 127
ASIC3	Human: 7q36.1 NM_004769.4 Mouse: chr 5 NM_183000.2 Identity: 83.3%	Na^+	MitTx (830 nM)	APETx2 (63 nM) Amiloride A-317567	GMQ (67 μM) Neuropeptide SF (50 μM) lactate	126,128–130
PAC/ ASOR	Human: 1q32.3 NM_001198862.2 Mouse: chr 1 NM_025864.4 Identity: 90.3%	Cl^-		pregnenolone sulfate		11
GPR4	Human: 19q13.32 NM_005282 Mouse: Chr 7 NM_175668 Identity: 91.5%	Gs $G_{12/13}$ Gq possibly Gi		Compound 3b (67 nM) NE 52-QQ 57 (70 nM hGPR4; 1.8 μM rGPR4)		84, 131
GPR65 (TDAG8)	Human: 14q31.3 NM_003608 Mouse: chr 12 NM_008152.3 Identity: 78.5%	Gs			ZINC13684400 (positive) ZINC62678696 (negative)	106

(continued)

Table 1. Continued.

Gene	Location and accession number	Ion selectivity or signaling	Agonist (EC_{50})	Antagonist (IC_{50})	Modulator	References for pharmacological reagents
GPR68 (OGR1)	Human: 14q32.11 NM_003485 Mouse: Chr 12 NM_175493 Identity: 92.1%	Gq, Gs possibly Gi and $G_{12/13}$	CARTPT (76–96) 3.2 μM Osteocrin (115–133) 0.4 μM Corticotropin (17–40) 1.8 μM	Cu2+ (μM range) Zn2+ (μM range)	Ogerin (positive modulator for Gs pathway) Lorazepam (non-specific) MS48107	72, 106, 132

CARTPT: cocaine- and amphetamine-regulated protein; GMQ: 2-guanidine-4-methylquinazoline; PcTx1: psalmotoxin 1.

However, homomeric ASIC1a, human ASIC1b, as well as ASIC1a/2b heteromers, exhibit a permeability to Ca^{2+}.[31,42] Though proton is the only known ligand, various cations, neuropeptides, and oxidants can have important modulatory effects on ASIC function.[34,37] As a neuronal and synaptic acid receptor, ASICs contribute to synaptic function, plasticity, and learning.[10,32,34] Detailed review of ASIC biophysical properties and regulation can be found in multiple review articles.[33,43]

ASIC and hypercapnia-induced cerebral vasodilation. Besides its function in neuron physiology, ASIC also contributes to cerebral blood flow regulation. In one study, ASIC1a deletion or local infusion of psalmotoxin (PcTx1), a specific inhibitor of homomeric ASIC1a and heteromeric ASIC1a/2b channels, attenuated hypercapnia-induced vasodilation.[44] The authors further generated a synapsin-cre driven syn-ASIC1a knockout mouse, which had reduced ASIC current in interneurons and principal neurons.[45] The syn-ASIC1a knockout mice had attenuated response to CO_2. This result suggests that ASIC1a activation in neurons contributes to hypercapnia-induced vasodilation. While the finding is interesting, it is worth noting that GPR4 in endothelium may play a more direct role in CO_2-induced vasodilation (see below).

ASICs in acidotoxicity and ischemic injury. In disease conditions which induce large pH reduction, ASICs are one important mediator of acid-induced neuronal injury. ASIC1a$^{-/-}$ exhibits significant reduction in ischemia-induced brain injury.[18,46] Inhibiting ASIC activity by either amiloride, a non-specific ASIC inhibitor, or either PcTx1 or Hi1a, two disulfide-rich spider venom peptides, all have protective effect following tMCAO.[46,47] Deleting the ASIC2 gene, through reducing ASIC1a trafficking and possibly biogenesis as well, also reduces tMCAO-induced brain injury.[48,49] The protective effect of ASIC inhibition is additive to that of NMDA receptor inhibition.[18,50] These data suggest that ASIC targeting can be combined with other approaches to offer enhanced protection against ischemia-induced brain injury. In most of the above studies, the pro-injury function of ASICs correlates with its channel activity. However, there are data showing that ASIC1a can elicit necroptosis independent of its channel activity.[51] This new mechanism requires the ASIC1a C-terminal tail, which elicits cell death through an interaction with serine/threonine kinase receptor interaction protein 1.

Table 2. Expression of proton-sensitive receptors in the brain and genetic tools available.

Family	Protein	Expression in brain	Mouse models available
ASIC	ASIC1a	Neuron-high expression Also reported in astrocyte & microglia	Asic1a$^{-/-}$ (JAX #013733)[133] Tg(Syn1-ASIC1a) (JAX #013734) ASIC1a$^{flox/flox}$ [45] ASIC1a$^{flox/flox}$ [134]
	ASIC1b	Protein undetected in the brain, though mRNA is present	ASIC1b$^{-/-}$[135] ASIC1b-Cre [135]
	ASIC2a	Neuron-high expression Also reported in astrocyte & microglia	Asic2$^{-/-}$ (JAX #013126)[136] Asic2$^{-/-}$[137]
	ASIC2b	Neuron-high expression Also reported in astrocyte & microglia	Tg(Syn1-Asic2a) (JAX #012878)[138] Tg(Syn1-Asic2a) (JAX #012877)[138]
	ASIC3	No to limited expression	Asic3$^{-/-}$ (JAX #013127)[139] ASIC3$^{-/-}$[140] ASIC3$^{-/-}$[141] ASIC3$^{flox/flox}$ [141] Tg(Syn1-Accn3*) (JAX #012879)[142] Tg(Syn1-Accn3*) (JAX #012880)[142]
PAC/ASOR	PAC/ASOR	Neuron Astrocyte Possibly in other cell types	PAC$^{-/-}$[12]
GPCR	GPR4	Endothelial cell-high expression Neurons-in some areas	Gpr4$^{-/-}$ (JAX #008580)[71] Gpr4$^{-/-}$[83]
	GPR65 (TDAG8)	Microglia or macrophages-limited expression	Gpr65$^{-/-}$ (JAX #008577)[95] Gpr65$^{-/-}$[85] Gpr65-cre (JAX #029282)[143]
	GPR68 (OGR1	Neuron-high expression Endothelial cell-sporadic expression	GPR68$^{-/-}$[114] GPR68$^{-/-}$[144] GPR68$^{flox/flox}$ [114] Gpr68-eGFP reporter (MMRRC #031057)

Proton-activated chloride channel

Discovery. It has been known for some years that protons induce an acid-sensitive outward rectifying anion channel (ASOR), which passes chloride.[52–54] With a cell-based RNA interference screening, two groups recently identified the molecular constituent of this anion current.[11,12] The ASOR channel turns out to be a previously uncharacterized protein, TMEM206 (transmembrane protein 206 or C1orf75). One group named it based on its function as "proton-activated chloride channel (PAC)".[12] PAC/ASOR/TMEM206 is present in multiple organisms, including zebrafish, chicken, rodents, and human. Ectopic expression of PAC in heterologous cells reconstitutes the typical proton-activated ASOR current.[11,12]

General properties. PAC/ASOR has two transmembrane domains, with the N- and C-termini inside the cell and a large extracellular domain.[11,12] A recent Cryo-EM study shows that PAC also forms a trimer.[55] Thus, the overall topology and stoichiometry resemble that of the ASICs, even though the two types of channels do not share any close sequence homology. At room temperature, PAC has an activation threshold of about pH 5.5 and a pH_{50} of close to pH 5. At 37 °C, PAC starts to get activated at about pH 6.2 with its pH_{50} shifted to around ~5.7. These data indicate that PAC is less pH sensitive than ASICs (also see Figure 1(b)). Consequently, PAC activation requires relatively more severe acidosis, i.e., when pH is reduced to 6 or lower.

Cells expressing PAC are present in multiple anatomical places, with high expression levels in brain, bone marrow, kidney, lung, lymph node, spleen, and bladder. In the brain, neurons possess robust PAC current.[12,35] In addition, PAC is also present in non-neuronal cells in the brain, including astrocytes and microglia.[36]

PAC/ASOR and intracellular trafficking. Besides its presence at cell membrane, PAC/ASOR can traffic to endosomes and forms a functional chloride channel there.[56] The trafficking to endosome apparently requires the YXXL motif of PAC/ASOR. PAC regulates endosomal pH and chloride concentration. Deleting PAC abolishes endosomal chloride leakage, which consequently raises chloride concentration and reduces endosomal pH.[56] In PAC deficient HEK293

cells, transferrin uptake exhibits a 30% increase.[56] This finding suggests that, through modulating endosomal pH and ion gradient across endosomal membrane, PAC/ASOR serves as one regulator of vesicle recycling and/or receptor trafficking.

Role in acidotoxicity and ischemic injury. PAC/ASOR activation in acidotic conditions leads to cell swelling, which suggests that its activation contributes to acidosis-induced cellular injury. In addition, its pH sensitivity indicates that the PAC/ASOR pathways contribute to injuries when the acidosis is more severe (e.g. when pH is lower than 6). In HEK293 cells, deleting PAC/ASOR protects the cells from pH 4.5-induced swelling and cell death.[11] In cultured cortical neurons, PAC deletion or shRNA knockdown offers protection against pH 6 and 5.6-induced cell death.[12,35] In a permanent model of brain ischemia, PAC null mice exhibit a reduction in MCAO-induced brain infarction.[12]

Proton-sensitive GPCRs

Introduction. Around mid-1990s, homologous cloning led to the identification of three proton-sensitive GPCRs: GPR4, GPR65, and GPR68.[57–59] These three GPCRs belong to the orphan family of GPCRs. Protons are the only well-established ligand which activates these receptors.[60] Phylogenic analysis shows that GPR4, 65, 68 evolved from a common ancestor, GPR132 (or G2A, G2 accumulation protein).[38] Early studies showed that GPR132 also responds to proton.[61,62] However, based on structural modeling and mutagenesis analysis, GPR132 does not contain the key proton-sensing residues which are conserved in the other three receptors.[38] In addition, GPR132 does not exhibit a robust pH-dependent signaling as compared to the other three GPCRs. In qPCR and RNA-Seq analysis, GPR132 had little expression in either mouse or human brain.[63,64] For these reasons, we focus here on GPR4, GPR65, and GPR68.

For all three receptors, when ectopically expressed together with various G alpha subunits, they are capable of coupling to most G alpha subunits tested.[38] This result indicates that the exact signaling these receptors conduct will depend on the specific system/cell type and the availability of the G alpha subunits. In the discussion below, we will mainly cover the results obtained from the brain and/or mammalian cells without the overexpression of G alpha. Table 1 presents a summary of the signaling and main pharmacological reagents which are currently available for these receptors.

GPR4.

GPR4 expression: GPR4 mRNA is present in multiple tissues throughout the body, with abundant expression in brain, heart, lung, placenta, spleen, skeletal muscle, testis, kidney, and ovary.[39,40,65] Cells expressing GPR4 include immune cells, peripheral and central endothelial cells, kidney epithelial cells, and certain types of neurons.[66–70] Compared to other proton-sensitive GPCRs, GPR4 is the only one that exhibits robust expression in multiple types of endothelial cells.

GPR4 signaling: GPR4 exhibits promiscuous signaling in various types of cells. The initial studies show that GPR4 signals through Gs, though it can also couple to $G_{12/13}$ and Gq.[40,67,71–76] Depending on the context, GPR4 recruits multiple downstream effectors, including the activation of cAMP-protein kinase A (PKA), exchange protein directly activated by cAMP (EPAC), and Rho-Rho-associated protein kinase (ROCK) pathways. What determines which pathway is turned on is not clear. Other than the cell type and/or treatment paradigm, the duration of acidosis appears to be one factor. The rise of cAMP occurs within minutes of acidic stimulation while ROCK activation typically associates with a longer (hours) acidosis.

Proinflammatory role in peripheral cells: In peripheral endothelial and epithelial systems, GPR4 activation initiates stress responses and contributes to intestinal inflammation, paracellular gap formation and ischemia-induced renal injury.[77–80] In human umbilical vein endothelial cells (HUVEC), lung microvascular endothelial cells, and lung arterial endothelial cells, acid activation of GPR4 increases the expression of pro-inflammatory chemokines, cytokines, and genes in the NF-κB pathway.[81] GPR4 inhibition inhibits acidosis-induced gap formation in endothelial cells.[80] The Rho-ROCK pathway is one mediator of acidosis-induced junctional disruption. Part of the GPR4 effect may be due to a change in cell-cell adhesion, which parallels with an increase in VCAM, E-selectin, and ICAM-1.[82] Consistent with an important role in vascular function, one global GPR4 null mouse line exhibits increased incidence of neonatal hemorrhage.[71] However, vascular malformation or spontaneous hemorrhage is not present with a different GPR4 null mouse line.[83]

GPR4 in hypercapnia-induced vascular responses: In the brain, GPR4 exhibits predominant vascular expression.[67,68] Systematic inhibition of GPR4 with NE 52-QQ57, a specific inhibitor of GPR4, has no effect on cerebral blood flow or hemodynamics.[67,84] However, GPR4 activity in cerebral endothelium is required for hypercapnia-induced vasodilation.[68] GPR4 deletion largely abolishes CO_2-induced release of prostacyclin and the vasodilating effect of hypercapnia. This effect does not appear to depend on cAMP but rather requires the activation of $G_{q/11}$. These data suggest that endothelial GPR4 activity has little impact on

baseline cerebral blood flow but mediates endothelial responses when the brain becomes acidic.

GPR4 in brain neurons: The overall expression of GPR4 is low in most types of brain neurons.[63,67] However, neurons in specific regions, including the retrotrapezoid nucleus, dorsal raphe, and lateral septum, express detectable GPR4 levels.[67,85] Consistent with GPR4 expression in retrotrapezoid nucleus, CO_2 induces Fos expression in these neurons while deleting GPR4 abolishes CO_2-induced hyperventilation.[65,85] This finding is consistent with the observation that systematic GPR4 inhibition reduces CO_2-induced hyperventilation.[67] Though the inhibitor and global knockout do not determine the site of action, lentiviral-mediated re-expression of GPR4 in retrotrapezoid nucleus is sufficient to rescue the deficit of CO_2 sensing in the global GPR4$^{-/-}$ mice.[85] This result indicates that the retrotrapezoid nucleus neurons are one key determinant of the central response to CO_2.

GPR4 and acidotoxicity: As discussed above, most brain neurons do not express detectable levels of GPR4. In our recent study, we examined organotypic cortical slices and found that WT and GPR4$^{-/-}$ slices exhibit comparable acidosis-induced neuronal injury.[63] In another study, following 30 min tMCAO, GPR4$^{-/-}$ mice did not exhibit statistically significant changes in brain infarct volume as compared to the wild-type mice.[86] These data suggest that GPR4 does not have a direct effect on acidotoxicity in neurons or acute brain injury following a mild stroke. However, GPR4 activation shows a consistent pro-inflammatory or pro-injury role in peripheral endothelial and epithelial cells. In SG-SY5Y neuroblastoma cell line, GPR4 silencing is also protective and reduces neurotoxin-induced cell injury.[87] These data suggest that GPR4 inhibition or silencing can have a protective effect. It will be of interest to assess this speculation in cells or regions which exhibit robust GPR4 expression.

GPR65.

GPR65 expression: GPR65 has limited expression in the brain.[64] One report showed that GPR65 is present in a limited number of microglia within the sensory circumventricular organs.[88] Given that the circumventricular organs do not have a blood-brain barrier, it raises a possibility that infiltrating macrophages, which express GPR65, account for some of the GPR65-positive microglia in brain. Consistent with the limited number of GPR65 positive cells, baseline mRNA level of GPR65 is over one order of magnitude lower as compared to GPR4 or GPR68.[64,86] However, following ischemia-reperfusion, GPR65 expression is increased.[64,86,89] Immunostaining shows that these GPR65 positive cells are IBA1 positive. Since ischemia reperfusion compromises the blood-brain barrier, it remains unclear whether these post-stroke GPR65/IBA1 positive cells reflect an upregulation of GPR65 in microglia or ischemia-induced macrophage infiltration.

GPR65 signals through cAMP: In CHO and HEK 293 T cells, acid activation of transfected GPR65 activates the Gs-cAMP pathway.[90–93] In macrophages, thymocytes, splenocytes, and microglia, acidosis activates cAMP through GPR65.[94,95] In dorsal root ganglion neurons, GPR65 mediates pH 6.4-induced cAMP increases following hyperalgesia.[93] These data demonstrate that, for both endogenous and overexpressed GPR65, cAMP is the major downstream effector.

GPR65 in acidotic injury: In heterologous cells, GPR65 overxpression reduces acidosis-induced LDH release.[90] In heart, GPR65 expression is elevated following myocardial infarction and GPR65 deletion leads to larger infarction.[96] In retina, GPR65 deletion accelerates the degeneration of photoreceptor cells in both a genetic and a light-induced injury in a mouse model.[97] In the rodent EAE model of multiple sclerosis, GPR65 null mice show worsened clinical outcome.[98] All these studies suggest that GPR65-dependent signaling elicits protection in acidotic and/or injurious paradigms. In rodent models of brain ischemia, GPR65 deletion worsens brain infarction.[86] In this case, GPR65 signaling in microglia/macrophage likely attenuates post-ischemia inflammatory responses and leads to post-ischemia protection in the brain.

GPR68.

GPR68 expression: GPR68 exhibits widespread expression in multiple types of cells, including immune cells, muscle cell, neurons, and some types of endothelial cells.[39,63,99–103] In the brain, GPR68 mRNA is detected in multiple regions.[59,63,70] Cerebellar granule cells express GPR68.[104] In brain neurons isolated by Thy1 labeling and FACS sorting, RT-PCR reveals robust GPR68 expression and diminished GPR4 level.[63] Using a GFP reporter mouse, which expresses GFP under the control of the GPR68 promoter, we recently showed that, in cortex, hippocampus, and striatum, GPR68 positive cells were NeuN positive.[63,102] Within the hippocampus, GFP expression is apparent throughout pyramidal neurons, with higher expression in CA3 neurons. These mRNA and reporter data clearly demonstrate that GPR68 is widely expressed in the brain and neurons are the main GPR68-expressing cells. Besides neurons, *in situ* hybridization reveals the presence of GPR68 mRNA in a fraction of endothelial cells in the small diameter arterioles in the brain.[103] Some studies have further examined the expression/localization of GPR68 at the protein level. However, we and others found that, using GPR68$^{-/-}$ tissue as the negative control, current GPR68 antibodies do not

generate specific signals.[63,103] In organotypic hippocampal slices, ectopically expressed GPR68 exhibits a relatively ubiquitous distribution in CA1 neuronal cell body, dendrites, dendritic spines, and axons.[102]

GPR68 signaling: GPR68 primarily couples to Gq and its activation by acidosis elevates IP3 and DAG.[72,105] Depending on the cell type, and typically associated with ectopic expression, GPR68 activation can increase cAMP level and/or recruit the $G_{12/13}$ effectors.[38,99,106,107] In cerebral granule cells, GPR68 mediates acidosis-induced calcium increase.[108] However, under the culture condition studied, revealing this GPR68-dependent mechanism requires the inhibition of calcium sensing receptors (CaSR). Reducing extracellular calcium and magnesium to close to 0 mM also largely alleviates the inhibitory effect of CaSR and facilitates acid-induced activation of GPR68.[108] Interestingly, another study in HEK293T cells shows that divalent metal ions can enhance GPR68 signaling.[109] In organotypic hippocampal and cortical slices, we found that acidosis induced the activation of PKC, and this effect requires GPR68.[63] These results suggest that, in both organotypic brain slices and cerebellar granule cells, GPR68 activation by acidosis activates the Gq-DAG-PKC axis.

Peripheral function of GPR68: GPR68 contributes to signaling and gene regulation in multiple peripheral cells, including smooth muscle cell, immune cell, and airway epithelial cells. In human airway smooth muscle cells, extracellular acidification elevates intracellular calcium levels, induces cell rounding, and stimulates IL-6 production.[100] siRNA-mediated knockdown of either GPR68 or Gq/11, or application of YM-254890, a Gq inhibitor, attenuates acidosis-induced changes.[100,101] In a mouse asthma model, GPR68 in dendritic cells is required for ovalbumin-induced asthma.[110] GPR68 also contributes to bone and cartilage function. GPR68 inhibition or silencing attenuates acid-induced calcium increase and acidification induced survival.[111,112] GPR68 deletion alters osteoclast number in mice, although the direction of change differs between two different knockouts.[113,114] For detailed review of GPR68 function in non-neuronal cells, see.[39,40,115]

GPR68 in synaptic function and learning: At the Schaffer collateral-CA1 synapse, deleting GPR68 does not alter paired-pulse facilitation but attenuates hippocampal long-term potentiation (LTP).[102] Consistent with a deficit in LTP, GPR68 null mice show a reduced dark-entry latency in the step-through passive avoidance test. In another study, however, $GPR68^{-/-}$ does not exhibit difference in a fear conditioning assay or the Morris water maze test.[106] However, Ogerin, a positive allosteric modulator of GPR68, attenuates context fear memory without affecting cued fear memory.[106] These data suggest that GPR68 likely regulates synaptic physiology and contributes to learning and memory. However, its exact effect depends on the training and testing paradigm.

GPR68 in neuroprotection: In organotypic hippocampal slices, GPR68 deletion worsened acidosis-induced neuronal injury.[63] At 24 hr following a 45-min tMCAO, we showed that GPR68 deletion exacerbated ischemia-induced brain injury.[63] However, using a 30 min tMCAO protocol, another study reported no difference between WT and $GPR68^{-/-}$ mice.[86] We speculate that the technical differences, including tMCAO duration and criteria for inclusion/exclusion (e.g. cerebral blood flow monitoring), contribute to the differences in outcome between the two studies. In our study, we further examined the outcome at day 3 after 45 min tMCAO. We found that $GPR68^{-/-}$ mice exhibited an increase in left (ipsilateral) rotation, which indicates a worsened left:right imbalance. Similar to the 24 hr result, brain infarction was larger in the knockout at 72 hr. The effect of GPR68 deletion on brain injury appeared to correlate with the incidence of hemorrhagic transformation (HT). When we quantified HT incidence following tMCAO, the difference in brain injury was apparent when there existed a diffuse, or moderate level, of HT.[116] It remains unclear whether this correlation with HT involves GPR68 in endothelial cells. Supporting a protective role of GPR68 in ischemia, AAV-mediated overexpression reduced tMCAO-induced brain injury.[63] In summary, our data obtained from both slice and animal studies, and with both knockout and overexpression, are in agreement to suggest that GPR68 activation offers protection in acidotic and ischemic conditions.

A recent study examined the contribution of GPR68 in a sevoflurane-induced neurotoxicity.[117] In this model, 4.9% sevoflurane for 2 hrs induces neuronal loss in hippocampus in neonatal rats. This change correlates with a reduction in GPR68 expression. AAV-mediate GPR68 overexpression reverted sevoflurane-induced loss of neurons. This effect correlates with a reduced neuronal apoptosis with GPR68 overexpression. Functionally, GPR68 overexpression improves the behavioral performance in the Morris water maze test. Compared to the group injected with control AAV, the group receiving AAV-GPR68 had reduced escape latency, increased time in target quadrant, and increased number of platform crossings.[117] The study further shows that GPR68 overexpressed group exhibits an increase in oligodendrocyte proliferation and myelination. Another interesting observation here is that sevoflurane treatment reduces BDNF expression while GPR68 overexpression partially rescues the

reduction in BDNF. Though the mechanism is unclear, the finding is consistent with a neuroprotective role of GPR68 in the brain, and suggests a potential link to neurotrophic signaling pathways.

In organotypic cortical slices, inhibiting PKC activities with Go6983 worsened acidosis-induced neuronal injury in WT slices, but had no significant effect in GPR68$^{-/-}$ slices.[63] In our RNA-Seq analysis, GPR68 deletion reduced the expression of Hsp70 and Grp78,[64] suggesting a link to protein misfolding/ER stress function. In previous studies, ischemia leads to upregulation of neuronal hemoglobin or neuroglobin, which is linked to increased antioxidant activities.[118,119] Interestingly, we found that deleting GPR68 abolished this increase.[64] These data suggest that GPR68 may contribute to neuroprotection through modulating chaperone and/or ER function, and PKC-mediated signaling likely mediates part of the effect in acidotic conditions. The exact downstream effector of GPR68 in ischemia or neuronal injury *in vivo* warrants further investigation.

Summary and speculations

We have gained much knowledge on brain acid signaling from the ASICs. With the emerging evidence regarding metabotropic proton receptor function in the brain, together with the discovery of PAC/ASOR anion channel, it is apparent that brain acid signaling is more complex than what was initially thought. As discussed above, the three classes of proton receptors differ in their pH sensitivity. The proton-sensitive GPCRs are most sensitive to acidification and are activated at ~pH 7.4[38–40]. The ASICs are in the middle and start to open at ~7.[34,37] The PAC is least pH sensitive and only activates when pH is at or below 6.[11,12,35]

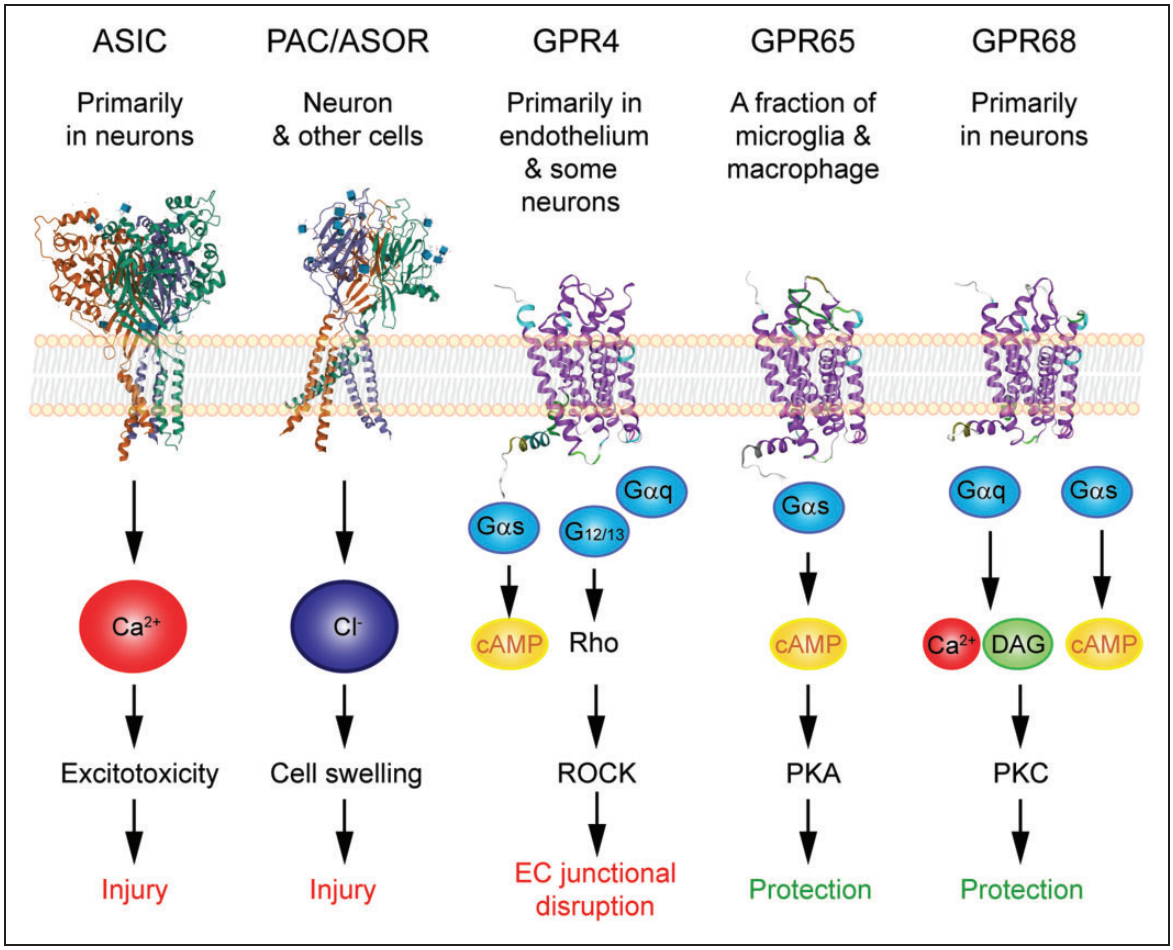

Figure 2. Summary of the expression, signaling, and impact on ischemic injury of the acid-sensitive receptors. 3D illustration of ASIC and PAC/ASOR was based on crystal structure deposited in NCBI PDB database (PBD ID 3S3W and 7JNA) and created with the NGL viewer.[145–147] Note that these structures do not contain most of the intracellular tails. For GPCRs, the 3D illustrations were generated using GPCR homology modeling located on GPCRdb.[148,149] The signaling illustrates the key pathways which have been either demonstrated or implicated in the receptor's contribution to neuronal injury, vascular dysfunction, or neuroinflammation.

Together, these receptors cover a wide range of pH changes, starting from the resting pH (~7.3–7.4) down to ~5. Besides their differences in pH sensitivity, these receptors differ in their expression and signaling (Figure 2). This combination suggests that these receptors work in concert to determine the outcome of cerebrovascular function, neuroinflammation, and brain injuries. Given that some receptors mediate protection while others elicit injury, discovering specific pharmacological reagents will be a key step and offer new opportunities for therapeutic targeting of these receptors in brain ischemia or other neurological diseases which involve acidotoxicity.

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Authors' contributions

XMZ reviewed the literature and wrote the manuscript. ZGX and RPS provided important discussions. All authors reviewed the manuscript.

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