BACKGROUND AND PURPOSE: Brain acidosis is prevalent in stroke and other neurological diseases. Acidosis can have paradoxical injurious and protective effects. The purpose of this study is to determine whether a proton receptor exists in neurons to counteract acidosis-induced injury.

METHODS: We analyzed the expression of proton-sensitive GPCRs (G protein-coupled receptors) in the brain, examined acidosis-induced signaling in vitro, and studied neuronal injury using in vitro and in vivo mouse models.

RESULTS: GPR68, a proton-sensitive GPCR, was present in both mouse and human brain, and elicited neuroprotection in acidotic and ischemic conditions. GPR68 exhibited wide expression in brain neurons and mediated acidosis-induced PKC (protein kinase C) activation. PKC inhibition exacerbated pH 6-induced neuronal injury in a GPR68-dependent manner. Consistent with its neuroprotective function, GPR68 overexpression alleviated middle cerebral artery occlusion–induced brain injury.

CONCLUSIONS: These data expand our knowledge on neuronal acid signaling to include a neuroprotective metabotropic dimension and offer GPR68 as a novel therapeutic target to alleviate neuronal injuries in ischemia and multiple other neurological diseases.

GRAPHIC ABSTRACT: An online graphic abstract is available for this article.

Key Words: acidosis • brain ischemia • mice • neurons • proton
**Nonstandard Abbreviations and Acronyms**

| Abbreviation | Definition |
|--------------|------------|
| AAV          | adenoassociated virus |
| ASIC         | acid-sensing ion channel |
| BAC          | bacterial artificial chromosome |
| CBF          | cerebral blood flow |
| GPCR         | G protein-coupled receptor |
| MCAO         | middle cerebral artery occlusion |
| PAC          | proton-activated chloride channel |
| PKC          | protein kinase C |
| pPKCSS       | phospho-PKC substrate |
| RT-PCR       | reverse-transcription-polymerase chain reaction |
| tMCAO        | transient MCAO |
| WT           | wild-type |

Focused on ion channels. It remains unclear whether a general acid-sensing metabotropic receptor exists in brain neurons. Answering these questions will provide novel insights into our understanding of proton signaling in health and disease.

Four proton-sensitive GPCRs (G protein coupled receptors): GPR4, GPR65, GPR68 (also termed OGR1 which stands for Ovarian cancer GPCR 1), and GPR132, exhibit acid-dependent activation within the pH range (7.4–6) normally observed in physiological and disease conditions. Although previous studies reported that some of these GPCRs are present in specific types of neurons, it is unclear whether metabotropic acid signaling is a general mechanism in brain neurons. Nor is it clear whether such signaling counterbalances acidosis-induced neuronal injury. Here, we started with a miniscale screening against the 4 known proton-sensitive GPCRs. Our result indicates that deleting GPR68 exacerbated pH 6-induced neuronal injury. Next, we determined the expression of GPR68 in the brain, investigated its role in acid-mediated signaling, and examined how GPR68 deletion or overexpression alters ischemic injury outcome.

**METHODS**

**Data Availability**

All data supporting the findings of this study are available within the paper and in the Data Supplement. Additional inquiries can be directed to the corresponding author.

**Human Cortical Tissue**

Human cortical tissue was obtained with consent. Obtaining and using of human tissue were approved by the IRB and IBC committees at the University of South Alabama (see the Data Supplement).

**Mice**

All procedures were approved by the University of South Alabama Animal Care and Use Committee (see the Data Supplement).

**Reagents and Methods**

Table I in the Data Supplement lists the antibodies and primers used. To determine gene expression, brain RNA was isolated and analyzed with standard reverse-transcription-polymerase chain reaction (RT-PCR). Immunostaining of a transgenic (Gpr68-GFP [green fluorescent protein]) mouse was performed to localize GPR68 expression in the brain. Flow cytometry analysis and sorting was used to isolated neurons from neonatal brain. Organotypic brain slices were used to study neuronal injury in vitro. Acid-induced signaling was examined by immunostaining and Western blot. Transient middle cerebral artery occlusion (MCAO) (tMCAO) in mice was performed as described earlier. Stereotoxic injection was used to deliver bicarbonate or overexpressing GPR68 in brain. See Methods in the Data Supplement for details.

**Statistical Analysis**

Statistics was performed in GraphPad Prism and Microsoft Excel. For 2 groups, we used 2-tailed Student’s t test or Mann-Whitney U test. For phospho-PKC substrates (pPKCSS) time course, we used Wilcoxon signed-rank test. For multiple comparisons, we used ANOVA followed by Tukey HSD post hoc correction. For histogram comparison, we used the Kolmogorov-Smirnov test. Data were reported as mean±SD. Differences were considered significant if P<0.05.

**RESULTS**

**GPR68 Mediates a Protective Effect Against Acidosis-Induced Neuronal Injury**

To determine the expression of proton-sensitive GPCRs in mouse and human brains, we isolated total RNA from mouse brain and acutely resected human cortical tissue and performed RT-PCR analysis. At 35 cycles, we detected the expression of GPR4, -65, and -68 (Figure 1A and 1B). GPR68 expression was evident at 30 cycles (Figure 1A). Compared with the WT, deleting GPR4 or GPR65 had no effect while deleting GPR68 significantly increased pH 6-induced neuronal injury. This result suggests that GPR68 mediates a protective function in acidicotic condition.
GPR68 Exhibits Ubiquitous Pattern of Expression in Brain Neurons

To understand how GPR68 exerts its protective effect, we first asked where is GPR68 expressed in the brain. RT-PCR analysis detected GPR68 expression across all brain regions examined (Figure 2A). Next, we examined GPR68 expression at the cellular level. The GPR68 antibodies which we tested were unspecific in detecting endogenous GPR68 (with negative controls using the GPR68 −/− brain, not shown). Therefore, we studied a reporter mouse line, a transgenic Gpr68-GFP mouse.21 This mouse carries a transgene of a bacterial artificial chromosome, which contains the Gpr68 gene locus but with the eGFP coding region inserted after the first methionine of GPR68 (Figure 2B; diagram). Hence, the expression of GFP reflects endogenous GPR68 expression. We prepared coronal sections of brains isolated from Gpr68-GFP and WT mice and performed immunostaining using anti-GFP antibodies. Gpr68-GFP brain exhibited widespread GFP immunofluorescence, which was absent in WT brain (Figure 2B). In cortex, superficial and deeper layers exhibited higher GFP immunofluorescence. At higher magnification, the majority of cortical neurons and essentially all striatal neurons exhibited positive GFP immunofluorescence (Figure 2C). As an alternative approach to determine whether neurons preferentially express GPR68, we dissociated neocortex and striatum from WT mice, labeled neurons with an APC-conjugated anti-Thy1 antibody, sorted APC (Thy1)-positive cells with flow cytometry analysis and sorting, and performed RT-PCR analysis. Compared with unsorted cells (total), Thy1+ cells exhibited higher GPR68 and diminished GPR4 expression (Figure 2D).

GPR68 Mediates Acidosis-Induced PKC Activation

GPR68 primarily couples to Gq, which increases inositol triphosphate and diacylglycerol,22 resulting in PKC (protein kinase C) activation. Therefore, to determine whether GPR68 mediates acid-induced signaling in brain slices, we probed for PKC-dependent activities using an antibody recognizing pPKCSS. First, we performed immunostaining in organotypic brain slices. Acidic pH (pH 6.5, 15-minute treatment) increased pPKCSS staining in WT cortical slices (Figure 3A). Essentially, all pPKCSS-positive cells were NeuN positive. Go6983, a selective inhibitor of several members of the conventional and novel PKCs: α, β, γ, δ, and ε,23 largely abolished the response. In GPR68 −/− slices, acidosis had diminished effect on pPKCSS staining.

To gain more quantitative measurement of acidosis-induced signaling, we performed Western blot analysis.
Figure 2. GPR68 expression in brain neurons.

A. Reverse-transcription-polymerase chain reaction (RT-PCR) result on GPR68 expression in different regions of mouse brain. −RT has no reverse transcriptase added into RT. B. GFP expression in the Gpr68-eGFP reporter mouse line. Diagram illustrates the organization of the BAC transgene, in which eGFP was inserted after the first methionine of GPR68. The GPR68 gene in the BAC chromosome was not expressed because of the addition of poly-A after eGFP coding. To visualize GFP signal, we performed immunofluorescence staining with an anti-GFP antibody, together with a NeuN antibody to identify neurons. Dashed lines outline the boundary between cortex and striatum. C. High magnification view of the boxed cortical and striatal regions in Gpr68-GFP image in (B). D. GPR68 expression in FACS-sorted neurons. Neurons were labeled with APC-conjugated Thy1 antibody. Left panels illustrate the gates and cell distribution of unstained (top) and antibody-stained samples. Gel images show RT-PCR result of total (unsorted) and Thy1+ (neuron) population. APC indicates allophycocyanin; BAC, bacterial artificial chromosome; eGFP, enhanced GFP; FACS, fluorescence-activated cell sorting; FSC, forward scatter; GFP, green fluorescent protein; and SSC, side scatter.
We treated organotypic cortical or hippocampal slices with acidic medium and blotted for pPKCSS. Acidosis increased pPKCSS signals in a time-dependent manner (Figure 3B). Go6983 abolished acidosis-induced increase of pPKCSS signal (Figure 3C). Besides activated PKC, acidosis also induced CaMKII phosphorylation on Thr 286 but had little effect on phosphorylation of Akt, Erk, or JNK (Figure II in the Data Supplement).
Deleting GPR68 attenuated acidosis-induced phosphorylation of PKCβII (Figure 3D) but had no significant effect on acidosis-induced CaMKII phosphorylation (Figure IIA in the Data Supplement). To determine whether PKC activity mediates the prosurvival effect of GPR68, we inhibited PKC with Go6983 and analyzed acidosis-induced injury. Go6983 worsened pH 6-induced injury in WT but not GPR68−/− slices (Figure 4A).

**GPR68 Deletion Worsens Ischemic Neuronal Injury**

Next, we investigated ischemia-induced neuronal injury. We first examined the effect of oxygen-glucose deprivation, an in vitro ischemia-reperfusion model. In mice, 60-minute tMCAO reduces brain pH to ≈6.5 for hours. Therefore, to better mimic in vivo ischemia paradigm, we added 3-hour pH 6.5 treatment after oxygen-glucose deprivation (Figure 4B diagram) and analyzed neuronal injury 24 hour after oxygen-glucose deprivation. As a control, we treated the slices with pH 6.5 alone for 3 hours, which did not induce neuronal injury (Figure 4B).

This lack of injurious effect by pH 6.5 is consistent with previous studies. Following oxygen-glucose deprivation-pH 6.5 incubation, GPR68−/− slices exhibited larger injury than WT slices (Figure 4C).

To determine ischemia outcome in vivo, we performed 45-minute tMCAO, sectioned the brain 24 hours later, and stained with vital dye: 2,3,5-triphenyltetrazolium chloride. Infarct volume was 24.4±7.1% in WT mice. In GPR68−/− mice, infarct volume was increased significantly (P=0.0020, Mann-Whitney U test) to 38.3±11.1% (Figure 5A). Since brain infarct may continue to develop during the first 48 to 72 hours after reperfusion, we further analyzed the outcome on the third day. In this experiment, we also performed behavioral assessment. In the absence of tMCAO, the 2 genotypes did not differ in baseline locomotor activities or the corner test (Figure III in the Data Supplement). Following tMCAO, GPR68−/− mice exhibited a trend (P=0.0861, Mann-Whitney U test) of higher total travel distance in home cage monitoring (Figure 5B), which fits with previously described post-stroke hyperactivity. Next, we quantified the number of left versus right rotations. To factor in the variation in distance traveled, we calculated a rotation index, which is defined as the number of rotations per 10 m distance traveled. This normalized index allows us to better compare animals with different activity levels. As expected for post-tMCAO animals, both WT and GPR68−/− animals exhibited more left (ipsilateral) than right rotations (Figure 5B, middle, upper bar graph, P from paired 2-tailed t test). However, the knockout mice showed a larger imbalance. When we compared the net rotation between

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**Figure 4. PKC (protein kinase C) inhibition or GPR68 deletion on neuronal injury in vitro.**

A. Organotypic cortical slices were treated with pH 7.4, 6.0+vehicle, or 6.0+5 µmol/L Go6983 as indicated for 120 min. Neuronal injury was determined by propidium iodide (PI) stain as described in Methods. B. pH 6.5 alone did not induce neuronal injury in organotypic cortical slices. C. Neuronal injury following oxygen-glucose deprivation (OGD) and pH 6.5 postincubation in vitro. Cortical slices were subjected for 30 min OGD, changed to medium buffered at pH 6.5 for 3 h, and examined for neuronal injury 24 h after OGD. Each circle indicates one slice from a total of 4 (A), 3 (B), or 7 (C) different experiments. ACSF indicates artificial cerebrospinal fluid; and WT, wild-type.

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Figure 5. GPR68 deletion worsens ischemia outcome in vivo.
A, Brain injury after middle cerebral artery occlusion (MCAO). Diagram on top right illustrates the MCAO procedure. Bottom panels show representative images and quantification of brain infarct in wild-type (WT) and GPR68−/− (KO) at 24 h after MCAO. WT and KO animals were subjected to 45 min MCAO. Brain injury was analyzed by TTC staining. B, Behavioral outcome at 3 d after MCAO. Diagram illustrates the experimental outline. On the third day after MCAO, locomotion of the mouse in a home cage was recorded for 90 min with an infrared-based SmartCage, followed by the corner test. The animals were euthanized at 72 h after MCAO, and brain injury was analyzed by TTC staining. Left panel shows the quantification of travel distance during the 60 (from 31 to 90 min) min recording. Middle shows rotation (entire 90 min) per 10 m traveled. For net rotation plot, positive and negative values indicate right and left rotations, respectively. See Methods in the Data Supplement for details. Right shows the average number of left turns (in 10 trials) in the corner test. C, Representative TTC stain and quantification of infarct percentage in WT and KO animals. D, Cerebral blood flow during occlusion and after reperfusion. Graph on the left shows a typical blood flow trace from the MCA territory, monitored with a laser doppler flowmetry. Red bars on top illustrate the window used to calculate the starting (0–1 min, set as 100%), occluded (sixth min—the end of occlusion), and reperfusion (last 1 min) of the blood flow. Histograms on the right show the distribution of blood flow changes for all animals where a successful occlusion was achieved (ie, suture reached the MCA position), which include both the animals met and those which did not meet the inclusion criteria for subsequent injury analysis (see Materials and Methods). Histograms were not different when compared for mean (Mann-Whitney U test) or frequency distribution (Kolmogorov-Smirnov test). P were from 2-tailed t test or Mann-Whitney U test as described in text. MCA indicates middle cerebral artery; and TTC, 2,3,5-triphenyltetrazolium chloride.
the 2 genotypes, GPR68<sup>−/−</sup> exhibited significantly more left rotations (P=0.0287, Mann-Whitney U test) than the WT animals (Figure 5B, middle, lower bar graph). Next, we performed the corner test.<sup>27</sup> Both genotypes exhibited a similar preferential turning to the ipsilateral (surgery) side (Figure 5B, right). Thus, both rotation analysis and the corner test confirmed that tMCAO resulted in sensorimotor deficit on the lesion/surgery side in both genotypes. However, the differences in rotation in home cage monitoring indicate that GPR68 deletion led to stronger left/right imbalance in locomotor function. Last, we performed 2,3,5-triphenyltetrazolium chloride staining at 72-hour after tMCAO. The percentage of infarct in WT was 31.71±14.3% (Figure 5C). Deletion of GPR68 increased the infarct percentage to 41.69±12.6% (P=0.0392, Mann-Whitney U test).

In this experiment, we noticed that brain infarct exhibited a large variation. We speculated that this may reflect the fact that some animals started to recover at 72 hours. However, this wide range of injury raised a question of whether GPR68 deletion alters collateral blood flow. To answer this question, we analyzed cerebral blood flow (CBF) changes during occlusion and after reperfusion. For this analysis, we included all the animals which underwent a successful surgery, which also include mice which did not meet the inclusion criteria for 2,3,5-triphenyltetrazolium chloride study and mice which met the inclusion criteria but died before the 72-hour time point. Figure 5D shows the histogram of the CBF during occlusion and after reperfusion. For either the occluded or reperfused CBF, there were no significant differences in either the mean (Mann-Whitney U test) or the frequency distribution (Kolmogorov-Smirnov test) between the occluded or reperfused CBF between the 2 genotypes.

**Attenuating Acidosis or GPR68 Overexpression Alleviates Ischemia-Induced Brain Injury**

Together with the literature, our data suggest the following model: a mild acidosis is sufficient to activate GPR68 while severe acidosis further recruits the injurious ASICs and PAC (Figure 6A). To assess this model, we first examined whether intracerebroventricular bicarbonate injection, which is a previously established protocol to attenuate brain acidosis,<sup>24</sup> leads to protection. In this and the next experiment, as we were assessing protection, we increased MCAO duration to 60 minutes, which would increase the initial injury and thus facilitate the detection of a protective effect. Mice receiving saline had an average infarct of 32.6±11.2% (Figure 6B). Similar to the previous report,<sup>24</sup> bicarbonate (2 mg/kg) reduced brain infarct to 18.7±10.2% (P=0.0289, Mann-Whitney U test). Next, we assessed the effect of GPR68 overexpression. We performed stereotaxic injection into mouse brain with adenovassociated virus (AAV)2/1 which expresses either eGFP (control) or GPR68 (Figure 6C, diagram). To ensure that we can target a large enough MCA territory, we performed injection at 2 depth and used a higher titer of AAV particles. Figure 6C, left illustrates the injection sites. At second week after injection, GFP expression was apparent in a large area in the MCA territory (Figure 6C, middle). To verify that the AAV-GPR68 virus correctly expresses GPR68, we infected organotypic slices and blotted the lysates using a custom-made GPR68 antibody, which detected overexpressed GPR68 (Figure 6C, right, top blot). Further, immunofluorescence showed that, at 3 to 4 weeks following AAV infection in vivo, the majority of GFP-positive cells were neurons (Figure 6C, right, images). To determine ischemia outcome, we performed MCAO at 3 to 5 weeks following AAV injection and analyzed brain infarction 24 hour later. Mice receiving AAV-GPR68 exhibited an average infarct of 21.97±12.4%, significantly (P=0.0022, Mann-Whitney U test) smaller than those receiving AAV-GFP (37.2±6.8%; Figure 6D).

**DISCUSSION**

Up until now, the literature on neuronal proton signaling focuses mostly on ion channels, including ASICs and PAC. Our data introduce a new metabolic dimension, mediated by GPR68, in neuronal proton signaling. Both ASICs and PAC contribute to acidosis- and ischemia-induced neuronal injury.<sup>5–9,10,12,24</sup> In contrast, GPR68 mediates a novel protective pathway in neurons. This finding provides new insights into our inquiry of acidosis in ischemic brain injuries. The expression of GPR68 in human cortical tissue further suggests that the similar protective mechanism may apply to human brain.

In previous studies, pH reduction to the range of 7.1 to 6.6 results in protection, further reduction down to 6.2 does not have major injurious effect by itself, and a severe acidosis to pH 6.0 or below is clearly deleterious.<sup>3–5</sup> This pH paradox<sup>12</sup> suggests that while acidosis is a well-established perpetrator of neuronal injury, a mild acidosis, probably in the range around 6.8, can have a protective effect. GPR68 starts to activate at pH 7.4, reaches maximal activation at ~6.8 to 6.5, and does not exhibit rapid desensitization.<sup>22</sup> In contrast, ASICs and PAC start to open at pH 7.2 to 7 with a pH<sub>50</sub> of ~6.4 (ASICs) and 5.0 (PAC).<sup>9,26,29</sup> These data together suggest that the magnitude of acidosis determines the balance between the protective GPR68 and the injurious ASICs and PAC (see Figure 6A). One caution is that acidosis is not the only contributor to neuronal injury in vivo. The exact effect of a specific pH will depend on additional factors. For example, while pH ~6.5 itself is not injurious, pairing it with ischemic insult can exacerbates the injury. In one study, 60-minute MCAO reduced brain pH to ~6.5 at 4 hours; and 2.5 mg/kg bicarbonate injection raised pH to ~7.2.<sup>24</sup> Although we did not measure brain pH here, our result is consistent with the previous finding on bicarbonate-induced protection. Given that acidosis
is a prominent feature accompanying multiple diseases, targeting GPR68 has its advantage to achieve the specificity (re: location and timing) needed for therapeutic interventions.

Previous studies have examined the expression of proton-sensitive GPCRs in the brain. GPR4 is predominantly present in cerebrovessels and in neurons within the retrotrapezoid nucleus and dorsal raphe nucleus;
GPR65 exhibits restricted expression in microglia within circumventricular organs; and GPR132 was undetectable at the messenger level.\textsuperscript{14,16,18,19,30} Our RT-PCR results are in good agreement with these reports. Nevertheless, we cannot rule out a low level of GPR132 or GPR65 expression in brain neurons. About GPR68, one study shows that GPR68 is present in cerebellar granule cells and contributes to calcium signaling.\textsuperscript{16} Here, we showed that GPR68 is present throughout the brain. Further, results from 3 different experiments—immunostaining of Gpr68-GFP mouse, RT-PCR of sorted neurons, and pPKCSS immunostaining in slice neurons—all support a primary neuronal role of GPR68 in the brain. Together, these data suggest that GPR68 serves as a ubiquitously expressed proton-sensitive receptor in brain neurons. Together with previous studies on proton-sensitive ion channels, our findings suggest a more dynamic picture of neuronal proton signaling through the activation of cationic ASICs, anionic PAC, and metabotropic GPR68.

Although our result here indicates that neurons are the main cell type which expresses GPR68, immune cells and endothelial cells have been reported to express GPR68 as well.\textsuperscript{21–23} It is likely that GPR68, depending on its expression, will have differential vascular impacts in different systems. For example, GPR68 is present in high percentage in third-order mesenteric vessels but sparsely in cerebral arterial endothelial cells.\textsuperscript{31} Similarly, we did not observe apparent GFP expression cerebral vessels in the Gpr68-GFP mouse brain (not shown). These data suggest that GPR68 may not have as big an impact on cerebral vessel as compared with mesenteric vessels.\textsuperscript{31} Our CBF result supports this speculation. Nevertheless, systematic analysis of PcomA plasticity in the GPR68\textsuperscript{-/-} warrants a future study.

Our results on Go6983 indicated that GPR68-dependent protection requires, at least in part, PKC activities. It will be important to determine the specific subtype of PKC which GPR68 activates, and its downstream targets. Poststroke functional outcome is another important question. Our 3-day behavioral assessment, although remains at the acute phase, showed that GPR68\textsuperscript{-/-} animals exhibited larger left:right imbalance and a trend of hyperactivity, which previously correlates with stroke-induced neurodegeneration.\textsuperscript{26,34,35} These data together suggest GPR68 may have an impact on cerebral vessel as compared with mesenteric vessels.\textsuperscript{31} Our CBF result supports this speculation. Nevertheless, systematic analysis of PcomA plasticity in the GPR68\textsuperscript{-/-} warrants a future study.

While the functional study here focuses on ischemic injury, pH reduction also occurs in physiological conditions. Previous studies have shown that protons regulate spine remodeling and plasticity.\textsuperscript{26–28} The high pH sensitivity makes GPR68 one excellent mediator of neuronal proton signaling. It will be interesting to examine whether GPR68 contributes to synaptic function and/or learning. Given the prevalence of pH reduction in physiology and disease, results obtained will generate essential information to better interpret how protons regulate normal brain function and pathological processes.

### ARTICLE INFORMATION

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**Disclosures**

None.

**Supplemental Materials**

Expanded Materials and Methods

Figures I–III

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