SUPPLEMENTAL MATERIAL

GPR68 is a neuroprotective proton receptor in brain ischemia

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Short title: Neuroprotective effect of GPR68

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SUPPLEMENTAL METHODS

Human cortical tissue

Human cortical tissue was obtained with consent, from patients who underwent surgical treatment of intractable epilepsy. In these patients, resecting a small piece of temporal lobe tissue is necessary in order to gain access to the epilepsy loci, which are typically located to hippocampus. Resected tissues were stored at -80 ºC until RNA extraction (see below). Obtaining and using of the human tissue were approved by the IRB and IBC committees at the University of South Alabama.

Mice

GPR4-/- and GPR65-/- mice have been described previously [39, 40] and purchased from The Jackson Laboratory. GPR68-/- mice have been described previously.[33] The GPR68-eGFP reporter mouse line was purchased from MMRRC. Wild-type C57BL/6, knockouts, and transgenic mice were maintained as breeding colonies at the University of South Alabama. The GPR68-/- mice [33] were on a congenic C57BL/6 background. The GPR4-/- and GPR65-/- mice were backcrossed for 7 generations to C57BL/6 background when recovered from Jackson Laboratory, and backcrossed further for a total of 10 generations to a congenic C57BL/6 background. All knockout and transgenic mice were refreshed (backcrossed to C57BL/6 wild-type) every 5–10 generations, according to the Jackson Laboratory's recommendations. Animal care met National Institutes of Health standards. All procedures were approved by the University of South Alabama Animal Care and Use Committee. For slice cultures, mice (both males and females) from postnatal day 5-7 day were used. For RT-PCR and immunolocalization studies, aged matched WT and transgenic mice (both sexes) from 1 to 15 months of age were used. For MCAO surgery, 8-12 week old aged matched male mice were used.

Antibodies and Reagents

Antibodies used: mouse anti-CaMKII alpha and beta (gift from Dr. Johannes Hell) and used at 1:5,000-10,000 dilutions. We have attempted to generate GPR68 antibodies against 6 different peptides. One rabbit GPR68 antibody shown in Figure 6 was raised against peptide "CSR" ($_{310}$CSRTSRAREAYPLGAPEA$_{327}$), which locates within the C-terminal intracellular tail of mouse GPR68. Antisera were generated by Syd Labs. The same peptide was conjugated to SulfoLink Resin (ThermoFisher), which was then used for affinity purification of the antibody, following the manufacturer's instructions. Affinity purified CSR antibody was used at 1:100-200 for Western blot and immunofluorescence to detect overexpressed GPR68. Supplemental Table I lists additional commercial primary antibodies used. Secondary antibodies used were: Alexa 405-, 488-, 568-, 680-, and 800-conjugated secondary antibodies, and Dylight 488-, 680- and 800-conjugated secondary antibodies (ThermoFisher Invitrogen, Rockland Immunologicals, & Jackson ImmunoResearch Lab).

Brain RNA isolation, Reverse Transcription (RT), and Polymerase Chain Reaction (PCR)

Total RNA from human cortical tissue or mouse brain (perfused with saline or PBS prior to isolation) was isolated using TRIzol and a RNeasy Kit (Qiagen), following manufacturer's instructions. For FACS sorted cells, RNA was isolated using a RNAqueous-4PCR Total RNA Isolation Kit (Ambion), following manufacturer's instructions. RNA was treated with DNA-free (ThermoFisher) to remove genomic DNA. For RT, 1 µg RNA (for tissue samples) or 50-100 ng RNA (for sorted cells) was used per 20 µl reaction, which was performed using Superscript II reverse transcriptase (ThermoFisher) and oligo(dT)$_{20}$ or random hexamer, following

manufacturer's instructions. Negative (-RT) control did not have reverse transcriptase. Standard PCR was performed using 2x Taq supermix (Syd Labs). Cycling condition was: 95°C 4'; 30 or 35 cycles of (94°C 20",53°C 20", 72°C 20"); 72°C 2';12°C hold.

Brain isolation, Cryosection, and Immunostaining

The mice were perfused with phosphate buffered saline (PBS) or saline and brain were rapidly isolated, snap-froze on dry ice, and stored at -80°C till use. For histology, saline perfusion was followed by 30-50 ml of 4% paraformaldehyde in PBS. The brains were then isolated and post-fixation in 4% paraformaldehyde (for one night) or 2% paraformaldehyde (for two nights). The brains were protected overnight in 15% and then 25 or 30% sucrose, and sectioned coronally at 12 or 16 μm thickness. For GFP staining in GPR68-GFP reporter line, 2-12 month old mice were used. The slices were adhered to SuperFrost plus glass slides, air-dried overnight, and stored at -20°C until use. For immuostaining, we used a protocol similar as previously described [17]. Briefly, the slices were rehydrated and permeabilized with PBS 1% Triton for 2 hrs. For GFP staining, a pre-treatment step with 1% SDS for 60 sec was adopted to reduce non-specific signals. The sections were blocked sequentially (20-30 min each at room temperature) with 50 mM NH_4Cl and blocking buffer (PBS, 10% Horse serum, 5 mg/ml BSA, 0.2% Triton X-100). For antibody dilution and washing, the buffer was a 1:4 dilution of the blocking buffer with PBS. We added primary antibodies (see supplemental Table I) to the slides and incubated the slides overnight at 4°C in a moisturized dark box, washed three times (5-10 min per wash with gentle rocking at room temperature) with washing buffer, and added Alexa- or DyLight-conjugated secondary antibodies (1:600-1000 dilutions) and incubated at room temperature for 2 hr. We mounted the slides with either a homemade 50% glycerol based mounting media, or with fluorescence mounting media (Agilent Dako), or Permount (EMS), and imaged with a Nikon A1R confocal microscope.

Flow Cytometry Analysis and Sorting (FACS)

Brain tissue (including neocortex, hippocampus and striatum) was isolated from postnatal day 0-1 mice. We used newborns due to the technical difficulty of neuron isolation from adult brain. Brain tissue was chopped to small pieces, digested in Neurobasal-A containing collagenase (1 mg/ml) and DNase (100 μg/ml) at room temperature with rotation for 10 min. Cells were dissociated with fire-polished Pasteur pipette, followed by labeling with APC-conjugated anti-Thy1 antibody (1:100) in Neurobasal-A containing 2% horse serum. Cell density during labeling was ~2 x 10^6 cells/ml. After 20 min incubation with gentle rocking at 4 ºC, cells were washed 3x with Neurobasal-A, and sorted by FACS. Unlabeled sample was acquired first to set gates.

Slice culture, acidosis, oxygen-glucose deprivation (OGD) and live/dead staining

Organotypic mouse cortical and hippocampal slices were cultured as described earlier [17]. Briefly, slices (350 μm thick) were cut with a tissue chopper. For organotypic cortical slices, to ensure that we are comparing the similar regions with in vivo MCAO study, we prepared sections from the motor-sensory cortex (approximately between Bregma +1 to -2). Slices were randomly grouped to ensure that each group (typically 4 slices) had a balanced combination of slices of different cortical thickness. Similarly, hippocampal slices were randomly grouped before plating so that each group (typically has 5-6 slices) has a balanced combination of sections from dorsal and ventral parts. When we did the treatment, we randomly assigned the transwells to different treatment groups. Adopting this approach will ensure the differences in layer thickness averaged out.

Following sectioning, the slices were transferred onto Falcon polyethylene terephthalate-etched membrane culture inserts with 1 μm pores (Fisher) in 6 well plates. Each well contains 1.2 ml FCM (25% HBSS, 25% horse serum and 50% MEM with 2 mM Glutamax, 1.5 mg/ml glucose, 5U/ml pen/strep and supplemented with extra 4.5 mM $NaHCO_3$). Slice cultures were kept in a humidified CO_2 incubator maintained at 37°C 5.5% CO_2 for 9-14 days. Medium was changed every 2-3 days.

For all slice injury studies, we compared sister cultures of WT and knockouts (i.e., the slices from WT and the knockout were cultured at the same time, and all post-processing, including medium change and treatment, were performed in parallel). For studying pH 6-induced injury, we used two types of media: 1) 74% MEM, 25% HBSS, 1% horse serum, with 1.5 mg/ml glucose; or 2) HBSS supplemented with 1% horse serum, 1× essential amino acid (Invitrogen), 1× vitamin mix (Invitrogen), 1× Glutamax and 1.5 mg/ml glucose. The results obtained from two media were similar and were combined in the analysis. To buffer pH, we used 20 mM HEPES for pH 7.4 and 20 mM MES for pH 6.0. On the day of treatment, the medium was warmed to 37°C, and the pH adjusted at least 2 times with 0.5-2 hr intervals to ensure that the pH stays stable. Due to the small volume of the slices (~1 mm x 2 mm x 0.3 mm = 0.6 μl), we did not measure pH inside the slices during treatment. However, to ensure sufficient buffering to clamp slice pH closely to the desired value, we used 1.5-2 ml of buffered medium during treatment. In addition, to ensure that pH in media does not drift significantly during the 2 hr treatment, we replaced media in the dish with fresh pH media at the 1 hr time point.

Oxygen-glucose deprivation (OGD) was similar to what was described earlier [17]. Briefly, slices grown on culture inserts were rinsed twice with XCSF (xylose substituted artificial cerebral spinal fluid); (in mM): 128 NaCl, 3.5 KCl, 23 $NaHCO_3$, 1.2 NaH_2PO_4, 26 Xylose, 1.8 $CaCl_2$, 1.2 $MgCl_2$, 1mg/ml BSA, osmolarity adjusted with additional Xylose to 320-325 mOsm). Up to 4 inserts were transferred into one 100 mm dishes containing 10-12 ml XCSF, with 1 ml of XCSF (balanced in a 37°C CO_2 incubator for 3 hr) added into the insert to cover the slices. Dishes were transferred to a cell culture chamber, with the lids of the dishes lifted to ensure efficient gas exchange. The chamber was fluxed for 5-6 mins at 1.6-1.8 psi with 95% N_2 5% CO_2, which was humidified through a 37°C water reservoir. The chamber was then transferred into a 37°C incubator, and maintained airtight for the duration as described in the corresponding experiments. Total time (including the flux time) was reported as the OGD duration in this study. At the end of OGD, slices were transferred back to 6 well plates. For controls, slices were changed into standard ACSF (same as XCSF above, except that the initial 10 mM xylose was substituted with glucose) and maintained in a 37°C CO_2 incubator for the same duration as OGD. Since in vivo MCAO reduces brain pH to ~6.5-6.6 at 3-4 hours after reperfusion[24], we added a 3-hr pH 6.5 treatment period following the OGD period to better mimic the *in vivo* tMCAO scenario.

Live/dead staining were performed on the second day as described earlier [17]. Briefly, at 22-24 hr following the treatment, 1 μl of Syto-13 and 13.5 μl of propidium iodide (1 mg/ml) were diluted with 2.5 ml FCM. 0.4 ml of this medium was mixed with the culture medium (1.2 ml) in the culture plate, and 0.8 ml of the resultant staining medium was added on top of the culture insert. Plates were incubated in the incubator for 1 hr, 0.8 ml of the staining medium in the well was transferred again to the top of the insert, and incubated for another hour. Staining medium was removed, and 1 ml of ice-cold HBSS with calcium and magnesium (HBSS+/+) containing 6 mg/ml glucose was

added to the top of the insert. The inserts were kept for 20-30 min on ice or at 4ºC to allow the HBSS buffer to pass through the insert. The slices were fixed for 10-15 min with 4% paraformaldehyde in HBSS+/+, 6mg/ml glucose, 20 mM HEPES, pH 7.4. Following washing (3× 10 min) with PBS, the slices were imaged with a fluorescence microscope.

Transient Focal ischemia

The induction of transient focal ischemia in mice was performed as described earlier [5, 17]. Briefly, 8-12 week old, body weight 25 ± 3 g, WT and GPR68-/- male mice (on congenic C57BL6 background) were used for this study. We focus our analysis here on males to remove the variation due to estrogen cycles in females. Mice were anesthetized and maintained with 1.5% isoflurane, 70% N_2O, and 28.5% O_2 with intubation and ventilation. Rectal and temporalis muscle temperatures were kept at 37°C ± 0.5°C with a thermostatically controlled heating pad. A probe for measuring cerebral blood flow was attached to the skull with cyanoacrylate adhesive. Under an operating microscope, the bifurcation of the common carotid artery (CCA) was exposed and the external carotid artery (ECA) ligated. The internal carotid artery (ICA) was isolated, the extracranial branch of the ICA was ligated, and then a 7-0 silicon coated monofilament nylon surgical suture (Doccol) was introduced through the open end of ECA into the ICA lumen and advanced ~7-8 mm past the CCA bifurcation. Once the suture reached the correct position, the tie at CCA was loosened to restore the blood flow to most of the ipsilateral side. Suture occlusion of the middle cerebral artery (MCAO) was performed for 45 min. We chose 45 min occlusion (as opposed to a longer duration) because our in vitro slice data suggested that GPR68 deletion leads to larger injury.

For *in vivo* studies, we followed the STAIR guideline[41, 42]. We randomly assigned animals to experimental groups. Other than the initial set of the 24 hr experiment (see below), we blinded genotypes to the operators. To ensure consistency, cerebral blood flow for all surgery animals was monitored for the entire duration by transcranial laser Doppler (MoorVMS-LDF2). We applied the following criteria to reduce the potential concern of variabilities introduced by blood flow variations. Blood flow traces were examined by at least two different researchers. Mice that failed to maintain a stable blood flow reduction between 5-20% of the original value, or failed to have at least 50% reperfusion of the original value following suture removal, or had a reperfusion rate exceeds 120% were excluded. Twenty four hour after MCAO, mice were sacrificed and brains were sectioned coronally at 1 mm intervals and stained by vital dye immersion: (2%) 2,3,5-triphenyltetrazolium hydrochloride (TTC) [17]. For 45 min MCAO-24 hr TTC analysis, the genotype of the first set of animals (7 WT 4KO) were not blinded, while that of the second cohort of the animals were blinded (5 WT 6 KO). The two sets generated comparable results and were combined and reported in Figure 2B. For the rest of MCAO experiments, the genotype or drug group was blinded to the operator for all manipulations and analyses. For percent infarct calculation, we used the method as described in one previous study [43]:

$$\% \text{ Infarct} = \frac{\Sigma(\text{Area on the contralateral side - healthy area on the ipsilateral side})}{\Sigma \text{ Area on the contralateral side}}$$

One technical note is that, for the initial WT KO comparison (Figure 2B), we quantified 6 slices for infarct calculation. Later, when we performed the 3-day and AAV studies, we noticed that the 7th slice tends to have some injury. Therefore, we quantified 7 sections in these later three sets of experiments.

For quantification of cerebral blood flow, raw blood flow before (0-2 min), during (from the 6th min till the end of occlusion), and after reperfusion (last 2 min of recording, typically at about 10 min after reperfusion) was measured by the MoorVMS software, exported and analyzed and plotted by Microsoft Excel and GraphPad software. For blood flow analysis, we included all animals which had successful surgery (i.e., with the suture reached the MCA position). The breakdown include a) 19 WT and 18 KO counted in the TTC analysis, b) 8 WT and 5 KO with good trace but died before the 72 hr time point, and c) 9 WT and 10 KO with traces not meeting the inclusion criteria. 3 WT and 2 KO which exhibited apparent unsatisfactory occluded blood flow were euthanized before suture withdraw and thus did not have reperfusion value.

Behavioral analysis

Behavioral analysis was performed with the operator blinded to the genotype of the mice. For locomotion assay, the mouse was be placed in a clean mouse cage with bedding, food and water. The mouse cage was placed inside an infrared monitored SmartCage (AfaSci Inc.), and the activities were recorded for 90 min. The first 30 min was considered habituation time and only the following 60 min (31-90 min) was used for locomotor analysis. The only exception is that, for post-stroke rotation analysis, we used the total 90 min. The reason is because some post-stroke animals had greatly reduced movement. Including the first 30 min, which tends to contain higher activities, will allow us to get more accurate quantification of the rotation index, as defined by number of rotations per 10 m traveled. Two of the WT animals had little overall movement and recorded no rotation (either left or right) during the 90 min period and thus were not included for rotation analysis. The corner test was performed similar to a previous study [27]. Briefly, the mouse was placed between two boards of (approximately 30 cm×20 cm) inside a large mouse/rat cage. The edges of the two boards were attached at approximately 30° angle with a small opening (gap) along the joint between the two boards to encourage entry into the corner. The mouse was placed between the two angled boards facing the corner and half way to the corner. When entering into the corner both sides of the vibrissae were stimulated, and the mouse then turned back to face the open end. The turning direction were recorded from 10 trials. Mice did not cooperate, or did not exhibit the rearing then turning behavior were excluded from this analysis.

Adeno-associated virus-mediated overexpression

The adeno-associated viral vector contained either a CMV promoter driving the expression of GPR68-FLAG and an internal ribosomal re-entry site (IRES) driving the expression of eGFP. Control vector contained only eGFP driven by CMV or CAG. Adeno-associated virus 2/1, which was chimeric virus with AAV1 capsids and AAV2 inverted terminal repeats, was produced by the University of Iowa Vector Core. Infection of organotypic slices were performed at 0 or 1 day in culture. AAV (~4 x 10^{12} particles) was mixed with 50 µl FCM and added directly on top of the slices. For Western blot analysis, slices were lysed 6-9 days after transfection. For stereotaxic injection, the injection was performed in mice at 4-5 week of age, using an automated stereotaxic injection instrument for mouse (Stoelting Co.). The coordinates were AP -1 or -1.5 and ML 3.2 ± 0.5 (the exact AP & ML depends on the size of the mouse). After moving to the intended X Y coordinates, the injection needle was lowered to the surface of the skull. From this point, the needle was moved down for 2.5 mm, 0.5-0.75 µl of AAV (about 1-2 x 10^{12} particles) injected, the needle was then backed up for 1.5-1.7 mm, followed by another injection of 0.5-0.75 µl of AAV. Since the mouse brain/skull size has larger variation at 4-5 weeks, we found that this approach allows better targeting of the striatum and part of the cortex (see Supplemental Figure S7A). The rate of

injection was 0.1-0.2 µl/min. The mice were returned to home cage and allowed to recover for 3-5 weeks, and subjected to 60 min MCAO surgery as described above.

Intracerebroventricular (i.c.v.) injection

We used a single dose and single time point in this experiment based on the time/dose analysis documented by a previous study.[24] As described above in the Transient Focal Ischemia section, animals were randomly assigned into the groups; the same cerebral blood flow criteria were adopted to decide inclusion/exclusion. Drug groups (bicarbonate or saline) were blinded to the operator. In this experiment, all 18 surgery animals survived the surgery and all met the blood flow inclusion criteria. Three of the 18 animals (1 received bicarbonate and 2 received saline) died before the 24 hr time point. At 1 hr following reperfusion, i.c.v. injection of saline or $NaHCO_3$ (25 mg/ml solution at a dose of 2 mg/kg) was performed using the similar stereotaxic injection procedure as described above, with the following coordinates: -1, -0.2, -2. The skin was stitched together and the mouse was allowed to recover for one hour in a temperature controlled stage. The rest of the procedure, including TTC analysis of brain injury, was performed as described above in the MCAO section.

Phosphorylation analysis in slices (immunostaining and Western blot).

For analysis of acid-induced phosphorylation, organotypic brain slices were treated with pH medium (either in MEM, ECF, or HBSS based medium, buffered with 20 mM HEPES or MES) for the durations as specified, and when needed, in the presence or absence of drugs. For immunolocalization of pPKCSS, slices were fixed with 4% paraformaldehyde for 10-15 min. The slices were washed with PBS, and permeabilized with PBS 1% Triton overnight at 4 °C. The subsequent blocking and immunostaining were similar to that described for cryosections.

For Western blot analysis, slices were scraped off from the culture inserts using a 1 ml pipette and 200-300 µl of lysis buffer (PBS with 1% Triton X-100, 0.5% SDS, and freshly added protease inhibitors and phosphatase inhibitors). Lysates were sonicated briefly and cleared by centrifugation. Before loading, 1/2 volume of 3x SDS sample buffer was added to the lysates, and incubated at ~50 °C for 15-20 min. The samples were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. Blotting was performed according to instructions of the Odyssey Imaging System (Li-cor). Briefly, membranes were blocked in blocking buffer (0.1% casein in 0.2×PBS pH 7.4) for 1 h. Primary antibodies were diluted with blocking buffer containing 0.1% Tween-20 and incubated at 4 °C overnight or at room temperature for 2 h. Secondary antibodies were diluted in blocking buffer containing 0.1% Tween-20 and 0.01% SDS and incubated at room temperature for 1 h. Blots were imaged using an Odyssey Infrared Imaging System according to manufacturer's instructions. Densitometry of imaged bands was performed as described earlier [44].

Microscopy

For confocal imaging, images were captured using a laser scanning microscope (Nikon A1R), similar to procedures described earlier [17]. Briefly, illumination was provided by an argon (Ar, 458, 488, 514 nm lines) and a 563 diode laser, and a 642 laser. Green, red, and far red channels were imaged sequentially to eliminate bleed-through, using 488 nm excitation and a 525/50 emission filter, 563 nm excitation and a 595/50 emission filter, and 642 nm excitation and 710/75 emission

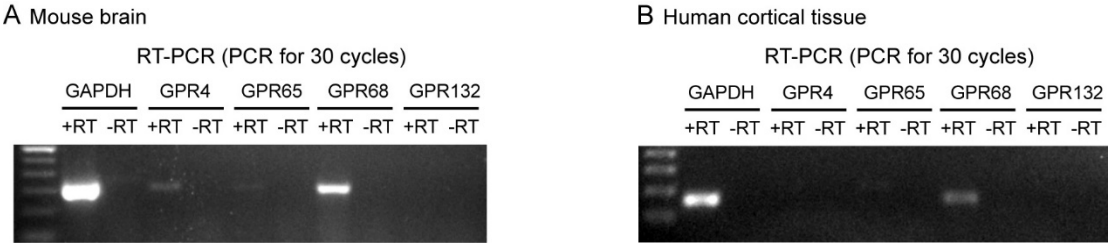
filter, respectively. Images were captured with a 4×, 20×, or 60×/1.2 PL APO water lens. Each captured image was an average of 4 scans in a single plane.

For live/dead staining, slices were imaged using an Olympus IX70 inverted epifluorescence microscope with band filters. Images were captured with a 4× or 10× lens with a SPOT cooled CCD camera and SPOT imaging software [44]. Exposure time was maintained the same for all slices in the same experiment. Images were imported into ImageJ for quantification. For cortical slices, we did not distinguish the layers and quantified the whole slice. We excluded the edges corresponding to about 1/16 thickness of a given slice. The reason is because the edge of the slice typically represents some cells or processes migrated out during the culture period. The raw average fluorescence intensity of red and green channels were obtained with ImageJ, exported to Microsoft Excel and GraphPad for further analysis. To determine neuronal injury, we calculated the ratio of the average fluorescence intensity of PI to that of Syto-13. By using ratio of PI:Syto (as opposed to the absolute PI fluorescence), the quantification takes away the concern that some slices may have better penetration of the dyes in general.

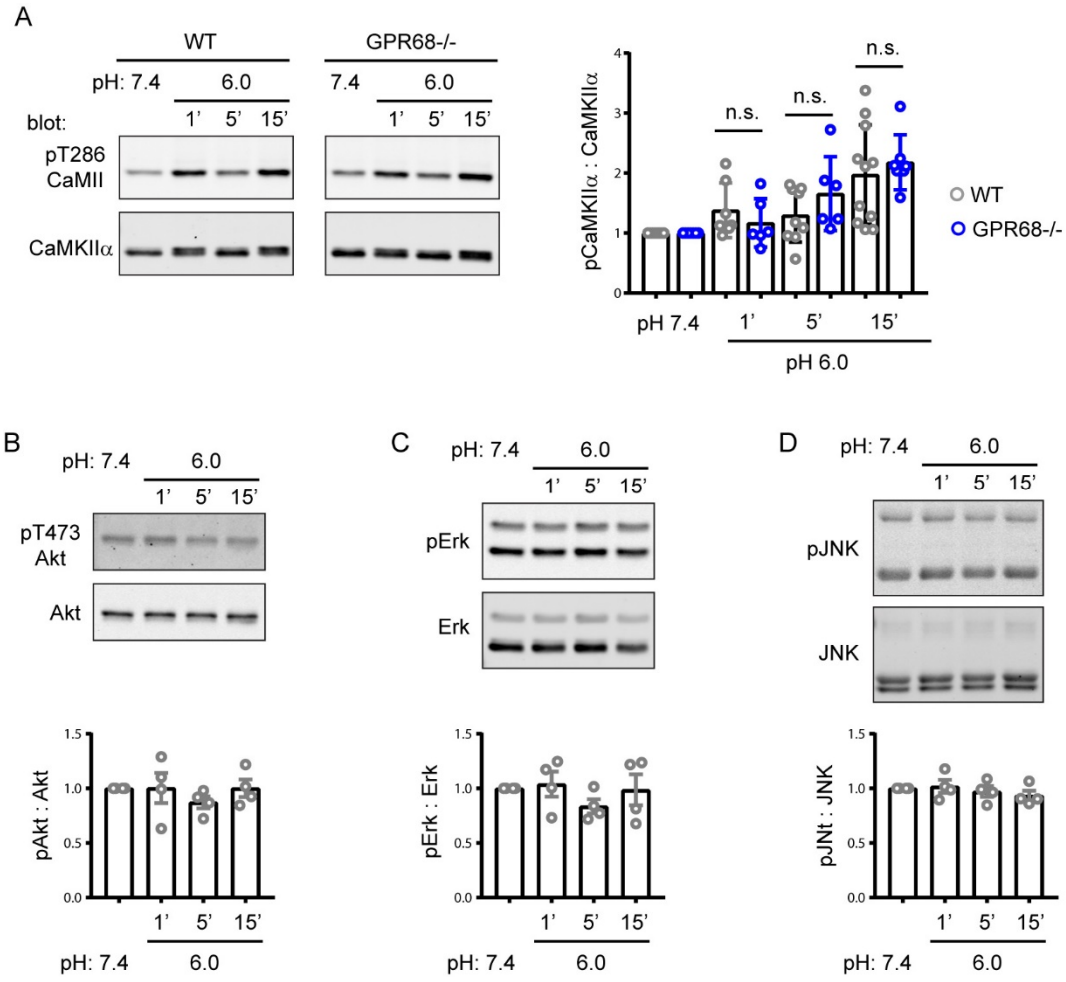
SUPPLEMENTAL TABLE I.
Summary of commercial primary antibodies (top part of the table) and primers (lower part of the table) used.

Target (host)	**Company**	**Catalog #**	**Dilution**
GFP (rabbit)	Invitrogen	A-11122	1:2000
GFP (sheep)	Invitrogen	OSE00001G	1:2000
GFP (rat)	Nacalai USA	GF090R	1:2000
NeuN (mouse)	Rockland Immunochemicals	200-301-X09	1:2000
NeuN (rabbit)	Cell Signaling Technology	12943S	1:2000
NeuN (chicken)	Abcam	ab134014	1:2000
pPKCSS (rabbit)	Cell Signaling Technology	6967S	1:2000
β-tubulin (mouse)	Sigma	T4026	1:60k
β-tubulin (mouse)	Sigma	T8578	1:30k
Erk 1/2 (mouse)	Invitrogen	MA5-15227	1:2000
pErk 1/2 (rabbit)	Sigma-Aldrich	05-797R	1:2000
JNK(mouse)	Cell Signaling Technology	3708S	1:2000
pJNK(rabbit)	Invitrogen	44-682G	1:2000
Akt(rabbit)	Cell Signaling Technology	9272S	1:2000
pAkt(mouse)	Cell Signaling Technology	4051S	1:2000
pCaMKII (rabbit)	Cell Signaling Technology	12716S	1:2000
Thy1-APC (rat)	Invitrogen	17-0902-82	1:100
Gene	***Forward primer***	***Reverse primer***	
Mouse GAPDH	GAACGGATTTGGCCGTATTG	TTCTCCATGGTGGTGAAGAC	
Mouse GPR4	CCACTTACTCACCTTCCTGTT	CAGCGGTGTCATGCTTATTG	
Mouse GPR65	CCAAGGAAACCCTAAGAGAGAA	GGGACAGACTGAAGAGGTAAAT	
Mouse GPR68	GAGAGCCTAAGGGAGAAGAAAG	CATCTCTGCCAACTCTCCTATAC	
Mouse GPR132	TGACATGAAGGTGGAGAAGAG	CATCCATGTCTCCTTGGTAGAA	
Human GAPDH	GTCAAGGCTGAGAACGGGAA	AAATGAGCCCCAGCCTTCTC	
Human GPR4	TACATCAGCATCGCCTTCCT	CAGAAGGTGTGGTTGTAGCG	
Human GPR65	CCTGATCTGCAACCGGAAAG	TCCCAGAATTGCTGTGGTCT	
Human GPR68	GGGTTGGGAGAATTGAGGGA	GTCCCAGTTCACTGACCAGA	
Human GPR132	GCCACAGTGCCCATGTCCCC	CACAGCCAAGGGAGCCAGCC	

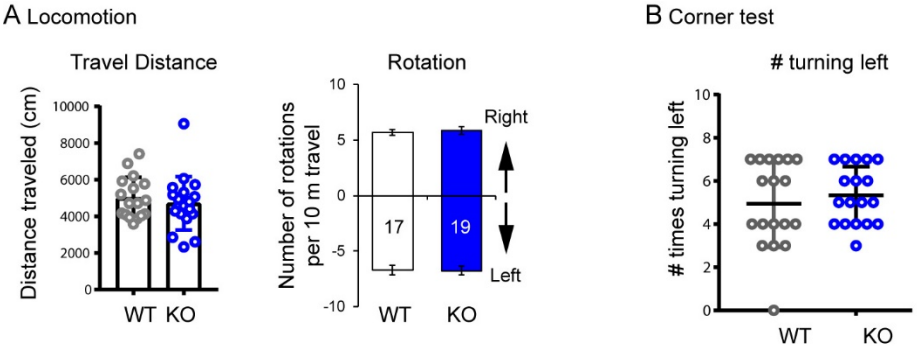
SUPPLEMENTAL FIGURE I. RT-PCR analysis of proton-sensitive GPCR expression in the brain. Representative gels showing the RT-PCR result of mouse brain (**A**) and human cortical tissue (**B**). Images shown were after 30 cycles of PCR. Note that GPR68 expression was evident in both mouse and human tissues.



SUPPLEMENTAL FIGURE II. Effects of acidosis on phosphorylation of CaMKII (A), Akt (B), Erk (C) and JNK (D). Organotypic hippocampal slices were treated with pH 7.4, or pH 6.0 for 1, 5, or 15 min. Lysates were blotted for different phospho-specific or control antibodies as indicated. Blots are representative from 4-6 different experiments.



SUPPLEMENTAL FIGURE III. Baseline behavioral analysis. (**A**) Locomotor activities. Animal locomotion was monitored for 90 min. The first 30 min is considered habituation period. The last 60 min (31-90 min) was used to calculate travel distance and rotation index, defined by number of left or right rotations per 10 m traveled. (**B**) Corner test was performed as described in Methods. Number of left turns out of 10 trials was plotted. One WT animal did not have locomotion recorded due to instrumental error. One knockout animal did not have corner test result because of un-cooperation during the test.



*** Preclinical Checklist**

Preclinical Checklist: Prevention of bias is important for experimental cardiovascular research. ***This short checklist must be completed, and the answers should be clearly presented in the manuscript.*** *The checklist will be used by reviewers and editors and it will be published. See "Reporting Standard for Preclinical Studies of Stroke Therapy" and "Good Laboratory Practice: Preventing Introduction of Bias at the Bench" for more information.*

This study invovles animal models:
Yes

Experimental groups and study timeline

The experimental group(s) have been clearly defined in the article, including number of animals in each experimental arm of the study:	Yes
An account of the control group is provided, and number of animals in the control group has been reported. If no controls were used, the rationale has been stated:	Yes
An overall study timeline is provided:	Yes

Inclusion and exclusion criteria

A priori inclusion and exclusion criteria for tested animals were defined and have been reported in the article:	Yes

Randomization

Animals were randomly assigned to the experimental groups. If the work being submitted does not contain multiple experimental groups, or if random assignment was not used, adequate explanations have been provided:	Yes
Type and methods of randomization have been described:	Yes
Methods used for allocation concealment have been reported:	N/A

Blinding

Blinding procedures have been described with regard to masking of group/treatment assignment from the experimenter. The rationale for nonblinding of the experimenter has been provided, if such was not feasible:	Yes
Blinding procedures have been described with regard to masking of group assignment during outcome assessment:	Yes

Sample size and power calculations

Formal sample size and power calculations were conducted based on a priori determined outcome(s) and treatment effect, and the data have been reported. A formal size assessment was not conducted and a rationale has been provided:	Yes

Data reporting and statistical methods

Number of animals in each group: randomized, tested, lost to follow-up, or died have been reported. If the experimentation involves repeated measurements, the number of animals assessed at each time point is provided, for all experimental groups:	Yes
Baseline data on assessed outcome(s) for all experimental groups have been reported:	Yes
Details on important adverse events and death of animals during the course of experimentation have been provided, for all experimental arms:	N/A
Statistical methods used have been reported:	Yes
Numeric data on outcomes have been provided in text, or in a tabular format with the main article or as supplementary tables, in addition to the figures:	Yes

Experimental details, ethics, and funding statements

Details on experimentation including stroke model, formulation and dosage of therapeutic agent, site and route of administration, use of anesthesia and analgesia, temperature control during experimentation, and postprocedural monitoring have been described:	Yes
Different sex animals have been used. If not, the reason/justification is provided:	Yes

Statements on approval by ethics boards and ethical conduct of studies have been provided:	Yes
Statements on funding and conflicts of interests have been provided:	Yes

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