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Pretreatment with GPR88 Agonist Attenuates Postischemic Brain Injury in a Stroke Mouse Model

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Stroke is one of the leading causes of neurological disability worldwide and stroke patients exhibit a range of motor, cognitive, and psychiatric impairments. GPR88 is an orphan G protein-coupled receptor (GPCR) that is highly expressed in striatal medium spiny neurons; its deletion results in poor motor coordination and motor learning. There are currently no studies on the involvement of GPR88 in stroke or in post-stroke brain function recovery. In this study, we found a decrease in GPR88 protein and mRNA expression levels in an ischemic mouse model using Western blot and real-time PCR, respectively. In addition, we observed that, among the three types of cells derived from the brain (brain microvascular endothelial cells, BV2 microglial cells, and HT22 hippocampal neuronal cells), the expression of GPR88 was highest in HT22 neuronal cells, and that GPR88 expression was downregulated in HT22 cells under oxygen-glucose deprivation (OGD) conditions. Moreover, pretreatment with RTI- 13951-33 (10 mg/kg), a brain-penetrant GPR88 agonist, ameliorated brain injury following ischemia, as evidenced by improvements in infarct volume, vestibular-motor function, and neurological score. Collectively, our results suggest that GPR88 could be a potential drug target for the treatment of central nervous system (CNS) diseases, including ischemic stroke.

Key words : Cerebral ischemia, GPCR, oxygen glucose deprivation, stroke

Introduction

Stroke is the leading cause of long-term disability and cognitive impairment worldwide; thus, it demands enormous resources from healthcare systems and incurs substantial social cost. Remarkable advances have been made in acute vascular treatments to reduce infarct size and improve neurological outcomes [10, 13]. Traditionally, the focus of stroke treatments has been on decreasing ischemic cell death, which has proven to be effective, given the progressive decline of stroke mortality in the past few decades [4]. However, stroke patients display motor, cognitive, and psychiatric impairments even after recovery [4]. The FDA-approved treatment for ischemic stroke is thrombolysis with tissue plasminogen activator (tPA) up to 4.5 hr after occlusion [1]. Although acute tPA treatment is beneficial against ischemic stroke, a more effective method of stroke treatment must be developed to further improve stroke recovery.

GPR88 is an orphan G protein-coupled receptor (GPCR) that is highly expressed in both the dorsal and ventral areas of the striatum [22], which is a major component of the basal ganglia circuitry. GPR88 expression has also been observed in other brain regions, including the cerebral cortex, amygdala, and hypothalamus [6, 15]. GPR88 is highly expressed in both D1 dopamine receptor (D1R)- and D2R-expressing medium spiny neurons (MSNs) [14]. Many basal ganglia functions are controlled by MSN networks, including voluntary movement, motor learning, motor planning, and decision making. The pathophysiology of neurological disorders, such as Parkinson's disease, Huntington's disease, bipolar disorder, schizophrenia, and attention deficit hyperactivity disorder (ADHD), are closely associated with the altered neural circuitry involving striatal MSN [21].

A study by Quintana et al reported that the lack of GPR88 in MSNs results in increased glutamatergic excitation and reduced GABAergic inhibition, which consequently enhances their excitability. GPR88 knockout mice exhibit behav-

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ioral hyperactivity, deficits in motor coordination, and impaired acquisition and integration of visual or auditory cues, leading to poor cue-based learning [19]. Reduced expression of GPR88 affects dopamine depletion, as observed in a unilateral 6-hydroxydopamine (6-OHDA)-lesioned rat model of Parkinson's disease [14]. In aged BACHD mice, a Huntington's disease model expressing the full-length human mutant huntingtin (mHTT) gene, dendritic spine loss was observed and this is attributable to the reduction in expression of the striatum-specific GPR88 [20]. GPR88 expression is not only confined to the striatum, but also expressed in the cerebral cortex, amygdala, and hypothalamus [6, 15]. GPR88 knockout mice thus acquired striatal deficits (hyperactivity, stereotypies, and motor impairment) and exhibited reduced anxiety-like behaviors [17]. Thus, the therapeutic potential of GPR88 extends to cognitive and anxiety disorders [17]. In addition, GPR88 knockout mice showed enhanced motivation for voluntary alcohol-drinking and -seeking behaviors [3]. However, there are currently no studies on the involvement of GPR88 in stroke, as well as in the post-stroke brain function recovery.

In this study, we investigated the possible role of GPR88 in ischemic stroke by determining the expression levels of GPR88 in ischemic mice and neural cells. We then used a GPR88 agonist, RTI-13951-33 [8, 9], and examined its effects on ischemic brain damage, particularly in terms of infarct volume, neurological deficits, and motor function, using a focal cerebral ischemia mouse model.

Materials and Methods

Animals

C57BL/6 mice (six-week-old, male) were purchased from Nara Biotechnology (Seoul, Korea). Mice were housed under a 12-hr light/dark cycle and allowed ad libitum access to food and water. All animal experiments were conducted in compliance with the guidelines set by the Pusan National University-Institutional Animal Care and Use Committee (PNU-IACUC; approval number PNU-2019-2167). GPR88 agonist (RTI-13951-33 hydrochloride: 5 and 10 mg/kg/total volume 100 µl, respectively) was intraperitoneally injected 30 min prior to ischemic brain injury [9].

Focal cerebral ischemia experiments

For the cerebral ischemia and reperfusion model, middle cerebral artery occlusion (MCAO) using the intraluminal filament technique was performed. The creation of the MCAO model was performed by inserting a 7-0 monofilament (Doccol Corporation, Redlands, CA) coated with silicone into the internal carotid artery to occlude the middle cerebral artery. The monofilament was withdrawn 60 min after occlusion to achieve reperfusion. The mice were sacrificed, and their brains were removed 4 hr, 24 hr, and 48 hr after occlusion. For the permanent ischemic occlusion model, focal cerebral ischemia was induced by photothrombosis of the cortical microvessels, as previously described [12]. Briefly, mice received an intraperitoneal (i.p.) injection of Rose Bengal (Sigma-Aldrich, St. Louis, MO; 0.1 ml of 10 mg/ml in 0.9% saline) 5 min prior to illumination. A fiber-optic bundle containing a CL 6000 LED cold light source (Carl Zeiss, Jena, Germany) was positioned over the sensorimotor cortex of the exposed skull (2.4 mm lateral from the bregma) and was used to illuminate the samples for 15 min. The mice were then returned to their home cages to recover under a heating lamp.

Measurements of infarct volume

The brains were removed 24 hr after ischemic injury, and the infarct size was determined by 2,3,5-triphenyltetrazolium chloride (TTC) staining of 2 mm-thick brain sections. Infarct size was quantified using the i-Solution software (Image & Microscope Technology, Vancouver, Canada). Measurements of the direct infarct volume included areas of the ipsilateral side that sustained direct damage. The indirect infarct volume was calculated according to the following formula: contralateral hemisphere (mm^3) - undamaged ipsilateral hemisphere (mm^3).

Behavior test

Neurological score and wire-grip tests were performed for brain functionality studies. *Neurological Score.* Neurological deficits were evaluated 24 hr after ischemic injury using the following scoring system: 1 = turning in the direction of the ipsilateral (nondamaged) side when held by the tail, 2 = turning in the direction of the contralateral (damaged) side and difficulty bearing weight, 3 = unable to bear weight on the contralateral side, and 4 = no spontaneous movement [11]. *Wire-Grip Test.* Vestibular-motor function was evaluated 24 hr after the ischemic injury using the wire-grip test. Each mouse was suspended on a metal wire and forced to hang with both forepaws. Wire grip was scored as follows: 1 = not holding onto the wire; 2 = holding onto the wire using

both forepaws and hind paws but not the tail; 3 = holding onto the wire using both forepaws and hind paws as well as the tail, without movement; 4 = moving on the wire using both forepaws, both hind paws, and tail; and 5 = moving well on the wire [11].

Cell cultures

Mouse primary brain microvascular endothelial cells (mEC) were purchased from Cell Biologics (C57-6023, Chicago, IL, USA) and cultured in EGM-2-MV complete medium (CC-3202, Lonza, Basel, Switzerland). HT22 cells (murine hippocampal neuronal cells) and BV2 cells (murine microglial cells) were cultured in Dulbecco's Modified Eagle Medium (DMEM; 11965, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; 16000, Thermo Fisher Scientific, Waltham, MA, USA), 100 U/ml penicillin, and 100 mg/ml streptomycin (1% of P/S; 15140, Thermo Fisher Scientific, Waltham, MA, USA). Cells were maintained at 37℃ in a humidified chamber under 5% CO_2. For oxygen glucose deprivation (OGD) studies, HT22 cells (2×10^5 cells/6 wells) were cultured in 10% DMEM/FBS for one day, washed twice with PBS (SH30264.01, Hyclone Laboratories, Logan, UT, USA), and resuspended in glucose-free DMEM (11966-025, Thermo Fisher Scientific, Waltham, MA, USA) containing 3% FBS. Cells were then incubated for 24 hr in a hypoxic chamber (SMA-30D, ASTEC, Fukuoka, Japan) filled with 1% O_2, 5% CO_2, and balanced with N_2.

RNA isolation and real-time PCR

Total RNA from brain tissues and cells was prepared using TRIzol™ Reagent (15596, Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's protocol. Two micrograms of total RNA were converted to cDNA using the PrimeScript™ 1st strand cDNA Synthesis Kit (6110, Takara, Shiga, Japan). PCR was performed with the Roter-Gene SYBR Green PCR kit (204074, Qiagen, Hilden, Germany), and PCR samples were run on a Rotor-Gene Q PCR system (Qiagen, Hilden, Germany). The primer sequences used were as follows: mouse GPR88 (Forward 5′ - GCCCCA AATCAAGCAGGCAA - 3′, Reverse 5′ - CCACGATTCTTC TTCCTCGCA - 3′) and m18S (Forward 5′ - GTAACCCGTT GAACCCCATT - 3, Reverse 5 - CCATCCAATCGGTAGTA GCG - 3′).

Cell lysis and Western blot analysis

Brain tissues and cells were lysed using RIPA buffer (9806, Cell signaling, Beverly, MA, USA) supplemented with a protease inhibitor mixture (P3100, Genedepot, Katy, TX, USA) and a phosphatase inhibitor mixture (P3200, Genedepot, Katy, TX, USA). The total protein contents (~25 µg) were separated in a 10% SDS-PAGE, transferred onto a nitrocellulose membrane (0.2 μm; 10600004, Amersham, Little Chalfort, UK), and immunoblotted with GPR88 antibody (ab110760, Abcam, Cambridge, UK). β-actin (A5316, Sigma-Aldrich, St. Louis, MO, USA) was used as an internal control. Chemiluminescence was quantified using an ImageQuant LAS 4000 apparatus (GE Healthcare Life Sciences, Uppsala, Sweden), and band intensity was analyzed using Image J software (NIH, Bethesda, MD. USA).

MTT cell viability assay

Cell viability was evaluated using an MTT assay kit (Sigma-Aldrich, St. Louis, MO, USA), following manufacturer's instructions. HT22 cells were seeded on a 96-well plate (1×10^4 cells/well) and incubated overnight for cell attachment. The cells were then washed twice with PBS, resuspended in glucose-free DMEM containing 3% FBS, and incubated for 24 hr in a hypoxic chamber (SMA-30D, ASTEC, Fukuoka, Japan) for OGD conditions. Subsequently, the MTT solution (10 µl per well) was added, and the plate was incubated at 37℃ for an additional 3 hr. Absorbance was measured at 570 nm using a SpectraMax 190 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA).

Statistical Analysis

All data were expressed as the mean ± standard error of the mean (SEM). The statistical differences between two groups were compared using a Student's t-test. Results with $p<0.05$ were considered statistically significant.

Results

GPR88 downregulation after focal cerebral ischemia and in neuron under OGD

To investigate the potential involvement of GPR88 in ischemic brain damage, we determined whether the expression of GPR88 in brain tissue is altered in an ischemic stroke model. GPR88 expression was significantly downregulated in ischemic brains at both the protein (24 hr; 59.66%, $p<0.05$; 48 hr; 27.24%, $p<0.001$) and mRNA levels (56.28%, $p<0.001$) (Fig. 1). To identify which type of cells among brain cells exhibits high GPR88 expression, we examined BV2 micro-

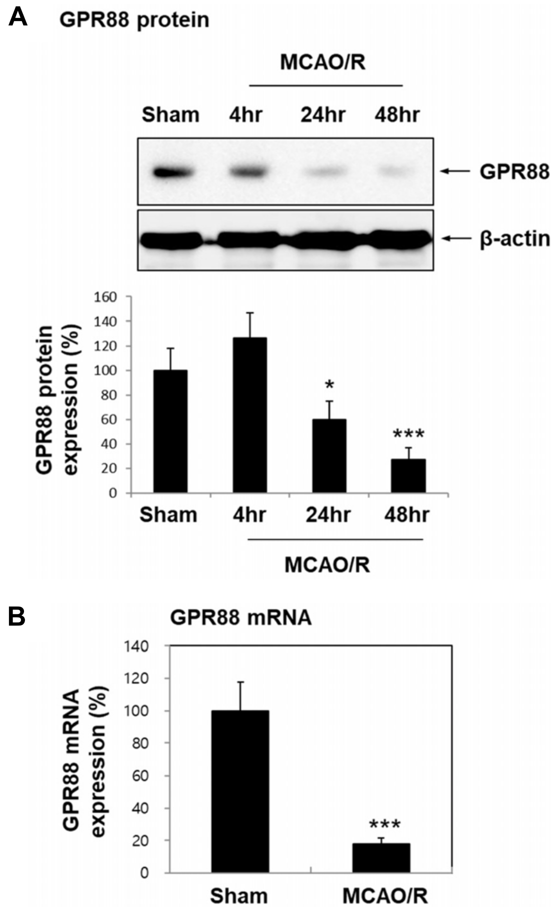

Fig. 1. Downregulation of GPR88 after transient MCAO in C57 BL/6 mice. Six-week-old C57BL/6 mice underwent transient focal cerebral ischemia by middle cerebral artery occlusion (MCAO) for 60 min, followed by reperfusion for 4 hr, 24 hr, and 48 hr (MCAO/R). (A) Ipsilateral brain tissues were lysed and analyzed for GPR88 expression by Western blotting (n=5 each). Quantification graph was presented as mean ± SEM. *p<0.05 and ***p<0.001 versus sham group. (B) GPR88 mRNA expression at 60 min MCAO/24 hr reperfusion by real-time PCR. Data was presented as mean ± SEM (n=4). ***p<0.001 versus sham group.

glial cells, HT22 neuronal cells, and brain microvascular endothelial cells (mEC) (Fig. 2). GPR88 protein expression was found to be high in HT22 hippocampal neuronal cells (p< 0.01 versus BV2 cells) (Fig. 2A). We confirmed that GPR88 was significantly downregulated in HT22 cells under oxygen glucose deprivation (OGD)-like ischemic conditions (mRNA 33.47%, p<0.001; protein 19.97%, p<0.05) (Fig. 2B, Fig. 2C). We then investigated the viability of HT22 cells under OGD conditions (Fig. 3). In comparison with control cells, OGD cells exhibited reduced confluence (Fig. 3A) and significantly lower levels of cell viability (32.26%, p<0.001) (Fig. 3B). These

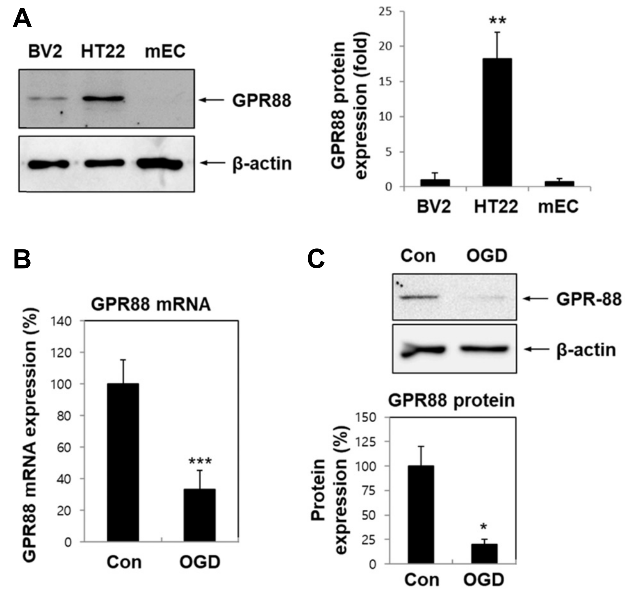

Fig. 2. GPR88 expression in three types of brain cells and under OGD conditions. (A) Western blots showing GPR88 expression in BV2 microglial cells, HT22 neuronal cells, and brain microvascular endothelial cells (mEC). Data was presented as mean ± SEM (n=4). **p<0.01 versus BV2 cells. (B, C) HT22 cells under OGD conditions for 24 hr were analyzed for GPR88 mRNA expression (B) by RT-PCR (n=3) and protein expression (C) by Western blot (n=3). Data was presented as mean ± SEM. *p<0.05 and ***p<0.001 versus control group.

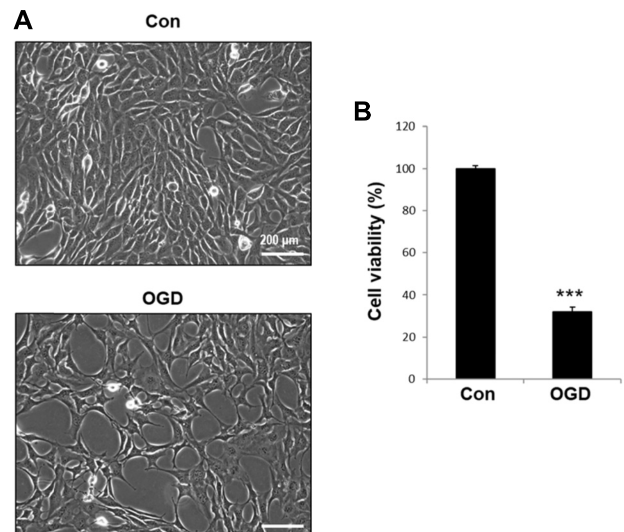

Fig. 3. Cell viability reduction in HT22 under OGD conditions. (A) HT22 cells were cultured under OGD conditions for 24 hr. Magnification: ×200; scale bar=200 μm. (B) Cell viability was determined via MTT assay. Three independent experiments with triplicates were performed. Data are presented as mean ± SEM. ***p<0.001 versus the control group.

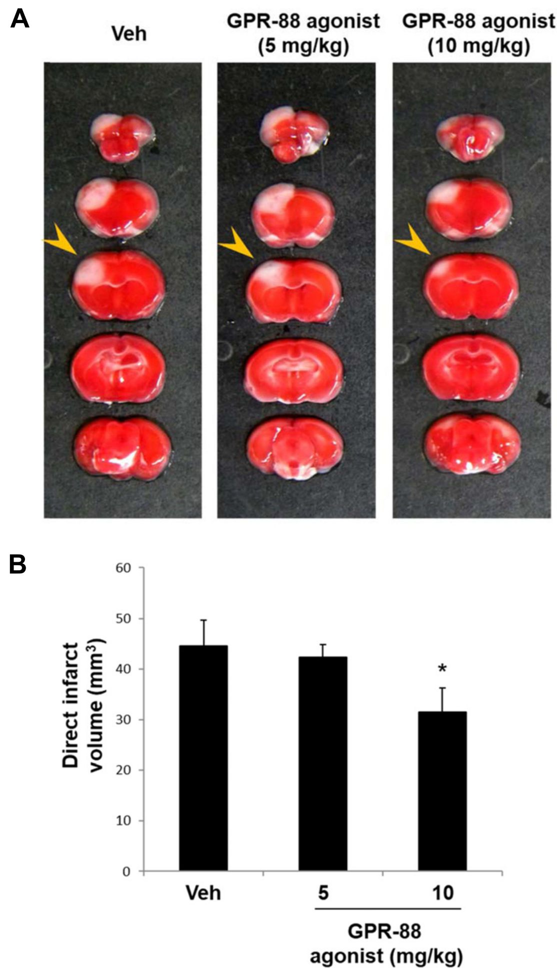

Fig. 4. Effect of GPR88 agonist on brain injury. (A) Mice were intraperitoneally injected with the GPR88 agonist RTI-13951-33 hydrochloride (5 or 10 mg/kg, n=5 each) or PBS (vehicle group, n=5) 30 min prior to ischemic insult. Representative photographs of brain sections stained with TTC. (B) Quantification of direct infarct volume 24 hr postischemia. *p<0.05 versus vehicle group.

results suggest the possible involvement of GPR88 in cell survival, i.e., GPR88 reduction affects cell death.

Effect of GPR88 activation using GPR88 agonist on ischemic brain damage

To determine whether GPR88 activation reduces ischemia-induced brain damage, mice were intraperitoneally injected with RTI-13951-33 hydrochloride (5 or 10 mg/kg), a strong GPR88 agonist, 30 min prior to ischemic injury (Fig. 4, Fig. 5). A significant reduction in infarct volume was observed in mice pretreated with 10 mg/kg of GPR88 agonist (31.57±4.67, p<0.05) (Fig. 4). We then investigated the effect of the GPR88 agonist on neurological deficits and motor function. Significant improvements in neurological score (1.43±0.2, p<0.001) and in wire-grip (3±0.2, p<0.05) were observed in mice pretreated with 10 mg/kg of GPR88 agonist, relative to those observed in the vehicle group (Fig. 5). These results suggest that GPR88 activation via an agonist may contribute to the amelioration of brain damage following ischemia.

Discussion

In this study, we found that GPR88 expression was decreased in ischemic brains and in neurons under OGD conditions. GPR88 activation with a GPR88 agonist ameliorated brain injury following ischemia, as evidenced by improvements in infarct volume, vestibular-motor function, and movement.

Motor disorders that manifest after a stroke are primarily associated with lesions in the basal ganglia and the thalamus. The basal ganglia consists of the principal subcortical component of the circuits that link the cerebral cortex and the thalamus [7]. GPR88 is highly expressed in striatal MSN, which is a major component of the basal ganglia circuitry striatum [22]. In the absence of GPR88, MSN exhibited enhanced excitability, resulting in hyperactivity, poor motor coordination, and impaired cue-based learning in mice.

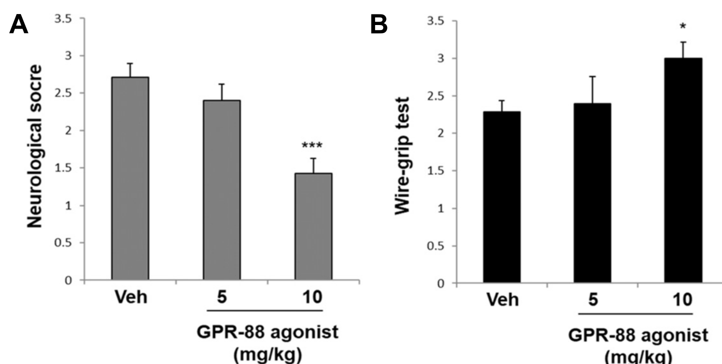

Fig. 5. Effect of GPR88 agonist on brain function and behavior following ischemic brain injury. Neurological score (A) and wire-grip tests (B) were performed to evaluate the recovery of neurological deficits and vestibular motor functions after ischemic brain injury. Data was presented as mean ± SEM (n=5~7 each). *p<0.05, ***p< 0.001 versus vehicle group.

Striatal GPR88 re-expression in GPR88$^{cre/cre}$ mice rescued their molecular and electrophysiological abnormalities and led to normalized behavior, suggesting the importance of GPR88 function in the regulation of MSN excitability in neurological and psychiatric diseases [19]. We observed that both GPR88 mRNA and protein levels were downregulated following ischemic stroke (Fig. 1). Neurons highly expressed GPR88, but OGD insult led to GPR88 downregulation (Fig. 2). Furthermore, administration of the GPR88 agonist RTI-13951-33 ameliorated ischemia-induced brain functional defects affecting vestibular-motor function and movement (Fig. 5). Therefore, these results suggest that the development of movement disorders after infarction could possibly be due to a decrease in GPR88 expression in the neurons.

The importance of GPR88 in the regulation of striatal function has been reported, and GPR88 has been suggested as a promising drug target for basal ganglia-associated disorders. Transcriptional profiling experiments have revealed that GPR88 gene expression is modulated either by treatment or conditions related to schizophrenia [16], bipolar disorder [18], depression [5], and drug addiction [2]. We also found that pretreatment with RTI-13951-33, a GPR88 agonist, reduced infarct volume and restored brain function after ischemic stroke (Fig. 4, Fig. 5). Jin C et al previously reported RTI-13951-33 as a potent, selective, and brain-penetrant GPR88 agonist, which has the advantage of having a higher aqueous solubility compared to 2-PCCA [2-(Pyridin-2-yl)cyclopropanecarboxylic acid], another pre-characterized GPR88 agonist [8, 9]. Taken together, although we cannot rule out the possibility that GPR88 has additional roles in brain regions other than the striatum, the behavioral restoration observed in the treatment groups subjected to administration with a GPR88 agonist following ischemia suggests that GPR88 may be a promising drug target for ischemic stroke recovery.

Data Availability

The dataset supporting the conclusions of this article is included within the article. All data generated or analyzed during this study are included in this published article or are available from the corresponding author upon reasonable request.

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The Conflict of Interest Statement

The authors declare that they have no conflicts of interest with the contents of this article.

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초록 : GPR88 효현제의 전처리에 의한 뇌졸중후 뇌손상 감소효과 연구

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뇌졸중은 전 세계적으로 신경계 장애를 일으키는 주요 원인 중 하나이며, 뇌졸중 환자는 다양한 운동, 인지 및 정신 장애를 나타낸다. GPR88은 orphan G protein coupled receptor이며 striatal medium spiny neurons에서 높게 발현이 되며, GPR88이 결손이 된 경우 motor coordination과 motor learning에 문제가 발생하게 된다. 본 연구에서는 Western blot 및 real-time PCR을 사용하여 허혈성 마우스 모델에서 GPR88 발현이 감소함을 발견 하였다. 또한, 뇌에서 유래한 세 가지 유형의 세포들, 뇌혈관내피세포(brain microvascular endothelial cells), 미세 아교세포(microglial cells) 및 신경 세포들에서 GPR88의 발현정도를 확인한 결과, HT22 신경 세포에서 GPR88의 발현이 가장 높음을 관찰하였고, 뇌졸중과 유사한 실험조건인 oxygen glucose deprivation (OGD) 조건에 배양한 HT22 신경세포에서 GPR88의 발현이 감소하였다. 또한 GPR88 효현제인 RTI-13951-33 (10 mg/kg)을 전처리후에 뇌허혈을 유발하였을 때, infarct volume의 감소, vestibular-motor function 및 neurological score의 개선효과를 관찰할 수 있었다. 이러한 결과는 GPR88이 허혈성 뇌졸중을 포함한 CNS 질환의 치료를 위한 잠재적인 약물표적이 될 수 있음을 제시한다.