THE NEW P2Y-LIKE RECEPTOR G PROTEIN-COUPLED RECEPTOR 17 MEDIATES ACUTE NEURONAL INJURY AND LATE MICROGliOSIS AFTER FOCAL CEREBRAL ISCHEMIA IN RATS

B. ZHAO, a G. Z. ZHAO,a,b X. Y. ZHANG,a X. Q. HUANG,a W. Z. SHI,a S. H. FANG,a Y. B. LU,a W. P. ZHANG,a Q. XIA,a AND E. Q. WEI*a,b

aDepartment of Pharmacology, School of Medicine, Zhejiang University, 866 Yuhangtang Road, Hangzhou 310058, China
bDepartment of Physiology, School of Medicine, Zhejiang University, 866 Yuhangtang Road, Hangzhou 310058, China

Abstract—G protein–coupled receptor 17 (GPR17), the new P2Y-like receptor, is phylogenetically related to the P2Y and cysteinyl leukotriene receptors, and responds to both uracil nucleotides and cysteinyl leukotrienes. GPR17 has been proposed to be a damage sensor in ischemic stroke; however, its role in brain inflammation needs further detailed investigation. Here, we extended previous studies on the spatiotemporal profiles of GPR17 expression and localization, and their implications for brain injury after focal cerebral ischemia. We found that in the ischemic core, GPR17 mRNA and protein levels were upregulated at both 12–24 h and 7–14 days, but in the boundary zone the levels increased 7–14 days after reperfusion. The spatiotemporal pattern of GPR17 expression well matched the acute and late (subacute/chronic) responses in the ischemic brain. According to previous findings, in the acute phase, after ischemia (24 h), upregulated GPR17 was localized in injured neurons in the ischemic core and in a few microglia in the ischemic core and boundary zone. In the late phase (14 days), it was localized in microglia, especially in activated (ED1-positive) microglia in the ischemic core, but weakly in most microglia in the boundary zone. No GPR17 was detectable in astrocytes. GPR17 knockdown by a small interfering RNA attenuated the neurological dysfunction, infarction, and neuron loss at 24 h, and brain atrophy, neuron loss, and microglial activation at 14 days after reperfusion. Thus, GPR17 might mediate acute neuronal injury and late microgliosis after focal cerebral ischemia. © 2011 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: GPR17, cerebral ischemia, neuronal injury, microgliosis, RNA interference.

Ischemic stroke induces a complex series of events and can be separated into three phases: acute (minutes to hours), subacute (hours to days), and chronic (days to months) (Dirnagl et al., 2003; Fagan et al., 2004). In the acute phase, neuron injury is dominant because of metabolic stressors such as energy failure, ion imbalance, excitotoxicity, and oxidative stress. In the late (subacute/chronic) phases, one of the ischemic responses is activation of microgliosis/macrophages (microgliosis) that release bioactive molecules and remove cellular debris (Stoll et al., 2002; Dirnagl et al., 2003; Schwartz, 2003; Fagan et al., 2004; Kriz, 2006; Candelario-Jallo, 2009). These responses in both the acute and late phases are regulated by various bioactive molecules, including nucleotides and cysteinyl leukotrienes (CysLTs, namely leukotriene C4 (LTC4), leukotriene D4 (LTD4), and leukotriene E4 (LTE4)), 5-lipoxygenase metabolites of arachidonic acid.

Extracellular adenine (ATP and ADP), uracil (UTP and UDP), and sugar nucleotides (such as UDP-glucose and UDP-galactose) are universal signaling molecules involved in many physiological processes, and their dysfunctions are associated with various diseases including cerebral ischemia. The actions of extracellular nucleotides are mediated by seven subtypes of ligand-gated purinergic P2X channels (P2X1-7) and eight subtypes of G protein–coupled P2Y receptors (P2Y1, 2, 4, 6, 11, 12, 13, and 14) (Abbracchio et al., 2003; Burnstock and Knight, 2004). On the other hand, CysLTs induce peripheral inflammation and post-ischemic brain inflammation, which is mediated by the G protein–coupled receptors cysteinyl leukotriene receptor 1 (CysLT1 receptor) and cysteinyl leukotriene receptor 2 (CysLT2 receptor) (Yu et al., 2005; Fang et al., 2006, 2007; Chu et al., 2007; Huang et al., 2008; Zhao et al., 2011). Both nucleotides and CysLTs accumulate at the sites of inflammation, and play roles in inflammatory and degenerative responses.

Recently, G protein-coupled receptor 17 (GPR17), the previously orphan receptor, has been reported to functionally link the nucleotides and CysLTs in ischemic/inflammatory responses (Ciana et al., 2006; Lecca et al., 2008; Ceruti et al., 2009; Daniele et al., 2010). The P2Y-like GPR17 is phylogenetically related to the already known P2Y receptors for nucleotides and the CysLT1 and CysLT2 receptors for CysLTs (Ciana et al., 2006; Lecca et al., 2008; Temporini et al., 2009). This receptor has been reported to respond to both uracil nucleotides (UDP, UDP-glucose, and UDP-galactose) and CysLTs (LTD4 and LTC4) (Ciana et al., 2006; Parravincini et al., 2008).

GPR17 is highly expressed in organs that undergo ischemic injury such as brain, heart, and kidney, and plays a role in the evolution of ischemic brain injury and traum
matic spinal cord injury (Ciana et al., 2006; Lecca et al., 2008; Ceruti et al., 2009). GPR17 is normally localized in neurons and upregulated after brain ischemia in both the ischemic core and boundary zone; its blockade by antisense oligonucleotides prevents the progression of acute brain injury (Ciana et al., 2006; Lecca et al., 2008). Similar GPR17 expression and modulation have been reported in traumatic spinal cord injury (Ceruti et al., 2009). GPR17 is also expressed in several other embryonically distinct cell types including oligodendrocytes as well as microglia/macrophages after permanent brain ischemia in mice, indicating a potential role of GPR17 during chronic ischemic injury (Lecca et al., 2008). However, the role of GPR17 in post-ischemic microgliosis needs detailed investigation because of the importance of microglial activation or microgliosis in the late phases after brain ischemia (Jin et al., 2010; Yenari et al., 2010).

In the present study, we determined whether GPR17 is involved in acute neuronal injury and late microgliosis after ischemic brain injury in rats. Our aim was to clarify the following issues: first, the spatiotemporal profiles of GPR17 expression during the acute and late (subacute/chronic) phases; second, the localization of GPR17 in different cells in the normal and ischemic brain; and third, the effects of GPR17 knockdown by small interfering RNA (siRNA) on ischemic brain injury, especially that in neurons and microglia. We focused on post-ischemic microglial activation or microgliosis.

**EXPERIMENTAL PROCEDURES**

**Focal cerebral ischemia**

Male Sprague–Dawley rats (250–300 g) were purchased from the Experimental Animal Center, Zhejiang Academy of Medicine Sciences, Hangzhou, China (Certificate No. 2008001603021). Rats were housed at 22±2 °C, relative humidity 50±10%, and a 12-h light/dark cycle. They were allowed free access to water and food. All experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. We made every effort to minimize the number of animals used and their suffering. The experimental protocols were approved by the Ethics Committee of Laboratory Animal Care and Welfare, School of Medicine, Zhejiang University.

Rats were anesthetized with an i.p. injection of chloral hydrate (400 mg/kg). Percent changes in regional cerebral blood flow (rCBF) were recorded as described previously (Yano et al., 2003) using a laser Doppler flowmeter (ML191, AD Instruments Pty. Ltd., Sydney, Australia), and the steady state baseline rCBF value before ischemia was defined as 100%. A polyethylene tube was inserted into the right femoral artery for continuous monitoring of blood pressure using a computer-assisted system (PC-Laboratory, Kelo Inc., Nanjing, China), and for measuring $P_O_2$, $P_CO_2$, and arterial pH (Blood Gas Analyzer ABL 300, Leidu Inc., Copenhagen, Denmark). Blood glucose was monitored by a one-touch basic blood glucose monitoring system (Lifescan Inc., Milpitas, CA, USA).

Transient focal cerebral ischemia was induced by middle cerebral artery occlusion (MCAO) as described by Longa et al. (1989). Briefly, after anesthesia rats were placed in dorsal recumbency. The left common carotid artery, external carotid artery (ECA) and internal carotid artery (ICA) were isolated. A 3-0 nylon suture was inserted from the ECA into the ICA for a length of about 18–19 mm until a slight resistance was felt, which indicated the suture had blocked the origin of the MCA. Thirty minutes after occlusion, the suture was withdrawn to allow reperfusion. Then the ECA was ligated and the incision was closed. In the sham group, the ECA was surgically isolated, but the suture was not inserted. After surgery, rats were kept for about 2 h in a warm box heated by lamps to maintain body temperature.

**i.c.v. administration of GPR17 siRNA**

After anesthesia, rats were placed on a stereotaxic apparatus (ASI instruments, Houston, TX, USA). A metal cannula was implanted into the left lateral ventricle (AP: 1.0 mm caudal to bregma; $V$: –4.0 mm from the dura mater; L: –0.9 mm from the mid-line), and secured to the skull with dental cement. Rats were placed in individual cages, and after 1 week of recovery, test agents were injected through the cannula.

Because no selective GPR17 antagonist is available, we used a siRNA strategy, which has been shown to efficiently downregulate proteins in the brain (Senn et al., 2005; Manrique et al., 2009; McCormack et al., 2010). The siRNA was specifically designed for the silencing of rat GPR17 (GenePharma Co. Ltd., Shanghai, China): CCG TAT AGA GAA GCA CCT CAA (target sequence) (Daniele et al., 2010). To rule out the possibility of non-specific effects of siRNA injection, an ineffective sequence of RNA was used as negative control (GenePharma Co. Ltd., Shanghai, China).

To evaluate its effect on acute injury, based on a preliminary study, 0.5 μg GPR17 siRNA mixed with Lipofectamine™ 2000 (Invitrogen, CA, USA) in 4 μl saline was injected through the cannula 48 and 24 h before MCAO and immediately after MCAO, respectively. To evaluate its effect on chronic injury, the same amount of GPR17 siRNA was injected through the cannula 48 and 24 h before MCAO, immediately after MCAO, then once per day from days 2–7, and once every 2 days from days 8–14; brain injury was assessed 14 days after reperfusion. Ineffective RNA was similarly injected as a negative control, and saline containing the same amount of Lipofectamine™ 2000 was injected in vehicle control rats. The effect of GPR17 siRNA was confirmed by both immunohistochemistry and immunoblotting.

**Behavioral assessment and histological analysis**

Neurological deficit scores were evaluated at the indicated time points after MCAO, based on a reported method (Longa et al., 1989), as follows: no neurological deficit – 0, failure to extend right paw fully – 1, circling to right – 2, falling to right – 3, no spontaneous walking, and depressed level of consciousness – 4. An observer who was blinded to the treatment performed all the assessments.

After neurological examination, rats were re-anesthetized and perfused transcardially with 4% paraformaldehyde after flushing with saline. Brains were removed, quickly photographed with a digital camera (FinePix S602 Zoom, Fuji, Japan), fixed in 4% paraformaldehyde for 24 h, and transferred to 30% sucrose for 3 days. Then, 10-μm and 20-μm sections at 2-mm intervals from the frontal to the occipital poles were cut by cryomicrotomy (CM1900, Leica, Weizlar, Germany). The 10-μm sections were used for Cresyl Violet staining and immunostaining, and the 20-μm sections were stained with 1% Toluidine Blue for histological examination (Yu et al., 2005; Fang et al., 2006).

In separate experiments, brains were quickly removed and sliced into six coronal sections of 2 mm thickness. The slices were incubated in phosphate-buffered saline containing 0.5% 2, 3, 5-triphenyltetrazolium chloride (TTC) at 37 °C for 20 min, and then photographed. The infarct was identified as an area of unstained tissue. Infarct volume was calculated by summing the volumes in the six slices, and expressed as the percentage of infarction in the cerebral hemisphere.
Immunohistochemistry and double immunofluorescence staining

For immunohistochemical analysis, 10-μm sections were rinsed with 0.01 M PBS, followed by incubation with 3% H₂O₂ for 30 min to eliminate endogenous peroxidase activity. Non-specific binding of IgG was blocked by incubation with 5% normal goat serum for 2 h at room temperature. Then, the sections were incubated overnight at 4 °C with rabbit anti-GPR17 (1:1000), rabbit anti-β-gal fibrillary acidic protein (GFAP) antibody (1:200, Zhongshan Biotechnology Co., Beijing, China), rabbit anti-Iba1 antibody (1:2000, Wako, Osaka, Japan), and mouse anti-ED1 (1:500, Millipore, Billerica, MA, USA). Sections were then washed extensively with 0.01 M PBS and sequentially incubated with biotinylated IgG (1:200, Vectorlab, USA) for 2 h, and with streptavidin HRP (1:200, Vectorlab, Burlingame, CA, USA) for 2 h. Finally, the sections were exposed for 2 min to 0.05% 3, 3-diaminobenzidine and 0.03% H₂O₂. For negative controls, we used PBS instead of the primary antibodies. The polyclonal rabbit antibody against rat GPR17 used in these experiments is specific as reported (Ciana et al., 2006; Qi et al., 2009).

To determine the localization of GPR17 in different cell types, double immunofluorescence was performed. The 10-μm sections were rinsed with 0.01 M PBS and then incubated for 2 h with 5% goat serum, and then incubated overnight at 4 °C with the following primary antibodies: rabbit anti-GPR17 (1:1000), mouse anti-neuronal nuclear antigen (NeuN) (1:800, Millipore, Billerica, MA, USA), mouse anti-GFAP (1:200, Millipore, Billerica, MA, USA), goat anti-Iba1 (1:800, Abcam, Cambridge, MA, USA), and mouse anti-ED1 (1:500, Millipore, Billerica, MA, USA). Sections were then incubated with Cy3- or FITC-conjugated secondary antibody (1:200, Millipore, Billerica, MA, USA), mouse anti-GFAP (1:800, Millipore, Billerica, MA, USA), and mouse anti-ED1 (1:500, Millipore, Billerica, MA, USA). Sections were then washed extensively with 0.01 M PBS, followed by incubation with 3% H₂O₂ for 30 min and then incubated with 0.01 M PBS and sequentially incubated with biotinylated IgG (1:200, Vectorlab, USA) for 2 h. Finally, the sections were exposed for 2 min to 0.05% 3, 3-diaminobenzidine and 0.03% H₂O₂. For negative controls, we used PBS instead of the primary antibodies. The polyclonal rabbit antibody against rat GPR17 used in these experiments is specific as reported (Ciana et al., 2006; Qi et al., 2009).

Reverse transcription–polymerase chain reaction (RT-PCR)

Brain tissue from the ischemic core, the boundary zone adjacent to the ischemic core, and the contralateral cortex corresponding to the ischemic core (Fig. 2A) were dissected on ice at the indicated time points, and stored at −80 °C until use. Total RNA was extracted using Trizol reagents (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions, and quantified spectrophotometrically. The specificity of the oligonucleotide primers was verified using the program BLASTN. RT-PCR analysis was performed as previously described (Fang et al., 2006). Amplifications were initially heated at 94 °C for 2 min; then at 94 °C for 60 s, 58 °C for 60 s, and 72 °C for 30 s; and finally stopped at 72 °C for 10 min. PCR products in 10 μl were separated by 2% agarose gel electrophoresis and visualized by Ethidium Bromide staining. The density of each band was measured by the UV gel analysis system (Bio-Rad, Richmond, CA, USA). This semi-quantitative measure was expressed as ratios compared with β-actin. The primer sequences were as follows: GPR17, forward 5’-GCT CTG TTC ACC CTT-3’ and reverse 5’-GAG TCA AGG CAC TGA-3’. The product sizes were 610 bp and 190 bp, respectively.

Immunoblotting analysis

Brain tissue isolated as described above was homogenized in RIPA buffer (Kangchen Biotechnology Inc., Shanghai, China) using a Polytron homogenizer. The homogenate was centrifuged at 12,000×g at 4 °C for 30 min, and the resultant supernatant was used. Protein samples (100 μg) were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were blocked by 10% fat-free milk, and sequentially incubated with goat polyclonal antibody against GPR17 (1:200, Santa Cruz, CA, USA), and mouse monoclonal antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:5000, Kangchen Biotech-
technology Inc., Shanghai, China). Then, the membranes were incubated with IRDye® 800CW donkey anti-goat IgG (1:3000, LI-COR Biosciences, Lincoln, NE, USA), and 800CW anti-mouse IgG (1:8000, Rockland, Gilbertsville, PA, USA) for 2 h at room temperature. Finally, the membranes were scanned to detect fluorescence. The optical densities of the GPR17 (41 kDa) and GAPDH (36 kDa) bands were quantitatively analyzed with Quantity One. Data are expressed as GPR17/GAPDH ratios.

**Statistical analysis**

Data are presented as mean±SEM. Significance of differences was analyzed by one-way analysis of variance (ANOVA), followed by Dennett’s post hoc test (SPSS 10.0 for Windows, 1999, SPSS Inc., USA). *P*<0.05 was considered statistically significant.

**RESULTS**

Pathological changes after MCAO

There were no significant differences in mean arterial blood pressure, \( P_{\text{aO}_2}, P_{\text{aCO}_2}\), arterial pH, and blood glucose between 20 min before and 20 min after the operation in the sham-operated, ischemic and GPR17 siRNA-in-

---

**Fig. 1.** Brain lesions and neurological dysfunctions over 28 d after focal cerebral ischemia in rats. (A) Photographs of whole brains and 2 mm-thick coronal slices stained with TTC show brain lesions from 6 h to 28 d after reperfusion. (B–D) Neurological deficit scores (B), infarct volumes (C), and left/right hemispheric ratio (D) confirm neurological dysfunction and ischemic lesions. Each value is the mean±SEM from eight rats per group. * * \( P<0.05 \) and ** * \( P<0.01 \) compared with sham operation, analyzed by one-way ANOVA. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.
jected groups. rCBF was reduced by ~60% during 30-min MCAO, and nearly recovered to baseline levels 20 min after reperfusion; GPR17 siRNA microinjection did not alter the reduction in rCBF (Table 1).

Fig. 2. Changes in densities of neurons and microglia over 28 d after focal cerebral ischemia in rats. (A) Sampling regions for microphotography or biochemical analysis. (B) Neurons stained with Cresyl Violet gradually decreased in both the ischemic core and boundary zone from 6 h to 28 d after reperfusion, and almost disappeared in the ischemic core from 5 d after reperfusion. (C) Microglia immunolabeled with an antibody against Iba-1 gradually increased in both the ischemic core and boundary zone from 3 d after reperfusion. Scale bars = 100 μm. Each value is the mean ± SEM from eight rats per group; * P<0.05 and ** P<0.01. *** P<0.001 compared with sham operation, analyzed by one-way ANOVA. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.
After MCAO, the brain showed mild swelling 6 h to 3 days after reperfusion, and thereafter a gradually atrophic appearance in the ischemic hemisphere (Fig. 1A). The neurological deficit scores (Fig. 1B), infarct volumes (Fig. 1A, C), and left/right hemispheric ratios (Fig. 1D) increased during 3 days after reperfusion with peaks at 24 h. Then, the neurological deficit scores and infarct volumes gradually recovered 3, 7, 14, and 28 days after reperfusion (Fig. 1B, C). However, the left/right hemispheric ratios were gradually reduced 7, 14, and 28 days after reperfusion (Fig. 1A, D), indicating brain atrophy.

MCAO induced obvious neuronal injury in the ischemic cortex, where the Nissl bodies disappeared, cell bodies shrank and were stained deeply or completely lost. In the ischemic core, neuron density was significantly decreased at 6, 12, and 24 h, and disappeared 3–28 days after reperfusion. In the boundary zone, neuron density was initially decreased at 6 h, and progressively decreased over 28 days after reperfusion (Fig. 1B). The microglial response was determined using ionized calcium binding adapter molecule 1 (Iba-1), an antibody against the microglial marker. In the normal brain of sham-operated rats, ramified Iba-1-positive microglia were diffusely distributed throughout the cortex. After MCAO, microglia with a rounded macrophage-like morphology progressively increased in the ischemic core from 3 to 28 days. In the boundary zone, microglia significantly increased from 24 h to 28 days (Fig. 2C). In the contralateral cortex, the number

![Fig. 3. Changes in activated microglia after focal cerebral ischemia in rats. Double immunolabeling shows that Iba-1-positive microglia were ED1-negative in both the ischemic core and boundary zone 24 h after ischemia. The ED-1-positive microglia were found in the ischemic core and in the near periphery of the boundary zone (inner boundary) 14 d after reperfusion. Experiments were repeated at least four times with similar results. Scale bars=100 μm.](image-url)
of microglia did not change significantly over 28 days after reperfusion (data not shown).

To label activated microglia, an antibody against the lysosomal phagocytic marker ED1 was used. No ED1-positive cells were detected in sham control, and only a few ED1-positive cells were found in the ischemic core 24 h after reperfusion. However, 14 days after reperfusion, ED1-positive cells were significantly increased in the ischemic core, and double immunofluorescence staining showed that approximately 60% of the Iba-1-positive microglia were co-labeled with ED1. In contrast, in the boundary zone, Iba-1-positive cells were greatly increased, whereas the ED1-positive cells were relatively few. Interestingly, the ED1-positive cells were primarily distributed closer to the ischemic core (inner boundary), but few or none in site distant from the core even if the numbers of Iba-1-positive cells were increased (Fig. 3). This differential pattern indicated that the microglia within and outside the ischemic core might function differently, with or without phagocytic activity.

Temporal and spatial profiles of GPR17 expression after MCAO

In the ischemic core, GPR17 protein expression increased initially at 12 and 24 h, then recovered at 3 days, and increased again at 7 and 14 days after reperfusion. In the boundary zone, the expression was only increased at 7 and 14 days after reperfusion (Fig. 4A). Similarly, in the ischemic core, GPR17 mRNA expression was increased with two peaks at 24 h and 7–14 days after reperfusion; in the boundary zone the increase was significant but relatively low at 24 h and 3 and 14 days after reperfusion (Fig. 4B).

Immunohistochemical analysis showed that the number of GPR17-immunoreactive cells in the ischemic core was significantly increased at 12 and 24 h, then recovered at 3 days, and increased again 5, 7, 14 and 28 days after MCAO (Fig. 5A, B). Interestingly, GPR17 was translocated from the cell membrane to the cytoplasm and nucleus after cerebral ischemia in the acute phase. In contrast, in the boundary zone, the number of GPR17-immunoreactive cells began to increase 24 h after reperfusion, and progressively increased thereafter (Fig. 5A, B). The expression in the contralateral cortex did not significantly change over 28 days after reperfusion (data not shown).

Cellular localization of GPR17 after MCAO

To determine the cellular localization of GPR17, double immunostaining was performed to detect the co-localization of GPR17 with the specific markers NeuN (neurons), GFAP (astrocytes), Iba-1 (microglia), and ED1 (activated microglia) at two time points, that is, 24 h for the acute phase and 14 days for the late phase. Despite previous studies reporting highly specific localization of GPR17 to oligodendrocyte precursor cells (Lecca et al., 2008; Ceruti et al., 2009, 2011; Chen et al., 2009b; Boda et al., 2011; Fumagalli et al., 2011), these cells were not analyzed in the present study.

Fig. 4. Expression of GPR17 protein and mRNA in rat brain after focal cerebral ischemia. GPR17 protein or mRNA was detected by immunoblotting (A) or RT-PCR analysis (B). In the ischemic core, the protein and mRNA levels of GPR17 increased at 24 h, recovered at 3 d, and increased again 7 and 14 d after reperfusion (left panels). In the boundary zone, the GPR17 protein increased at 7 and 14 d but the mRNA level increased at 24 h, 3 and 14 d after reperfusion (middle panels). In the contralateral cortex, the expression had no significant changes over 28 d (right panels). Each value is the mean±SEM from six rats per group; *P<0.05, **P<0.01 compared with sham operation, analyzed by one-way ANOVA.
In the normal brain from sham-operated rats, GPR17 was localized in the plasma membrane of NeuN-positive neurons in intact cortex (Fig. 6), as well as in the striatum and hippocampus; GPR17 immunoreactivity was also found in ependymal cells around the ventricles (data not shown). In contrast, co-localization was found neither in cortical GFAP-positive astrocytes nor in Iba1-positive resting microglia (Fig. 6).

After MCAO, GPR17 immunoreactivity in the ischemic core was localized in NeuN-positive neurons 24 h after reperfusion, but some was localized in Iba1-positive microglia (Fig. 7). The GPR17 on neurons in the ischemic core was translocated from the membrane to the nucleus and cytoplasm 24 h after reperfusion. In contrast, 14 days after reperfusion, most of the GPR17 was localized in hypertrophic Iba-1- and ED1-positive microglia in the ischemic core, where no neurons or astrocytes were visible (Fig. 7). On the other hand, in the boundary zone, the GPR17 was localized in neurons, but some in Iba1-positive microglia 24 h after reperfusion (Fig. 7). However, 14 days after reperfusion, it was localized in neurons, increased in some Iba-1-positive microglia, and remarkably in ED1-positive microglia in the inner boundary, but not in proliferated astrocytes (Fig. 7).

These findings indicated that, in the acute phase of ischemic injury, the GPR17 was upregulated in injured neurons in the ischemic core, whereas in the late phase, it was expressed in activated microglia in the ischemic core and inner boundary. In contrast, in the boundary zone, the GPR17 was expressed in neurons. The relatively resting Iba1-positive microglia (in the ischemic core and the boundary zone at 24 h, and most microglia in the boundary zone at 14 days) expressed some GPR17, but astrocytes did not express it.

GPR17 knockdown by siRNA ameliorates both acute and chronic ischemic injury

To determine the knockdown efficiency, we assessed GPR17 expression in the brain after siRNA injection in rats...
with MCAO 24 h and 14 days after reperfusion. Immunoblotting results showed that GPR17 siRNA successfully downregulated GPR17 protein expression in the ischemic cortex at both time points (Fig. 8A, B), and also were confirmed by immunohistochemical analysis (data not shown). These results confirmed a substantial and stable GPR17 knockdown.

The ischemic infarction and moderate edema in the ischemic hemisphere at 24 h (Fig. 8C), and atrophy (Fig. 8D) 14 days after reperfusion were significantly attenuated by GPR17 siRNA. In addition, compared with vehicle or negative control sequence, GPR17 siRNA significantly decreased the neurological deficit score (Fig. 9A) and infarct volume (Fig. 9B) 24 h after reperfusion. It attenuated the increase in left/right hemispheric ratio (edema) at 24 h and the reduction in this ratio (atrophy) 14 days after reperfusion (Fig. 9C).

To determine the effect of GPR17 knockdown on neuron and microglia, we further assessed the changes in neuronal density and microglial responses in ischemic brain after GPR17 siRNA injection. The results showed that the reduction in the number of neurons was ameliorated by GPR17 siRNA in the ischemic core at 24 h, and in the boundary zone 24 h and 14 days after reperfusion, whereas neurons completely disappeared in the ischemic core 14 days after reperfusion (Fig. 9F). The number of microglia did not change significantly in the ischemic core but increased slightly in the boundary zone 24 h after reperfusion. Both Iba1-positive microglia and Iba1/ED1-positive activated microglia dramatically increased in the ischemic core 14 days after reperfusion (Figs. 9E and 10). However, in the boundary zone, Iba1-positive cells increased greatly, whereas increased ED1-positive cells were restricted to the inner boundary but few were seen in

Fig. 6. Cellular localization of GPR17 in the brain of sham-operated rats. GPR17 localization in cortex was determined by double immunofluorescence staining. In normal brain, GPR17 is localized in NeuN-positive neurons, but not in GFAP-positive astrocytes, or in Iba1 or ED1-positive microglia. Experiments were repeated at least four times with similar results. Scale bars=100 μm.

Markers GPR17 Merge Merge
NeuN
GFAP
Iba1
ED1
most other sites (Figs. 9E and 10). GPR17 siRNA did not significantly reduce the number of Iba-1-positive microglia in the ischemic core, but slightly reduced the microglia in the boundary zone 24 h after reperfusion, whereas it significantly reduced both Iba-1- and ED1-positive microglia in the ischemic core and boundary zone 14 days after reperfusion.

Fig. 7. Cellular localization of GPR17 in rat brain after focal cerebral ischemia. At 24 h after reperfusion, GPR17 is localized in injured neurons and in some Iba-1-positive microglia, but not in astrocytes in both the ischemic core and boundary zone. At 14 d after reperfusion, neurons and astrocytes disappear, but microglia increase in the ischemic core; increased astrocytes and microglia are found in the boundary zone. GPR17 is localized in Iba-1- and ED1-positive microglia in the ischemic core, and in neurons and partly in Iba-1-positive microglia in the boundary zone 14 d after reperfusion. GPR17-expressing ED1-positive microglia are also distributed in the inner boundary (square in lower right panels). Experiments were repeated at least four times with similar results. Scale bars = 100 μm.
Therefore, the effects of GPR17 siRNA indicated that GPR17 might be involved in the acute and chronic ischemic lesions, especially in acute neuronal injury and late microgliosis.

**DISCUSSION**

In the present study, we confirmed and extended previous studies showing that upregulated GPR17 was temporally and spatially related to acute neuronal injury and late microgliosis after focal cerebral ischemia, which was verified by GPR17 knockdown with siRNA. We provided more evidential details and revealed the role of GPR17 in the development of post-ischemic inflammation (microgliosis). Currently, the main focus is on the roles of GPR17 in regulating oligodendrocyte precursor cell maturation (Lecca et al., 2008; Ceruti et al., 2009, 2011; Chen et al., 2009b; Boda et al., 2011; Fumagalli et al., 2011) or as a cell-intrinsic timer of myelination (Chen et al., 2009b). In ischemic brain injury and spinal cord injury, GPR17 has been reported to be a damage sensor, which is activated in several embryonically distinct cell types, and might play key roles in both inducing ischemic neuronal death and local remodeling/repair responses (Lecca et al., 2008; Ceruti et al., 2009). GPR17 also acts as a pro-survival neurotrophic regulator in PC12 cells, a neuronal cell line (Daniele et al., 2010). Our study revealed the details of its role in microgliosis in addition to acute neuronal injury, suggesting that GPR17 may also regulate post-ischemic inflammation, as reported in the pulmonary inflammation induced by house dust mites (Maekawa et al., 2010).

Our results confirmed the temporally sequential responses after focal cerebral ischemia, that is, acute neuronal injury and late microgliosis, as well as neurological dysfunction and morphological changes. The temporal pat-
Fig. 9. Effect of GPR17 siRNA on neurological dysfunction, infarct size, and densities of neurons and microglia after focal cerebral ischemia. (A, B) GPR17 siRNA significantly decreased neurological deficit score and infarct volume 24 h after reperfusion compared with vehicle or negative control RNA (NC); however, it did not affect the already reduced score and infarct volume 14 d after reperfusion. (C) The left/right hemispheric ratio increased (edema) at 24 h and reduction (atrophy) at 14 d after reperfusion were attenuated by GPR17 siRNA. (D) In the ischemic core, the reduced neuron density was attenuated by GPR17 siRNA at 24 h, whereas neurons completely disappeared at 14 d after reperfusion. In the boundary zone, reduction of neuron density was attenuated by GPR17 siRNA both 24 h and 14 d after reperfusion. (E) At 24 h after reperfusion, Iba-1-positive microglia did not change in the ischemic core but were mildly increased in the boundary zone, and this was attenuated by GPR17 siRNA. At 14 d after reperfusion, both Iba-1- and ED1-positive microglia were substantially increased, and these were attenuated by GPR17 siRNA. Each value is the mean±SEM from six rats per group; * P<0.05, ** P<0.01, and *** P<0.001 compared with control, * P<0.05 and ** P<0.01 compared with vehicle, analyzed by one-way ANOVA.
tern of GPR17 expression well matched the acute and subacute/chronic responses; moreover, GPR17 knockdown ameliorated both acute neuronal injury and late microgliosis. These findings indicated the regulatory roles of GPR17 in the evolution of ischemic brain injury.

GPR17 was normally localized in the neuron and its expression in neurons increased after ischemic injury, especially in the ischemic core 24 h after reperfusion; however, it was not expressed in astrocytes, as reported elsewhere (Ciana et al., 2006; Lecca et al., 2008). As GPR17 was reported to respond to both uracil nucleotides and CysLTs (Ciana et al., 2006; Lecca et al., 2008; Ceruti et al., 2009), its expression in the acute phase might be a response to the changes in extracellular nucleotides and CysLTs. Such changes are supported by the following line of evidence. Oxygen-glucose deprivation (an in vitro ischemic model) increases UDP release in cortical cell cultures (Günther et al., 2002); various stimuli induce an in vitro release of uracil nucleotides (UTP) (Lazarowski et al., 1997) and nucleotide sugar (UDP-glucose) (Lazarowski et al., 2000).
al., 2003; Kreda et al., 2008) in 1321N1 human astrocytoma cells; cardiac ischemia induces in vivo UTP release (Erlinge et al., 2005) and kainic acid, an excitatory amino acid, increases extracellular UTP in vivo and in vitro (Kozumi et al., 2007). The levels of CysLTs in ischemic brain sharply increase at 3 h, are maintained over 24 h after MCAO, and decline afterward (Ciceri et al., 2001; Zhou et al., 2006). Therefore, upregulated GPR17 in injured neurons might mediate responses to the endogenous ligands. How GPR17 modulates neuronal injury remains to be investigated.

One important event in ischemic brain injury is microgliosis and the related inflammatory responses. Microglia are the most important inflammatory modulators and are necessary for normal or injured brain, but uncontrolled or over-activated microglia are detrimental (Schwartz, 2003; Kriz, 2006; Block et al., 2007). In the present study, we revealed the role of GPR17 in microgliosis. GPR17-positive activated microglia are still present in the late phases. In regard to the distribution profiles, upregulated GPR17 was restricted to regions where microglia increased greatly, that is, the ischemic core and the inner boundary 14 days after reperfusion. Moreover, the GPR17 was mainly localized in ED1-positive microglia. The markers of microglia used in our study were Iba-1, a calcium-binding protein that is specifically expressed in microglia in the brain (Ito et al., 2001), and ED1, which is a lysosomal phagocytic marker of macrophages and microglia (Zhang et al., 1997; Ito et al., 2001; Sugama et al., 2003; Sanagi et al., 2010). We found that most of the microglia in the ischemic core and inner boundary were ED1-positive, and might possess phagocytic activity in the late phases; whereas the microglia unaffected or less affected by ischemia were ED1-negative. It has also been reported that GPR17 expression increases in IB4-positive microglia in mouse brain 72 h after MCAO (Lecca et al., 2008). Thus, GPR17 might regulate the activity of activated microglia, and our study provided details for the role of GPR17 in post-ischemic microgliosis.

Supporting this notion, GPR17 knockdown attenuated the chronic injury and microgliosis as indicated by reduction in both Iba-1- and ED1-positive microglia. As the endogenous ligands of GPR17, the levels of CysLTs in ischemic brain increase at 7 days after focal cerebral ischemia in rats (Zhou et al., 2006). The changes in endogenous nucleotides in the late phases of brain ischemia are not clear; however, i.p. injection of kainic acid increases extracellular UTP for up to 5 days in rat brain (Kozumi et al., 2007). Because kainic acid is involved in post-ischemic brain injury (Liu et al., 1996; Chen et al., 2009a; Hasson et al., 2010), uracil nucleotides might undergo late phase changes similar to CysLTs. Therefore, in the late phases, the endogenous ligands would be increased in the ischemic brain, and GPR17 expression be induced to regulate microgliosis.

How GPR17 mediates microglial activation remains to be elucidated. One possibility is that GPR17 on microglia directly mediates the responses to uracil nucleotides and CysLTs released from ischemic brain tissue. UTP/UDP activate microglial phagocytosis; however, this is reported to be mediated by the P2Y1 receptor (Kozumi et al., 2007). CysLTs are also associated with microglial activation, but this may be mediated by the CysLT2 or CysLT1 receptors (Fang et al., 2006; Zhao et al., 2011). Therefore, direct evidence is required to clarify the regulatory mode. Another possibility is that GPR17 regulates microglial activation by interacting with other G protein–coupled receptors. It has been reported that GPR17 is a negative regulator that inhibits CysLT1 receptor-mediated allergic pulmonary inflammation in mice (Maekawa et al., 2010) and LTD4-elicited calcium flux in mouse bone marrow-derived macrophages (Maekawa et al., 2009). We have found that the CysLT1 and CysLT2 receptors show a pattern of expression and cellular localization similar to that of GPR17, that is, they are mainly upregulated and localized in greatly increased microglia in the ischemic core in the late phases of focal brain ischemia (Fang et al., 2006; Zhao et al., 2011). Therefore, it could be presumed that GPR17 interacts with CysLT1 or CysLT2 receptors in post-ischemic microgliosis. This interaction may occur in acute ischemic neuronal injury as well.

**CONCLUSION**

In summary, the present study showed the spatiotemporal profiles of GPR17 expression and localization in rat brain during 28 days after focal cerebral ischemia. Upregulated GPR17 was associated with acute neuronal injury in the ischemic core, and late microgliosis both in the ischemic core and the boundary zone. Furthermore, GPR17 knockdown attenuated the neuronal injury and inhibited the microgliosis after focal cerebral ischemia. Based on these findings, we suggest that GPR17 may mediate post-ischemic inflammation in addition to acute neuronal injury, so it may be a potential target for the drug therapy of ischemic stroke.

**Acknowledgments—**This study was supported by the National Natural Science Foundation of China (81072618, 81173041, 30772561, and 30873053), the Zhejiang Provincial Natural Science Foundation (Y2090069), the Zhejiang Provincial “QianJiangRenCai Research Plan” (2010R10055), and the Fundamental Research Funds for the Central Universities (2009QNA7008). We thank Dr. I.C. Bruce for critically reading and revising this manuscript.

**REFERENCES**


effects of injury-induced inflammation and cytokine expression in
Sugama S, Cho BP, Degrigo LA, Shimizu Y, Kim SS, Kim YS, Shin DH,
analysis of microglia in the substantia nigra following medial forebrain
Temporini C, Ceruti S, Calleri E, Ferrario S, Moaddel R, Abbracchio
MP, Massolini G (2009) Development of an immobilized GPR17
receptor stationary phase for binding determination using frontal
affinity chromatography coupled to mass spectrometry. Anal
effect of urinary trypsin inhibitor against focal cerebral ischemia-
Yenari MA, Kauppinen TM, Swanson RA (2010) Microglial activation in
Yu GL, Wei EQ, Wang ML, Zhang WP, Zhang SH, Weng JQ, Chu LS,
a cysteinyl leukotriene receptor-1 antagonist, protects against
chronic ischemic brain injury and inhibits the glial scar formation in
response following transient (2 h) middle cerebral artery occlusion.
Zhao CZ, Zhao B, Zhang XY, Huang XQ, Shi WZ, Liu HL, Fang SH, Lu
receptor 2 is spatiotemporally involved in neuron injury, astrocyto-
sis and microgliosis after focal cerebral ischemia in rats. Neurosci-
ence 189:1–11.
YL, Lin SC, Chen Z (2006) Spatio-temporal properties of 5-lipoxy-
genase expression and activation in the brain after focal cerebral

(Accepted 28 November 2011)
(Available online 6 December 2011)