
Joji Yui; Lin Xie, MD, PhD; Masayuki Fujinaga, PhD; Tomoteru Yamasaki; Akiko Hatori; Katsushi Kumata; Nobuki Nengaki; Ming-Rong Zhang, PhD

Background and Purpose—Recent pharmacological evidence shows that antagonists for the metabotropic glutamate 1 (mGlu1) receptor exhibit neuroprotective effects in an ischemic brain. The aim of this study was to visualize the mGlu1 receptor and to monitor neuroprotective effects in a rat model of mild focal ischemia using positron emission tomography (PET) with N-[4-[6-(isopropylamino)pyrimidin-4-yl]-1,3-thiazol-2-yl]-4-[11C]methoxy-N-methylbenzamide ([11C]ITMM), a radiotracer for mGlu1.

Methods—Rats were subjected to a 30-minute transient right middle cerebral artery occlusion. Saline or minocycline, a neuroprotective agent, was intravenously injected immediately after surgery and then daily during the subsequent 7 days. PET imaging with [11C]ITMM was performed on the rats on days 1 to 7 after ischemia. In vitro autoradiography and histopathologic staining were conducted to confirm the results of in vivo PET.

Results—PET with [11C]ITMM demonstrated a gradual decrease of radioactivity in the ipsilateral sides of the ischemic brains. The radioactivity uptake ratio between the ipsilateral and contralateral sides also decreased with time. Minocycline treatment slowed down the decrease in the radioactivity level in the ipsilateral sides. Pretreatment with JNJ16259685, an mGlu1-selective ligand, significantly reduced brain radioactivity, confirming that the uptake of [11C]ITMM primarily reflects mGlu1 levels in the brain regions, including the ischemic area. In vitro autoradiography and histopathology confirmed the changes in mGlu1 levels in the brains.

Conclusions—[11C]ITMM-PET may be a useful technique for characterizing the change in mGlu1 level during the occurrence and progression of neuronal damage and for evaluating the neuroprotective effects of drugs after ischemia. (Stroke. 2013;44:2567-2572.)

Key Words: autoradiography ■ [11C]ITMM ■ ischemia ■ metabotropic glutamate receptor type 1 ■ positron-emission tomography

Glutamate is a major excitatory amino acid neurotransmitter in the brain, and its release has been shown to significantly increase in ischemic animal models. After ischemia, excess release of glutamate may result in a large increase in the free calcium-ion concentration in neurons and eventual neuronal death.1 It has been suggested that the large concentrations of glutamate in the extracellular spaces of ischemic brain may activate the metabotropic glutamate 1 (mGlu1) receptor.2 The mGlu1 receptor, cloned in 1991, is 1 of 8 subtypes within the metabotropic glutamate receptor family. mGlu1 is widely disturbed in rodent and primate brain regions, such as the cerebellum, thalamus, striatum, and cerebral cortex.3

Recent results suggest that blockade of the mGlu1 receptor may provide an effective approach for inhibiting glutamate-mediated neurotoxicity in the ischemic brain.4,5 Furthermore, some mGlu1 antagonists have shown a neuroprotective effect in the ischemic brain.6,9 However, cerebral infarction volumes after ischemia in mGlu1-knock-out mice were not different from those in wild-type mice.8 In different neurotoxic and ischemic models, mGlu1 agonists have also been shown to reduce neuronal damage in vitro.7,10 Despite these findings, the precise physiological role and locations of mGlu1 in the living brain and the therapeutic effects of mGlu1 antagonists on various diseases of the central nervous system have not been well-characterized.

It has been reported that minocycline, an antibiotic and neuroprotective agent, reduces neurotransmission of glutamate, activation of the N-methyl-D-aspartate receptor, and signaling or release of calcium ions in neurons.9 Minocycline can prevent transient ischemia-induced neuronal damage in rat and gerbil ischemic models.10,11

Received February 14, 2013; final revision received May 30, 2013; accepted June 4, 2013.

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The online-only Data Supplement is available with this article at http://stroke.ahajournals.orglookup/suppl/doi:10.1161/STROKEAHA.113.001178/-/DC1.

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Stroke is available at http://stroke.ahajournals.org

DOI: 10.1161/STROKEAHA.113.001178
Positron emission tomography (PET) is a molecular and functional imaging modality that permits noninvasive and repeatable determination and quantification of receptors, enzymes, transporters, and plaques in the living human brain. Recent work has shown that N-4-[6-(isopropylamino)pyrimidin-4-yl]-1,3-thiazol-2-yl]-4-[14C]methoxy-N-methylbenzamide ([11C]ITMM) can be used as a new mGlu1-specific and selective PET radiotracer, with potential for imaging the mGlu1 receptor in rodent and primate brains.12 [11C]ITMM is being used clinically for studying the human brain to elucidate the distribution and density of mGlu1 receptor.13 In this study, we aimed to determine whether [11C]ITMM-PET can be used for monitoring the mGlu1 receptor levels in the rat brains with mild focal ischemia. PET imaging was performed to elucidate the changes in mGlu1 in the brain 1 week after ischemic surgery. The decrease in brain mGlu1 slowed the occurrence and progression of neuronal damage and for characterizing the neuroprotective effects of minocycline in the ischemic brain.

Methods

Animals and Surgery

This study was approved by the Animal Ethics Committee of the National Institute of Radiological Sciences. The animals were maintained and handled in accordance with the recommendations of the National Institute of Health and the institutional guidelines of the National Institute of Radiological Sciences.

In male Sprague Dawley rats (7–8 weeks; Japan SLC, Shizuoka, Japan), mild focal ischemia was produced by intraluminal occlusion of the middle cerebral artery for 30 minutes using an intraluminal thread model.14 Briefly, a rat was anesthetized with 4% (v/v) isoflurane and maintained with 1.8% isoflurane. The right internal carotid artery was ligated. A 4.0-monofilament nylon suture coated with silicon was inserted (16–18 mm) into the internal carotid artery up to the level where the middle cerebral artery branches. The neck incision was closed with a silk suture. Then, rats were allowed to wake up from anesthesia. After 30 minutes, rats were reanesthetized and the filament was carefully removed for reperfusion. The body temperature was monitored and maintained throughout the surgery. All rats were evaluated during the awake period of ischemia and during filament removal by 2 blinded investigators using the neurological examination score system (Table 1 in the online-only Data Supplement). Rats with a score of 2 to 3 in ischemia and an increase of 0 to 1 in the score after reperfusion were included for further study (8 of 50 rats were excluded after surgery). The rats were randomly divided into 2 groups and injected intravenously with either saline (n=7) or minocycline hydrochloride (n=7; Sigma-Aldrich, St. Louis, MO) at 10 mg/kg immediately after surgery and then daily during the subsequent 7 days.

Production of [11C]ITMM

[11C]ITMM was produced according to our previously reported method.12 [11C]ITMM with specific activity of 70±32 GBq/μmol (n=30) and radiochemical purity of 98.5±1.1% (n=30) was provided for animal experiments.

PET Scans and Data Acquisition

PET scans were performed using a small-animal PET scanner (Inveon, Siemens Healthcare, Erlangen, Germany). The time schedule of PET scans is shown in Figure 1.

Ischemic rats (n=7) were imaged using PET with [11C]ITMM at 1, 2, 4, or 7 days after saline or minocycline treatment. Each rat underwent the full PET protocol and was serially scanned at the appointed times after ischemia. For comparison, normal rats (n=4) also underwent the same PET scanning. Each rat was anesthetized with 5% isoflurane and maintained with 1% to 2% isoflurane. After a transmission scan for attenuation using a cobalt-57 point source, an emission scan was acquired for 60 minutes after intravenous injection of [11C]ITMM with a radioactivity of 37±10 MBq (0.6–1 nmol) in 200-μL saline. In inhibitory experiments, immediately before injection of [11C]ITMM (33±4 MBq), JNJ1625968516 (Sigma-Aldrich), an mGlu1-selective ligand (dose 3 mg/kg in 300-μL saline containing 10% ethanol), was injected. All list-mode acquisition data were sorted into 3D sinograms, which were then Fourier-rebinned into 2D sinograms (frames/min: 4x1, 8x2, 8x5). PET images were analyzed using ASI Pro VM (Analysis Tools and System Setup/Diagnostics Tool; Siemens Healthcare) with reference to the template MR images. A region of interest (size, 23.5±0.3 mm2) was manually positioned on the ischemic area, including the striatum and cerebral cortex, which were defined for each experiment on a summation image 0 to 60 minutes after injection. The ipsilateral region of interest was copied and symmetrically pasted into the contralateral striatum and cerebral cortex in the same section. Brain radioactivity uptake was decay-corrected to the injection time and expressed as the standardized uptake value (SUV=radioactivity per cm3 tissue/inserted radiotracer activity×grams body weight), normalized for injected radioactivity and body weight. Quantitative measurement of specific uptake in the brain was calculated and expressed as uptake ratio of SUVn (standardized uptake value at n minutes) to SUVc (standardized uptake value at control at n minutes).

In Vitro Autoradiography

Rats were euthanized at 5 minutes, 2 hours, 12 hours, 1 day, 2 days, and 7 days (n=5–8) after ischemia by decapitation under diethyl ether anesthesia, and their brains were quickly removed and frozen on powdered dry ice. Normal rats (n=4) were used as controls. Coronal brain sections (10 μm) were cut with a cryostat (HM560, Carl Zeiss; Oberkochen, Germany) at −20°C and mounted on air plasma spraying–coated glass slides (Matsunami; Tokyo, Japan). After the brain sections were preincubated in a 50-mmol/L Trizma buffer for 20 minutes at 25°C, [11C]ITMM (3.7 MBq) was added to each incubated solution. The free ligand concentrations for the binding experiment were 0.2 to 0.4 nmol/L. To determine the specific binding of [11C]ITMM to mGlu1 receptor, JNJ16259685 (10 μmol/L) was added to the incubated solutions before [11C]ITMM. The brain sections were incubated with [11C]ITMM or [11C]ITMM/JNJ16259685 for 30 minutes at 25°C. After incubation, brain sections were exposed to imaging plates (BAS-MS 2325; Fuji Photo Film, Tokyo) for 60 minutes and analyzed using a Bio Imaging Analyzer System (BAS5000; Fuji Photo Film). The radioactivity on the sections was quantified and expressed as photo-stimulated luminescence per unit area (photo-stimulated luminescence/mm2).

Histopathology

To confirm the ischemic areas, all rats were euthanized via exsanguination under ether anesthesia after PET scanning on the day 7.
PET image (A) of 0 to 60 minutes summation and time-activity curves of brain regions (Figure I in the online-only Data Supplement) show a high accumulation of radioactivity in different regions. The highest uptake was seen in the cerebellum, followed by the thalamus, striatum, and cerebral cortex. This distribution pattern of uptake reflected the distribution of mGlu1 in the brain regions, as previously reported.12,17 Pretreatment with the mGlu1-selective ligand JNJ16259685 significantly reduced the brain uptake (B and Figure II in the online-only Data Supplement). For the control (C) and JNJ16259685-treated (D) brain sections, in vitro autoradiography showed a distribution of radioactivity that was consistent with that in the corresponding PET images.

The high inhibition by JNJ16259685 in the controls implied that the in vitro and in vivo uptake of \([^{11}C]\)ITMM was mostly reflective of mGlu1 in the brain regions, including the striatum and cerebral cortex, the 2 target regions of this study.

Figure 3 shows representative PET- and 2,3,5-triphenyltetrazolium chloride–staining brain images of the same ischemic individuals. PET images of the striatum and cerebral cortex were summed between 0 and 60 minutes after injection of \([^{11}C]\)ITMM. Figure 4 shows the uptake ratios of radioactivity (standardized uptake value) between the ipsilateral and contralateral sides of the same sections at 57.5 minutes. The time-activity curves are shown in Figure III in the online-only Data Supplement.

A time-dependent decrease of radioactivity accumulation was observed in the ipsilateral side (Figure 3B) of the brain at 1, 2, 4, and 7 days after ischemia but not in normal section (Figure 3A). Differences in the uptake ratio between the ipsilateral and contralateral sides became significant with time progression (Figure 4). At 7 days after ischemia, the uptake ratio between the 2 sides decreased to 0.72±0.05 for the striatum (P<0.01) and 0.74±0.05 for the cerebral cortex (P<0.01). The pattern of lesions observed by 2,3,5-triphenyltetrazolium chloride–staining was similar to that seen in the corresponding PET image.

Figure 3C shows the effect of minocycline treatment on \([^{11}C]\)ITMM uptake in the ischemic brain. Through daily treatment with minocycline, the decrease in radioactivity in the ipsilateral side was slowed down. At 7 days after treatment, the uptake

**Results**

**PET Studies**

Figure 2 shows representative PET and in vitro autoradiographic images of normal brains without ischemia. The control

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**Figure 2.** PET for normal brain. Representative sagittal PET (A and B) and autoradiographic (C and D) images for \([^{11}C]\)ITMM in normal rat brains without ischemia (n=4). A and C, \([^{11}C]\)ITMM only. B and D, JNJ16259685 treatment. PET images were generated by summation of whole scans (0–60 minutes) and were overlaid on MR images, which were used as anatomic brain templates. \([^{11}C]\)ITMM indicates N-[4-[6-(isopropylamino)pyrimidin-4-yl]-1,3-thiazol-2-yl]-4-[\(^{11}\)C]methoxy-N-methylbenzamide; PET, positron emission tomography; and SUV, standardized uptake value.

The brains were removed quickly, coronal sections were cut, and half of the sections were stained with 2,3,5-triphenyltetrazolium chloride, whereas the remaining half of the same brain sections were stained with cresyl violet and immunofluorescence. After fixation in cold-acetone for 10 minutes and immersion in peroxidase-blocking solution for 30 minutes, the brain sections were incubated with rabbit–anti-mGlur1 primary antibodies (1:200; LSBio, Seattle, WA) overnight at 4°C and then incubated with the secondary antibodies Alexa Fluor 488 goat anti–rabbit IgG (1:500; Life Technologies, Carlsbad, CA) for 1 hour at room temperature. The sections were washed 3x in phosphate-buffered saline for 5 minutes after each step. Histopathologic images of coronal sections were acquired with an epifluorescence microscope (Olympus, Tokyo) and e-tiling software (Mitani, Tokyo).

**Statistical Analysis**

Data are expressed as the mean±SEM. For autoradiography, changes in values were compared using the Student paired t test. PET data were examined using 2-way repeated-measures ANOVA. The threshold for statistical significance was set at P<0.05.
ratio between the ipsilateral and contralateral sides of the same sections increased to 0.86±0.03 for the striatum (P<0.05) and 0.92±0.01 for the cerebral cortex (P<0.01). Compared with the saline group, the minocycline group showed improvement of the [11C]ITMM uptake in the ipsilateral sides at the same time points. Pretreatment with JNJ16259685 significantly reduced the brain uptake and produced a uniform and low distribution pattern. The decreasing percentage of brain uptake reached 80%, not only in the ipsilateral, but also in the contralateral side.

In Vitro Autoradiography

Figure 5 shows representative in vitro autoradiograms of the brain sections at 5 minutes, 2 hours, 12 hours, 1 day, 2 days, and 7 days after ischemia, when the same radioactivity of [11C]ITMM was used for the binding experiments.

For the brain sections taken at 5 minutes, 2 hours, and 12 hours after ischemia, no statistical difference (P>0.05; Figure 5A) in radioactivity level was observed between the ipsilateral and contralateral sides. At 1 day, radioactivity in the ipsilateral side seemed to decrease (Figure 5B), which was consistent with the corresponding PET image. At 7 days, the difference in radioactivity level between the 2 sides was 0.46±0.02 for the striatum (P<0.01) and 0.26±0.01 for the cerebral cortex (P<0.01). As shown in brain sections treated with minocycline, the ipsilateral side showed improvement of radioactivity compared with the ipsilateral side of saline groups. Coincubation with excess JNJ16259685 significantly...
reduced the radioactivity level in the day-7 ischemic brains and produced a uniform distribution of radioactivity among all brain regions.

**Histopathologic Staining**

Figure 6 illustrates the distributions of the mGlu1 and neurons in the different regions of the normal brain and the day-7 ischemic brains treated with saline or minocycline after the PET scans, as observed using an immunohistochemical assay and cresyl violet staining.

In the normal brain, dense fluorescence representing mGlu1 was detected over the striatum and cerebral cortex. Decrease in mGlu1 density was observed on the ipsilateral side of the saline group (also see Figure IV in the online-only Data Supplement), whereas mGlu1 level was improved by minocycline treatment. Furthermore, the zone expressing mGlu1 seemed to closely match with the neuron distribution identified by cresyl violet staining, and a similar pattern of reduced neurons with mGlu1 was seen in the ischemic brain.

**Discussion**

This is the first imaging study of mGlu1 receptor in the ischemic brain using PET with [11C]ITMM, a novel specific radiotracer for mGlu1. In the ischemic brain, decrease in mGlu1 was demonstrated. Treatment with the neuroprotective agent minocycline slowed down the reduction of mGlu1 in the brain. The in vivo PET results were supported by findings of in vitro autoradiography and histopathologic assay. This indicates that [11C]ITMM-PET can be used to monitor the occurrence and progression of neuronal damage and evaluate the effects of neuroprotective drugs after ischemia.

To visualize the change in mGlu1 level in the brain, a rat model of mild focal ischemia was prepared using occlusion of the middle cerebral artery, a relevant method for representing human ischemic stroke. This model had been proven to be successful without a longitudinal breakdown of the blood–brain barrier from the ischemic surgery. The reliability of this model ensures that the brain uptake of [11C]ITMM, as measured in this PET study, is not influenced to a large degree by transient disruption of the blood–brain barrier.

In this study, PET with [11C]ITMM demonstrated decreased uptake of radioactivity in the ipsilateral side of the ischemic brains (Figures 3 and 4). The most significant difference in uptake was observed between the ipsilateral and contralateral sides on day 7 after ischemia. The specific binding of [11C]ITMM to mGlu1 receptor was confirmed by blocking this receptor with the mGlu1-selective ligand JNJ16259685. Treatment with JNJ16259685 significantly decreased the radioactivity in the whole rat brain. This result, along with the same inhibitory result for the normal brain (Figure 2B and 2D), demonstrated that the uptake of [11C]ITMM in different brain regions, including the ischemic area, primarily reflected mGlu1 density. In vitro autoradiography (Figure 5) and histopathologic analysis (Figure 6) also confirmed the decrease in mGlu1 density and the neuroprotective effect of minocycline.

Ischemia has been reported to accelerate a rapid release of glutamate, which then immediately activates mGlu1, resulting in excess release of calcium ion and eventual neuronal damage in the brain. However, the in vitro autoradiographic images from 5 minutes to 12 hours after ischemia did not show transient increase in the radioactivity levels in the ipsilateral side (Figure 4). This result suggests that ischemia-induced activation of mGlu1 may be very rapid and short-lived to be monitored using autoradiography. From day 1 after ischemia, a decrease in mGlu1 was seen on the ipsilateral side, illustrating the occurrence and progression of neuronal damage in the brain.

The neuroprotective effect of minocycline has been confirmed using models of posts ischemic neuronal death. Minocycline may depress glutamatergic neurotransmission and the excess release of calcium ions, which represents one of the key events in the processes leading to posts ischemic neuronal death. Our present study suggests that minocycline may also inhibit activation of the mGlu1 receptor, which is linked to neuronal damage in the ischemic brain. Moreover, minocycline treatment produced a dose-dependent neuroprotective effect. In fact, daily administration of minocycline for 0 to 7 days after ischemia demonstrated a better neuroprotective effect than a single dose of minocycline (data not shown). These results suggest that mGlu1 may play a crucial role in the early phase of neuronal damage and that the neuroprotection
conferred by minocycline during the critical phase is associated with timely improvement of mGlu1 expression in the ischemic area.

Our present study revealed the presence of mGlu1 in neurons (Figure 6b) but not in microglia and astrocytes (Figure 5 in the online-only Data Supplement). Because mGlu1 is a postsynaptic and purely neuronal receptor, reduction in specific binding of [11C]ITMM and mGlu1 density in this ischemic model most likely reflects the process of neuronal damage. The decrease in mGlu1 density at the ischemic area was seen even on day 1 after surgery and likely reflects an irreversible synaptic degeneration. However, the exact mechanism needs to be further elucidated. Nonetheless, PET with [11C]ITMM may be used to monitor early neuronal damage.

To evaluate the clinical changes after stroke, several techniques have been developed for imaging regional cerebral blood flow, regional cerebral metabolism, and molecular responses in the infarcted area.19,20 In particular, assessment of neuronal integrity, apoptosis, and neuroinflammation.21,22 The present findings indicate that mapping of mGlu1 with PET is a useful alternative tool for characterizing neuronal integrity and has potential to be used as a biomarker in future therapeutic regimens. As a specific and selective radiotracer for mGlu1, [11C]ITMM coupled with PET may provide a more sensitive assessment of neuronal damage than the conventional visualization of metabolism.

In this PET study, what we monitored with [11C]ITMM is the density of mGlu1, which may reflect both (and together) a loss of synapses/receptors secondary to ischemic damage and a functional change in mGlu1 receptor density because of functional down-regulation, but the 2 aspects may not be differentiated. Moreover, we did not determine if imaging of mGlu1 receptor with [11C]ITMM is superior to imaging of central benzodiazepine receptor with [11C]flumazenil, an established and validated PET tracer for monitoring of neuronal density after stroke.23

In conclusion, this study demonstrated that [11C]ITMM-PET is a useful technique for monitoring the progress of neuronal damage and for characterizing mGlu1 with early and small changes in the ischemic brain. Recently, the first trial of using [11C]ITMM-PET has been performed in a clinical study on humans.13 We think that this technique can be used to evaluate the neuroprotective effects of mGlu1 antagonists and other drugs used for treating ischemic stroke, such as N-methyl-D-aspartate antagonists, antineuroinflammation agents, and tissue plasminogen activators.

Acknowledgments
We thank the staff at National Institute of Radiological Sciences for their technical support in radiosynthesis and animal experiments.

Disclosures
None.

References
Monitoring Neuroprotective Effects Using Positron Emission Tomography With $^{11}$CITMM, a Radiotracer for Metabotropic Glutamate 1 Receptor

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*Stroke*. 2013;44:2567-2572; originally published online August 1, 2013;
doi: 10.1161/STROKEAHA.113.001178

*Stroke* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/44/9/2567

Data Supplement (unedited) at:
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SUPPLEMENTAL MATERIAL

Monitoring neuroprotective effects using PET with $[^{11}C]$ITMM, a radiotracer for metabotropic glutamate 1 receptor

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Supplemental data: Table I
   Figure I
   Figure II
   Figure III
   Figure IV
   Figure V
Supplemental Table I. Neurological examination score system

<table>
<thead>
<tr>
<th>Assessment</th>
<th>Score</th>
<th>Comments</th>
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<tr>
<td>Normal</td>
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<td>No observable neurological deficits</td>
</tr>
<tr>
<td>Mild</td>
<td>1</td>
<td>Mild left forelimb flexion</td>
</tr>
<tr>
<td>Moderate</td>
<td>2</td>
<td>Decreased resistance to lateral push without circling</td>
</tr>
<tr>
<td>Severe</td>
<td>3</td>
<td>Decreased resistance to lateral push with circling</td>
</tr>
</tbody>
</table>

Modified from the neurological deficits score system by Bederson JB, et al.¹

Reference:

Supplemental Figure I. Time–activity curves of PET in different regions of the NORMAL brains between 0 and 60 min after injection of $[^{11}\text{C}]$ITMM.
Supplemental Figure II. Time–activity curves of PET in different regions of the NORMAL brains between 0 and 60 min after injection of $^{[11]}$CITMM and JNJ16259685 (3 mg/kg).
Supplemental Figure III. Time–activity curves of PET in different regions of the ischemic brains treated with saline (A) or minocycline (B) between 0 and 60 min after injection of \([^{11}\text{C}]\text{ITMM}\).
Supplemental Figure IV. In vitro autoradiography and immunofluorescence representing the mGlu1 receptor in serial ischemic brain sections. (A) Representative autoradiographic images for \(^{11}\text{C}\)ITMM. (B) Immunofluorescence images of mGlu1 receptor (green). Arrows indicate ischemic areas. Scale bars: 1 mm.
Supplemental Figure V. Distribution of mGlu1 expression on day 7 ischemic brain section with saline treatment. Double immunofluorescence staining was performed with anti-mGlu1 (green; 1:200; LSBio)/anti-CD11b (red; 1:100; AbD Serotec) for identifying microglia, anti-mGlu1 (green)/anti-GFAP (red; 1:100; Life Technologies) for identifying astrocytes, and anti-mGlu1 (green)/anti-neuron specific beta III tubulin (red; 1:200; R&D) for identifying neurons. The mGlu1 signals were observed in majority of neurons, but not in microglia and astrocytes. Scale bars: 200 µm.