Chronic brain ischemia induces the expression of glial glutamate transporter

EAAT2 in subcortical white matter

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Running title: EAAT2 expression in chronic brain ischemia

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Abstract

Glutamate plays a central role in brain physiology and pathology. The involvement of excitatory amino acid transporters (EAATs) in neurodegenerative disorders including acute stroke has been widely studied, but little is known about the role of glial glutamate transporters in white matter injury after chronic cerebral hypoperfusion. The present study evaluated the expression of glial (EAAT1 and EAAT2) and neuronal (EAAT3) glutamate transporters in subcortical white matter and cortex, before and 3-28 days after the ligation of bilateral common carotid arteries (LBCCA) in rat brain. K-B staining showed a gradual increase of demyelination in white matter after ischemia, while there was no cortical involvement. Between 3 and 7 days after LBCCA, a significant increase in EAAT2 protein levels was observed in the ischemic brain and the number of EAAT2-positive cells also significantly increased both in the cortical and white matter lesions. EAAT2 was detected in glial-fibrillary acidic protein (GFAP)-positive astrocytes in both the cortex and white matter, but not in neuronal and oligodendroglial cells. EAAT1 was slightly elevated after ischemia only in white matter, but EAAT3 was at almost similar levels both in the cortex and white matter after ischemia. A significant increase in EAAT2 expression level was also noted in the deep white matter of chronic human ischemic brain tissue compared to the control group. Our findings suggest important roles for up-regulated EAAT2 in chronic brain ischemia especially in the regulation of high-affinity of extracellular glutamate and minimization of white matter damage.
Introduction

Glutamate is an essential neurotransmitter but its excess in the extracellular space is excitotoxic to neuronal cells (Choi, 1992). Extracellular glutamate concentration is regulated mainly through the reuptake of glutamate from the synaptic cleft via glial excitatory amino acid transporters (EAATs). EAATs are Na\(^+\)-dependent transporters; three Na\(^+\) and one H\(^+\) are co-transported with each negatively charged glutamate molecule and one K\(^+\) is counter-transported. In the normal brain, the high-affinity plasma membrane glutamate transporters actively clear glutamate released in the synaptic cleft to maintain glutamatergic homeostasis and prevent neurotoxicity (Anderson and Swanson, 2000, Camacho and Massieu, 2006). Glutamatergic synaptic dysfunction is the major mediator of excitotoxic neuronal death in neurodegenerative disorders such as amyotrophic lateral sclerosis, Alzheimer’s disease (Francis, 2005), and cerebral infarction (Kato and Kogure, 1999).

Five subtypes of glutamate transporters (EAAT1, 2, 3, 4 and 5) have so far been identified in the central nervous system (Kanai and Hediger, 1992, Pines et al., 1992). EAAT1 is predominantly found in the glial cells, EAAT2 is predominantly present in astrocytes, EAAT3 and EAAT4 are predominantly found in neurons, and EAAT4 and EAAT5 are, respectively, present in the retina and cerebellum (Danbolt et al., 1998). EAAT2 is the most active glutamate transporter, and it regulates more than 90% of extracellular glutamate reuptake (Yamada et al., 2006).

Studies using cell cultures and experimental models described increases in EAAT1 and EAAT2 after acute ischemia or hypoxia-ischemia (Arranz et al., 2010), with selective overexpression of EAAT2 after moderate hypoxia-ischemia (Weller et
al., 2008). Another study reported that administration of antisense oligonucleotides of EAAT2 increased infarct volume and mortality after cerebral ischemia (Rao et al., 2001b). These studies and others (Rao et al., 2001a, Kim et al., 2011) stress the importance of EAAT2 changes in cerebral ischemia.

While changes in glial glutamate transporters have been reported in acute ischemic models, there is little or no information on the regulation of excess glutamate and changes in glial glutamate transporters in models of vascular dementia. The main hypothesis of the present study was that chronic brain ischemia induces the expression of glial glutamate transporter EAAT2 in subcortical white matter. To test this hypothesis, we determined first the expression of glial glutamate transporters EAAT1, 2 and 3 by immunohistochemistry and immunoblotting both in the cortex and white matter at different times after chronic cerebral hypoperfusion, ligation of bilateral common carotid arteries (LBCCA), in rat brain. We also analyzed EAAT2 expression in the human brain with damaged white matter in patients with Binswanger’s disease and multiple lacunar infarction.

Experimental procedures

Animals and experimental design

Adult male Wistar rats (11-week-old) weighing 350-450 g were purchased from the Charles River Institute (Kanagawa, Japan) and maintained on a 12-h light/dark cycle with free access to food and water. To occlude both common carotid arteries, the rats were anesthetized with 1–2% isoflurane in 30% oxygen and then anesthesia was maintained with 70% nitrous oxide. During surgery, a temperature probe was inserted
into the rectum, and a heat lamp was applied to maintain the body temperature at 37.0–37.5°C. Through a midline incision, each common carotid artery was carefully separated from the cervical sympathetic and vagal nerves and ligated permanently. Rats of the control group were sham-operated, which involved bilateral exposure of the common carotid arteries.

Twenty-five rats were examined for histological changes and kept in cages with food and water *ad libitum*. At days 3, 7, 14 and 28 after LBCCA, the rats were re-anesthetized with 1% isoflurane and 70% N₂O:30% O₂, and then transcardially perfused with phosphate-buffered saline (PBS) and 4% paraformaldehyde. The brain was dissected out immediately, postfixed in 4% paraformaldehyde for 48 h, and stored in 30% sucrose in 0.1 M PBS. For immunohistochemistry, 20-μm-thick free-floating coronal sections of the corpus callosum were prepared for staining.

All animals were acquired and cared for according to the guidelines published by the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All experiments described in this study were conducted after approval of the Animal Care Committee of the Juntendo University.

*Measurement of cerebral blood flow (CBF)*

After LBCCA, CBF was measured in a left temporal window using laser Doppler flowmetry (Laser tissue Blood Flow Meter FLO-C1; Omega Wave, Inc., Portland, OR). The probe in the shape of straight rectangular sheet (7.5 mm in length and 1.0 mm in-depth) was positioned between the temporal muscle and the lateral aspect of the skull, as described previously (Harada et al., 2005). In these experiments, there
was no need for craniotomy. CBF was monitored continuously for 3-5 min at each time, before, immediately and after, and at days 3, 7, 14 and 28 after LBCCA. Reproducible recorded CBF velocity was obtained.

**Immunohistochemistry**

Immunohistochemistry was performed on 20-μm-thick free-floating coronal sections, which were prepared as described previously (Miyamoto et al., 2010). After incubation in 3% H₂O₂ followed by 10% block ace in 0.1% PBS(−), the brain sections were immunostained overnight at 4°C using rabbit antibody against excitatory amino acid transporter 1 (EAAT1; dilution, 1:50; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit antibody against excitatory amino acid transporter 2 (EAAT2; dilution, 1:300; (Sasaki et al., 2009)), rabbit antibody against excitatory amino acid transporter 3 (EAAT3; dilution, 1:500; Abcam, Cambridge, MA), anti-glutathione-S-transferase π antibody (GSTπ, dilution, 1:500, Medical and Biological Laboratories, Nagoya, Japan), anti-NG2 chondroitin sulfate proteoglycan (NG2, dilution, 1:100, Millipore, Bedford, MA), anti-glial-fibrillary acidic protein (GFAP, dilution, 1:500; Dako Corp., Carpinteria, CA), anti-ionized calcium-binding adapter molecule-1 (Iba-1, dilution, 1:500, Wako Pure Chemical Industries, Osaka, Japan) and anti neuronal nuclear antigen (NeuN, dilution, 1:100, Millipore), and finally treated with secondary antibodies (dilution, 1:500, Vectastain; Vector Laboratories, Burlingame, CA). Immunoreactivity was visualized using the avidin–biotin complex method (Vectastatin). Negative control sections were stained using the above-mentioned immunohistochemical technique with the omission of the primary antibodies. The
images were captured using a digital camera (DXM1200, Nikon, Tokyo, Japan) attached to an Olympus CX40 microscope (Olympus, Tokyo) and analyzed using the ACT-1 image system (version 2.20, Nikon).

**Double immunofluorescence histochemistry**

Double immunofluorescence staining was performed by simultaneous incubation of the sections overnight at 4°C with mouse anti-GFAP (dilution, 1:500; Medical and Biological Laboratories), mouse anti-adenomatous polyposis coli (APC, dilution, 1:100; Calbiochem, Darmstadt, Germany), mouse anti-NG2 (dilution, 1:50; Santa Cruz Biotechnology), anti-platelet derived growth factor receptor-α (PDGFRα, dilution, 1:100; Santa Cruz Biotechnology), anti-NeuN (dilution, 1:500; Millipore), and goat anti-Iba-1 (dilution, 1:500; Abcam) antibodies. After overnight incubation with the primary antibody, the sections were immunostained with fluorochrome-conjugated secondary antibody (Cy3- or fluorescein isothiocyanate, dilution 1:500, Jackson Immunoresearch Laboratories, West Grove, PA), and then covered with Vectashield mounting medium (Vector Laboratories).

**Klüver-Barrera staining**

The corpus callosum was evaluated for white matter lesion by Klüver-Barrera staining. The myelin areas in three sections per animal and both sides of the selected areas were stained with Luxol Fast Blue. The severity of white matter lesion was graded as 0 (normal), 1 (disarrangement of nerve fibers), 2 (formation of marked vacuoles), and 3 (disappearance of myelinated fibers), according to the previously described grading
system (Wakita et al., 2002).

**SDS-PAGE and immunoblotting**

Rats of each group were decapitated at 3, 7, 14 and 28 days after LBCCA (n=5 per time point for each group). Proteins were extracted from the white matter area and analyzed as described previously (Kobayashi et al., 2002). Briefly, aliquots containing 30 μg of protein were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The protein bands were electrotransferred onto polyvinylidene fluoride membrane (Bio-Rad Laboratories, Hercules, CA). The membranes were subsequently incubated with the following primary antibodies: anti-EAAT1 (dilution, 1: 500 ), anti-EAAT2 (dilution, 1 :1000), anti-EAAT3 (dilution, 1 :1000) or anti-α-tubulin antibody (dilution, 5000:1, Santa Cruz Biotechnology). After incubation with the appropriate horseradish peroxidase- conjugated secondary antibody (dilution, 20,000:1, Amersham Life Science, Buckinghamshire, UK) for 1 h at room temperature, immunoreactive bands were visualized in the linear range by using an enhanced chemiluminescence Western blotting system (Amersham). Equal protein loading was confirmed by measuring α-tubulin.

**Cell count and statistical analysis**

In each of the EAAT1-, 2-, 3-, GFAP-, GSTπ-, and Iba-1-stained sections, the numbers of stained cells in white matter lesions (0.25 mm²) in predefined areas (SVZ, RMS, granular cell layer of olfactory bulb (Grl)), were counted (n=5 rats for each group) by an investigator blinded to the experimental group, using Axio-Vision.
software (Carl Zeiss, Jena, Germany). One-way analysis of variance (ANOVA) followed by post hoc Fisher’s protected least significant difference test was used to examine the differences in various parameters among the different groups. A $P$ value $<0.05$ denoted the presence of a statistically significant difference.

**Autopsy Specimens**

Postmortem human brains of six patients (three with chronic ischemia and three control) were obtained from Juntendo University School of Medicine. The clinical data of these six subjects are summarized in Table 1. The Human ethics review committee of Juntendo University approved this part of the study. Formalin-fixed paraffin-embedded samples from the white matter frontal lobe were immunostained. Cerebral hemispheres were fixed in 4% paraformaldehyde in 0.1 mol/L phosphate buffer, pH 7.4, and embedded in paraffin. The cortex and white matter from the frontal lobe were cut into 30-μm-thick sections. After removal of paraffin, antigens were retrieved with autoclaving and endogenous peroxidase activity was quenched for 30 min with 3% $\text{H}_2\text{O}_2$ in methanol. After incubation in 3% $\text{H}_2\text{O}_2$ followed by 10% block ace in 0.1% PBS(-) for 1 h, the brain sections were immunostained overnight with rabbit antibody against EAAT2 (dilution, 1:300; (Sasaki et al., 2009)) at 4°C followed by treatment with secondary antibodies (dilution, 1:500, Vectastain; Vector Laboratories). Immunoreactivity was visualized using the avidin–biotin complex method (Vectastatin). The images were captured using a digital camera (DXM1200, Nikon) attached to an Olympus CX40 microscope (Tokyo) and analyzed using the ACT-1 image system (version 2.20, Nikon). The numbers of stained cells found in the white matter were counted in EAAT2 immunohistochemistry section. One way $t$-test
followed by post hoc Fisher’s protected least significant difference test was used to examine differences in various parameters among the different groups.

Results

Histological findings

Hypoperfusion was confirmed by reduction in CBF after LBCCA relative to the baseline (before LBCCA), whereas no significant change was noted in control rats (post-LBCCA: 35% immediately after, 55% at day 3, 66% at day 7, 78% at day 14 and 83% at day 28, relative to the baseline) (Fig. 1). Extensive changes in the Klüver-Barrera staining pattern appeared in the corpus callosum at 7 days after LBCCA. After the initial changes of disarrangement of the nerve fibers in the corpus callosum, the myelinated fibers were reduced in number while the remaining fibers showed further and gradual disorganization. The numbers of intracellular vacuoles increased in the corpus callosum at day 28 after LBCCA. There was no significant hemorrhage or infarction in any brain region. The overall grade of white matter injury was significantly higher from day 14 after ischemia compared with the control (Fig. 2). In comparison, no changes were noted in the cerebral cortex after ischemia (data not shown).

The density of astrocytes positive for GFAP in the white matter at 3 and 7 days in the LBCCA group was significantly higher than in the sham group, although the density gradually returned to the baseline level. In the cerebral cortex, the number of GFAP-positive cells was significantly higher at day 3 after ischemia, and the increase
continued until day 28 post-LBCCA. The number of GSTπ-positive oligodendrocytes was transiently higher at days 7 and 14 days after ischemia but was significantly lower at day 28 post-LBCCA than the control (Fig. 3C). On the other hand, the number of NG2-positive oligodendrocyte progenitor cells in the white matter increased gradually and significantly from 7 days after ischemia relative to the control (Fig. 3D). Although the number of Iba-1 positive microglial cells was significantly higher at day 3 post-LBCCA, it decreased gradually after day 7 post-LBCCA (Fig. 3E).

Changes in expression of EAATs

The expression of EAAT1, EAAT2 and EAAT3 was evident in both the cerebral cortex and white matter of the normal brain. The expression level of EAAT2 was relatively intense in both areas compared to EAAT1 and EAAT3. In the normal brain, immunostaining for EAAT2 was observed mainly in astrocytes in both areas, while immunostaining for EAAT1 and EAAT3 was detected in oligodendrocytes present in the white matter area and also in cortical neurons (Fig. 4a, f, k, p).

The detection of EAAT1, EAAT2 and EAAT3 signals in western blot analysis prompted a time-course study of EAAT expression at days 3, 7, 14, and 28 post-ischemia. EAAT2 protein appeared as a band of ~62 kDa in the subcortical white matter tissue, and quantitative analysis indicated a significant increase in the protein at day 3 post-ischemia (to 123% of the baseline), which persisted up to day 14 (P < 0.001), consistent with the results of immunohistochemical analysis. Further analysis showed a gradual reduction at day 14 post-ischemia, with EAAT2 protein level reduced at ~90% of the control at day 28 post-LBCCA. Analysis of the cerebral cortex
for serial changes in EAAT2 protein level showed a pattern of changes in this protein similar to that observed in the white matter, with a moderate increase of EAAT2 protein at days 3 and 7 post-LBCCA.

Analysis of EAAT1 protein level showed a slight increase at 3 days (to 114% of the baseline), and a further increase at day 7 post-ischemia (to 116% of the baseline) ($P < 0.05$). However, EAAT1 protein level decreased after day 14 post-LBCCA. In comparison, EAAT1 protein level was observed in 84% of the control animals at day 28 post-ischemia. Only a negligible increase in EAAT3 protein level was observed in the white matter and cortex, but the change was not significant compared to the control (Fig. 5).

Immunohistochemical staining showed more intense and coherent staining for EAAT2 in the white matter than in other areas. Staining for EAAT2 at day 1 post-LBCCA was similar to the control brain (data not shown), but a significant increase in EAAT2 level was observed in the white matter at days 3-7 post-ischemia. Immunohistochemical analysis showed no significant changes in EAAT1 and EAAT3 expression levels after hypoperfusion (Fig. 4).

**Localization of EAAT2**

Double-labeling immunofluorescence analysis was conducted to determine the distribution of EAAT1, EAAT2 and EAAT3. The expression of EAAT2 was localized to astrocytes in both the white matter and the cortex throughout the study period. Staining for EAAT2 was not observed in other glial cells such as oligodendrocytes or microglia (Fig. 6A).
The expression of EAAT1 was limited in the white matter to mature oligodendrocytes stained with antibodies to APC, a marker of oligodendroglia. However, in the cortex, staining for EAAT1 was noted in neurons. Both astrocytes and microglias were negative for EAAT1 staining (Fig. 6B).

Staining for EAAT3 was observed in the white matter in immature oligodendrocytes stained with antibodies to PDGFRα, a marker of oligodendroglial progenitor cells. In the cortex, staining for EAAT3 was limited to neurons. Both astrocytes and microglias were negative for EAAT3 (Fig. 6C).

**Changes in expression of EAAT2 in human brain**

Finally, we analyzed the change in EAAT2 expression pattern in human ischemic brain tissue. EAAT2 was expressed in the white matter of the control brains. The number of EAAT2-positive cells was significantly higher in ischemic brain than the control (Fig. 7). Furthermore, morphological variation and hypertrophy of EAAT2-positive cells were noted in the ischemic brain, compared to the control EAAT2-positive cells with few axons.

**Discussion**

The results of this study showed that chronic cerebral hypoperfusion resulted in transient up-regulation of glial glutamate transporters in the subcortical white matter and cortex. Among the three glutamate transporters examined in this study, EAAT2 increased significantly in both the rat brain ischemia model used in this study and
human brain tissue.

Glutamate-mediated excitotoxicity is one of the major etiologies of neurodegenerative diseases, such as ALS and AD. In fact, selective loss of EAAT2 was reported in both the motor cortex and spinal cord of patients with ALS (Rothstein et al., 1995), whereas overexpression of EAAT2 in ALS-associated mutant SOD mice improved L-glutamate induced cytotoxicity and delayed motor neuron loss (Guo et al., 2003). In Alzheimer’s disease, EAAT2 expression in the brain is significantly decreased (Masliah et al., 1996), even in the early stages of the disease (Jacob et al., 2007). Interestingly, partial loss of EAAT2 was recently reported to result in acceleration of the disease process in animal models of Alzheimer’s disease (Mookherjee et al., 2011). These results indicate that EAAT2 plays an important role in the pathogenesis of neurodegenerative disease.

Several studies have examined the effects of ischemia on EAATs, which resulted in and variable changes in their expression patterns according to the protocol of acute ischemia model (Gottlieb et al., 2000, Cimarosti et al., 2005).

EAAT2 levels were reported to decrease in the cortex and caudate-putamen at day 3 of recovery from brain ischemia (Rao et al., 2001a), increase in the white matter at day 1 after reperfusion following middle cerebral artery occlusion (MCAO), but decrease at 7 days after ischemia (Arranz et al., 2010). However, overexpression of EAAT2 induced by intraperitoneal injection of ceftriaxone (beta-lactams) or intracerebral injection of viral vector is reported to exert significant neuroprotective effects against focal cerebral ischemia (Chu et al., 2007, Harvey et al., 2011). Thus, overexpression of EAAT2 seems to protect against neuronal death induced by
ischemic insult. In the present study, we used ligation of the left and right common carotid arteries in rats, which is reported to be an appropriate model for human ischemic white matter lesions, as confirmed histologically (Kobayashi et al., 2002, Thomas et al., 2002, Tomimoto et al., 2003, Chida et al., 2011). Our results showed increased amounts of EAAT2 up to 7 days after ischemia. Glutamate is known to be released from damaged oligodendroglia and axons following reversal of uptake carriers in conditions of energy deprivation (Karadottir and Attwell, 2007, Tekkok et al., 2007). In this regard, excitotoxic injury of the white matter (infiltration of immunocytes, loss of myelin and oligodendrocytes, and axonal damage) is considered an important pathophysiology in multiple sclerosis, and is enhanced by deficiency of glutamate detoxification. EAAT2 was selectivity lost from oligodendrocytes found around active MS lesions, especially in early active lesions, extending into adjacent white matter (Werner et al., 2001). These results suggest that excess glutamate plays a critical role in tissue injury not only in the cortical area but also in white matter region. In periventricular white matter injury, hypoxia-ischemia induces glutamate production from oligodendrocyte lineage cells and axons, which in turn activates receptors on adjacent oligodendrocytes, such as NMDA receptors, with cause white matter injury (Back et al., 2007).

Considered together, the results suggest that overexpression of EAAT2 after chronic hypoperfusion was induced by high extracellular levels of glutamate. Thus, prevention of such up-regulation could protect against white matter damage. These results are in agreement with the previous finding of up-regulation of EAAT2 expression in reactive astrocytes in human periventricular leukomalacia (Desilva et al.,
Our results also showed large numbers of EAAT2-positive cells in ischemic white matter of autopsy cases.

One recent study showed that elimination of EAAT2 by antisense oligonucleotide worsened the neurological status after transient MCAO (Rao et al., 2001b). In contrast, the present study demonstrated that pretreatment with β-lactam antibiotics, like ceftriaxon, which is reported to induce up-regulation of EAAT2 (Rothstein et al., 2005, Lee et al., 2008), reduced infarct volume after transient MCAO (Chu et al., 2007). These results suggest that long-term up-regulation of EAAT2 after chronic hypoperfusion might result in minimization of white matter lesion by removal of extracellular glutamate. Further studies are needed to understand the effects of changes in EAATs expression after chronic hypoperfusion.

Acknowledgment

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References


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Werner P, Pitt D, Raine CS (2001) Multiple sclerosis: altered glutamate homeostasis in lesions...


**Figure Legends**

Fig. 1. Temporal changes in cerebral blood flow (CBF). Pre: before ligation of bilateral common carotid artery (LBCCA); post: immediately after LBCCA, and at 3, 7, 14 and 28 days after LBCCA. Values are mean ± SEM of five rats in each group.

*P* < 0.05, **P** < 0.001, compared with the baseline (before occlusion, pre).

Fig. 2. Klüver-Barrera staining of the corpus callosum. Pre: before ligation of bilateral common carotid artery (LBCCA); post: immediately after LBCCA, and at 3, 7, 14 and 28 days after LBCCA. Vacuolation and demyelinated fibers were prominent at 28 days after LBCCA, compared with the control group. Scale bar = 20 μm.

Fig. 3. (A) Photomicrographs of GFAP-positive cells (a–j), GSTπ-positive cells (k-o), NG2-positive cells (p-t) and Iba-1-positive cells (u-y) after ligation of bilateral common carotid artery (LBCCA). Scale bar = 50 μm. (B) Density of GFAP-positive cells in white matter and cortex, (C) density of GSTπ-positive cells in the white matter, (D) density of NG2-positive cells in white matter and (E) density of Iba-1-positive cells in the white matter. Data are mean ± SEM of five rats in each group. *P* < 0.05, **P** < 0.001, compared with the baseline (pre).

Fig. 4. Photomicrographs of EAAT2-positive cells in the white matter (a–e) and
cortex (f-j), EAAT1-positive cells in the white matter (k-o), and EAAT3-positive cells (p-t) after ligation of bilateral common carotid artery (LBCCA). Scale bar = 50 μm. EAAT2 labeling increased significantly at 3 days after LBCCA.

Fig. 5. (A, B) Western blotting analysis of EAAT1, EAAT2 and EAAT3 after ligation of bilateral common carotid artery (LBCCA) in the: (A) cortex and (B) white matter. Note the 60-, 62- and 57-kDa bands corresponding to EAAT1, EAAT2, EAAT3 proteins, respectively. Equal protein loading was confirmed by measuring α-tubulin. (A’, B’). Densitometric analysis of EAAT1, EAAT2 and EAAT3 protein after LBCCA (A’) in the cortex and (B’) white matter. Representative results of three experiments with similar results. Data in A’ and B’ are mean ± SEM of five rats in each group, *P < 0.05, **P < 0.001, compared with the baseline (pre).

Fig. 6. (A) (a) Proportion of EAAT2/GFAP double-stained cells, (b) EAAT2/APC double-stained cells, (c) EAAT2/NG2 double-stained cells and (d) EAAT2/Iba-1 double-stained cells in the white matter. (B) Proportion of EAAT1/APC double-stained cells. (C) Proportion of EAAT3/PDGFRα double-stained cells in the white matter after ligation of bilateral common carotid artery.

Fig. 7. (A) Photomicrographs of EAAT2-positive cells in human brain. Scale bar = 500 μm. (B) Density of EAAT2-positive cells in the white matter. Data are mean ± SEM, **P < 0.001, compared with the control group.
Table 1. Characteristics of autopsy cases.

<table>
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<th>Case</th>
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<th>Diagnosis</th>
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<td>1</td>
<td>76</td>
<td>M</td>
<td>1</td>
<td>Multiple cerebral infarction</td>
</tr>
<tr>
<td>2</td>
<td>80</td>
<td>F</td>
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<td>M</td>
<td>1</td>
<td>Binswanger disease</td>
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<td>5</td>
<td>41</td>
<td>M</td>
<td>2</td>
<td>Lipid aturage myopathy, chronic renal failure</td>
</tr>
<tr>
<td>6</td>
<td>67</td>
<td>M</td>
<td>2</td>
<td>Left putaminal hemorrhage</td>
</tr>
</tbody>
</table>

Cases were divided into two groups based on the presence (or absence) of white matter damage: group 1: patients with chronic hypoperfusion, group 2: patients without white matter damage.
A. 

<table>
<thead>
<tr>
<th></th>
<th>pre</th>
<th>3 day</th>
<th>7 day</th>
<th>14 day</th>
<th>28 day</th>
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<tr>
<td>Iba-1 White matter</td>
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Bars=50µm

B. 

**GFAP positive cells (cells/0.25mm²)**
- White matter
- Cortex

C. 

**GST-pi positive cells in WM (cells/0.25mm²)**
- Time after LBCCA (days)

D. 

**NG2 positive cells in WM (cells/hemisphere)**
- Time after LBCCA (days)

E. 

**Iba-1 positive cells in WM (cells/0.25mm²)**
- Time after LBCCA (days)
A. Ischemic brain
   Case 1  Case 2  Case 3
   a  b  c

   Bars=100μm

B. EAAT2 positive cells (cells/0.25mm²)

<table>
<thead>
<tr>
<th>Ischemic brain</th>
<th>control</th>
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<tr>
<td><strong>[100]</strong></td>
<td>[60]</td>
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</table>

** indicates statistical significance.