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Dynamic expression of cerebral cortex and hippocampal glutamate transporters in a rat model of chest compression-induced global cerebral ischemia☆

Qinhua Guo[1], Jin Lan[1], Weiqiao Zhang[1], Pin Guo[1], Liemei Guo[1], Zhiqiang Li[2], Yongming Qiu[1]

1*Department of Neurosurgery, Renji Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200127, China*
2*Central Hospital of Fengxian District, Shanghai Neurological Research Institute of Anhui University of Science & Technology, Shanghai 201400, China*

Abstract

The present study established a rat model of global cerebral ischemia induced by chest compression for six minutes to dynamically observe expressional changes of three glutamate transporters in the cerebral cortex and hippocampus. After 24 hours of ischemia, expression of glutamate transporter-1 significantly decreased in the cerebral cortex and hippocampus, which was accompanied by neuronal necrosis. At 7 days post-ischemia, expression of excitatory amino acid carrier 1 decreased in the hippocampal CA1 region and cortex, and was accompanied by apoptosis. Expression of glutamate-aspartate transporter remained unchanged at 6 hours–7 days after ischemia. These results suggested that glutamate transporter levels were altered at different periods of cerebral ischemia.

Key Words: apoptosis; excitatory amino acid carrier 1; global cerebral ischemia; glutamate-aspartate transporter; glutamate transporter; glutamate transporter-1; neuroprotection

Qinhua Guo☆, Studying for doctorate, Department of Neurosurgery, Renji Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200127, China

Corresponding authors: Yongming Qiu, Doctor, Professor, Department of Neurosurgery, Renji Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200127, China; Zhiqiang Li, Doctor, Central Hospital of Fengxian District, Shanghai Neurological Research Institute of Anhui University of Science & Technology, Shanghai 201400, China
Qiuzhoub@hotmail.com; lzq_999@163.com

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INTRODUCTION

Dysfunctional cerebral blood flow and cerebral microcirculation has been thought to be the main source of pathological damage in global cerebral ischemia[1]. However, functional imaging has shown that dysfunctional energy metabolism and disseminated inhibition is the primary mechanism of pathological damage following cerebral ischemia[1]. Excessive glutamate accumulates in the synaptic cleft, acts on postsynaptic receptors, and plays a role in excitotoxicity by triggering a series of downstream mechanisms of injury that ultimately lead to neuronal insults[2-3]. The glutamate transporters mediate ambient glutamate concentrations through functional changes of reuptake and reversal transport[4]. Studies focused on the effect of single glutamate transporters on cerebral ischemia have shown that glutamate transporters are potential therapeutic targets for neurological diseases, such as motoneuron degeneration and ischemic injury[5-6]. However, global cerebral ischemia is a result of multiple factors and involves different functional mechanisms and pathological changes during the various phases of disease[7]. Very little is known about successive expression of multiple glutamate transporters following global cerebral ischemia.

The present study analyzed expression of three major glutamate transporters following global cerebral ischemia induced by chest compression to provide scientific evidence for the management of global cerebral ischemia.

RESULTS

Quantitative analysis of experimental animals

A total of 42 rats were randomly assigned to three groups: control (n = 6; routinely fed), sham-surgery (n = 6; free of chest compression, but subjected to the same procedures as the global cerebral ischemia group), and global cerebral ischemia (n = 30; global cerebral ischemia) which was further subdivided into 6 hours and 1, 2, 3, 5, and 7 days subgroups (n = 5 for each time point). All 42 rats were included in the final analysis.

Cerebral cortical and hippocampal glutamate transporter-1 (GLT-1), glutamate-aspartate transporter (GLAST), and excitatory amino acid carrier 1 (EAAC1) expression following global cerebral ischemia

Three major glutamate transporters,

including two glial (GLT-1 and GLAST) transporters and one neuronal (EAAC1) high-affinity transporter, were analyzed[8]. Neurons with a yellowish-brown cell membrane were regarded as positive (Figure 1).

Glutamate transporter expression in hippocampal CA1 region

At 6 hours after ischemia, GLAST expression was decreased in the global cerebral ischemia group compared with the control group, but there was no statistical difference. GLAST expression remained low thereafter, but reached the lowest level at 3 days ($P < 0.05$). At 7 days, GLAST expression returned to normal levels ($P > 0.05$). GLT-1 expression was decreased by 6 hours after ischemia and reached the lowest level at 1 day ($P < 0.05$). From 3–7 days, GLT-1 expression was significantly increased in the global cerebral ischemia group ($P < 0.05$). At 6 hours after ischemia, EAAC1 expression was slightly decreased in the global cerebral ischemia group compared with the control group, but the differences were not significant ($P > 0.05$). EAAC1 expression continued to decrease after 1 day ($P < 0.05$; Figure 2A).

Glutamate transporter expression in the hippocampal CA3 region

Following global cerebral ischemia, GLAST expression slightly increased compared with the control group ($P > 0.05$). At 6 hours after ischemia, GLT-1 expression was significantly decreased in the global cerebral ischemia group compared with the control group ($P < 0.05$), but expression was significantly increased at 3 days compared with the control group ($P < 0.05$). From 6 hours to 2 days, EAAC1 expression was decreased in the global cerebral ischemia group compared with the control group ($P < 0.05$). There was no significant difference between global cerebral ischemia and control groups after 3 days of ischemia (Figure 2B).

Glutamate transporter expression in the cortical motor area

At 6 hours after ischemia, GLAST expression was similar between the global cerebral ischemia and control groups, but expression was significantly decreased at 3 days in the global cerebral ischemia group ($P < 0.05$). At 7 days, GLAST expression returned to normal levels compared with the control group ($P > 0.05$). At 6 hours after ischemia, GLT-1 expression was significantly decreased in the global cerebral ischemia group compared with the control group ($P < 0.05$). After 3 days, GLT-1 expression was significantly increased in the global cerebral ischemia group compared with the control group ($P < 0.05$); at 6 hours, EAAC1 expression was slightly decreased in the global cerebral ischemia group compared with the control group ($P > 0.05$), and expression continued to decrease after 3 days in the global cerebral ischemia group ($P < 0.05$; Figure 2C).

Pathological changes in the cortex and hippocampus in a rat model of global cerebral ischemia

At 6 hours after ischemia, hematoxylin-eosin staining showed neuronal necrosis in the hippocampal CA1 region and significantly less neurons (Figure 3). The number of pyramidal cells was decreased and lacked unity and coherence. In addition, the cell bodies were swollen. However, eosinophilia stain reactions or necrotic, pyknotic pyramidal cells were not observed. At 1 day after ischemia, the number of neurons further decreased, representing only 30% of the control group. However, by 3 days after ischemia, the number of neurons was slightly recovered.

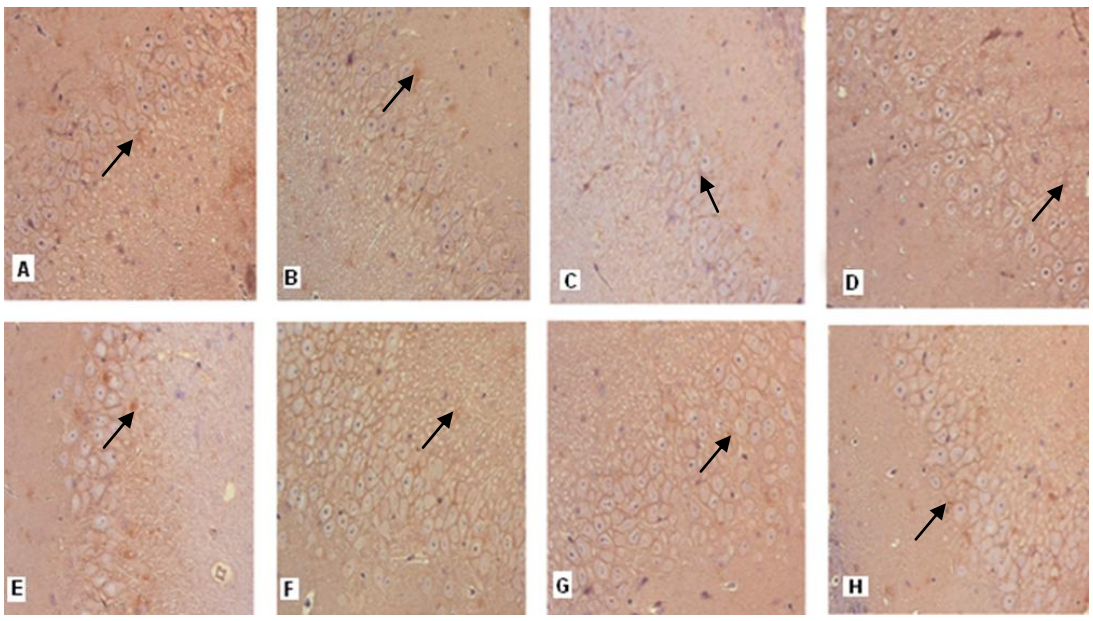

Figure 1 Glutamate transporter-1 expression in hippocampal CA1 in different groups and at different time points (immunohistochemical staining, light microscope, × 400). Cells with yellowish-brown membranes are positive (arrows).

(A) Control group; (B) sham-surgery group; (C–H) global cerebral ischemia for 6 hours, 1, 2, 3, 5, and 7 days.

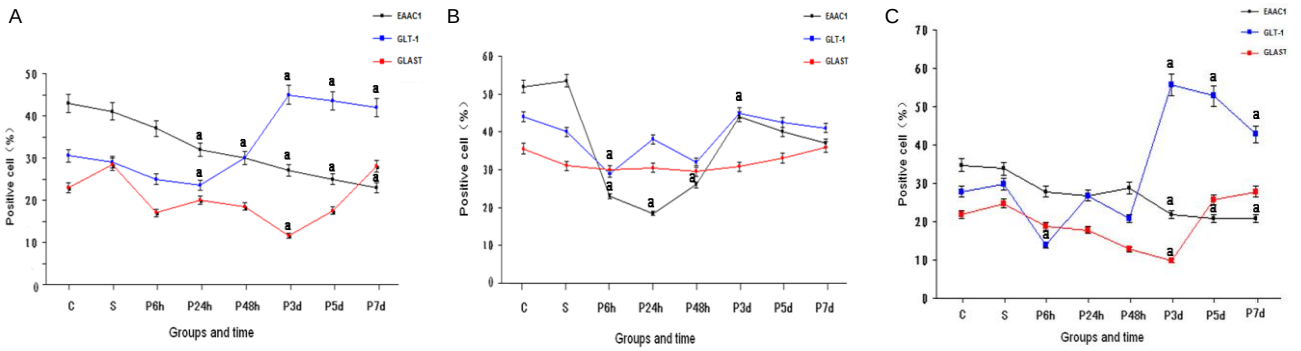

Figure 2 Expression of glutamate transporters in hippocampal CA1/CA3 regions and cortex of rats.

(A) CA1 region; (B) CA3 region; (C) motor cortex. Data are expressed as mean ± SD from six rats in the control and sham-surgery groups, respectively; in the global cerebral ischemia group, there are five rats for each time point. $^{a}P < 0.05$, *vs.* control group (analysis of variance). Positive rate (%) = EAAC1/GLT-1/GLAST positive neural/glial cells/total neural/glial cells. GLT-1: glutamate transporter-1; GLAST: glutamate-aspartate transporter; EAAC1: excitatory amino acid carrier 1. C: Control; S: sham-surgery; P: post-ischemia.

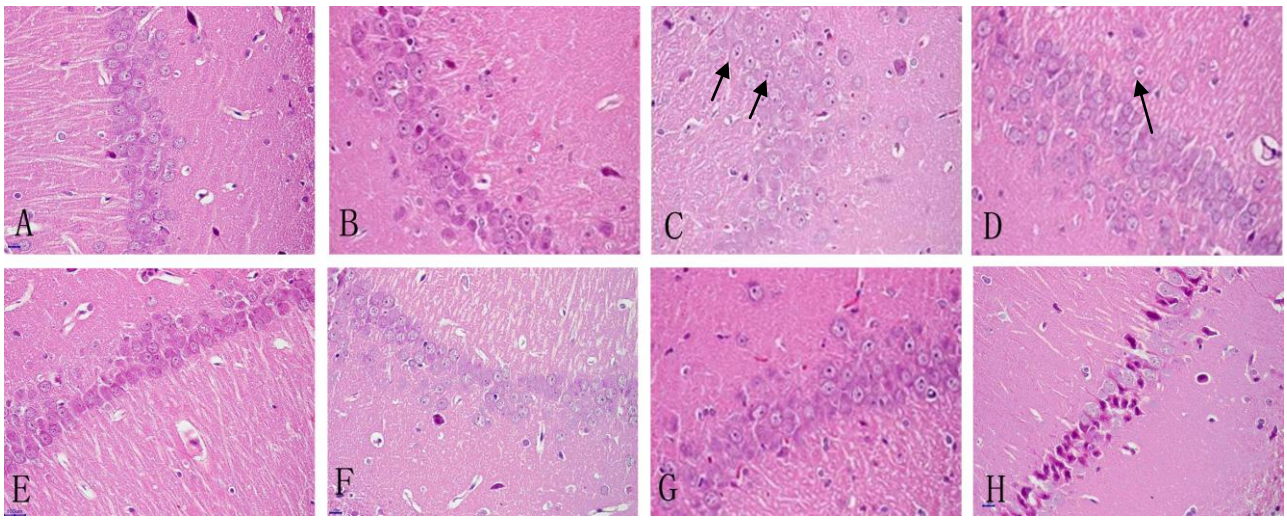

Figure 3 Pathological changes in hippocampal CA1 region in different groups and time points (hematoxylin-eosin staining, light microscopy, × 400). Necrotic neurons (arrows) are observed in earlier periods of cerebral ischemia, but are partially recovered during later periods of cerebral ischemia.

(A) Control group; (B) sham-surgery group; (C–H) global cerebral ischemia for 6 hours, 1, 2, 3, 5, and 7 days.

In the hippocampal CA3 region, the number of neurons decreased by 6 hours after ischemia, but no necrotic neurons were observed. At 1 day after ischemia, the number of neurons significantly decreased, and necrotic pyramidal cells were observed. At 2 days after ischemia, the number of neurons significantly decreased and the nuclei were deeply stained. At 3 days after ischemia, neuronal morphology was partially recovered. At 7 days after ischemia, neuronal morphology was similar to the control group.

Cortical neurons were significantly injured by ischemia. Necrosis of cortical neurons was observed at 6 hours after ischemia, nuclei exhibited an eosinophilia stain reaction, and necrosis was concentrated in cortical layers III-V. At 1 and 2 days after ischemia, the amount of necrosis increased. At 3 days after ischemia, the number of necrotic neurons decreased, and at 7 days after ischemia, dying cells were eliminated.

Ultrastructural changes in cortical and hippocampal neurons in a rat model of global cerebral ischemia

Transmission electron microscopy suggested pathological changes in cortical neurons at 6 hours after ischemia, which was manifested by decreased synaptic numbers, mitochondrial swelling, and loss of membrane cristae (Figure 4). At 2 days after ischemia, neuronal damage was most severe, but gradually decreased at 3–7 days. At 7 days after ischemia, apoptotic bodies emerged. Intracellular ultrastructural changes in the hippocampal CA1 and CA3 regions were similar to the cortex.

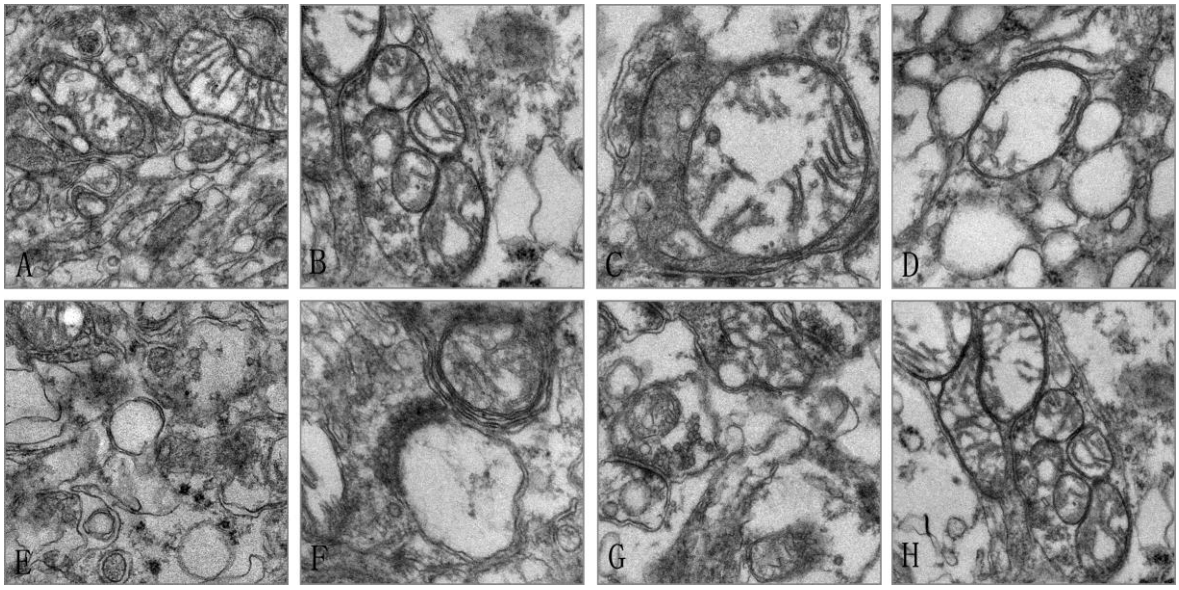

Figure 4 Neuronal ultrastructure in cortical motor area (transmission electron microscope, × 5 000). Mitochondrial swelling and loss of membrane cristae are obvious during the earlier stage, but gradually recovers with time.

(A) Control group; (B) sham-surgery group; (C–H) global cerebral ischemia for 6 hours, 1, 2, 3, 5, and 7 days.

Neuronal apoptosis in the rat cerebral cortex and hippocampus

At 2 days after global cerebral ischemia, necrosis, swelling, and neuronal depletion was observed. However, apoptotic bodies were not present. At 3 days after ischemia, apoptosis was significant. The number of apoptotic cells significantly increased at 7 days compared with 3 days after ischemia.

DISCUSSION

A previous study from our group showed that the chest compression model accurately simulates global cerebral ischemic events, such as cardiac arrest[9]. The intervention of glutamate transporter expression is thought to induce changes in nervous system structure and function. For example, Kawahara *et al* [10] reported that sublethal ischemia leads to an increased tolerance against subsequent prolonged cerebral ischemia *in vivo*. Preconditioning-induced neuronal ischemic tolerance in cortical neuron/astrocyte co-cultures resulted in significantly increased extracellular glutamate concentrations. Treatment with a GLT-1 blocker significantly suppressed the glutamate increase and reduced preconditioning-induced neuronal ischemic tolerance. Mitani *et al* [11] demonstrated a relationship between ischemic severity and glutamate transporter function in GLT-1 mutant mice, concluding that mild ischemia (5 minutes) results in a re-absorption function in the glutamate transporters. Neuronal damage in the mutant mice was exhibited by delayed necrosis. However, when ischemia lasted for 20 minutes, the transporters altered from a re-absorption function to reversal transport during late ischemia (12.5 minutes). Consequently, extracellular glutamate concentrations in wild-type mice were significantly greater than mutant mice. In addition, early neuronal necrosis was apparent. These results demonstrated that, during the early stage of ischemia, GLT-1 exerts a re-absorption function, but GLT-1 also functions as a reverse transporter and releases glutamate to the extracellular space, thereby inducing neuronal necrosis. Glutamate transporter function highly correlates with the ischemic period. Nelson *et al* [12] studied glutamate release following ischemia using a variety of ion channel blockers and inhibitors of glutamate transporters, demonstrating that ischemia-induced glutamate is calcium-dependent during the early stage of release, but later release involves volume-activated ion currents. Glutamate transporters are involved in the entire glutamate-releasing process, and changes in glutamate transporter functions have been observed after complete cerebral ischemia through the use of the competitive glutamate transporter blocker, DL-threo-β-benzyloxyaspartate[13], suggesting that glutamate transporters induce reverse transport following ischemia, which leads to increased extracellular glutamate.

The diversity and extent of these changes are dependent on different models and management. It is possible to alter cerebral ischemic injury by intervening with glutamate transporters. The present study systematically analyzed expression of three important glutamate transporters, GLT-1, GLAST, and EAAC1, in an animal model of cerebral ischemia induced by chest compression. The relationships between variations in glutamate transporters expression and neuropathological

changes after ischemia were also analyzed.
Results revealed expression trends of glutamate transporters in different brain regions, resulting in varying pathological changes. Because of the close relationship between glutamate transporter expression and neurological deficits following global cerebral ischemia[8], altered expression of glutamate transporters could result in neurological deficits. Results from the present study showed that GLAST and GLT-1 expression was decreased at 1 day after global cerebral ischemia, which was accompanied by neuronal necrosis. However, at 7 days after ischemia, GLT-1 expression increased and EAAC1 expression decreased, and these changes were accompanied by apoptosis. These results suggested that changes in GLT-1 and EAAC1 expression played an important role in the observed neuropathological changes.
In summary, glutamate transporters were shown to be involved in neuropathology following global cerebral ischemia. Results suggested that glutamate transporters could serve as effective targets for ischemia treatment. During the early stage of ischemia, measures should be taken to restore GLT-1 expression and increase reabsorption efficiency of glutamate transporters, and the primary strategy should be to decrease neuronal necrosis. During the late stage of ischemia, measures should be taken to restore EAAC1 expression, as well as provide effective anti-apoptotic measures.

MATERIALS AND METHODS

Design

A randomized, controlled, animal experiment.

Time and setting

The present study was performed at the Laboratory Animal Center, Shanghai Jiao Tong University School of Medicine, China between December 2009 and September 2011.

Materials

A total of 42 male, Sprague-Dawley rats, aged 8–9 weeks and weighing 220–250 g, were provided by the Shanghai Laboratory Animal Center (SCXK 2007-0005). All experimental procedures were performed in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China[14].

Methods

Establishment of a global cerebral ischemia model

A rat model of global cerebral ischemia was induced by chest compression as previously described[10, 15]. Briefly, rats were anesthetized with 2% pentobarbital (0.3 mL/kg, i.p.). During surgery, body temperature was maintained at 37 ± 0.5°C with a heating pad. Endotracheal intubation was performed under direct vision, and a ventilator was utilized. The respiratory rate was maintained at 75 times/min. A multi-channel physiological monitor (78354C, Hewlett Packard, Palo Alto, CA, USA) was connected to the right femoral artery to monitor blood pressure, heart rate, and blood gas levels. The weight of compression was eight times the rat body mass and lasted for 6 minutes[9]. Following chest compression, blood pressure decreased to 15 mm Hg (1 mm Hg= 0.133 kPa), which subsequently decreased to nearly 0 mm Hg with cardiac arrest and respiration, resulting in global cerebral ischemia. After 6 minutes of chest compression, cardio-pulmonary resuscitation was immediately applied until spontaneous breathing was recovered and the rat was fully awake. The rats were returned to animal housing, and room temperature was maintained at 25 ± 1°C. The ischemic time was equal to chest compression time (6 minutes)[11].

Preparation of brain tissue

The rats were intraperitoneally anesthetized with pentobarbital, and a perfusion needle was inserted into the ascending aorta. A constant infusion pump was used to infuse 200 mL isotonic ice-cold saline and 400 mL 4% paraformaldehyde in 0.05 M phosphate-buffered saline (PBS; pH 7.4). Following sacrifice at ice-cold temperature, the brain was post-fixed in the same fixative at room temperature. Coronal sections (2-mm thick) were dissected beginning at bregma and continued in a caudal direction.

Hematoxylin-eosin pathology of hippocampal CA1/CA3 regions and cortical motor area

Hippocampal CA1/CA3 regions and motor cortex were dissected, and following dehydration with ethyl alcohol, the tissue was immersed in paraffin for embedding. Ten-μm thick sections were cut for hematoxylin-eosin staining. A tissue microarray was made, and six points from each tissue slice were selected. Morphological changes were observed under a light microscope (Olympus, Tokyo, Japan).

GLT-1, EAAC1, and GLAST immunohistochemistry in the hippocampus and cortex

Glutamate transporter expression was analyzed by immunohistochemistry utilizing the streptavidin-biotin complex method. The UltraSensitive SP immunohistochemistry kit (Maixin Biotechnology Company, Fuzhou, Fujian Province, China) was used for immunohistochemical staining. Paraffin-embedded sections were dewaxed, rehydrated in water, incubated in 150 μL peroxidase blocking solution (0.3% H_2O_2) for 10 minutes to eliminate endogenous peroxidase activity. Samples were incubated in 150 μL goat anti-rat GLT-1 (or anti-GLAST, anti-EAAC1) monoclonal antibody (1: 200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) in a humidity chamber at 4°C overnight (about 24 hours), followed by 150 μL biotinylated rabbit anti-goat IgG (1: 2 000; Santa Cruz Biotechnology) at room temperature for 10 minutes. The sections were then incubated in 100 μL high-sensitivity peroxidase complex (Santa Cruz Biotechnology) at room temperature for 45 minutes, and the staining was developed with diaminobenzidine. PBS (0.01 M) was used, rather than primary antibody, for the negative control. Positive neuronal membranes were stained brown. A light

microscope was used to observe, and a KS400 image analysis system (Ver 3.0; Zeiss, Oberkochen, Germany) was used for image analysis. The image analysis system identified and calculated the number and proportion of positive cells. Successive expressional changes were analyzed in the CA1 and CA3 regions of the hippocampus and in the cortical motor area. The percentage of positive neural cells to glial cells was used as a parameter for analysis.

Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling detection in the hippocampus and cortex

Paraffin-embedded tissues sections were placed in a staining jar, rinsed twice with xylene, rinsed with PBS, and incubated with 20 μg/mL protease K solution for 10 minutes. Subsequently, 54 μL TdT enzyme reaction fluid was dropped onto the tissue sections and the sections were incubated at 37°C in a humidity chamber, followed by two drops of peroxidase-labeled anti-digoxin antibodies (Santa Cruz Biotechnology) and freshly prepared 0.05% diaminobenzidine solution at room temperature. The sections were then counter-stained with methyl green for 10 minutes at room temperature. Following dehydration with xylene, the sections were observed under a light microscope. The hippocampus and cortex samples were cut into 1-mm^3 cubes, fixed in gluteraldehyde dehydrated in acetone, embedded in epoxide resin, cut into 100-nm thick ultrathin sections, stained with uranyl acetate and lead citrate, and observed using a transmission electron microscope (Olympus).

Statistical analysis

Data were analyzed using SPSS 10.0 software (SPSS, Chicago, IL, USA) for statistical analysis and were expressed as mean ± SD. The analysis of variance test was used to evaluate differences between groups. The Dunnett calibration and analysis of variance were used for data analysis between groups. $P < 0.05$ was considered statistically significant.

Author contributions: Qinhua Guo conducted the majority of the experiment and wrote the manuscript. Jin Lan contributed to data analysis. Weiqiao Zhang conducted the experiment. Pin Guo and Liemei Guo completed statistical analyses. Zhiqiang Li and Yongming Qiu conceived and designed the study, revised the manuscript, and were responsible for funding.

Conflicts of interest: None declared.

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Ethical approval: The protocol of this study was approved by the Animal Care and Use Committee and was conducted in accordance with the Guide for the Care and Use of Laboratory Animals of Shanghai Jiao Tong University.

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