NOTE *Anatomy*

Transient Increases of Glutamic Acid Decarboxylase 67 Immunoreactivity and Its Protein Levels in the Somatosensory Cortex after Transient Cerebral Ischemia in Gerbils

In Koo HWANG[1)], Ki-Yeon YOO[2)], Hua LI[2)], Ok Kyu PARK[2)], Choong Hyun LEE[1)], Jung Hoon CHOI[2)], Dae Young KWON[3)] and Moo-Ho WON[2,4)]*

[1)]*Department of Anatomy and Cell Biology, College of Veterinary Medicine and BK21 Program for Veterinary Science, Seoul National University, Seoul 151–742,* [2)]*Department of Anatomy and Neurobiology, College of Medicine, Hallym University, Chuncheon 200–702,* [3)]*Korea Food Research Institute, Songnam 463–746 and* [4)]*Institute of Neurodegeneration and Neuroregeneration, Hallym University, Chuncheon 200–702, South Korea*

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ABSTRACT. In this study, we investigated changes in glutamic acid decarboxylase 67 (GAD67) immunoreactivity and its protein levels in the gerbil somatosensory cortex after ischemia/reperfusion. GAD67 immunoreactivity was significantly increased in layers III and V of the somatosensory cortex 12 hr after ischemia/reperfusion. Thereafter, GAD67 immunoreactivity was decreased with time after ischemia/reperfusion. GAD67 immunoreactivity in the somatosensory cortex 4 days after ischemia/reperfusion was similar to that in the sham-operated group. In addition, GAD67 protein levels were also significantly increased 12 hr after transient forebrain ischemia. These results suggest that the transient increase of GAD67 immunoreactivity in layers III and V may be associated with responses to transient ischemia-induced neuronal damage.

KEY WORDS: GABA, GAD67, gerbil, somatosensory cortex, transient forebrain ischemia.

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Transient cerebral ischemia causes extensive neuronal injuries in several regions such as neocortex, striatum and hippocampus: The progression of neuronal damage in the neocortex is moderate [23], while in the hippocampal CA1 region the neuronal death is delayed [2, 22]. In the neocortex, neuronal damage is observed mainly in layers III and VI of the gerbil and rat somatosensory cortex after ischemia/reperfusion [3, 6, 14, 23].

Transient cerebral ischemia depletes oxygen and ATP in brain neurons. In this condition, excessive glutamate is released, and glutamate transporter is not able to reabsorb this glutamate because of intracellular ATP depletion. Finally, glutamate is concentrated in the extracellular space. This excessive glutamic acid binds to *N*-methyl-*D*-aspartate (NMDA) and/or non-NMDA receptors in the postsynaptic membrane, increasing cell permeability to Ca^{2+} [12, 28, 31]. A high concentration of free Ca^{2+} is thought to cause neuronal death by activation of calcium-dependent proteinase in cells [1, 30, 33].

Glutamatergic neurons are the vast majority of neocortical neurons with long axons; GABAergic neurons account for 15–25% of the neocortical neurons, and they function as inhibitory neurons [11, 34]. It has been generally accepted that GABAergic neurons in the brain are resistant to ischemic damage [29], because GABAergic inhibition serves to control the NMDA receptor-mediated excitatory system in the cortex [19, 24–27].

* CORRESPONDENCE TO: Prof. WON, M. H., Department of Anatomy and Neurobiology, College of Medicine, Hallym University, Chuncheon 200–702, South Korea.
e-mail: mhwon@hallym.ac.kr

It is well known that glutamic acid decarboxylase (GAD) is a rate-limiting enzyme for GABA synthesis and that it is used as a reliable marker for inhibitory interneurons [7, 9]. GAD65 is important for the local control of GABA synthesis at the synaptic sites; whereas GAD67 is responsible for maintaining GABA baseline levels for both neurotransmitter and metabolite [37, 38]. It has been reported that the administration of GABA improves the emotional states and signs caused by neuronal dysfunction in aged animals [15].

There are some reports on changes in GAD67 in the hippocampus after transient forebrain ischemia in rats [16, 32] and gerbils [17, 29], however, there is no study about changes in GAD67 in the neocortex after transient forebrain ischemia. In the brain, somatosensory area is very important to control the complex movements [10] and to modulate the neural rehabilitation in patients with brain lesions [5]. In the present study, therefore, we investigated changes in GAD67 immunoreactivity and its protein levels in the somatosensory cortex after transient forebrain ischemia in gerbils.

The progeny of male Mongolian gerbils (*Meriones unguiculatus*) were obtained from the Experimental Animal Center, Hallym University, Chuncheon, South Korea. Gerbils were used at 6 months (B.W., 65–75 g) of age. The animals were housed in a conventional state under adequate temperature (23°C) and humidity (60%) control with a 12-hr light/12-hr dark cycle, and provided with free access to food and water. The procedures for handling and caring for animals adhered to the guidelines that are in compliance with the current international laws and policies (NIH Guide for the Care and Use of Laboratory Animals, NIH Publication

No. 85–23, 1985, revised 1996), and they were approved by the Institutional Animal Care and Use Committee (IACUC) at Hallym's Medical Center. All of the experiments were conducted to minimize the number of animals used and the suffering caused by the procedures used in the present study.

The animals were anesthetized with a mixture of 2.5% isoflurane in 33% oxygen and 67% nitrous oxide. Bilateral common carotid arteries were isolated and occluded using non-traumatic aneurysm clips. The complete interruption of blood flow was confirmed by observing the central artery in retinae using an ophthalmoscope. After 5 min of occlusion, the aneurysm clips were removed from the common carotid arteries. The body (rectal) temperature under free-regulating or normothermic (37 ± 0.5°C) conditions was monitored with a rectal temperature probe (TR-100; Fine Science Tools, Foster City, CA, U.S.A.) and maintained using a thermometric blanket before, during and after the surgery, until the animals completely recovered from anesthesia. Thereafter, animals were kept on the thermal incubator (Mirae Medical Industry, Seoul, South Korea) to maintain the body temperature of animals, until the animals were euthanized. Sham-operated animals were subjected to the same surgical procedures except that the common carotid arteries were not occluded.

To obtain the accurate data for GAD67 immunoreactivity, the sections from sham- and ischemia-operated animals (n=7 at each time point) were used at designated times (3 hr, 12 hr, 2 days and 4 days) under the same condition. The animals were anesthetized with sodium pentobarbital and perfused transcardially with 0.1 M phosphate-buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde in 0.1 M phosphate-buffer (PB, pH 7.4). The brains were removed and cryoprotected by infiltration with 30% sucrose overnight. Thereafter, frozen tissues were serially sectioned on a cryostat (Leica, Wetzlar, Germany) into 30-μm coronal sections, and they were then collected into six-well plates containing PBS.

Sections were sequentially treated with 0.3% hydrogen peroxide (H_2O_2) in PBS for 30 min and 10% normal goat serum in 0.05 M PBS for 30 min. They were then incubated with diluted rabbit anti-GAD67 antibody (1:50, Chemicon, Temecula, CA, U.S.A.) overnight at 4°C and subsequently exposed to biotinylated goat goat anti-rabbit IgG and streptavidin peroxidase complex (diluted 1:200, Vector, Burlingame, CA, U.S.A.). They were then visualized by staining with 3,3'-diaminobenzidine in 0.1 M Tris-HCl buffer (pH 7.2) and mounted on gelatin-coated slides.

A negative control test was carried out using pre-immune serum instead of primary antibody in order to establish the specificity of the immunostaining. The negative control resulted in the absence of immunoreactivity in all structures.

To obtain the accurate data for change in GAD67 levels in the somatosensory cortex after transient forebrain ischemia, sham- and ischemia-operated animals (n=5 at each time point) were used for western blot analysis at designated times (3 hr, 12 hr, 2 days and 4 days) after the ischemic surgery. After sacrificing them and removing the brain, it was serially and transversely cut into a thickness of 400 μm on a vibratome (Leica), and the somatosensory cortex was then dissected with a surgical blade. The tissues were homogenized in 50 mM PBS (pH 7.4) containing 0.1 mM ethylene glycol bis (2-aminoethyl Ether)-N,N,N',N' tetraacetic acid (EGTA) (pH 8.0), 0.2% Nonidet P-40, 10 mM ethylendiamine tetraacetic acid (EDTA) (pH 8.0), 15 mM sodium pyrophosphate, 100 mM β-glycerophosphate, 50 mM NaF, 150 mM NaCl, 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM dithiothreitol (DTT). After centrifugation, the protein level in the supernatants was determined using a Micro BCA protein assay kit with bovine serum albumin as a standard (Pierce Chemical, Rockford, IL, U.S.A.). Aliquots containing 50 μg of total protein were boiled in loading buffer containing 150 mM Tris (pH 6.8), 3 mM DTT, 6% SDS, 0.3% bromophenol blue and 30% glycerol. The aliquots were then loaded onto an 8.75% polyacrylamide gel. After electrophoresis, the gels were transferred to nitrocellulose transfer membranes (Pall Crop, East Hills, NY, U.S.A.). To reduce background staining, the membranes were incubated with 5% non-fat dry milk in PBS containing 0.1% Tween 20 for 45 min, followed by incubation with rabbit anti-GAD67 antiserum (1:50), peroxidase-conjugated goat anti-rabbit IgG (Sigma, St. Louis, MO, U.S.A.) and an ECL kit (Pierce Chemical).

In order to quantitatively analyze GAD67 immunoreactivity, the corresponding areas of the somatosensory cortex were measured from 15 sections per animal. Cellular immunoreactivity of GAD67 was graded in the somatosensory cortex. Digital images of the hippocampus were captured with an AxioM1 light microscope (Carl Zeiss) equipped with a digital camera (Axiocam, Carl Zeiss) connected to a PC monitor. Semi-quantification of the immunostaining intensity of GAD67 was evaluated with digital image analysis software (MetaMorph 4.01, MDS Inc., Downingtown, PA, U.S.A.). The mean intensity of GAD 67 immunostaining in each GAD67 immunoreactive neuron was measured by a 0–255 gray scale system (white to dark signal corresponded from 255 to 0). Based on this approach, the level of immunoreactivity was scaled as –, ±, + or ++, representing no staining (gray scale value: ≥200), weakly positive (gray scale value: 150–199), moderate (gray scale value: 100–149), or strong (gray scale value: ≤99), respectively.

The result of the Western blot analysis was scanned, and the quantification of the Western blotting was done using Scion Image software (Scion Corp., Frederick, MD, U.S.A.), which was used to count the ROD: A ratio of the ROD was calibrated as %.

Data are expressed as the mean ± SEM. The data were elevated by a one-way ANOVA SPSS program and the means assessed using Duncan's multiple-range test. Statistical significance was considered at P<0.05.

In the sham-operated group, weak GAD67 immunoreactivity was detected in cells in the somatosensory cortex (Fig. 1A and 1B). In the ischemia-operated groups, GAD67 immunoreactivity was markedly changed in layers III and V

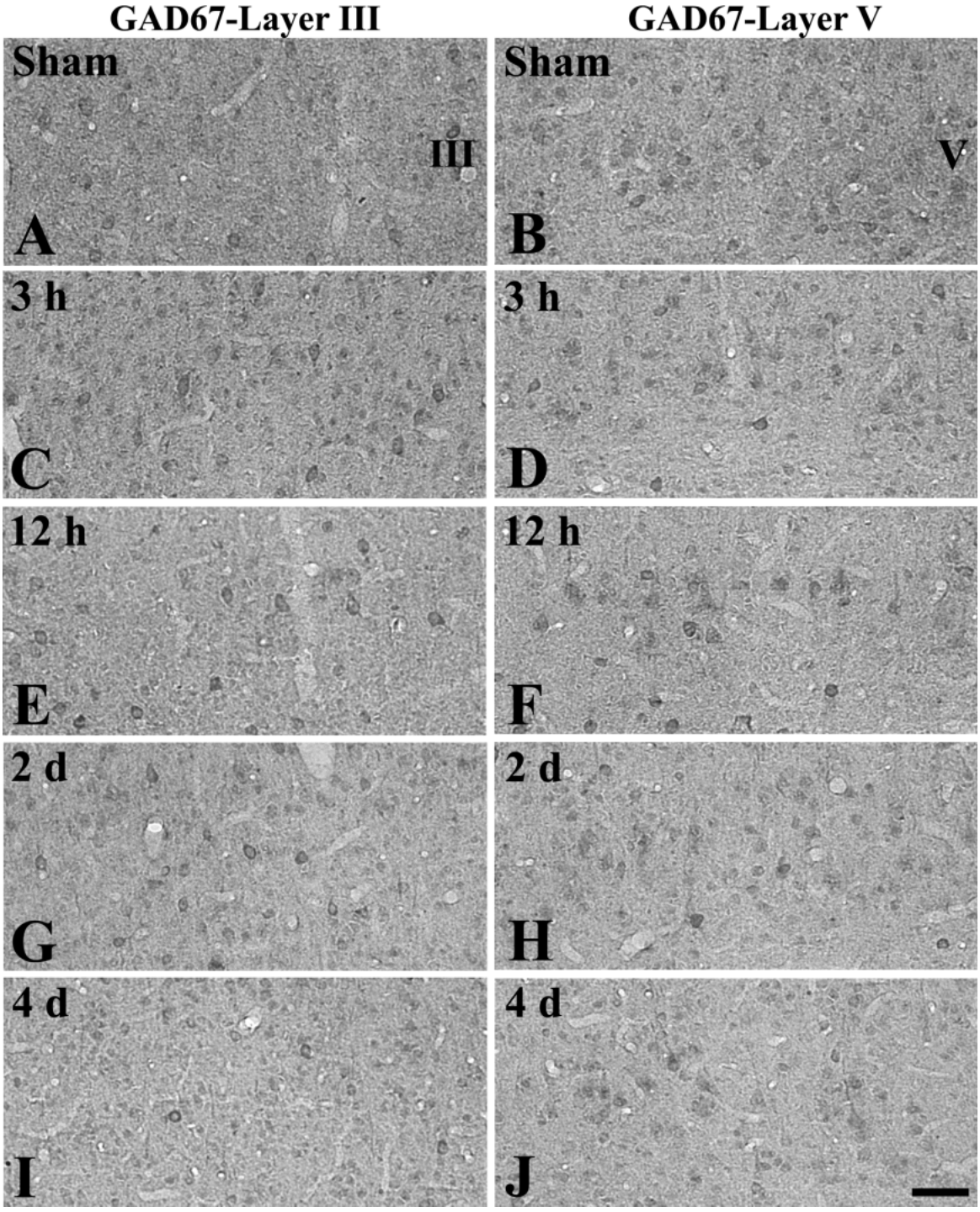


Fig. 1. GAD67 immunoreactivity in layers III and V of the somatosensory cortex in sham- (A and B) and ischemia-operated (C-J) groups. GAD67 immunoreactivity is increased in layers III and V of the somatosensory cortex 12 hr after ischemia/reperfusion. Four days after ischemia/reperfusion, GAD67 immunoreactivity is similar to that in the sham-operated group. Bar=50 μm.

(Table 1, Fig. 1), whereas, in layers I, II, IV and VI, we could not find significant changes in GAD67 immunoreactivity.

Three hours after ischemia/reperfusion, GAD67 immunoreactivity was increased in layers III and V of the somatosensory cortex (Table 1, Fig. 1C and 1D): GAD67

Table 1. The time-course levels of GAD67 immunoreactivity in neurons in the gerbil somatosensory cortex following transient ischemia

	Time after ischemia/reperfusion				
Layers	Sham	3 hr	12 hr	2 d	4 d
Layer III	±	+ ~ ++	++	± ~ +	±
Layer V	+	+ ~ ++	++	+ ~ ++	+

The levels of immunoreactivity were defined as four grades, negative (–), weakly positive (±), moderate (+) and strong (++).

immunoreactive neurons in layer III were more than those in layer V. Twelve hours after ischemia/reperfusion, neurons in layers III and V showed strong GAD67 immunoreactivity (Table 1, Fig. 1E and 1F); however, at this time point the number of GAD67 immunoreactive neurons was lower than that in the 3 hr post-ischemic group. Thereafter, GAD67 immunoreactivity was decreased with time after ischemia/reperfusion (Table 1, Fig. 1G-1J). Four days after ischemia/reperfusion, GAD67 immunoreactivity was decreased in layer III: GAD67 immunoreactivity was similar to that in the sham-operated group (Fig. 1I and 1J).

In the present study, the result of Western blot analysis for GAD67 levels in the ischemic somatosensory cortex showed a similar pattern compared with that of immunohistochemical change after 5 min of transient forebrain ischemia. GAD67 protein level was significantly increased 12 hr after ischemia/reperfusion, thereafter decreased with time (Fig. 2).

Neuroanatomical studies in a wide variety of mammals have revealed that the cerebral cortex contains many glutamatergic and GABAergic neurons. In cerebral ischemia, glutamatergic neurons in the hippocampal CA1 region are degenerated, while GABAergic interneurons are survived [8, 29]. Investigations regarding susceptibility of GABAergic neurons or GAD transcript regulation within the context of ischemic injury to neocerebral cortex are rare. In a mouse model, GABAergic neurons in the cerebral cortex were rather insensitive to short middle cerebral artery occlusion (MCAO) [20].

In the present study, GAD67 immunoreactivity in the sham-operated group was detected in the neuronal cytoplasm in the gerbil somatosensory cortex. The regional location of GAD67 immunoreactive neurons had a good agreement with a previous study using GAD67-green fluorescent protein knock-in mice [36]. In addition, GAD67 is mainly found in the cytoplasm and is responsible for maintaining GABA baseline levels for both neurotransmitter and metabolite [7].

In this study, we found that the intensity of GAD67 immunoreactivity changed markedly in layers III and V, but not in layers I, III, IV and VI. The GAD67 immunoreactivity was significantly increased in the layers III and V of the somatosensory at cortex 12 hr after ischemia/reperfusion. We previously observed that GAD67 immunoreactivity in the hippocampal CA1 region was significantly increased 24 hr after ischemia/reperfusion [17]. This regional difference of GAD67 increase between hipocampal CA1 region and somatosensory cortex may be associated with the vulnera-

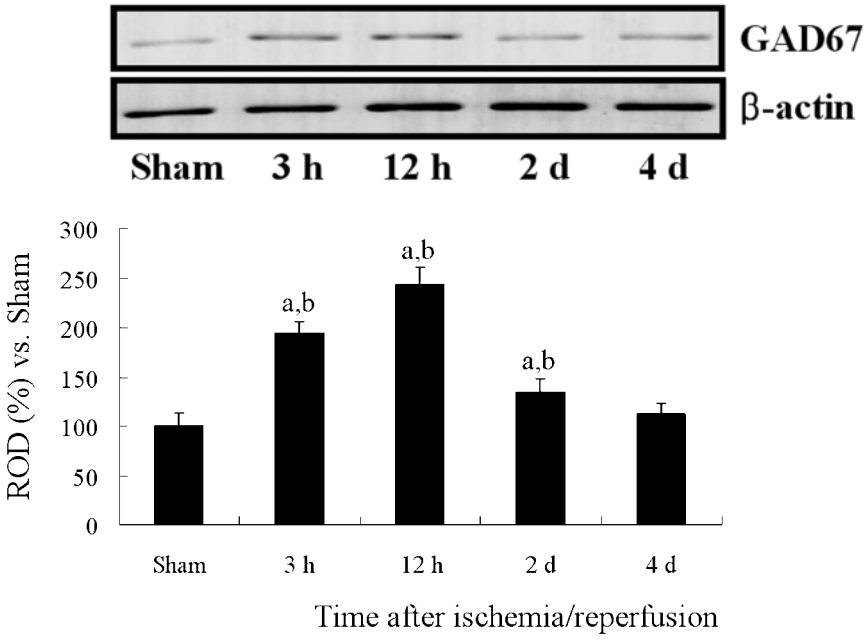


Fig. 2. Changes in GAD67 protein levels in the somatosensory cortex derived from ischemic somatosensory cortex. The relative optical density (ROD) as % values of immunoblot band is represented (n=5 per group, [a] P<0.05, significantly different from the sham-operated group, [b] P<0.05, significantly different from the pre-adjacent group). The bars indicate the means ± SEM.

bility of neurons concerned. In previous studies, neuronal damages are detected in layers III and VI of the gerbil somatosensory cortex at 2 days after ischemia/reperfusion [14, 23], while the neuronal death in the hippocampal CA1 region was observed 4 days after ischemia/reperfusion [13, 14, 21].

On the other hand, it was reported that pyridoxine 5'-phosphate oxidase (PNPO), which may enhance the GABAergic activity, immunoreactivity and protein levels were significantly increased in layers III and V of the gerbil somatosensory cortex 12 hr after ischemia/reperfusion [14]. This may be related with the synthesis of pyridoxal phosphate (PLP) after the ischemic insult [13]. GABA is synthesized by GAD and catabolized by GABA transaminase [18, 35]: Both the enzymes absolutely require PLP as cofactor [4]. In conclusion, the transient increase of GAD67 in layers III and V of the ischemic somatosensory cortex in gerbils may be associated with responses to a transient ischemia-induced neuronal damage.

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