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Research Report

Correlation between extracellular glutamate release and neuronal cell death in an eleven vessel occlusion model in rat

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ABSTRACT

The aim of this study was to define the effects of glutamate release on cell death in an eleven vessel rat occlusion model. Male Sprague–Dawley rats (250–350 g) were used for the 11 vessel occlusion ischemic model, which was induced by a 5- and 10-min transient occlusion. During the surgical procedure, the extracellular glutamate concentration was measured in real-time using a microdialysis amperometirc biosensor with cerebral blood flow. In order to confirm neuronal cell death, brains were removed 72 h after ischemia for the detection of the neuron-specific nuclear protein and cleaved caspase-3 levels, using double-immunofluorescence. A significant decrease in % cerebral blood flow was observed in both the 5- and 10-min 11 vessel occlusion models, while an increase in glutamate release was detected after the onset of ischemia that continued to rise during the ischemic period. However, a significantly higher level of glutamate release was observed in the 10-min ischemia group compared to the 5-min group. Unlike the small amount of brain damage in the 5-min group, the increased glutamate levels in the 10-min group resulted in ischemic cell death in the hippocampal region with the activation of cleaved caspase-3 and the inhibition of neuron-specific nuclear protein expression. This study suggests that the increased level of glutamate release induces apoptotic cell death in the 11 vessel occlusion ischemic model.

1. Introduction

For many years, several modified models have produced a more consistent decreased cerebral blood flow. The eleven vessel rat occlusion (11VO) model introduced in 1998 has been suggested to better mimic complete ischemia by measuring the glutamate levels in real-time (Caragine et al., 1998). It has been reported that the cerebral glutamate levels are higher in 4VO animals than in the 11VO model because 11VO causes a rapid decrease in cerebral blood flow (CBF) to consistently low

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Abbreviations: 11VO, 11 vessel occlusion; NeuN, neuron-specific nuclear protein; C.caspase-3, cleaved caspase-3; CBF, cerebral blood flow; EEG, electroencephalography

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levels, which may reduce the level of brain damage. It was suggested that 11VO may more closely stimulate complete forebrain ischemia in rats (Caragine et al., 1998). Recently, we developed an accurate vascular 11VO model for global ischemia (Choi et al., 2008). Overall, these studies suggest that the 11VO model can easily induce an infarction because of the reproducibility of the CBF plateau to ischemic levels and the faster decrease in CBF compared to other ischemic models.

In ischemia, excitotoxic neuronal damage is affected by glutamate release through the overactivation of its receptors (Taghibiglou et al., 2009). Accumulated extracellular glutamate promotes death-inducing molecules in neurons, which amplifies the impact of the ischemic injury (Taoufik and Probert, 2008). The changes in the local level of L-glutamate release in cultured nerve cells was determined continuously using a glassy carbon electrode (Niwa et al., 1997). The determinations of glutamate release using a sensor system that combines microdialysis sampling have been developed for *in vivo* analysis and continuous monitoring. Real time *in vivo* monitoring of glutamate has been investigated using amperometirc biosensor technology with successful applications in research (Yao et al., 2001). Our previous studies developed a method for detecting extracellular glutamate levels in an 11VO model in real time using either microdialysis (Lee et al., 2008) or enzyme-immobilized carbon nanotube-field effect transistor (CNT-FET) (Lee et al., 2009c). The level of glutamate release between 5- and 10-min 11VO ischemia groups was previously reported (Lee et al., 2009a). Furthermore, although the CNT technique has not been used extensively, this study confirmed that model of 11VO global ischemia is a suitable model for L-glutamate oxidase-immobilized CNT-FET probe network junctions with a fast response time and high sensitivity for glutamate, which are essential for real-time glutamate monitoring (Lee et al., 2009b). This study conducted microdialysis measurements, which are used more widely to determine the glutamate levels. However, in the 11VO global ischemia model, the damaged area and neuron cell death by the increased level of glutamate release have not been studied.

In this study, the level of glutamate released was monitored in real time and compared with the extent of neuronal damage in the hippocampal region of an 11VO model.

2. Results

2.1. *%CBF levels between 5- and 10-min ischemia*

Since the CBF is essential for cerebral brain ischemia, this study examined whether the 11VO model decreased the CBF with the release of glutamate to induce the brain ischemia. The pattern of the ischemia response in the 11VO model was similar in the 5- and 10-min groups (Fig. 1). A rapid decrease in %CBF to 13.5±6.1% of the control levels was observed immediately after 11VO (Table 1), which is consistent with the development of a flat electroencephalography (EEG) (Fig. 1). The time elapsed from the onset of ischemia to the ischemic plateau was within 15.3±2.5 s, and was successfully maintained during the ischemic period. No fluctuations in CBF were observed during the ischemic period in the 5- and 10-min 11VO models. After the initiation of reperfusion, the %CBF increased significantly to the pre-ischemic levels. The time elapsed to reach the peak %CBF levels after the onset of the reperfusion period was 157.8±11.8 s and 668.2±318.3 s in the 5-min and 10-min ischemia group, respectively. The maximum level of %CBF during reperfusion period was 195.6±70.2% and 273.8±56.7% in 5-min and 10-min 11VO group, respectively.

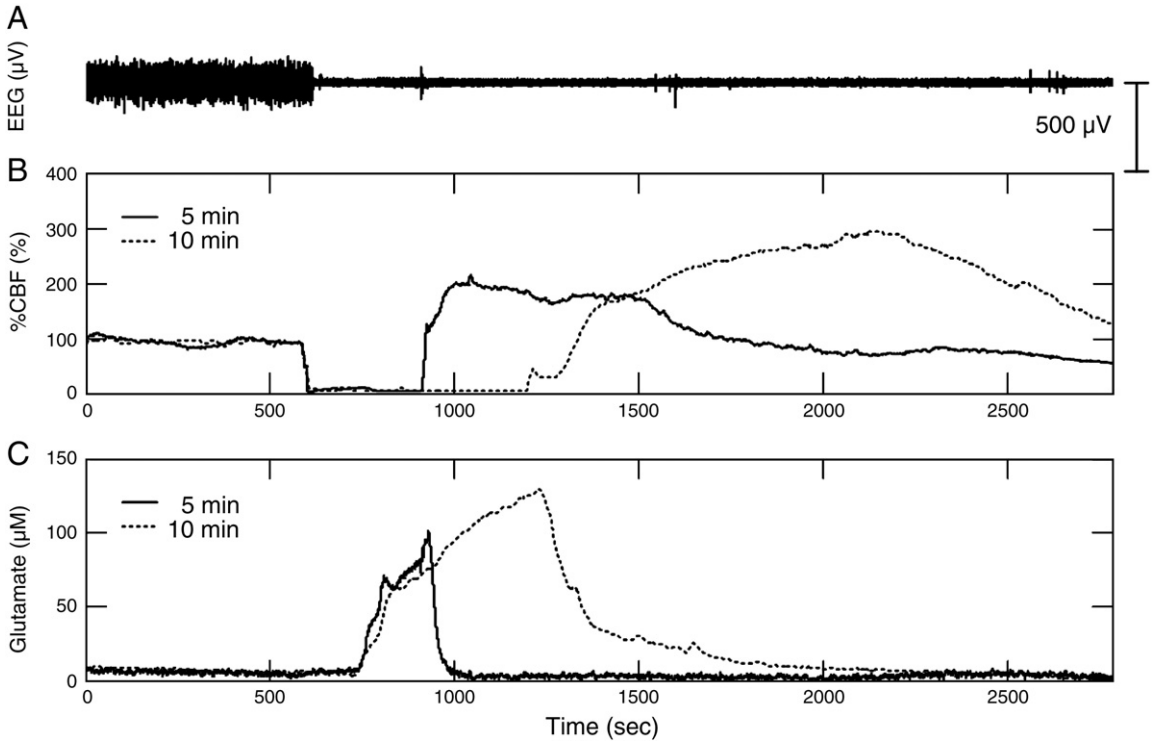

Fig. 1 – Changes in EEG, glutamate and CBF dynamics in the 5- and 10-min 11VO ischemia models.

Table 1 – Changes in %CBF and level of glutamate release in the 5- and 10-min ischemia groups of global ischemia induced by an eleven vessel occlusion method. ($n=4$).

Variable	Averages between 5- and 10-min groups
Average ischemic %CBF level	13.5±6.1
Average elapsed time from the onset of ischemia to the ischemic plateau (s)	15.3±2.5
Average time delay of the beginning of glutamate release after the onset of ischemia (s)	111.3±30.0

Variable	5 min	10 min
Maximum level of ischemic glutamate release (μM)	84.0±32.8	140.0±24.3
Time elapsed to reach maximum level of reperfusion %CBF after the onset of reperfusion period (s)	157.8±11.8	668.22±318.3
Maximum level of reperfusion CBF (%)	195.6±70.2	273.8±56.7
Maximum level of reperfusion glutamate release (μM)	95.0±30.7	141.7±21.6

2.2. *Glutamate release between 5- and 10-min ischemia*

An elevation in level of glutamate release was observed from 111.3±30.0 s after the onset of ischemia with a dramatic increase occurring throughout the entire ischemic period in both groups (Table 1). The glutamate levels in the 5- and 10-min groups decreased quickly to the pre-ischemic levels after reperfusion. The maximum change in the glutamate concentration in 5- and 10-min group during the ischemic period was 84.0±32.8 μM and 140.0±24.3 μM, respectively. After reperfusion, the peak glutamate concentration in the 5- and 10-min group was 95.0±30.7 μM and 141.7±21.6 μM, respectively.

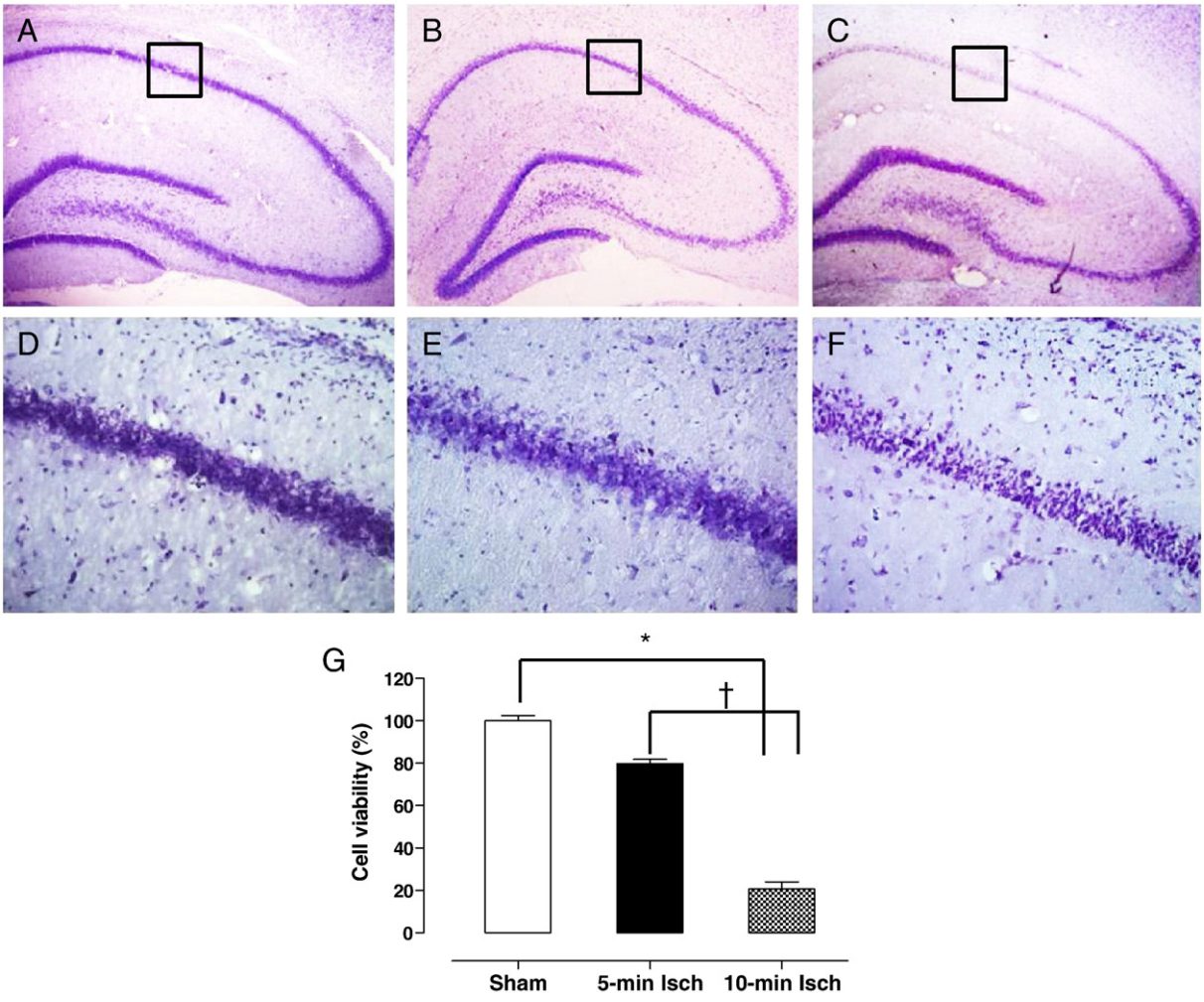

Fig. 2 – Representative morphology of Cresyl violet staining in the hippocampal region from the sham group (A and D), 5-min ischemia groups (B and E), and 10-min ischemia group (C and F) at 72 h of reperfusion. The graph (G) shows the quantitative results of the CA1 neuronal cell counts. $^{*}p<0.005$, $^{\dagger}p<0.005$. ($n=4$).

2.3. *Histological analysis*

To evaluate the ischemic cell death in the 11VO model, histological analysis was performed in the hippocampal region at 3 days after ischemia. The normal pyramidal neurons from four hemispherical sections were counted and averaged. Nissl staining revealed neuronal cell damage in the 10-min group at the CA1 region but a small amount of damage was found in the 5-min group (Fig. 2). The percentage of visible cells in the hippocampus in the 10- and 5-min group was 20.5±4.8 % and 79.2±6.7 %, respectively, compared to the normal group. The mean number of stained cells was determined by three researchers who were blinded to the experimental conditions. The cell viability from each group was presented as a percentage of the mean number of viable cells from sham animals. Mean number of stained cells was obtained by three researchers blinded to the experimental conditions.

2.4. *Expression of NeuN and C.caspase-3 in hippocampal region*

According to Nissl staining showing a small damage in the 5-min ischemia group, immune-staining for the neuronal specific nuclear protein (NeuN) and cleaved caspase-3 (C. caspase-3) in the normal and 10-min groups was compared to confirm neuronal apoptotic cell death. The cell viability of the NeuN was significantly higher in the normal group than in the 10-min group (38.0±3.4 % of normal group; $p<0.005$). In contrast, greater immunoreactive cells were observed in the hippocampal region of the 11VO group than in the normal group (7.0±1.3 % of ischemia group; $p<0.005$, see, Fig. 3). The immunoreactive cell from each group was presented as a percentage of the mean number of florescent cells. Mean number of immunoreactive cells was also obtained by three researchers blinded to the experimental conditions.

3. Discussion

This is the first study to evaluate the correlation between glutamate release and neuronal cell death in an eleven vessel rat occlusion model. In this study, the %CBF during the ischemic period was significantly lower in the 5- and 10-min 11VO models than in the controls, and there was an increase in the level of glutamate release during the occlusion period. The glutamate concentrations were significantly higher in the 10-min group

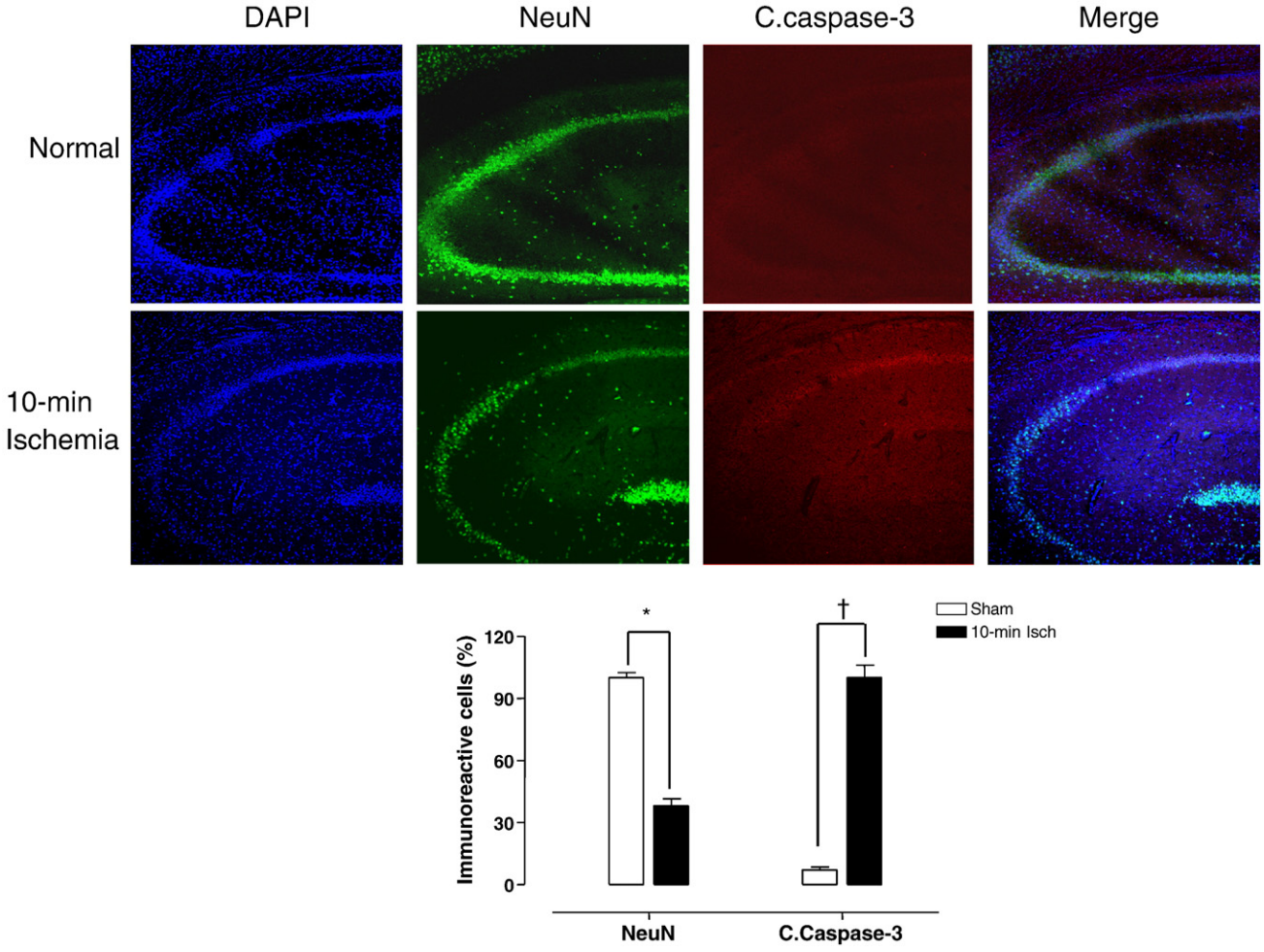

Fig. 3 – The quantitative analysis of NeuN and C.caspase-3 in the hippocampal region after global ischemia by eleven vessel occlusion. DAPI is a fluorescent stain both live and fixed cells. Merge between NeuN, C.caspase-3 and DIPI staining. The graph shows the quantitative results of the CA1 neuronal cell counts. $^{*}p<0.005$, $^{\dagger}p<0.005$. ($n=4$).

than the 5-min group. Three days after the induction of ischemia, ischemic cell death was observed in the hippocampal region of the 10-min 11VO model with increased C.caspase-3 positive and decreased NeuN positive cells.

As observed in current study, a reproducible near unit decrease in %CBF to a consistently low level after a carotid artery occlusion was observed during the ischemic period. This suggests that the 11VO ischemic model may achieve a level of profound reversible ischemia that better mimics complete forebrain ischemia. Moreover, this model gives a reproducibility of peracute excitotoxic damage with elevated glutamate levels in agreement with previous studies (Caragine et al., 1998; Lee et al., 2009a,b).

Glutamate is the most abundant excitatory neurotransmitter in the brain, and a high extracellular level of glutamate release might play an important role in neuronal death (Kirino, 1989; Zhao et al., 1998; Taoufik and Probert, 2008). In 1986, microdialysis was initially used to measure the neurotransmitters in the rat brain (Tossman and Ungerstedt, 1986). In the next year, the first application of microdialysis in humans was carried out through detection of the interstitial glucose concentrations (Lonnroth et al., 1987). Recently, most microdialysis applications were used for either human metabolic or animal neuroscience studies. In particular, an ischemia-induced rat during the ischemia period increases with increasing extracellular glutamate concentration, which is strongly related to neuronal cell death (Ogawa et al., 2007). A previous study in our lab demonstrated that an understanding of the excitotoxic processes requires an accurate assessment of the changes in extracellular glutamate levels in real time, during and after the various insults suspected of initiating ischemic damage (Lee et al., 2009b). Three days after the occlusion, the higher glutamate level caused neuronal cell damage to the hippocampal region of the 10-min group. In contrast, the 5-min 11VO model after 3 days reperfusion showed only a small amount of neuronal cell death. Indeed, the excitotoxicity damage of glutamate had recovered functionally in the 5-min 11VO ischemic group but not in the 10-min group. This suggests that high glutamate levels induced for 10 min might cause more neuronal cell damage in the brain than 5-min occlusion. Thus, a shorter occlusion time stimulates a small amount of glutamate, resulting in less damage to the hippocampal area.

It has been suggested that increased glutamate levels cause neuronal cell damage with neuronal degenerative ischemic disease. However, the time when the maximum neuronal cell death occurs after the occlusion is controversial. Significant increases in TUNEL-positive and Bax-positive cells in the hippocampal CA1 subfield appeared in the 5-min transient global ischemia followed by 4 days reperfusion group (Li et al., 2006), and an increased in TUNEL-positive cells were observed in gerbil hippocampal CA1 area from 3 days after a bilateral occlusion of the carotid arteries for 5 min (Shimizu et al., 2007). On the other hand, several recent studies have indicated a significant increase in CA1 neuronal apoptosis at 72 h after reperfusion due to the detection of TUNEL, C.caspase-3 (Ostrowski et al., 2008), DNA fragmentation (Racay et al., 2009), and NeuN and p53-upregulated modulator of apoptosis (Niizuma et al., 2009). The present study investigated hippocampal CA1 neuronal apoptotic cell death at 72 h in an 11VO ischemic model. In this study, brain apoptosis was measured by the level of NeuN and C.caspase-3 expression in the hippocampal area. Caspase-3 is a key enzyme for the apoptosis and survival of mammalian cells (Miura et al., 2004). In the brain, caspase-3 is a key effector of neuronal apoptosis in the hippocampus region (Gianinazzi et al., 2003) and C.caspase-3 is not detected in unprocessed caspase-3. C.caspase-3 immunohistochemistry detects the apoptosis of the lymphoma cells more sensitively than TUNEL and ssDNA immunohistochemistry (Arai et al., 2005). The NeuN expression was observed in most neuronal cell types throughout the nervous system (Mullen et al., 1992). In this study, 10 min of 11VO transient global cerebral ischemia induced neuronal damage in the hippocampal region after 72 h of reperfusion, which was accompanied by the activation of C.caspase-3 and the inhibition of NeuN. This immunofluorescence evidence suggests that excitotoxic glutamate release correlates neuronal apoptotic cell death in the hippocampal area of 11VO model.

Animal models for rat forebrain ischemia can provide various benefits for humans, e.g. testing of drugs and evaluating surgical techniques. In conclusion, the release of glutamate was detected in real-time in an eleven vessel occlusion for 10-min ischemic period. The results showed that glutamate may induce the activation of C.caspase-3 positive and the inhibition NeuN positive cells at 72 h after reperfusion, resulting in neuronal cell death in the hippocampal region.

4. Experimental procedures

4.1. *Animal preparation*

Adult male Sprague–Dawley rats weighing 250–350 g from Orient Bio. INC were kept in a central animal care facility, under 12-h light, 12 h-dark cycle (light on at 0600 h) and controlled temperature (24±0.5 °C). Food and water were provided *ad libitum*, but the animals were fasted at 1 day before the surgical procedure. All animal experiments were approved by the Committee of Animal Experiments in College of Medicine, Kyung Hee University, and were in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

During the entire preparation, the body temperature was maintained at 37.1±0.10 °C with a homoeothermic blanket control unit (Harvard Apparatus, Holliston, MA, USA). The rats were anesthetized with chloral hydrate (0.1 ml/100 g, i.p.) with an additional intermittent injection (0.1 ml/300 g) for maintenance when necessary. After confirming full-anesthesia, the surgical steps were performed. During surgery, the arterial blood pressure was monitored with an arterial line, and the animals were not affected by the blood gas values, rectal temperature, hematocrit, hemoglobin or injury.

4.2. *Eleven vessel occlusion*

Rats were placed in the supine position. After a midline vertical incision from the cricoids cartilage to the sternal border with a platysma incision, the common carotid artery was defined between the sternocleidomastoid and sternohyoid muscles. The occipital artery, ascending pharyngeal

artery and superior thyroid artery of the external carotid artery were occluded with bipolar electrocautery. The pterygopalatine artery of the internal carotid artery was detected just medial of the tympanic bulla and coagulated. Before occlusion of the basilar artery, a tracheostomy was made at the level of the third or fourth tracheal ring, and then a silastic tube (inner diameter: 1.8–2.1 mm) was inserted and tied up with 4-0 black silk. After sequential retraction of the trachea, esophagus and both longus colli muscles, we ascertained the clivus and drilled it out with a 3 mm diameter. The basilar artery was selected at the mid pontine groove at the caudal level of the anterior cerebellar artery after the dura incision and arachnoid dissection, and then the basilar artery was permanently coagulated. We sealed the pore with bone wax to prevent leakage of cerebrovascular fluid. Finally, the carotid arteries were prepared for the induction of ischemia. For safe exposure, the carotid artery was dissected with caution so as not to injure the vagus nerve and autonomic nervous chains. On ischemia induction, we hung a rubber ring on the common carotid artery and internal carotid artery on both sides to make blood flow decrease over time, which enabled vascular patency upon subsequent reperfusion.

4.3. *Real-time monitoring of glutamate*

The animals were placed in stereotaxic head holder (David Kopf Instruments, Tujunga, CA, USA), in order to determine the glutamate levels in real-time. Eight burr holes were made using hand drill (FOREDOM, Bethel, CT, USA). Two with 1.5 mm in diameter were placed 4 mm apart from the midline and 1 mm in front of the coronal suture: one for glutamate sensor insertion and the other for brain temperature measurement. Two burr holes were made for electroencephalography (EEG) electrodes at 4 mm apart from the sagittal suture and 1 mm behind the coronal suture. Two burr holes were made for the CBF probe at both the side and 2 mm behind the EEG sites. Two additional burr holes were made for EEG 2 mm behind the CBF probe on both sides. EEG electrodes were attached with four screws to acquire two channels of EEG signals that were amplified with a 511 AC amplifier (Grass Product Group, Astro-Med, Inc., West Warwick, RI, USA). The signals were converted to digital information at 256 times per second using a data acquisition system designed in our laboratory. An EEG signal represented the electrical failure of a neuronal cell during an ischemic episode. The probes for the CBF were attached at both hemispheres with BLF21D laser Doppler flowmetry (Transonic Systems Inc., Ithaca, NY, USA).

$$\%\mathrm{CBF} = \frac{\text{CBF level on ischemia/reperfusion period (ml g}^{-1}\text{ min}^{-1})}{\text{CBF level on pre}-\text{ischemic period (ml g}^{-1}\text{ min}^{-1})}$$

The microdialysis electrode, purchased from Sycopel International Ltd. (Type: General 20-10-4-4, Tyne & Wear NE32 3DT, UK), was filled with phosphate buffered saline to electropolymerize the O-phenylenediamine on the platinum electrode at 0.65 V for 20 min with Sycopel BD2000 potentiostat. Fresh phosphate buffered saline containing glutamate oxidase was then perfused in the dialysis electrode at a flow rate of 0.5 μl/min. The dialysis electrode displayed a linear response in the concentration range of 50–450 μM standard glutamate solution with a sensitivity of 0.22 nA/μM (R2, coefficient of regression of 0.998). A calibration was constructed, according to repetitive addition of glutamate solution. After sensor calibration, the microdialysis electrode was inserted into the motor cortex at coordinates A 1: L 4: V 4 mm (from the bregma and the dura) through a small incision in the dura. Five- and 10-min 11VO cerebral ischemia was initiated by pulling the snares on the common carotid artery and external carotid artery. The snares were released and withdrawn after 5 and 10 min.

4.4. *Fixation*

At 72 h after brain ischemia, the rat brains were fixed with cold 4% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4. The brains were postfixed overnight at 4 °C in the same solution and soaked in 0.5 M phosphate-buffered saline, containing 30% sucrose for cryoprotection. Serial 40-μm-thick coronal sections were cut on a freezing microtome (Leica, Nussloch, Germany). The sections were stored in a cryoprotectant (25% ethylene glycol, 2 5% glycerol, 0.05 M PB, pH 7.4) at –20 °C until ready for use.

4.5. *Nissl staining*

The sections were mounted on gelatin-coated slides and stained with cresyl violet for a histological assessment of the neuronal cell damage, which identifies viable and nonviable stained cells. Viable neurons were defined as cells with a normal morphology, exhibiting round nuclei stained with cresyl violet. The number of viable neuronal cells were counted using Image Plus 2.0 (Motic, Xiamen, China) in a $500 \times 500\ \mu m^2$ area of the hippocampal CA1 regions approximately 300 μm from the hillus of the three coronal sections (approximately 1.4 to 1.8 mm posterior to bregma).

4.6. *Immunofluorescence*

For the detection of neuronal apoptosis, double-immunofluorescence of NeuN and C.caspase-3 was performed in hippocampal region in the sham and 10-min ischemia groups. The slides were incubated at room temperature for 30 min. Free-floating sections were pre-incubated for 15 min in a 1% H_2O_2 solution before being incubated overnight at 25 °C in 0.3% Triton X-100 and 0.5 mg/ml bovine serum albumin with one of the following primary antibodies: NeuN mouse monoclonal antibody at a 1:2000 dilution (Millipore, #MAB377) and C. caspase-3 rabbit polyclonal antibody at 1:500 (Cell signaling, #9661). The sections were incubated for 60 min in the dark with the secondary antibodies; Alex fluor 488 goat anti-mouse IgG antibody at 1:1500 (Invitrogen, #A-11001) and Texas Red goat anti-rabbit IgG antibody at 1:1500 (Invitrogen, #T2767). Following another washing period, the tissues were covered with a DAPI mounting medium (Vector, #H-1200).

4.7. *Statistical analysis*

The data for the %CBF and glutamate changes is expressed as the mean ± the standard error of the mean. A two-tailed Student's *t*-test was used to compare the %CBF and glutamate

changes in the two groups. The significant differences in neuronal cell viability were assessed by one-way analysis of variance, followed by a Tukey's post hoc test using SPSS statistical software (version 17.0 for Windows, SPSS Inc., Chicage, IL). *P*-values <0.05 were considered significant.

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