

# Time-course pattern of neuronal loss and gliosis in gerbil hippocampi following mild, severe, or lethal transient global cerebral ischemia

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## Abstract

Transient ischemia in the whole brain leads to neuronal loss/death in vulnerable brain regions. The striatum, neocortex and hippocampus selectively lose specific neurons after transient ischemia. Just 5 minutes of transient ischemia can cause pyramidal neuronal death in the hippocampal cornu ammonis (CA) 1 field at 4 days after transient ischemia. In this study, we investigated the effects of 5-minute (mild), 15-minute (severe), and 20-minute (lethal) transient ischemia by bilateral common carotid artery occlusion (BCCAO) on behavioral change and neuronal death and gliosis (astrocytosis and microgliosis) in gerbil hippocampal subregions (CA1–3 region and dentate gyrus). We performed spontaneous motor activity test to evaluate gerbil locomotor activity, cresyl violet staining to detect cellular distribution, neuronal nuclei immunohistochemistry to detect neuronal distribution, and Fluoro-Jade B histofluorescence to evaluate neuronal death. We also conducted immunohistochemical staining for glial fibrillary acidic protein and ionized calcium-binding adapter molecule 1 (Iba1) to evaluate astrocytosis and microgliosis, respectively. Animals subjected to 20-minute BCCAO died in at least 2 days. BCCAO for 15 minutes led to pyramidal cell death in hippocampal CA1–3 region 2 days later and granule cell death in hippocampal dentate gyrus 5 days later. Similar results were not found in animals subjected to 5-minute BCCAO. Gliosis was much more rapidly and severely progressed in animals subjected to 15-minute BCCAO than in those subjected to 5-minute BCCAO. Our results indicate that neuronal loss in the hippocampal formation following transient ischemia is significantly different according to regions and severity of transient ischemia. The experimental protocol was approved by Institutional Animal Care and Use Committee (AICUC) of Kangwon National University (approval No. KW-180124-1) on May 22, 2018.

**Key Words:** transient global brain ischemia; delayed neuronal death; glial activation; ischemic duration; hippocampus; spontaneous motor activity; Mongolian gerbil; histology; neural regeneration

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## Introduction

Transient ischemia in the brain is caused by temporary obstruction of blood flow in the cerebrovascular system. Temporary hindrance of blood supply to the brain can increase the risk of ischemia/reperfusion (IR) injury to the brain (Park et al., 2014; Zhang et al., 2017). Transient global brain ischemia occurs under particular ischemic conditions such as return of spontaneous circulation after cardiac arrest. It leads to selective neuronal death/loss in the specific vulnerable substructures (region or areas) of the brain (*i.e.*, the neocortex, striatum and hippocampus) (Nakayama et al., 2013; Park et al., 2017b). Among these hippocampal structures, pyramidal cells or neurons in the CA1 region will die several

days after transient global brain ischemia. This is called “delayed neuronal death” (Kirino, 2000).

It is well known that astrocytes perform many functions, including biochemical support of endothelial cells to constitute the blood-brain barrier (BBB), maintenance of extracellular ion balance, provision of nutrients to nervous tissues, and participation in the repair of the central nervous system (CNS) following injuries (Horner and Palmer, 2003; Daneman and Prat, 2015). In the BBB, astrocytes form neurovascular coupling by provision of cellular link between neuronal circuitry and blood vessels (Daneman and Prat, 2015). Astrocyte activity is related to blood flow in the CNS. Thus it can be measured by functional magnetic resonance imaging

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(fMRI) (Swaminathan, 2008; Figley and Stroman, 2011).

Microglia are immune cells in the CNS that secrete various kinds of inflammatory cytokines under some conditions such as cerebral ischemia (Yan et al., 2012b; Gulke et al., 2018). In addition, microglia communicate with neurons to maintain a healthy environment in the CNS (Suzumura, 2013). Both astrocytes and microglia are activated in the pathological status of the CNS, such as ischemic insults and neurodegenerative diseases. They release various factors to contribute to neuronal death as well as neuronal survival when nervous tissues are in the pathological status (Burda and Sofroniew, 2014).

It is well known that just 5-minute bilateral common carotid artery occlusion (BCCAO) in gerbils can cause death/loss of pyramidal neurons in the hippocampal CA1 region 4–5 days later (Kirino, 1982; Kirino and Sano, 1984; Park et al., 2015, 2017a). Furthermore, spontaneous hyperactivity following 5-minute BCCAO is generally shown in gerbils (Janac et al., 2006; Park et al., 2017b). To our best knowledge, previous studies on ischemic injuries after mild, severe, or lethal transient ischemia were performed in the septum and striatum (Ohk et al., 2012; Park et al., 2013). Chronological alterations of neuronal loss and gliosis in the hippocampal formation, which consists of hippocampal CA1–3 regions and dentate gyrus, following mild, severe, or lethal transient ischemia have not been fully investigated yet. We chose 5, 15 and 20 minutes of BCCAO to induce mild, severe and lethal ischemic injury, respectively, as previously reported (Park et al., 2013). The main objective of this study was to investigate the differences in degree of behavioral alterations, neuronal death/loss, and gliosis in the hippocampus CA1–3 region and dentate gyrus of gerbils subjected to 5, 15 and 20 minutes of BCCAO. Gerbils were chosen because they had been demonstrated as an excellent animal model of transient global cerebral ischemia (Li et al., 2011; Kim et al., 2017).

## Materials and Methods

### Experimental animals

One hundred and five 6-month-old male gerbils, weighing ~70 g were provided by the Experimental Animal Center of Kangwon National University located at Chuncheon, Republic of Korea. These animals were randomly divided into sham operated ( $n = 28$ ), 5-minute BCCAO ( $n = 28$ ), 15-minute BCCAO ( $n = 28$ ), 20-minute BCCAO ( $n = 21$ ) groups. They were housed with an optimum status under suitable temperature (about 23°C) and humidity (about 60%). Twelve hours of light and dark cycle was controlled. They were provided free access to water and feed. The experimental protocol was approved by Institutional Animal Care and Use Committee (AICUC) of Kangwon National University (approval No. KW-180124-1) on May 22, 2018. The study protocol adhered to guidelines from the current international laws and policies in “Guide for the Care and Use of Laboratory Animals” (The National Academies Press, 8<sup>th</sup> Ed., 2011).

### BCCAO procedure

Surgical procedure of BCCAO was carried out as we previously described (Yoo et al., 2016; Park et al., 2017d). Briefly,

using an inhaler, gerbils were anesthetized with a mixture of 2.5% isoflurane (Hana Pharmaceutical Co., Ltd., Seoul, Republic of Korea) in 33% oxygen and 67% nitrous oxide (Carpenter, 2013). Common carotid arteries (CCA) were exposed and ligated with aneurysm clips (0.69 N; Yasargil FE 723K; Aesculap, Tuttlingen, Germany) for 5, 15, or 20 minutes. Thereafter, these clips were removed for reperfusion. Complete occlusion and reperfusion of the CCA was confirmed by interruption of blood flow in the central retinal artery using an ophthalmoscope (HEINE K180<sup>®</sup>, Heine Optotechnik, Herrsching, Germany). Normal rectal temperature ( $37 \pm 0.5^\circ\text{C}$ ) was controlled during the surgery. Temperature was monitored using a rectal temperature probe (TR-100; Fine Science Tools Inc., Foster City, CA, USA). Sham operated animals underwent the same surgical procedure without BCCAO. After the surgical procedure, to maintain body temperature, animals were kept at 23°C with 60% humidity in a thermal incubator (Mirae Medical Industry, Seoul, Republic of Korea) until they were euthanized.

### Determination of spontaneous motor activity

To elucidate hyperactivity induced by BCCAO, spontaneous motor activity (SMA) was measured according to a published study (Yan et al., 2011) at 1 day after BCCAO operation because SMA peaked at 1 day after transient ischemia/reperfusion. Briefly, each gerbil was placed in a Plexiglas cage (25 cm × 20 cm × 12 cm) located inside a soundproof chamber (Kinder Scientific, Poway, CA, USA). SMA was recorded using a Photobeam Activity System-Home Cage (San Diego Instruments, San Diego, CA, USA). Each gerbil was examined continuously *via* a 4 × 8 photobeam configuration. Score for each gerbil was generated under live observation. Video sequences were used for subsequent analysis.

### Tissue preparation for histological examination

Tissue preparation was done at sham, 1, 2 and 5 days after ischemia/reperfusion according to our previous study (Lee et al., 2016a). Briefly, the ischemic gerbils ( $n = 7$  at each time point after ischemia/reperfusion) were anesthetized by intraperitoneal injection of pentobarbital sodium (70 mg/kg, JW Pharm. Co., Ltd., Seoul, Republic of Korea) (Carpenter, 2013) and perfused *via* the ascending aorta with 0.1 M phosphate-buffered saline (PBS; pH 7.4) followed by fixation with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were removed after fixation and post-fixed in the same fixative for 7 hours. The fixed brains were infiltrated with 30% sucrose solution (in 0.1 M phosphate buffer, pH 7.4) for 12 hours for cryoprotection. Finally, these brains were coronally and serially sectioned (25 μm in thickness) in a cryostat (Leica, Nussloch, Germany).

### Cresyl violet staining

To observe cellular distribution and change in the hippocampus following BCCAO, Cresyl violet (CV) staining was done according to a published method (Park et al., 2015). Briefly, sections were mounted onto microscopy slides coated with gelatin. These sections were stained with 1.0% (w/v) CV acetate (Sigma, St. Louis, MO, USA) and subsequently

incubated in serial ethanol bath for dehydration. Finally, cover glasses were mounted onto these stained sections with Canada balsam (Kanto, Tokyo, Japan).

### Fluoro Jade B histofluorescence

Fluoro Jade B (FJB), a high affinity fluorescent marker for degenerative neuron, histofluorescence staining was carried out to investigate neuronal death/degeneration after BCCAO as described previously (Ahn et al., 2016a). Briefly, brain sections were immersed in a solution of 0.06% potassium permanganate. Subsequently, these sections were incubated in a solution of 0.0004% FJB (Histochem, Jefferson, AR, USA). These stained sections were dehydrated and mounted by cover glasses using dibutylphthalate polystyrene xylene (DPX, Sigma, St. Louis, MO, USA) as a mounting medium. For observation, we used an epifluorescent microscope (Carl Zeiss, Oberkochen, Germany) equipped with 450–490 nm blue excitation light.

### Immunohistochemistry

Neuronal damage/death and gliosis after BCCAO operation were examined by immunohistochemistry according to a published protocol (Park et al., 2015). Briefly, sections were exposed to 0.3% H<sub>2</sub>O<sub>2</sub> (in 0.01 M PBS, pH 7.4) for 25 minutes and then treated with 10% normal horse or goat serum (in 0.01 M PBS, pH 7.4) for 40 minutes at room temperature. Subsequently, they were incubated for 24 hours at room temperature with primary antibodies: mouse anti-NeuN (1:1100; Chemicon, Temecula, CA, USA) for analyzing neuronal cells, mouse anti-gial fibrillary acidic protein (GFAP) (1:700; Chemicon) for measuring astrocytes, and rabbit anti-Iba1 (1:700, Wako, Osaka, Japan) for examining microglia. These incubated sections were reacted with biotinylated horse anti-mouse IgG or goat anti-rabbit IgG (1:250, Vector, Torrance, CA, USA) as a secondary antibody and exposed to avidin-biotin complex (1:300, Vector). Finally, these immunoreacted sections were visualized by reacting with 3,3'-diaminobenzidine tetrahydrochloride (Sigma) in 0.1 M PBS (pH7.4).

### Data analysis

Data were analyzed as described previously (Park et al., 2017c). For quantitative analyses of NeuN-immunoreactive (ir) neurons, FJB-positive cells, GFAP-ir astrocytes and Iba1-ir microglia, we selected seven sections/gerbil (a total of 49 sections) and took digital images of each immunostained structure in hippocampal sub-regions using an optical (BX53, Olympus, Hamburg, Germany) or epifluorescent microscope (Carl Zeiss, Göttingen, Germany) equipped with a camera (Olympus). We carried out cell counting in a 250 μm × 250 μm area of each hippocampal subregion using an Optimas 6.5 software (CyberMetrics, Scottsdale, AZ, USA) for image analysis.

To quantitatively analyze GFAP and Iba1 immunoreactivity, we captured digital images of each immunoreactivity in a tissue area of 140 μm × 140 μm and calibrated the image into an array of 512 × 512 pixels. We measured the immunoreactivity of each abovementioned index by 0 to 255 gray-scale system. The ratio of relative optical density (ROD) of each immunoreactivity was calibrated as percentage using

Adobe Photoshop (version 8.0, Adobe, San Jose, CA, USA). Finally, we analyzed RODs using NIH Image software version 1.59 (National Institutes of Health, Bethesda, MD, USA). A ratio of the ROD was calibrated as percentage of the sham operated group, which was designated as 100%.

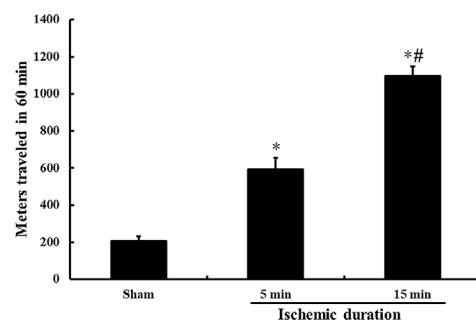
### Statistical analysis

Data shown in this study represent the mean ± standard error of the mean (SEM) among experimental groups. They were statistically analyzed using SPSS 18.0 (SPSS, Chicago, IL, USA). One-way analysis of variance (ANOVA) with a *post hoc* Bonferroni's multiple comparison test was done to determine differences among groups. Statistical significance was considered at  $P < 0.05$ .

## Results

### SMA

SMA was determined at 1 day after BCCAO operation (Figure 1). Seven animals were applied in each group. SMA was significantly increased in the 5-minute BCCAO group compared with the sham group ( $P < 0.05$ ). SMA was significantly increased in the 15-minute BCCAO group than in the 5-minute BCCAO group ( $P < 0.05$ ). In the 20-minute BCCAO group, we did not obtain any data because these animals died.



**Figure 1** SMA of gerbils subjected to 5- and 15-min BCCAO at 1 day after BCCAO.

\* $P < 0.05$ , vs. sham operated (sham) group, # $P < 0.05$ , vs. 5-min BCCAO group. The bars indicate the mean ± SEM ( $n = 7$ /group). SMA: Spontaneous motor activity; BCCAO: bilateral common carotid artery occlusion; min: minute.

### CV-stained cells

As shown in Figure 2A, in the sham operated group, CV-stained cells were easily identified in all sub-regions of hippocampal formation (hippocampal CA1–3 region and dentate gyrus) (Figure 2Aa). CV-stained cells were typically distributed in the pyramidal layer in hippocampal CA1–3 regions (Figure 2Ab and Ac) and in the granule cell layer in the dentate gyrus (Figure 2Ad).

One day after BCCAO operation, the distribution of CV-stained cells in BCCAO groups was not altered compared to the sham operated group (Figure 2B).

Two days after BCCAO, the distribution of CV-stained cells in the 5-minute BCCAO group was not changed (Figure 2Ca–Cd). CV staining in the 15-minute BCCAO group was weakened in hippocampal CA1–3 regions while CV staining in the dentate gyrus was not changed compared to that in the sham operated group (Figure 2Cd).

Five days after BCCAO, CV-stained cells in the 5-minute BCCAO group were rarely observed in the hippocampal CA1 pyramidal layer. However, the distribution of CV-stained cells in the hippocampal CA2–3 region and dentate gyrus was similar to that in the sham operated group (Figure 2Da–Dd). In the 15-minute BCCAO group, CV-stained cells were rarely detected in the hippocampal CA1–3 pyramidal layer. They were significantly decreased in the dentate granule cell layer (Figure 2De–Dh).

#### FJB-positive cells

As shown in Figure 3, FJB-positive cells in the sham operated group were not observed in any sub-regions of the gerbil hippocampal formation (Figure 3A).

One day after BCCAO, FJB-positive cells were not found in the hippocampal CA1–3 regions in both 5-minute and 15-minute BCCAO groups. However, a few FJB-positive cells were found in the dentate polymorphic layer (Figure 3Bc, Bf and G).

Two days after BCCAO, in the 5-minute BCCAO group, FJB-positive cells were slightly increased in the dentate polymorphic layer (Figure 4Cc and G). In the 15-minute BCCAO group, many FJB-positive cells were found in the hippocampal CA1 pyramidal layer, and the numbers of FJB-positive cells were increased in the dentate polymorphic layer compared with the 5-minute BCCAO group (Figure 3Cc, Cd–Cf and E–G).

Five days after BCCAO, in the 5-minute BCCAO group, FJB-positive cells were increased in the dentate polymorphic layer (Figure 3Dc and G) and many FJB-positive cells were found in the hippocampal CA1 pyramidal layer alone (Figure 3Da and E). In the 15-minute BCCAO group, the numbers of FJB positive cells were significantly increased in all hippocampal CA regions (Figures 3Dd, De and E–4G). In addition, FJB-positive cells in the dentate polymorphic layer were significantly increased in number, and many FJB-positive cells were shown in the lower blade of the granule cell layer (Figure 3Df and G).

#### NeuN-ir neurons

NeuN-ir neurons, in sham operated gerbils, were easily observed in all sub-regions of the hippocampal formation. Hippocampal CA1–3 pyramidal cells, dentate granule cells and polymorphic cells were all immunoreactive for NeuN (Figure 4A).

One day after BCCAO, NeuN-ir neurons in the gerbils from 5- or 15-minute BCCAO groups were not different from those identified in the sham operated group (Figure 4B).

Two days after BCCAO, in the 5-minute BCCAO group, the numbers of NeuN-ir neurons in all hippocampal sub-regions were similar to those in the sham operated group (Figure 4Ca–Cc). However, in the 15-minute BCCAO group, the numbers of NeuN-ir neurons were significantly decreased in the hippocampal CA1–3 regions compared with the 5-minute BCCAO group, although no significant difference in such number was found in the dentate gyrus (Figure 4Cd–Cf and E–G).

Five days after BCCAO, NeuN-ir neurons in the 5-minute BCCAO group were rarely detected in the hippocampal CA1

region. The number of NeuN-ir neurons was not altered in the hippocampal CA2–3 regions or dentate gyrus (Figure 4Da–Dc and E–G). In the 15-minute BCCAO group, the numbers of NeuN-ir neurons were significantly reduced in all hippocampal subregions compared to those in the sham operated group. Especially, NeuN-ir neurons in the dentate granule cells layer were significantly reduced in the lower blade of the granule cell layer (Figure 4A, D and E–G).

#### GFAP-ir astrocytes

In the sham operated group, GFAP-ir astrocytes were in the resting form and distributed in all layers of all subregions of the gerbil hippocampal formation (Figure 5A).

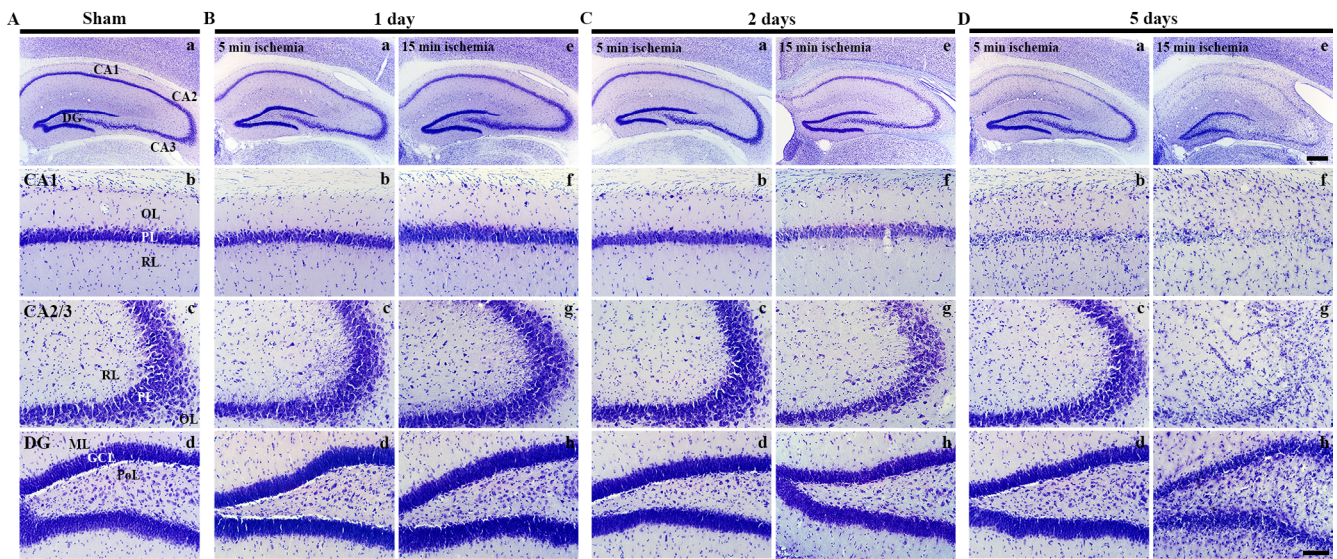
One day after BCCAO, GFAP immunoreactivity in 5- and 15-minute BCCAO groups was slightly increased. They showed bulky cytoplasm (hypertrophic state) in all subregions (Figure 5B and E–G). In addition, the numbers of GFAP-ir astrocytes in both groups were significantly increased in all subregions (Table 1). In the hippocampal CA2–3 regions, the numbers of GFAP-ir astrocytes were significantly increased in the 15-minute group than in the 5-minute BCCAO group (Table 1).

Two days after BCCAO, GFAP-ir astrocytes were significantly hypertrophied in all hippocampal subregions of the gerbils from the 15-minute BCCAO group. The ROD was 125.6% in the CA1 region, 119.6% in the CA2–3 regions, and 135.9% in the dentate gyrus of gerbils in the 15-minute BCCAO group compared to the 5-minute BVVAO group (Figure 5Ca–Cf and E–G). In addition, the numbers of GFAP-ir astrocytes in both groups were significantly increased in all hippocampal subregions compared to the pre-time point (Table 1). Furthermore, in the 15-minute BCCAO group, their numbers at each time point after BCCAO were significantly increased in all subregions compared to those in the 5-minute BCCAO group (Table 1).

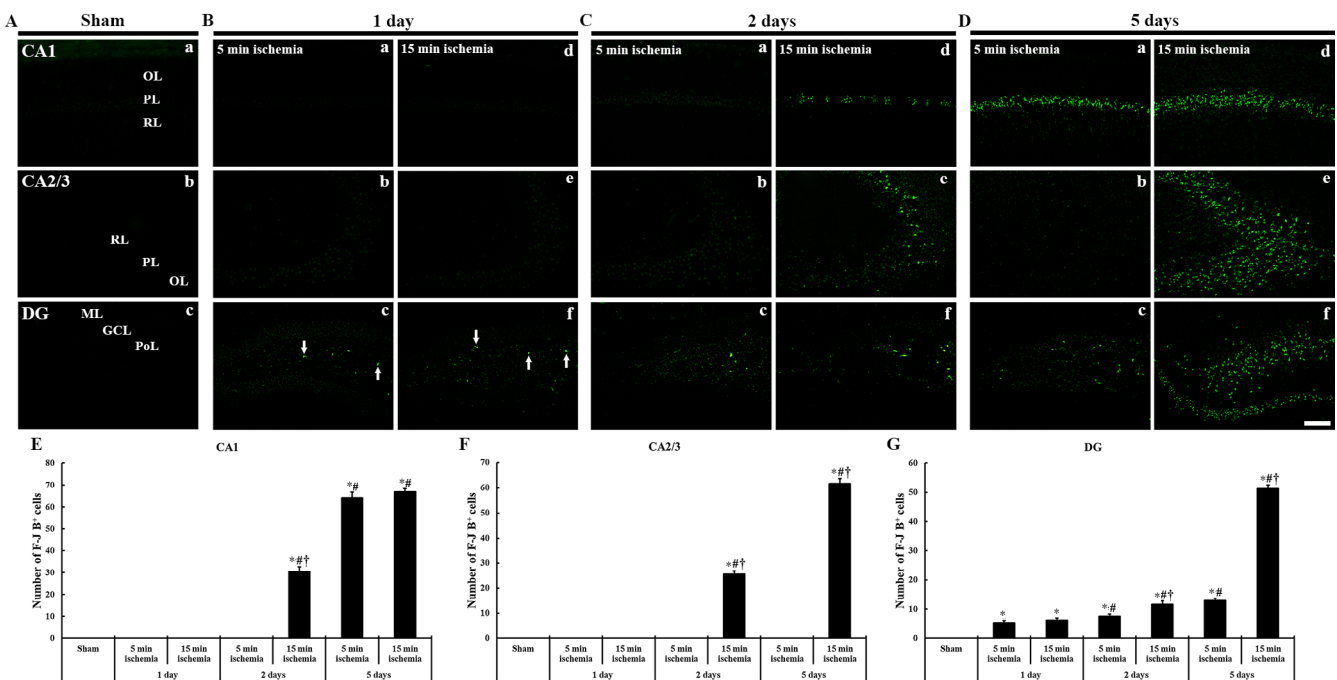
In the 5-minute BCCAO group, GFAP immunoreactivity at 5 days after BCCAO was increased significantly compared to the prior time point (Figure 5C and D). However, GFAP immunoreactivity in the 15-minute BCCAO group was slightly lower than that in the 5-minute BCCAO group. The ROD of GFAP immunoreactivity in the 15-minute BCCAO group was 96.7% in the hippocampal CA1 region and 99.8% in the hippocampal CA2–3 regions of gerbils and it was 92.9% in the 5-minute BCCAO group (Figure 5Ca–Cf and E–G). In addition, the numbers of GFAP-ir astrocytes were significantly increased in the hippocampal CA1–3 regions and dentate gyrus of gerbils in the 5-minute BCCAO group compared with those at 2 days after BCCAO operation (Table 1).

#### Iba1-ir microglia

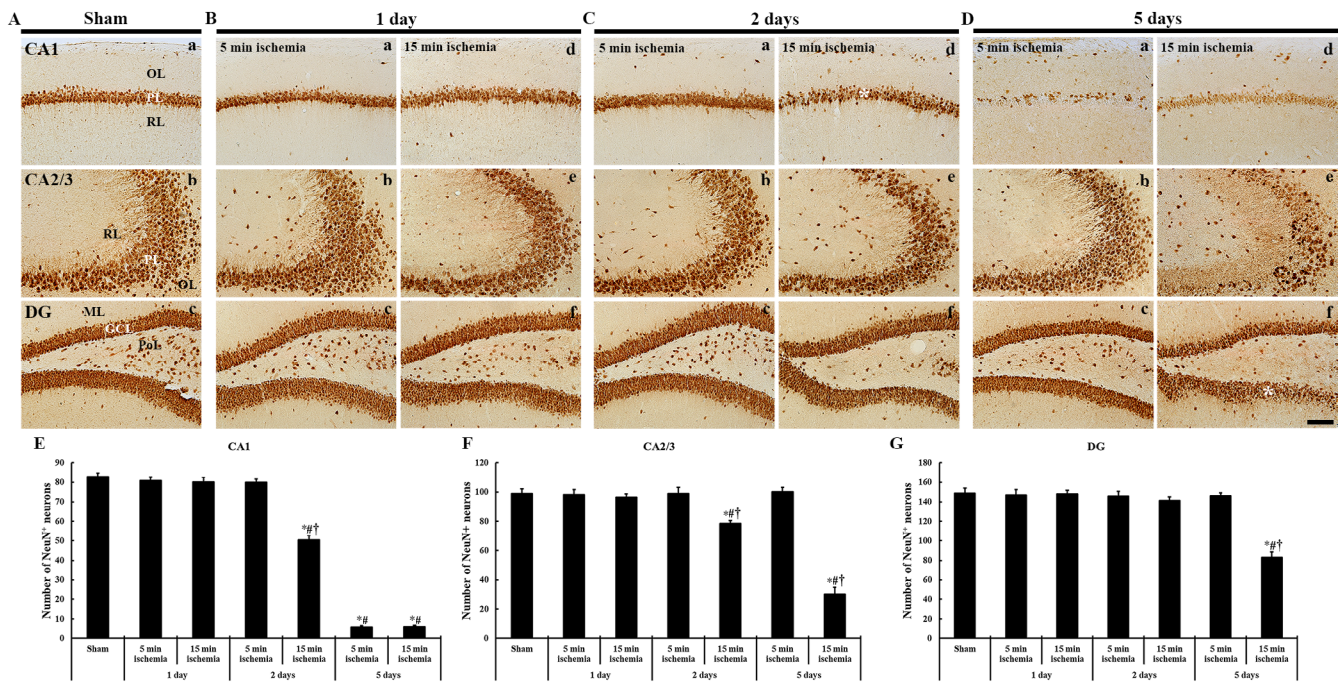
Iba1-ir microglia in the sham operated gerbils were in the resting form and evenly distributed throughout all layers in all hippocampal subregions (Figure 6A). One day after BCCAO, Iba1-ir microglia in both 5- and 15-minute BCCAO groups were slightly activated in all subregions compared to those in the sham operated gerbils (Figure 6B and E–G). Moreover, the numbers of Iba1-ir microglia in both 5- and 15-minute BCCAO groups were significantly increased in all hippocampal subregions (Table 2). In the 15-minute BCCAO group, the numbers of Iba1-ir microglia were more



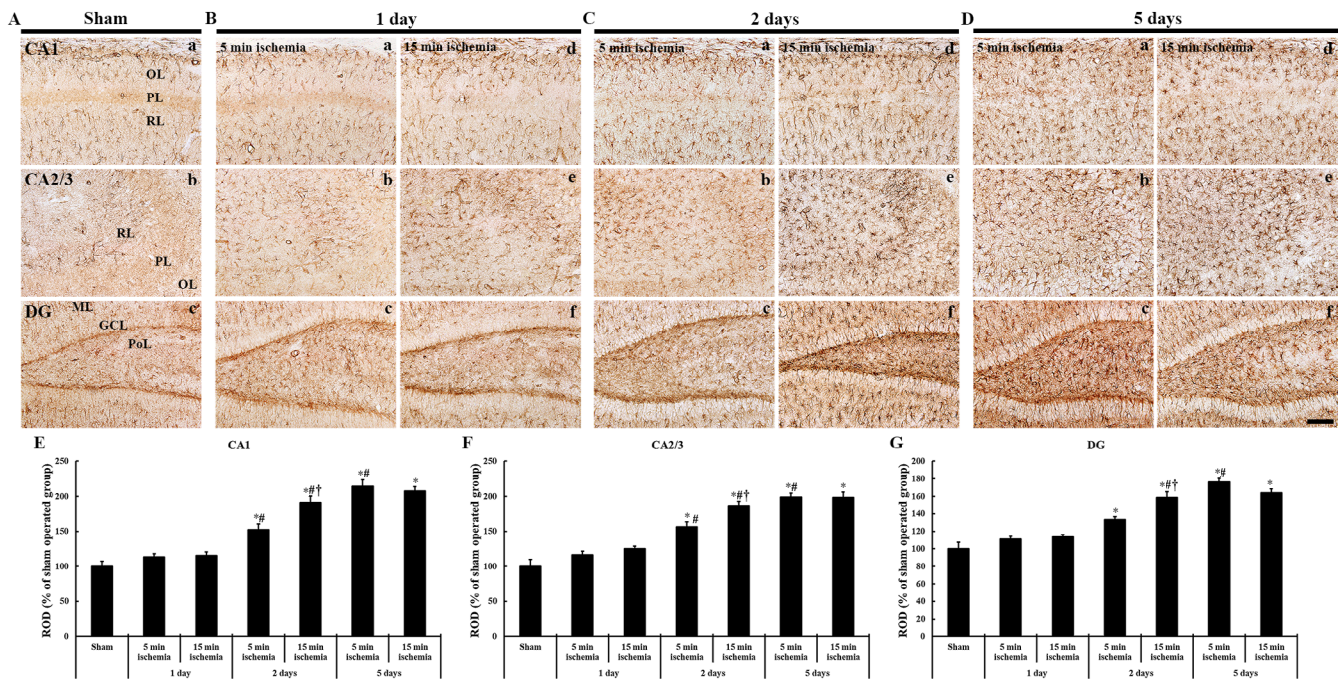
**Figure 2 CV staining in the hippocampal formation of gerbils at different time after 5- and 15-min BCCAO.** (A) Sham operation; (B) 1 day after BCCAO, (C) 2 days after BCCAO, (D) 5 days after BCCAO. CV-stained cells are not changed in all subregions in the hippocampus at 1 day after BCCAO. At 2 days after BCCAO, CV staining is markedly weakened in the PL of the CA1 region of gerbils from the 15-min BCCAO group. Five days after BCCAO, CV-stained cells are decreased in number in the PL of the CA1 region of gerbils from the 5-min BCCAO group. The numbers of CV-stained cells in the 15-min BCCAO group were reduced in the PL of the CA1–3 regions. In the DG, changes in the number of CV-stained cells are detected only in the 15-min BCCAO group. Scale bars: 400  $\mu$ m (Aa, Ba, Be, Ca, Ce, Da, De) and 100  $\mu$ m (Ab–Ad, Bb–Bd, Bf–Bh, Cb–Cd, Cf–Ch, Db–Dd, Df–Dh). CV: Cresyl violet; BCCAO: bilateral common carotid artery occlusion; PL: pyramidal layer; DG: dentate gyrus; GCL: granule cell layer; ML: molecular layer; OL: oriens layer; PoL: polymorphic layer; min: minute.



**Figure 3 FJB histofluorescence in the hippocampal formation of gerbils at different time after 5- and 15-min BCCAO.** (A) Sham operation; (B) 1 day after BCCAO, (C) 2 days after BCCAO, (D) 5 days after BCCAO. Degenerative neurons are positive to FJB and marked in green fluorescence. No FJB-positive cells are shown in the sham operated group. One day after BCCAO, FJB-positive cells (arrows) are detected in the dentate PoL. Two days after BCCAO, FJB-positive cells are additionally detected in the CA1 PL of gerbils from the 15-min BCCAO group. Five days after BCCAO, FJB-positive cells in the 5-min BCCAO group are significantly increased in the CA1 PL, and FJB-positive cells in the 15-min BCCAO group are significantly increased in the CA1 PL, dentate PoL and GCL. Scale bar: 100  $\mu$ m. (E–G) The mean number of FJB-positive cells in the CA1 (E), CA2/3 (F) and DG (G) ( $n = 7$  at each time after BCCAO; \* $P < 0.05$ , vs. the sham operated group, † $P < 0.05$ , vs. the prior time point of corresponding BCCAO group, # $P < 0.05$ , vs. the same time point of 5-min BCCAO group). The bars indicate the mean  $\pm$  SEM. FJB: Fluoro Jade B; BCCAO: bilateral common carotid artery occlusion; OL: oriens layer; PL: pyramidal layer; PoL: polymorphic layer; RL: radiant layer; DG: dentate gyrus; GCL: granule cell layer; ML: molecular layer; min: minute.



**Figure 4 Immunohistochemistry (brown color) for NeuN in the hippocampal formation of gerbils at different time after 5- and 15-min BCCAO.** (A) Sham operation; (B) 1 day after BCCAO, (C) 2 days after BCCAO, (D) 5 days after BCCAO. At 1 day after BCCAO, NeuN-ir neurons are not altered in all subregions. At 2 days after BCCAO, NeuN-ir neurons are decreased in the pyramidal layer (PL, asterisk) of the CA1 region alone. At 5 days after BCCAO, NeuN-ir neurons are rarely shown in the CA1 PL of gerbils from both 5- and 15-min BCCAO groups. In the 15-min BCCAO group, NeuN-ir are significantly decreased in number in the CA2/3 PL, dentate polymorphic layer (PoL) and lower granule cell layer (GCL, asterisk). Scale bar: 100  $\mu$ m. (E-G) The mean number of NeuN-ir neurons in the CA1 (E), CA2/3 (F) and dentate gyrus (G) ( $n = 7$  at each time after BCCAO;  $*P < 0.05$ , vs. the sham operated group,  $\#P < 0.05$ , vs. the prior time point of corresponding BCCAO group,  $\dagger P < 0.05$ , vs. the same time point of 5 min-BCCAO group). The bars indicate the mean  $\pm$  SEM. BCCAO: Bilateral common carotid artery occlusion; OL: oriens layer; PL: polymorphic layer; RL: radiant layer; DG: dentate gyrus; GCL: granule cell layer; ML: molecular layer; min: minute.



**Figure 5 Immunohistochemistry (brown color) for GFAP in the hippocampal formation of gerbils at different time after 5- and 15-min BCCAO.** (A) Sham operation; (B) 1 day after BCCAO, (C) 2 days after BCCAO, (D) 5 days after BCCAO. One day after BCCAO, the distribution of GFAP-ir astrocytes is not significantly altered in all subregions. At 2 days after BCCAO, GFAP-ir cells are significantly hypertrophied in all subregions. Five days after BCCAO, GFAP-ir cells are significantly hypertrophied in all subregions of gerbils from the 5- and 10-min BCCAO groups. Scale bar: 100  $\mu$ m. (E-G) ROD of GFAP-ir cells in the CA1 (E), CA2/3 (F) and DG (G) ( $n = 7$  at each time after BCCAO;  $*P < 0.05$ , vs. the sham operated group,  $\#P < 0.05$ , vs. the prior time point of corresponding BCCAO group,  $\dagger P < 0.05$ , vs. the same time point of 5 min-BCCAO group). The bars indicate the means  $\pm$  SEM. GFAP: Glial fibrillary acidic protein; ir: immunoreactive; BCCAO: bilateral common carotid artery occlusion; OL: oriens layer; PL: polymorphic layer; RL: radiant layer; DG: dentate gyrus; GCL: granule cell layer; ML: molecular layer; PoL: polymorphic layer; min: minute.

**Table 1** The mean number of GFAP-ir astrocytes in the gerbil hippocampal formation of gerbils from the 5- and 15-minute BCCAO groups at sham, 1, 2 and 5 days after BCCAO

Region	Sham	1 day		2 days		5 days	
		5-minute ischemia	15-minute ischemia	5-minute ischemia	15-minute ischemia	5-minute ischemia	15-minute ischemia
CA1	43±2.9	81±3.1*	84±3.4*	107±2.0**	121±2.4**†	131±2.3**	126±1.8*
CA2/3	22±1.9	68±2.4*	80±3.5*†	95±3.1**	121±2.7**†	137±2.3**	134±3.5**
DG	48±2.5	73±3.7*	75±3.2*	88±3.5**	123± 4.0**†	130±3.4**	127±2.8*

GFAP-ir astrocytes, expressed as the mean ± SEM, are counted in a 250 µm x 250 µm square in hippocampal subregions at sham, 1, 2 and 5 days after 5- and 15-minute BCCAO operation. *n* = 7 at each time point in each group. \**P* < 0.05, vs. sham operated group; #*P* < 0.05, vs. prior time point of the corresponding BCCAO group; †*P* < 0.05, vs. the same time point of the 5-minute BCCAO group. GFAP-ir: Glial fibrillary acidic protein-immunoreactive; BCCAO: bilateral common carotid artery occlusion; DG: dentate gyrus.

**Table 2** The mean number of Iba1-ir microglia in the gerbil hippocampal formation of gerbils from the 5- and 15-minute BCCAO groups at sham, 1, 2 and 5 days after BCCAO

Region	Sham	1 day		2 days		5 days	
		5-minute ischemia	15-minute ischemia	5-minute ischemia	15-minute ischemia	5-minute ischemia	15-minute ischemia
CA1	33±3.7	54±2.7*	59±3.5*	68±3.1**	81±3.1**†	106±3.0**	123±3.3**†
CA2/3	30±2.6	59±2.5*	62±2.6*	72±2.4**	78±2.8**	95±4.0**	103±6.1**†
DG	31±3.0	57±2.5*	61±2.4*	74±3.5**	74±3.1**	102±3.0**	118±1.3**†

Iba1-ir microglia, expressed as the mean ± SEM, are counted in a 250 µm x 250 µm square of the hippocampal subregions. *n* = 7 at each time point in each group. \**P* < 0.05, vs. the sham operated group, #*P* < 0.05, vs. the prior time point of corresponding BCCAO group; †*P* < 0.05, vs. the same time point of 5-minute BCCAO group. BCCAO: bilateral common carotid artery occlusion; ir: Immunoreactive; DG: dentate gyrus.

significantly increased in any subregion compared to those in the 5-minute BCCAO group (Table 2).

Two days after BCCAO, Iba1-ir microglia in the 15-minute BCCAO group were more activated compared to those in the 5-minute BCCAO group. The ROD of Iba1 immunoreactivity in the 15-minute BCCAO group was 109.0% in the hippocampal CA1 region, 111.9% in the hippocampal CA2–3 regions and 110.5% in the dentate gyrus compared to the 5-minute BCCAO group (Figure 6B, C and E–G). Furthermore, the numbers of Iba1-ir microglia in both 5- and 15-minute BCCAO groups were significantly increased in all hippocampal subregions compared to pre-time point after BCCAO (Table 2).

Iba1-ir microglia in both 5- and 15-minute BCCAO groups were more activated at 5 days after BCCAO than at 2 days after BCCAO (Figure 6D). At this time point after BCCAO, the ROD of Iba1 immunoreactivity in the 15-minute BCCAO group was 121.6% in the hippocampal CA1 region, 125.0% in the hippocampal CA2–3 regions, and 121.9% in the dentate gyrus compared to the 5-minute BCCAO group (Figure 6E–G). In addition, the numbers of Iba1-ir microglia in both 5- and 15-minute BCCAO groups were significantly increased in all hippocampal subregions compared to those at 2 days after BCCAO (Table 2).

## Discussion

Mongolian gerbil has a unique hallmark in arteries of the cerebrovascular system. In the base of the brain, the Willis' circle frequently has no posterior communicating arteries (Kuchinka et al., 2008). Therefore, only ligation of bilateral common carotid arteries in the gerbil can lead to ischemia in the forebrain which is supplied by common carotid arteries

(Martinez et al., 2012). Based on this characteristic, gerbils have widely been used to study ischemic damage and related mechanisms (Candelario-Jalil et al., 2001; Yan et al., 2012a; Park et al., 2014, 2017a; Ahn et al., 2016b; Lee et al., 2016b). In particular, neuronal loss or death occurs selectively in vulnerable gerbil brain regions, such as the striatum, cerebral cortex and hippocampal formation, by transient ligation of bilateral CCA. In the case of transient ischemia for 5 minutes, hippocampal CA1 pyramidal neurons will die at 4–5 days after 5 minutes of transient ischemia (Kirino, 1982; Nakayama et al., 2013; Park et al., 2017b).

In this study, we modulated durations of transient ischemia to be 5, 15 and 20 minutes to compare the effects on locomotor activity, neuronal death, and glial activation among the three groups through SMA test, CV staining, immunohistochemistry for NeuN, FJB histofluorescence and immunohistochemistry for GFAP and Iba1. Above all, in this study, we found that animals died in at least 2 days after 20 minutes of BCCAO. This finding indicates that transient ischemia for 20 minutes in the forebrain or telencephalon is lethal in gerbils.

The SMA test has been considered as a useful behavioral estimation after transient global cerebral ischemia. Many researches have reported significant hyperactivity after BCCAO operation in gerbils (Janac et al., 2006; Park et al., 2017b). In the present study, SMA was evaluated at 1 day after BCCAO operation. It was found that SMA was significantly increased in gerbils subjected to 15-minute BCCAO.

The present study revealed that pyramidal neurons of CA1–3 regions showed NeuN immunoreactivity. In addition, NeuN immunoreactivity in the pyramidal neurons of gerbils in the 15-minute BCCAO group was significantly

weaker than that in the 5-minute BCCAO group at 5 days after BCCAO. Such a phenomenon might be related to the severe neuronal damage induced by longer ischemia duration. Previous studies have reported that hyperthermia can evoke severer damage of pyramidal neurons after 5 minutes of BCCAO in gerbils (Noor et al., 2003; Kim et al., 2015). NeuN immunoreactivity reported in the previous studies was similar to our present result. In this study, 15 minutes of BCCAO operation led to loss of pyramidal neurons in the hippocampal CA1–3 regions at 2 days after BCCAO. This study is the first to use FJB histofluorescence staining. It has been reported that pyramidal cells of the CA1 will die at 5 days after 5 minutes of BCCAO. However, at this time point after BCCAO, pyramidal neurons of CA2–3 regions were not lost (Cho et al., 2013). This finding indicates that 15 minutes of BCCAO is severe enough to evoke pyramidal cell death in all hippocampal CA regions. The above-mentioned studies and our present study indicate that 15 minutes of transient ischemia in the forebrain under hyperthermia could evoke much severer damage or death compared to that under normothermic condition.

On the other hand, neuronal death or loss in the dentate gyrus occurs in the polymorphic layer at about 6 hours after transient cerebral ischemia. These cells are considered to be very vulnerable to transient ischemic insults (Moon et al., 2009). In this respect, we have found that polymorphic cells of the dentate gyrus were lost from 1 day after 5 minutes of BCCAO (Ahn et al., 2016a). This is consistent with our present finding observed by FJB histofluorescence staining. Furthermore, for the first time, we found that granule cells of the dentate gyrus died at 5 days after 15 minutes of BCCAO, rather than after 5 minutes of BCCAO in this study.

Granule cells located in the dentate gyrus give mossy fibers to the CA3 region. They are very resistant against transient cerebral ischemia (Schmidt-Kastner and Freund, 1991; McAuliffe et al., 2011). It is well known that the subgranular zone, which is a narrow zone of the granule cell layer, offers neurogenesis for granule cells in lifespan (Kempermann et al., 2015). To study neurogenesis of granule cells in the dentate gyrus, researchers have applied adrenalectomy to induce death of granule cells (Brunson et al., 2005; Spanswick et al., 2011). However, it is very hard to study neurogenesis because animals subjected to adrenalectomy cannot survive for long time. In this regard, our present findings might be a tool to study the mechanisms of neurogenesis in the dentate gyrus (Brunson et al., 2005). Based on this finding, gerbils subjected to 15-minute BCCAO might be used as a tool to study neurogenesis in the dentate gyrus, since granule cell loss is induced in the 15-minute BCCAO group.

Several studies have demonstrated that brain ischemia leads to excessive activation of glial cells (astrocytes and microglia) (Ordy et al., 1993; Ahn et al., 2016a; Park et al., 2018). This phenomenon is termed gliosis. It is accompanied by neuroinflammatory responses through secretion of various inflammatory factors induced by ischemic insults (Lambertsen et al., 2004; Yoo et al., 2011). Many studies have reported that neuroprotective materials against brain ischemic insults can attenuate gliosis as a yardstick of neuroprotection against ischemic insults (Melani et al., 2014; Park

et al., 2015, 2017d).

In this study, we compared patterns of astrocyte and microglial activations among the three ischemia groups. Results from the 5-minute BCCAO group were consistent with the findings in the hippocampal CA1 region from a precedent study (Park et al., 2017d). That is to say, with time after 5 minutes of ischemia, astrocytes (GFAP-ir cells) and microglia (Iba1-ir cells) were increased in numbers. They became hypertrophied in the CA1 region, suggesting that gliosis was significantly weak in other subregions.

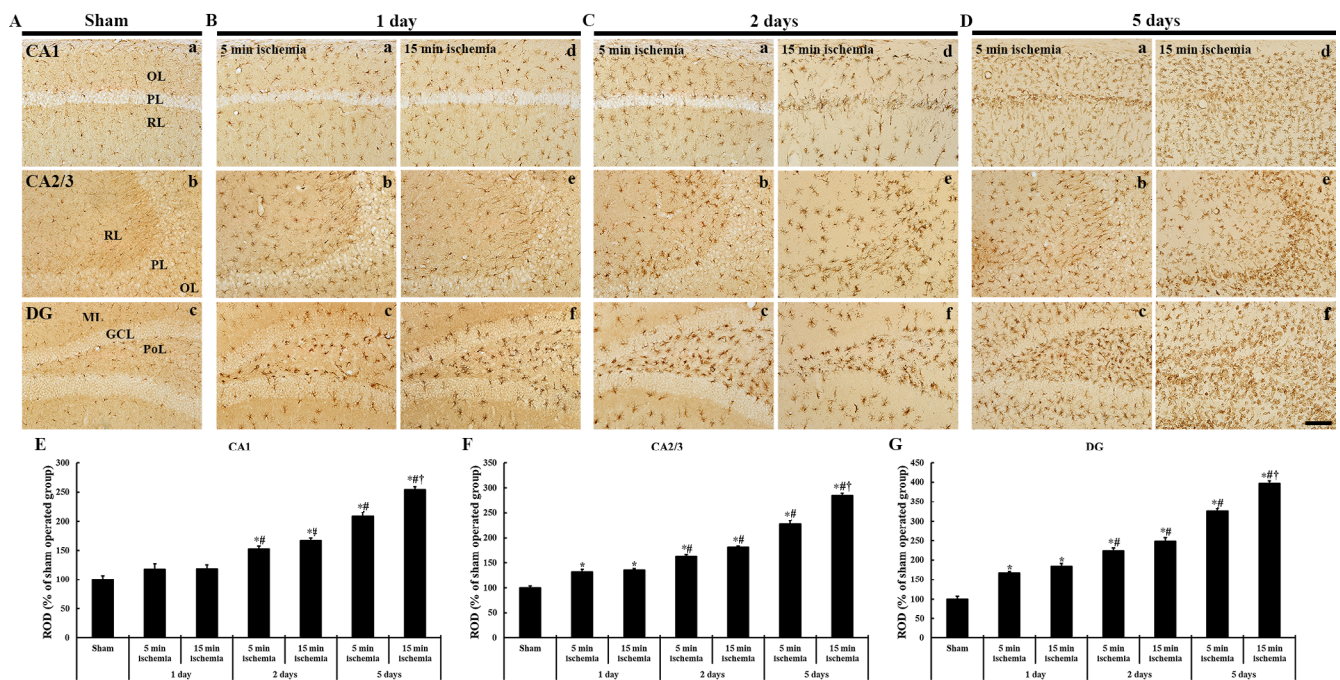
Results of this study revealed that in the 15-minute BCCAO group, GFAP-ir astrocytes were hypertrophied earlier, and their GFAP immunoreactivity was slightly reduced at 5 days after BCCAO, compared to the 5-minute BCCAO group. Sugawara et al. (2002) have reported that GFAP immunoreactivity in astrocytes is decreased after onset of neuronal death induced by transient ischemia. In this respect, our results suggest that earlier neuronal death in the 15-minute BCCAO group might have occurred at 2 days after BCCAO. Such death might have caused a decrease in GFAP immunoreactivity at 5 days after 15-minute BCCAO.

Microglial cells participate in the clearance of dead cells and tissue debris after ischemic insults (Schilling et al., 2005). In the present study, we found that pyramidal cells of the CA1–3 regions in the gerbils from the 15-minute BCCAO group died at 2 days after BCCAO and granule cells of the dentate gyrus died 5 days after BCCAO. Together with neuronal loss, many Iba1-ir microglia became hypertrophied significantly earlier in all hippocampal subregions of gerbils in the 15-minute BCCAO group than in the 5-minute BCCAO group. In particular, many hypertrophied microglia in the 15-minute BCCAO group accumulated in the pyramidal layer of CA1–3 regions at earlier time points compared to those in the 5-minute BCCAO group. When neuronal death occurred, many hypertrophied microglia accumulated in the pyramidal layer or granule layer to remove tissue debris. Furthermore, congregation of microglia in those layers occurred earlier in the 15-minute BCCAO group than in the 5-minute BCCAO group. This finding indicates that, to remove tissue debris, microglia in the 15-minute BCCAO group became hypertrophied and proliferated more rapidly than those in the 5-minute BCCAO group.

Our results focused on the behavioral and histological analysis and showed the differences among mild, severe or lethal transient global cerebral ischemia. However, these results are difficult to reveal molecular mechanisms underlying abovementioned differences. Therefore, in-depth molecular mechanism studies about differences among mild, severe or lethal transient cerebral ischemia are needed.

In summary, this study showed that 15 minutes of BCCAO evoked earlier neuronal death/loss in hippocampal subregions than 5 minutes of BCCAO. Together with neuronal death/loss, astrocytes and microglia were markedly proliferated and hypertrophied in hippocampal regions after 15 minutes of BCCAO compared to those after 5 minutes of BCCAO. Furthermore, gerbils died in at least 2 days after 20 minutes of BCCAO. These findings imply that we must consider the effect of different ischemic durations when we treat transient brain ischemia injury.





**Figure 6 Immunohistochemistry for Iba1 (brown color) in the hippocampal formation of gerbils at different time after 5- and 15-min BCCAO.** (A) Sham operation; (B) 1 day after BCCAO, (C) 2 days after BCCAO, (D) 5 days after BCCAO. At 1 day after BCCAO, Iba1-ir cells are hypertrophied in all subfields. At 2 and 5 days after BCCAO, Iba1-ir microglia are more hypertrophied in all subregions of both groups: in particular, they are much more hypertrophied in the 15-min BCCAO group than in the 5-min BCCAO group. (E-G) ROD of Iba1-ir cells in the CA1 (E), CA2/3 (F) and DG (G) ( $n = 7$  at each time point after BCCAO;  $*P < 0.05$ , vs. the sham operated group,  $\#P < 0.05$ , vs. the prior time point of corresponding BCCAO group,  $\dagger P < 0.05$ , vs. the same time point of 5 min BCCAO operated group). The bars indicate the mean  $\pm$  SEM. BCCAO: Bilateral common carotid artery occlusion; ir: immunoreactive; OL: oriens layer; PL: polymorphic layer; RL: radiant layer; DG: dentate gyrus; GCL: granule cell layer; ML: molecular layer; PoL: polymorphic layer; ROD: relative optical density; min: minute.

**Author contributions:** Experimental design, data collection, data analysis, and manuscript writing: MHW, JHC; experiment implementation: HK, MS, JCL, JHP, JHA; data analysis and prudential comments on the whole process of this work: TKL, GEY, HK, TGO, MCS. All authors have read and approved the final version of this paper.

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