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Original Article

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Absence of Delayed Neuronal Death in ATP-Injected Brain: Possible Roles of Astrogliosis

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Although secondary delayed neuronal death has been considered as a therapeutic target to minimize brain damage induced by several injuries, delayed neuronal death does not occur always. In this study, we investigated possible mechanisms that prevent delayed neuronal death in the ATP-injected substantia nigra (SN) and cortex, where delayed neuronal death does not occur. In both the SN and cortex, ATP rapidly induced death of the neurons and astrocytes in the injection core area within 3 h, and the astrocytes in the penumbra region became hypertropic and rapidly surrounded the damaged areas. It was observed that the neurons survived for up to 1-3 months in the area where the astrocytes became hypertropic. The damaged areas of astrocytes gradually reduced at 3 days, 7 days, and 1-3 months. Astrocyte proliferation was detectable at 3-7 days, and vimentin was expressed in astrocytes that surrounded and/or protruded into the damaged sites. The NeuN-positive cells also reappeared in the injury sites where astrocytes reappeared. Taken together, these results suggest that astroycte survival and/or gliosis in the injured brain may be critical for neuronal survival and may prevent delayed neuronal death in the injured brain.

Key words: brain injury, astrogliosis, delayed neuronal death

INTRODUCTION

It has been generally accepted that, in injured brain, neurons die in two phases: acutely in the injury core and slowly in the penumbra. However, delayed neuronal death does not occur in all types of injury [1, 2]. Delayed neuronal death occurs in contusion-induced spinal cord injury [2], but not in ATP-induced brain injury [1]. Many studies have suggested brain inflammation played by microglia and/or monocytes as a cause of delayed neuronal death [3-5]. However, no correlation appears between inflammatory responses and secondary injury since microglia are activated and monocytes are infiltrated in both ATP-induced brain injury and contusion-induced spinal cord injury [1, 2, 6, 7]. It has been reported that brain inflammation is rather neuroprotective and functions to repair the damaged sites [1, 2, 6-10].

Astrocytes constitute the majority of brain cells and function for the well-being and well-function of neurons. EAAT1/2 and Kir4.1 expressed in astrocytes maintain extracellular homeostasis through uptake of glutamate and potassium, respectively [11-13]. Aquoaporin-4 in astrocytes regulates the extracellular water content [14]. Astrocytes also provide neurons with neurotrophic factors and glucose, and protect neurons [15-17]. Therefore, neurons can not live without support of astrocytes. Accordingly, it has been reported that selective ablation of reactive astrocytes exacerbates traumatic neuronal damage and that transplantation

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of astrocytes diminishes brain damage [18, 19]. We also found spatial-temporal correlation between delayed neuronal death and functional loss and/or death of astrocyte in the spinal cord injury [2]. These findings suggest that loss of astrocytes may cause delayed neuronal death; we therefore sought to determine how astrocytes behave in the ATP-injected brain where delayed neuronal death does not occur.

In this study, we injected ATP into the cortex, and investigated the astrocyte behavior and its effects on neuronal damage. Hyper-reactive astrocytes surrounded the injury core, and neurons with these astrocytes were healthy, which strongly suggests that astrogliosis is a mechanism to prevent delayed neuronal death in the ATP-injected brain.

MATERIALS AND METHODS

Ethics statement

All experiments were performed in accordance with the approved animal protocols and guidelines established by the Ajou University School of Medicine Ethics Review Committee for animal experiments, and all animal work was approved by the Ethical Committee for Animal Research of Ajou University (Amc-28).

Stereotaxic surgery and drug injection

SD rats were anesthetized by injection of chloral hydrate (0.4 mg/kg, i.p.), and positioned in a tereotaxic apparatus (David Kopf Instruments, Tujunga, CA). ATP (10~1000 nmol in 2 μl sterile PBS; Sigma, St. Louis, MO) was unilaterally administered into the right cortex (AP, +0.7 mm; ML, –2.0 mm; DV, –2.0 mm from bregma) and the right SNpc (AP, 25.3 mm; ML, 22.3 mm; DV, 27.6 mm from bregma), according to the atlas of Paxinos and Watson [20]. All animals were injected using a Hamilton syringe equipped with a 30-gauge blunt needle to minimize mechanical damage attached to a syringe pump (KD Scientific, New Hope, PA). ATP was infused at a rate of 0.4 μl/min. After injection, the needle was held in place for an additional 5 min before removal. The contralateral sides were used as a control.

Tissue preparation

Rats were anesthetized and transcardially perfused with saline solution containing 0.5% sodium nitrate and heparin (10 U/ml), followed by 4% paraformaldehyde in a 0.1 M phosphate buffer, pH 7.2, for tissue fixation. Brains were obtained and post-fixed overnight at 4°C in 4% paraformaldehyde. Fixed brains were stored at 4°C in 30% sucrose solution until they sank in the solution. Six separate series of 30 μm coronal brain sections were obtained using a sliding microtome (Microm, Walldorf, Germany).

Immunohistochemistry

For 3, 3'-diaminobenzidine (DAB) staining, serial sections were rinsed three times with PBS, treated with 3% H_2O_2 for 5 min, and rinsed with PBS containing 0.2% Triton X-100 (PBST). Non-specific binding was blocked with 1% BSA in PBST. The sections were incubated overnight at room temperature with primary antibodies against Ki-67 (1:100; Abcam, Cambridge, UK) or NeuN (1:300; Chemicon, CA, USA). Following rinsing in PBST, the sections were incubated with biotinylated secondary antibodies (Vector Laboratories, Burlingame, CA) for 1 h and the avidin/biotin system (Vector Laboratories, Burlingame, CA) for 1 h and visualized using DAB solution (0.05% DAB and 0.003% hydrogen peroxide in 0.1 M PB). For double-labeling with GFAP/NeuN, GFAP/Ki67, or vimentin/Ki67, the sections were firstly stained with NeuN or Ki67 antibodies, and visualized with DAB (brown product), and then washed in PBS, blocked with 1% BSA, and secondarily stained with GFAP or vimentin antibodies, and visualized using DAB/nickel sulfate solution (dark purple products) according to the manufacturer's guidance. Next, the sections were mounted on gelatin-coated slides, and examined under a bright field microscope (Olympus Optical, BX51, Tokyo, Japan). Bright field images were obtained using PictureFrame Application 2.3 software. For double-immunofluorescence staining, sections were washed twice in PBS, treated with 1% BSA, and incubated with combinations of antibodies for GFAP and vimentin. Visualization was performed with Alexa Fluor488- or Alexa Fluor555- conjugated secondary antibodies (1:600 dilution; Invitrogen, Eugene, OR, USA). DAPI (Vector Laboratories, Burlingame, CA) was used to detect nuclei. The sections were analyzed under a confocal microscope (Carl Zeiss, Germany) using 40× water and 63× oil immersion objectives.

RESULTS

Time-dependent behavior of astrocytes in ATP injected-substantia nigra (SN)

Previously, we reported spatial and temporal correlation between astrocyte death and delayed neuronal death in contusion-induced spinal cord injury [2]. However, delayed neuronal death was not detectable in ATP-injected SN and cortex [1]. Thus, we investigated the behavior of astrocytes in the ATP-induced injury model. At 3 h after ATP injection (100 nmol in 2 μl PBS; '*', injection site) into the SNpc (areas inside the dotted lines in 'contra'), GFAP+ astrocytes disappeared in the damaged core (areas inside the dotted lines) and surrounding astrocytes, including in the SNr region increased

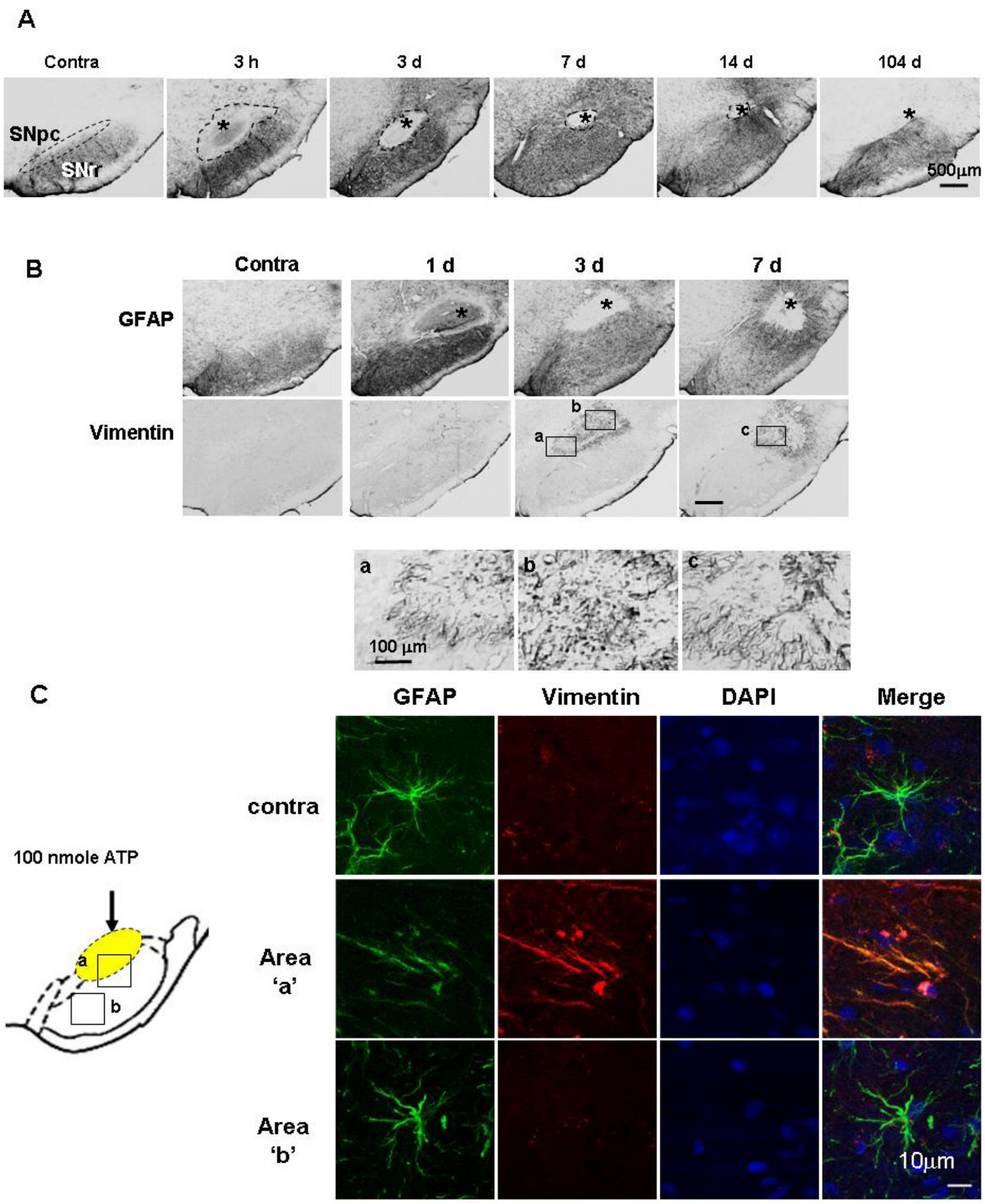

Fig. 1. Time-dependent behavior of astrocyte in the ATP-injected SN. ATP (100 nmol in 2 µl PBS) was unilaterally injected into SNpc (*, injection sites), and brains were obtained at the indicated times after the injection. Brain sections (30 µm thickness) of the midbrain including the entire SN were prepared, and every sixth serial section was selected. (A) Sections were stained with GFAP antibodies, and visualized with biotin-conjugated secondary antibodies. Photographs of the most damaged sections were taken. The contralateral side (contra) and PBS-injected rat brain sections were used as control. (B) Adjacent sections were stained with GFAP and vimentin antibodies and visualized as for (A). The lower panels show the higher magnification of the boxed areas (a~c) in the upper panels. (C) Sections were double-labeled with GFAP and vimentin antibodies, and visualized using Alexa Fluor555- and Alexa Fluor488-conjugated secondary antibodies, respectively. Nuclei were labeled with DAPI. Area 'a' is adjacent to the damage core, and area 'b' is in the undamaged area. Scale bars, 500 µm (A, B upper panel); 100 µm (B lower panel); 10 µm (C). All data are representative of at least three independent experiments.

GFAP expression (Fig. 1A). However, astrocyte-damaged areas gradually reduced at 3, 7, and 104 days (Fig. 1A). At 104 days, astrocytes appeared similar to those in the contra-lateral side (Fig. 1A). An interesting finding was that vimentin, a marker of early developmental astrocytes, was expressed in the damaged core (Figs. 1Bb, 1Bc) and areas surrounding the core (Fig. 1Ba, 1Bc) at 3 and 7 days after the injection. In the double-labeling experiments using GFAP and vimentin antibodies, GFAP-positive astrocytes near the core region expressed vimentin (area 'a' in Fig. 1C), but the GFAP-positive astrocytes in SNr located some distance from the core were vimentin-negative (area 'b' in Fig. 1C).

Next, we examined whether astrocytes proliferate and fill the damaged areas. The number of Ki67-positive proliferating cells dramatically increased at 2-5 days after ATP injection (arrows in Fig. 2A). We further confirmed that Ki67-positive cells were astrocytes, since Ki67 immunoreactivity was found in GFAP-positive and/or vimentin-positive astrocytes (Fig. 2B). Taken together, these results showed that astrocytes in the penumbra region were healthy and proliferate in ATP-injected SN. In addition, vimentin-positive astrocytes may infiltrate and fill the damaged core, which results in a gradual decrease in astrocyte-damaged areas.

Spatial and temporal correlation between damage/repair of astrocytes and neurons in ATP injected-cortex

Previously, we reported that NeuN-positive cells acutely died

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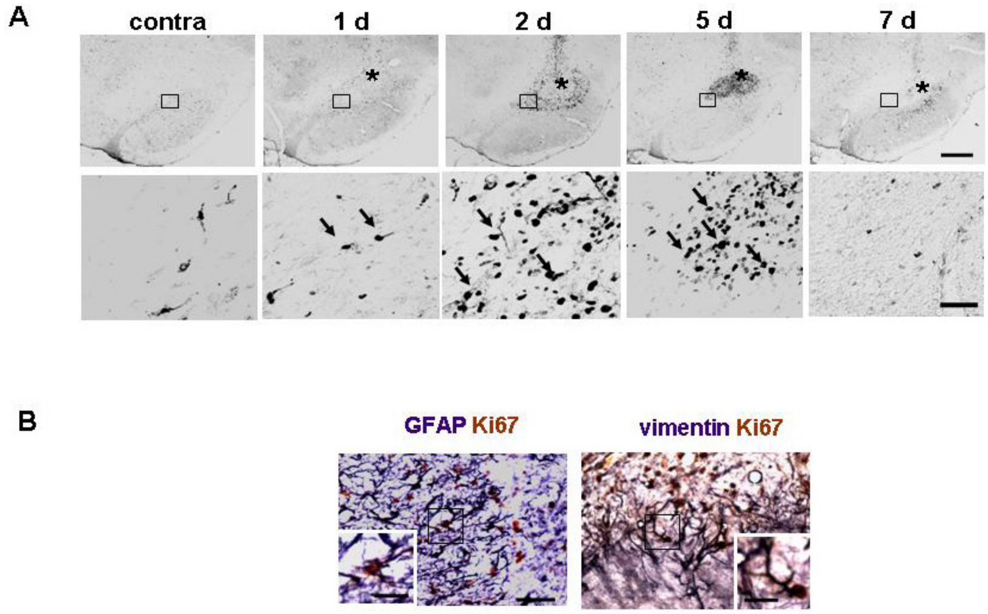

Fig. 2. Astrocyte proliferation in ATP-injected SNpc. (A) Serial sections were obtained at the indicated times after ATP injection into the SN (*, injection sites) and processed for Ki67 immunostaining, as described for Fig. 1. Photographs of the most damaged sections were obtained. The lower panel represents higher magnification of the indicated area in the upper panel. Arrows indicate Ki67+ cells. (B) Sections obtained at 2 d after injection were double-labeled with GFAP/Ki67 or vimentin/Ki67. GFAP and vimentin were visualized with biotin-conjugated secondary antibodies and purple-color reaction, and Ki67 were visualized with biotin-conjugated secondary antibodies and brown-color reaction. Inset: higher magnification of the boxed area. Scale bars, 500 μm (A, upper panel); 50 μm (A, lower panel); 50 μm (B); 20 μm (B, inset). All data are representative of at least three independent experiments.

within 3 h after ATP-injection, and further neuronal death did not occur [1]. In this study, we examined the time-dependent behavior of astrocytes and MAP-2-positive neurite in ATP-injected cortex. Astrocytes disappeared within 3 h after ATP injection, and the astrocyte-damaged areas gradually decreased at 7, 14, and 30 days (Fig. 3A). MAP-2-positive neurites were damaged within 3 h, and MAP-2-negative areas gradually and time-dependently reduced, similar to the astrocytes (Fig. 3A), while astrogliosis surrounding the damaged core appeared at 3 h (Fig. 3). Using NeuN and GFAP antibodies, we further examined the location of the neurons and astrocytes (Fig. 3B). At 3 h, many live neurons were detectable (black arrows in Fig. 3Bh), where astrocytes were healthy (white arrows in Fig. 3Bh). However, the neurons died, as demonstrated by the shrunken cell bodies (black arrowheads in Fig. 3Bh) where the astrocyte processes had broken (white arrowheads in Fig. 3Bh). At 3 days, both the neurons and astrocytes disappeared and appeared to be unhealthy at 3 h (Figs. 3Bc, 3Bi). Interestingly, however, the neurons were still alive (black arrows in Fig. 3Bi) where the astrocytes remained (white arrows in Fig. 3Bi). At 7 days, the damaged area was rather slightly reduced compared to that at 3 days (Fig. 3Bb compared to Fig. 3Bd), because the astrocytes filled part of the damaged area (white arrows in Fig. 3Bj), and some neuronal cell bodies had reappeared (black arrows in Fig. 3Bj). At 14 days and 30 days, the damaged area was further reduced (Figs. 3Be, 3Bf), due to the reappearance of astrocytes (white arrows in Figs. 3Bk, l) and neurons (black arrows in Figs. 3Bk, l).

In the cortex, as in SN, vimentin-positive astrocytes appeared around the damaged core at 3, 7, and 14 days (Fig. 4A). Ki67 immunoreactivity also increased at 3, 7, and 14 days in the ATP-injected cortex (Fig. 4B). These results showed that in an ATP-injected brain, astrocytes underwent gliosis in the penumbra region surrounding the damage core. Importantly, the neurons were healthy if the astrocytes were healthy. In addition, the size of the damaged areas was reduced as the astrocytes in the penumbra region proliferate and express vimentin.

DISCUSSION

While delayed secondary neuronal death occurs in some injured brain cases such as contusion-induced spinal-cord injury [2], it does not occur in other cases such as ATP-induced brain injury [1]. In this study, we found that astrogliosis in the penumbra region is critical in determining the absence or presence of delayed neuronal death. Thus, in ATP-injected SN and cortex, astrocytes become hypertrophic in the penumbra region where neurons are healthy without delayed neuronal death. However, in contusion-

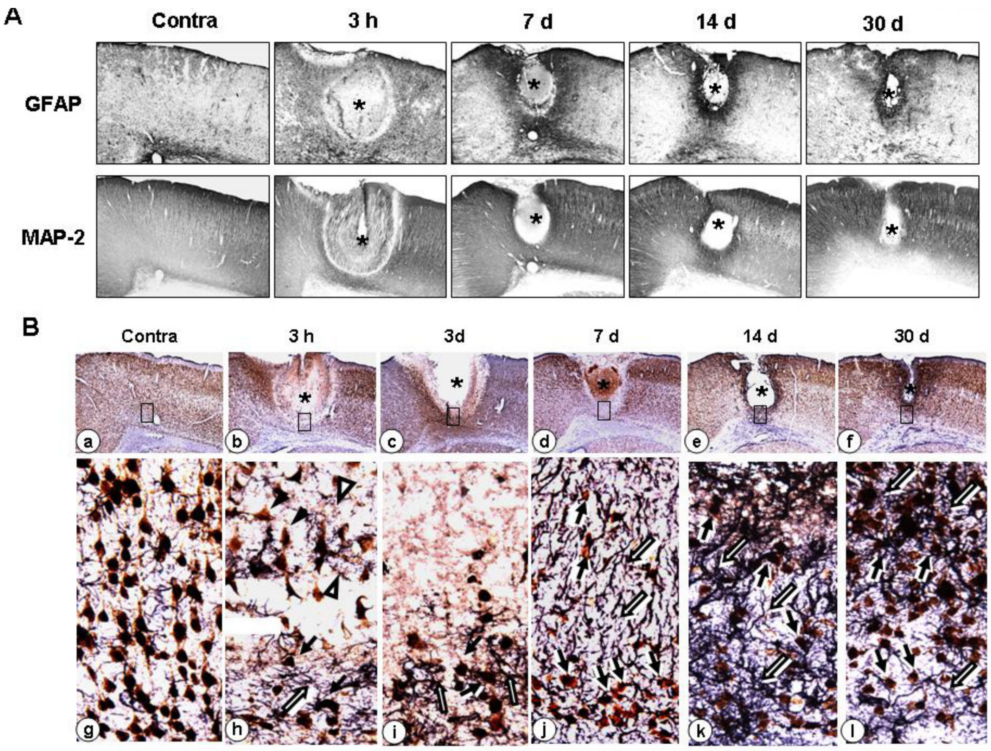

Fig. 3. Time-dependent behavior of astrocyte and neurite in ATP-injected cortex. ATP (1,000 nmol in 2 μl PBS) was unilaterally injected into the cortex, and brains were obtained at the indicated times. Brain sections (30 μm thickness) were prepared, and every sixth serial section was selected. (A) Adjacent sections were stained with GFAP and MAP-2 antibodies, and visualized with biotin-conjugated secondary antibodies. Photographs of the most damaged sections were taken. The contralateral side (contra) was used as the control. (B) Sections were double-labeled with GFAP/NeuN. GFAP was visualized with a purple-color reaction (white arrows), and NeuN with a brown-color reaction (thin black arrows). The black arrow heads and thick black arrows in 'h' indicate unhealthy neurons and astrocytes, respectively. The lower panels show the higher magnification of the boxed areas in the upper panels. All data are representative of at least three independent experiments.

induced spinal-cord injury, astrocyte death and neuronal death have a certain spatial and temporal correlation [2].

Astrocytes protect neurons in both the injured and intact brain [11, 21-27]. Furthermore, astrocytes increase their roles to protect neurons in the injured brain: hypertropic reactive astrocytes increase expression of AQP [28], Kir4.1, and GLAST (unpublished observation). Astrocytes also rapidly respond to ROS that can be produced in an injured brain and produce anti-inflammatory factors [29, 30]. Although microglia are the first line of cells that isolate damage sites [1, 31, 32], astrocytes also become hypertropic and surround the damaged area (Figs. 1, 3). In addition, to prevent delayed neuronal death, astrocytes also participate in recovery of the injured brain. The astrocyte-absent areas became smaller at 7 d after the injection, and disappeared at about 3 months due to the proliferation of astrocytes that express vimentin, similarly to an ischemic brain [33, 34]. We also detected Ki67 immunoreactivity in resident GFAP+ astrocytes (Fig. 2). It has been suggested that astrocytes de-differentiate into stem-like cells in a damaged brain, then proliferate and re-differentiate into astrocytes [35]. There may have been another source of astrocytes that filled the damage sites from SVZ since vimentin-positive cells were detected from SVZ to the injury sites (Fig. 4A). Therefore, the absence of astrogliosis, neurons in the penumbra region, was not able to be sufficiently supported, which may cause delayed injury. Furthermore, repair cannot properly occur, which also contributes to further damage of the neurons.

Although brain inflammation has been suggested as a cause of delayed neuronal death, little correlation has been found in microglial activation and delayed neuronal death, since neurons and neuritis were healthy where microglia were activated [1]. More importantly, the microglia died prior to the neurons in the penumbra region, where the delayed neuronal death occurs [2, 35]. We previously reported that LPS did not induce neuronal death in the cortex but induced it slowly in the SN, where astrocytes density was high and low, respectively [35]. Although ATP induced mRNA and/or protein expression of IL-1β, TNF-α, and IL-6 [1], these cytokines were also induced by PBS that did not cause neuronal death [1]. In the injured brain, the activated

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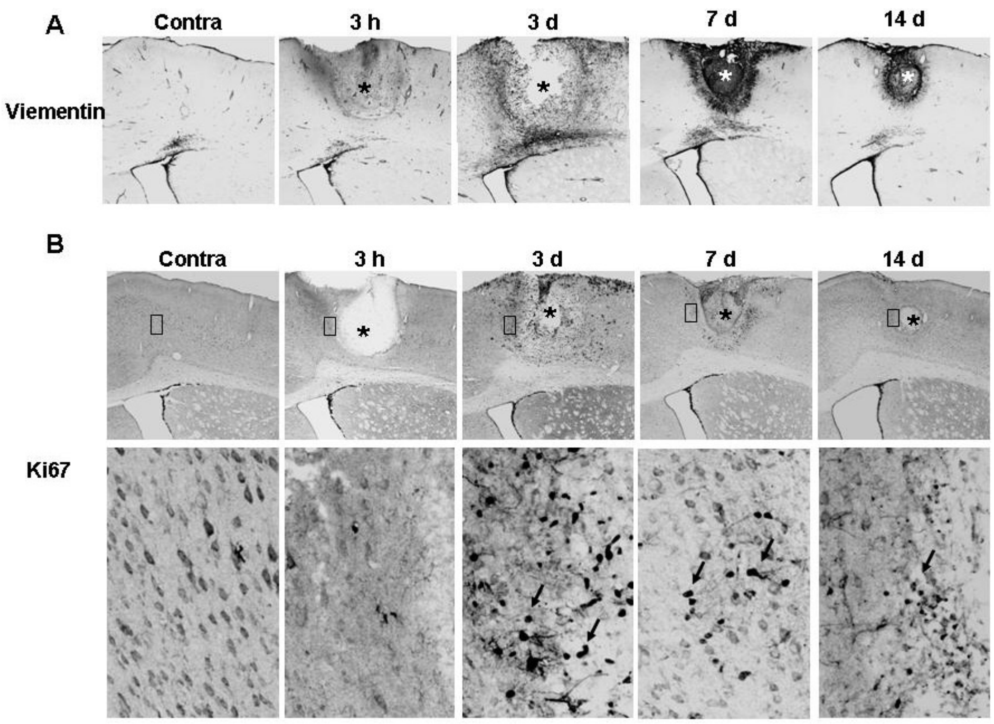

Fig. 4. Increase in vimentin expression and Ki67+ cells in ATP-injected cortex. Sections obtained at the indicated times after ATP injections were processed for vimentin (A) and Ki67 (B) immunostaining. Photographs of the most damaged sections were obtained. The lower panel s in (B) represents higher magnification of the indicated area in the upper panel. Arrows in (B) indicate Ki67+ cells. All data are representative of at least three independent experiments.

microglia the damaged sites, which prevents propagation of the disrupted microenvironmental effect on the surrounding tissues [1]. Furthermore, neuron-loss areas were correlated with astrocyte-loss areas in contusion-induced SCI [2], similarly to the ATP-induced damaged brain (Fig. 3). Taken together, these findings suggest the importance of astrocytes in the injured brain. Therefore, determining a way to increase astrocyte survival would be a new therapeutic target in the area of acute and degenerative brain diseases.

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