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Effects of retinoic acid on ischemic brain injury-induced neurogenesis

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Abbreviations: BrdU, bromodeoxyuridine; DCX, doublecortin; DG, dentate gyrus; EPO, erythropoietin; GFAP, glial fibrillary acidic protein; MCAO, middle cerebral artery occlusion; NeuN, neuron specific nuclear protein; RA, retinoic acid; SGZ, subgranular zone; SVZ, subventricular zone

Abstract

Neurogenesis can be induced by pathological conditions such as cerebral ischemia. However the molecular mechanisms or modulating reagents of the reactive neurogenesis after the cerebral ischemia are poorly characterized. Retinoic acid (RA) has been shown to increase neurogenesis by enhancing the proliferation and neuronal differentiation of forebrain neuroblasts. Here, we examined whether RA can modulate the reactive neurogenesis after the cerebral ischemia. In contrast to our expectation, RA treatment decreased the reactive neurogenesis in subventricular zone (SVZ), subgranular zone (SGZ) and penumbral region. Furthermore, RA treatment also decreased the angiogenesis and gliosis in penumbral region.

Keywords: angiogenesis; cerebral infarction; gliosis; tretinoin

Introduction

New neurons can be generated from neural stem cell in adult brain (McKay, 1997; Gage, 2000; Alvarez-Buylla *et al*., 2001). Generation of new neurons from neural stem cell (neurogenesis) physiologically occurs in discrete regions of the adult brain, including the rostral subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus (DG). New neurons that arise in the SVZ travel via the rostral migratory stream to the olfactory bulb and also enter association neocortex. New neurons leaving the SGZ migrate into the adjacent DG granule cell layer. Even some newly formed neurons integrate functionally into the neuronal circuitry. This neurogenesis in SVZ and SGZ is subject to physiological regulation by glucocorticoids (Cameron and McKay, 1999), sex hormones (Tanapat *et al*., 1999), growth factors (Wagner *et al*., 1999; O'Kusky *et al*., 2000), excitatory neurotransmission (Cameron *et al*., 1998), learning (Gould *et al*., 1999), and stress (Gould and Tanapat, 1999). Moreover neurogenesis in these regions can be modified pharmacologically (Chen *et al*., 2000).

In addition to the physiological neurogenesis, the reactive neurogenesis after injuries such as targeted ablation of cortical neurons, gross trauma, or stroke, has been reported. In global cerebral ischemia in the gerbil and mouse, neurogenesis increased in the SGZ. The number of BrdU labeled cells were increased and these BrdU-labeled cells co-expressed the neuronal marker NeuN, MAP-2, and calbindin (Liu *et al*., 1998; Takagi *et al*., 1999). These cells migrated into the granule cell layer, where they became mature neurons. Moreover, the reactive neurogenesis in the SVZ also has been reported. After middle cerebral artery (MCA) occlusion in the rat, BrdU labeling was increased in the ipsilateral SVZ and rostral migratory stream and co-localized with the neuronal markers polysialylated neural cell adhesion molecule (Parent *et al*., 2002). Importantly, neurogenesis at penumbral area after focal cerebral ischemia was observed. Photothrombotic infarction in the rat enhanced incorporation of BrdU in peri-infarction cortex, with some BrdU-labeled cells co-expressing MAP-2 or NeuN (Gu *et al*., 2000). These findings suggest directed migration of neuronal precursors toward the infarct, possibly in response to a chemical signal. These reactive neurogenesises might contribute to functional recovery after the

ischemic injury.

How is neurogenesis regulated after ischemic insults? The molecular mechanism regulating ischemia-induced neurogenesis is only partly understood. Hypothetically, neurogenesis following ischemic insults could proceed as it does during embryonic development, involving the same concerted action of transcription factors, signaling molecules, and growth factors. In accordance, both the stroke-generated striatal cells (Arvidsson *et al*., 2002) and the cells in the posterior periventricle that had proliferated in response to global ischemia initially expressed several developmental transcription factors (Nakatomi *et al*., 2002).

Recently, several molecules including FGF-2 (Yoshimura *et al*., 2001), stem cell factor (SCF) (Jin *et al*., 2002), brain-derived neurotrophic factor (BDNF) (Pencea *et al*., 2001), erythropoietin (EPO) (Larsson *et al*., 2002), have been associated with ischemia-induced neurogenesis. For example, the increase of neurogenesis in the SGZ following the middle cerebral artery occlusion (MCAO) was attenuated in homozygous FGF-2 knockout mice. Moreover, intraventricular administration of SCF and EPO gave rise to an increased number of proliferated cells co-expressing a neuronal marker in the SVZ. In addition, increased expression of the SCF receptor, c-kit, was observed in the SVZ and SGZ after MCAO.

Retinoic acid (RA) plays an important role in the developing mammalian nervous system (Maden, 2002). RA is essential for initial anteroposterior neural patterning and the subsequent development of spinal cord and hindbrain structures. RA also influences the embryonic development of forebrain structures such as the striatum and olfactory bulb (Valdenaire *et al*., 1998; Toresson *et al*., 1999).

Some evidence suggests that RA has roles in the postnatal brain. Retinoid binding proteins are expressed in olfactory bulb and lateral ventricle ependyma (Zetterstrom *et al*., 1994, 1999), and RA receptors persist into adulthood in the olfactory bulb (Krezel *et al*., 1999). The RA synthesizing enzyme retinaldehyde dehydrogenase-3 (RALDH3) is expressed in the SVZ, RMS and olfactory glomerular layer (Wagner *et al*., 2002). Importantly, adult transgenic mice expressing a RA response element (RARE)-reporter construct showed reporter expression in the SVZ and olfactory granular and glomerular layers, indicating RA-induced transcriptional activation in this pathway (Thompson *et al*., 2002).

In addition to expressions of RA signaling in the adult brain, recent work suggests that RA modulates cell proliferation or neurogenesis in regions where neural progenitors persist postnatally. RA exposure stimulates neurogenesis in neural stem cell cultures isolated from the embryonic striatal SVZ or adult hippocampus (Wohl and Weiss, 1998; Takahashi *et al*., 1999; Wang *et al*., 2005). Moreover, prolonged oral RA administration also increases adult rat SVZ cell proliferation (Giardino *et al*., 2000).

However, it is unknown whether RA plays a role under the pathological conditions such as brain ischemia. Because RA can induce neurogenesis during development and *in vitro* culture, there is possibility that RA may induce neurogenesis after ischemic injury. To test this possibility, the effects of RA on neurogenesis after the cerebral ischemia was examined. In contrast to our expectation, RA reduced ischemia-induced neurogenesis in this study. In addition, RA also reduced ischemia-induced angiogenesis and gliosis.

Materials and Methods

Animals

Male Sprague-Dawley rats (body weight 280-320 g) were caged in an air-conditioned room maintained at 22 $\pm$ 2^{o}C, 50 $\pm$ 10% relative humidity, with a 12/12 h light/dark cycle. Animals had free access to tap water and were fed a conventional rat chow diet. They were acclimated for 1 wk prior to the beginning of the study. Procedures related to animal care were in accord with the guidelines in 'Guide for the Care and Use of Laboratory Animals' (Bayne, 1996). Following acclimation, cerebral ischemia was induced by photothrombosis. Rats were injected with all-trans RA (1 mg/kg), disulfiram (5 mg/kg) or DMSO intraperitoneally. Rats were then sacrificed, whole brains removed, sliced in a contour-fit brain matrix.

Induction of focal cerebral infarction by photothrombosis

The procedure used for photothrombosis was as described by Watson *et al*. (1985), with slight modification. Briefly, Rose Bengal was dissolved in sterile saline at a concentration of 10 mg/ml, and then 0.1 ml containing 1 mg of Rose Bengal was injected intravenously 5 min before illumination. Rats were placed in a stereotactic frame with rectal temperature monitored and controlled at 36.5-37.5^{o}C with the help of a heating pad. At the dorsal aspect of the head, the skull was exposed by a median incision of the skin. The periosteum was gently removed and bregma and lambda points were identified. 532 nm green laser light (1.5 mm aperture) was centered at 4 mm posterior and 4 mm laterally from bregma after a hole was made in the skull. The duration of laser exposure was 15 min. The cerebral infarction was confirmed by 2,3,5 triphenyltetrazolium chloride staining the next day.

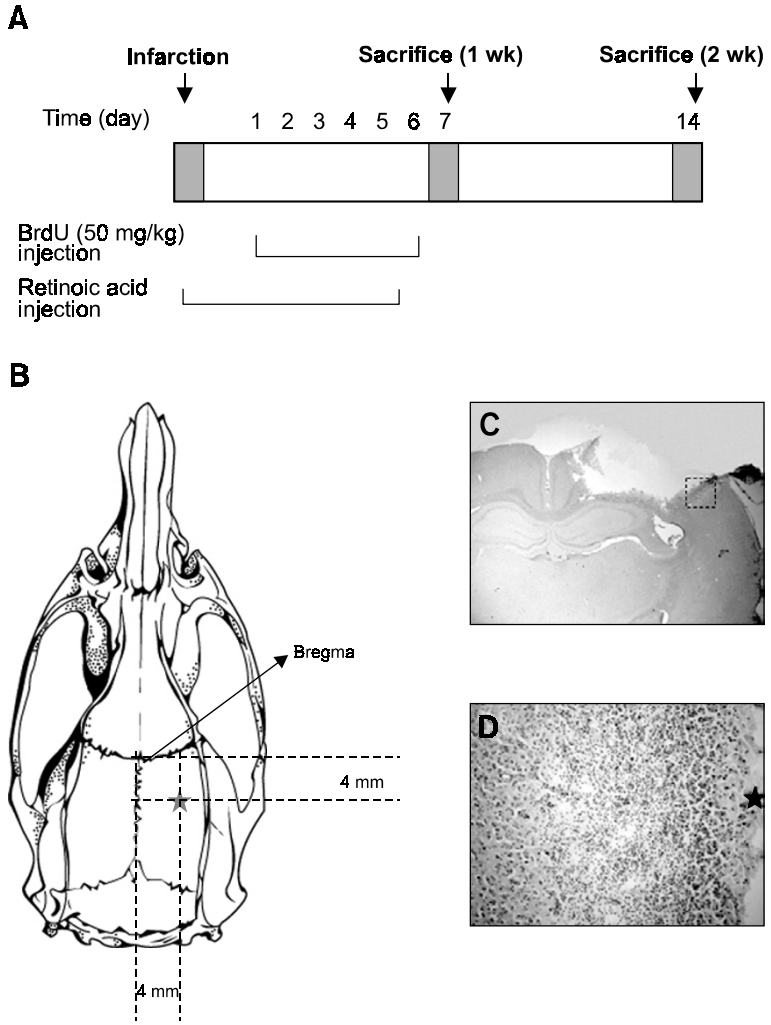

Figure 1. (A) Experimental paradigms of BrdU labeling. Rats were injected with all-trans RA or DMSO once a day for 6 d from the cerebral ischemia. Together with this RA treatment, rats were injected with BrdU (50 mg/kg) twice a day for 6 d from the next day after the cerebral ischemia. The rats were sacrificed 7 or 14 d after the cerebral ischemia. The brains were then perfused, sectioned, and immunostained, and the number of BrdU-labeled cells was counted in penumbral region, SVZ and SGZ. (B) Anatomical location of photothrombotic infarction was indicated by star mark. (C) H&E staining of cerebral cortex 1 wk after photothrombosis at a magnification of ×16. Rectangle indicates the magnified area at D. (D) H&E staining of cerebral cortex 1 wk after photothrombosis at a magnification of ×200. Star mark indicates the infract area by photothrombosis.

Immunohistochemistry

Animals were anesthetized with pentobarbital sodium (50 mg/kg) and sacrificed by intracardiac perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Brains were post-fixed for 4 h at 4 °C and cryoprotected with 30% sucrose. Tissues were frozen using OCT compound embedding medium in dry ice powder. Coronal sections were cut (30 μm) using a Leica CM3050 cryostat (Leica, Nussloch, Germany) and processed for immunohistochemistry. Every 6th section from the corpus striatum through to caudal hippocampal region was immunostained. Sections were incubated in a blocking buffer (0.3% Triton X-100 and 10% goat or horse serum in PBS) for 1 h, and then incubated overnight with primary antibodies in an incubation buffer (0.1% Triton X-100, 1% goat or horse serum and 1% BSA in PBS) at 4°C. The sections were then washed three times for 10 min each with PBS and incubated in secondary anti-mouse IgG, goat anti-rabbit IgG, or anti-rat IgG antibody (1:200, Alexa Fluor 488 or 594) for 2 h at room temperature. They were then washed, mounted on coated slides with Vector-shield (Vector Laboratories, Burlingame, CA). For all incubation and rinse steps, sections were agitated on a shaker table. The following primary antibodies were used; anti-doublecortin (DCX) (1:500, Santa Cruz Biotechnology, Santa Cruz, CA), anti-neuron specific nuclear protein (NeuN) (1:500, Chemicon, El Segundo, CA), anti-bromodeoxyuridine (BrdU) (1:500, Megabase Research Products, Lincoln, NB), anti-glial fibrillary acidic protein (GFAP) (1:500, Chemicon, El Segundo, CA) antibodies. Lectin (1:200, tomato, Vector Laboratories, Burlingame, CA) was used to detect endothelial cells. For staining with anti-DCX, anti-GFAP antibody or Lectin, rats were sacrificed 1 wk after cerebral ischemia and for anti-NeuN antibody staining, rats were sacrificed at 2 wk after cerebral ischemia.

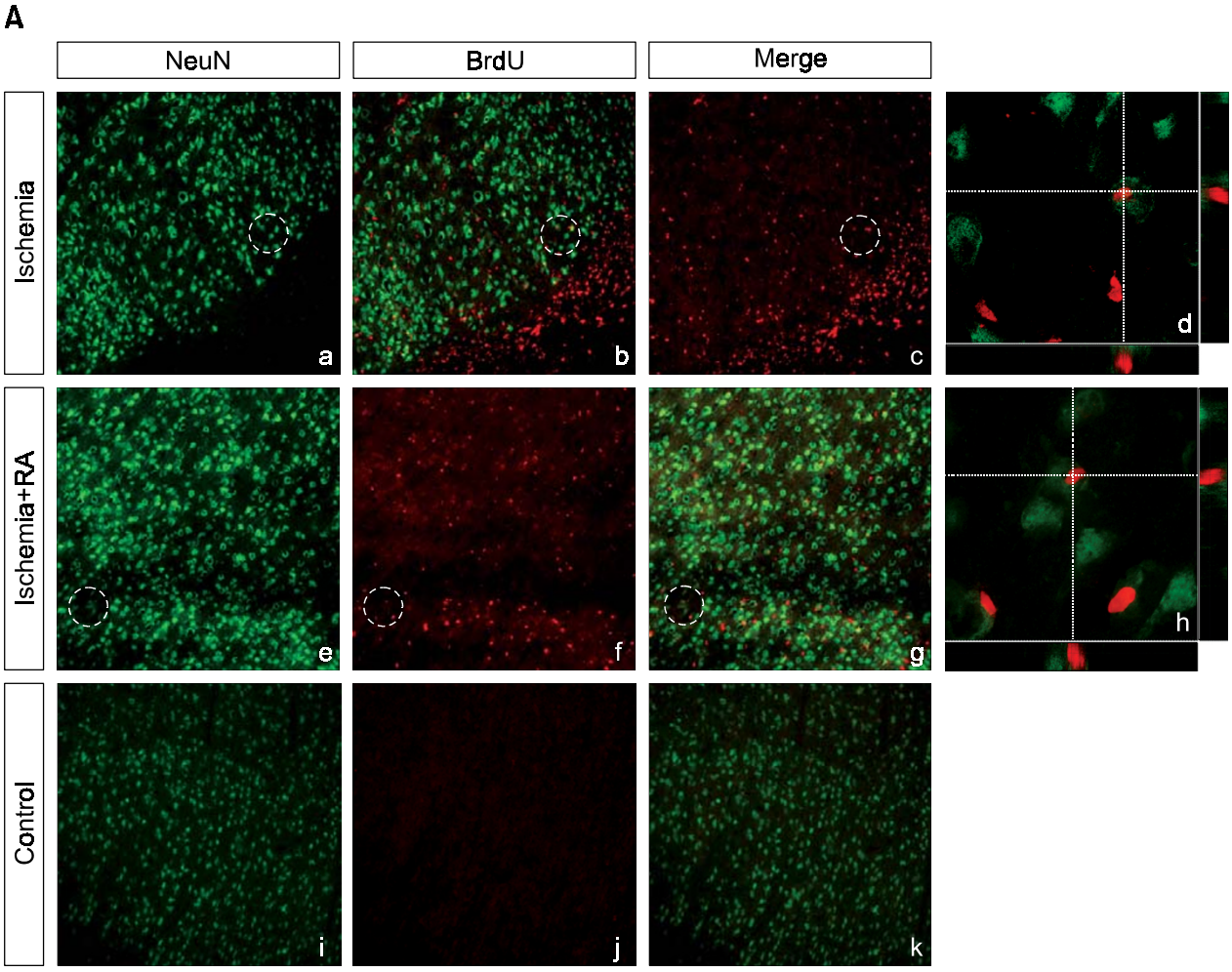

Figure 2. Retinoic acid (RA) reduced the neurogenesis in the penumbral region. The photothrombosis-induced cerebral ischemia increased the neurogenesis. This induction of the neurogenesis was reduced by RA treatment. The rats received RA and BrdU as described in Figure 1. Control rats received DMSO instead of RA. The rats were sacrificed 14 d after the ischemia. (A) The brain sections were stained with anti-BrdU (red, b, c, d, f, g, h, j, k) and anti-NeuN (green, a, c, d, e, g, h, i, k) antibodies. The doubly labeled cells were interpreted as a newly generated neuron. Confocal 3D reconstructed images (d, h) are presented as viewed in the x-z (bottom) and y-z (right) planes. Circles indicate the magnified area in d and h. Magnification × 200 (a-c, e-g, i-k), × 1600 (d, h). (B) The newly generated neurons were counted in the penumbral region. Data are means ± SEM. **P* < 0.05.

BrdU immunohistochemistry

Rats were injected with BrdU (50 mg/kg) intraperitoneally twice a day for 6 d from the next day after the cerebral ischemia (Figure 1). Brain sections were pretreated with 50% formamide/280 mM NaCl/30 mM sodium citrate at 65°C for 2 h, and incubated in 2 M HCl at 37°C for 1 h. The remaining procedure used was the same as that described in "immuno-

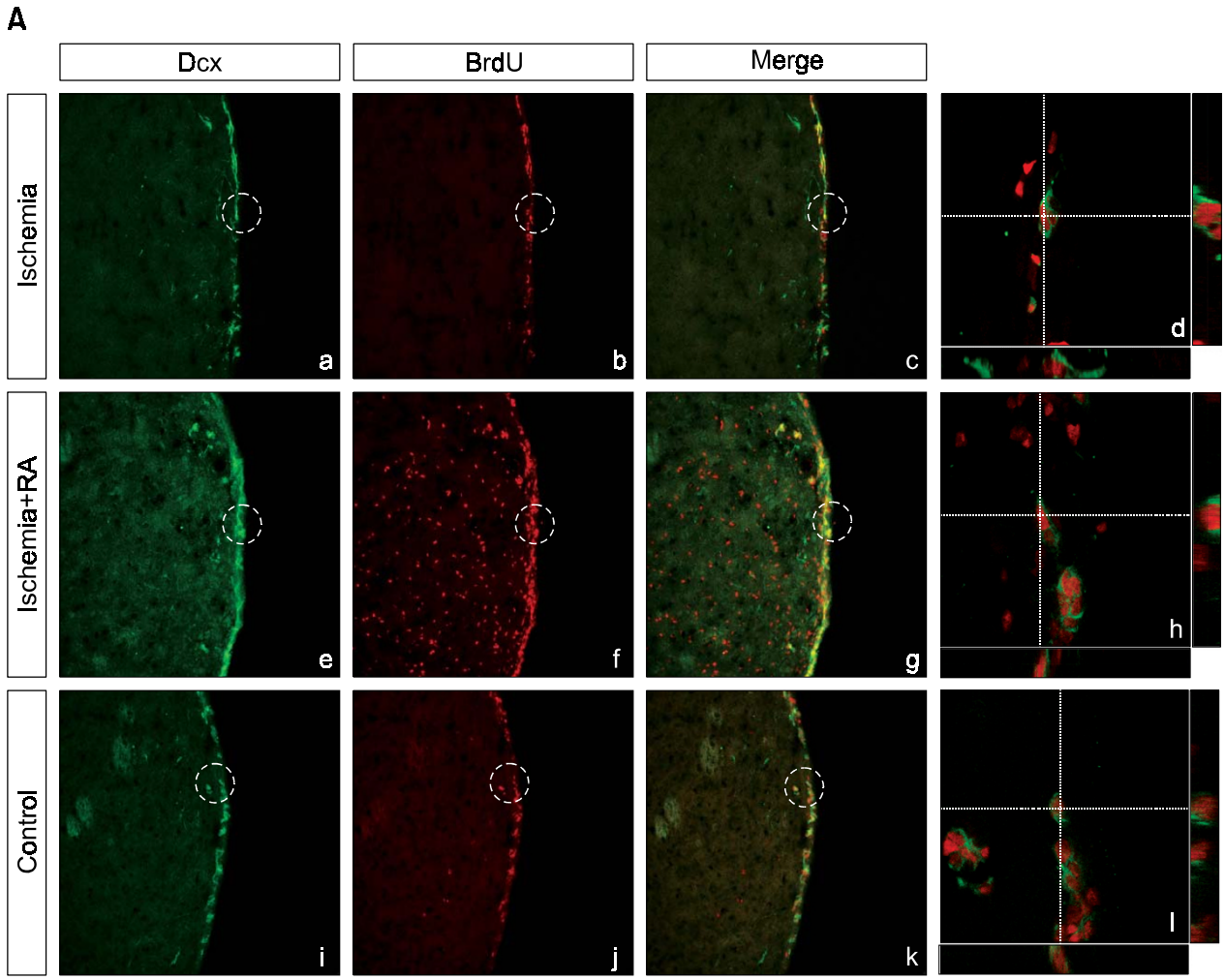

Figure 3. Retinoic acid (RA) reduced the neurogenesis in sub ventricular zone (SVZ). The photothrombosis-induced cerebral ischemia increased the neurogenesis in SVZ. This induction of the neurogenesis was reduced by RA treatment. Control rats received DMSO instead of RA. The rats were sacrificed 7 d after the ischemia. (A) The brain sections were stained with anti-BrdU (red, b, c, d, f, g, h, j, k, l) and anti-doublecortin (DCX, green, a, c, d, e, g, h, i, k, l) antibodies. The doubly labeled cells were interpreted as newly generated neuroblasts. Confocal 3D reconstructed images (d, h, l) are presented as viewed in the x-z (bottom) and y-z (right) planes. Circles indicate the magnified area in d and h. Magnification × 200 (a-c, e-g, i-k), ×1,600 (d, h, l). (B) The newly generated neuroblasts were counted in SVZ. Data are mean ± SEM. *$P < 0.05$.

histochemistry" above.

Morphological analysis

Every 6th sections that included SGZ, SVZ or penumbral region were immunostained as described above. In case of SVZ or penumbral region, BrdU- and phenotype-specific marker-double positive cells were counted at a magnification of × 200 in five regions,

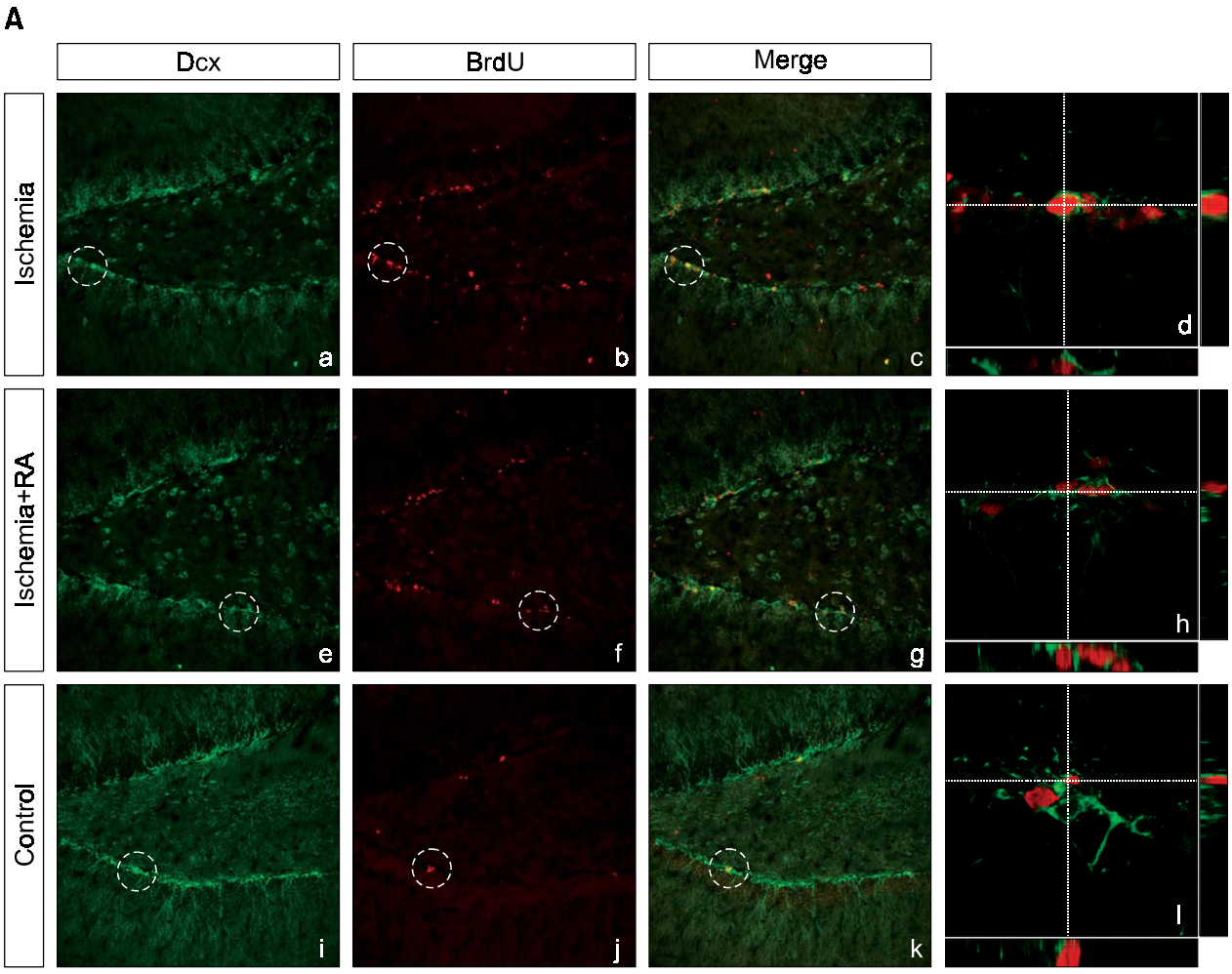

Figure 4. Retinoic acid (RA) reduced the neurogenesis in subgranular zone (SGZ). The photothrombosis-induced cerebral ischemia increased the neurogenesis in hippocampus. This induction of the neurogenesis was reduced by RA treatment. Control rats received DMSO instead of RA. The rats were sacrificed 7 days after the ischemia. (A) The brain sections were stained with anti-BrdU (red, b, c, d, f, g, h, j, k, l) and anti-doublecortin (DCX, green, a, c, d, e, g, h, i, k, l) antibodies. The doubly labeled cells were interpreted as newly generated neuroblasts. Confocal 3D reconstructed images (d, h, l) are presented as viewed in the x-z (bottom) and y-z (right) planes. Circles indicate the magnified area in d and h. Magnification × 200 (a-c, e-g, i-k), × 1,600 (d, h, l). (B) The newly generated neuroblasts were counted in SGZ. Data are mean ± SEM. **P* < 0.05.

each 0.85 mm^2 area. In case of SGZ, double positive cells were counted at a magnification of × 200 in all immunostained regions where SGZ was included. At least three different rats were used for counting. To confirm the double labeling, a confocal microscope Leica TCS SP2 (Leica, Nussloch, Germany) was used. At least 50 BrdU+ cells per animal were analyzed using Z-plane sectioning (1 μm).

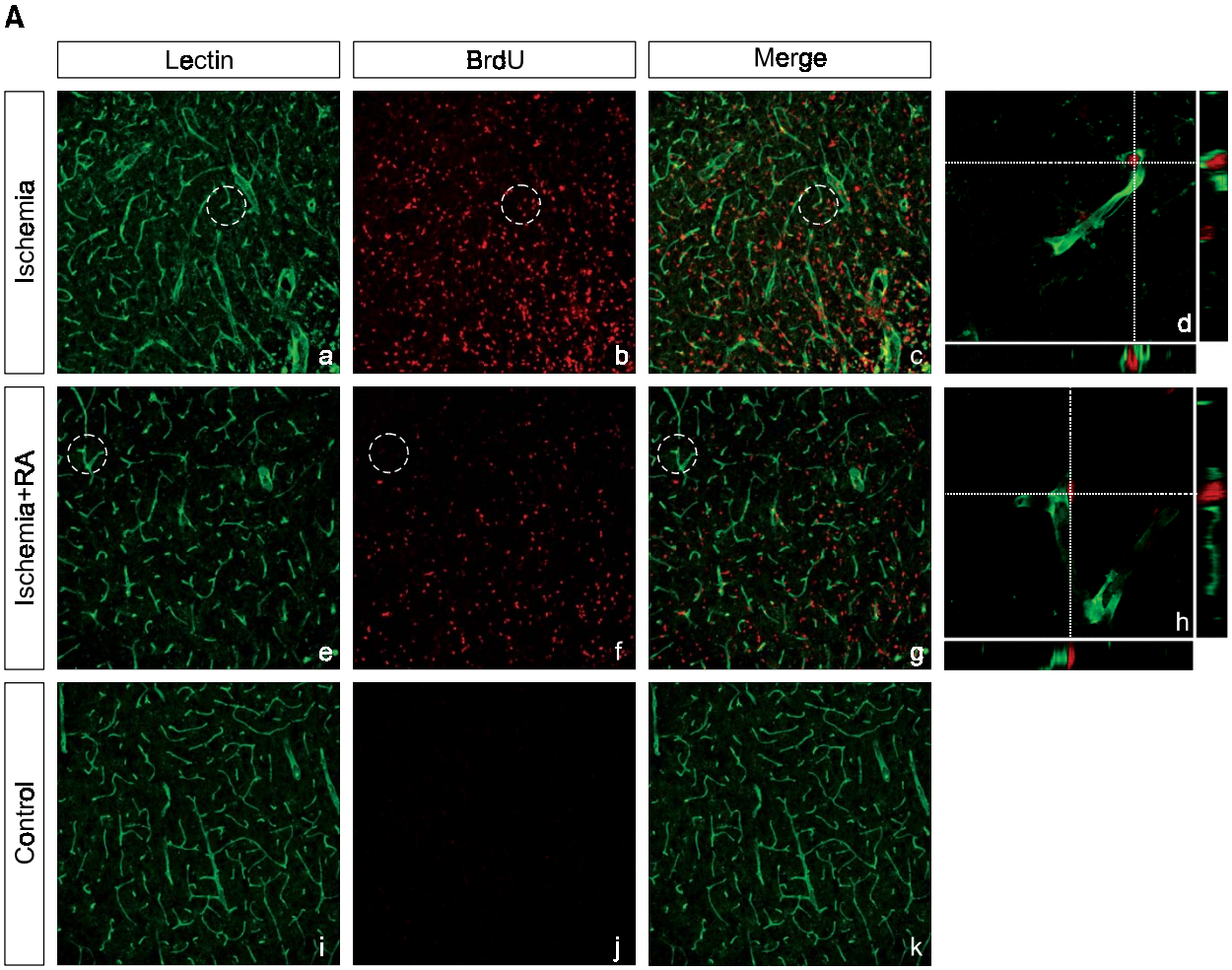

Figure 5. Retinoic acid (RA) reduced the angiogenesis in the penumbral region. The photothrombosis-induced cerebral ischemia induced angiogenesis in the penumbra region. This induction of the angiogenesis was reduced by RA treatment. Control rats received DMSO instead of RA. The rats were sacrificed 7 d after the ischemia. (A) The brain sections were stained with anti-BrdU (red, b, c, d, f, g, h, j, k) antibody and tomato Lectin (Lec, green, a, c, d, e, g, h, i, k). The doubly labeled cells were interpreted as newly generated endothelial cells. Confocal 3D reconstructed images (d, h) are presented as viewed in the x-z (bottom) and y-z (right) planes. Circles indicate the magnified area in d and h. Magnification × 200 (a-c, e-g, i-k), ×1,600 (d, h). (B) the newly generated endothelial cells were counted in the penumbral region. Data are mean ± SEM. *$P < 0.05$.

Data analysis

Data are presented as means ± SEM. Differences between mean values were evaluated using the Student's *t*-test (unpaired comparison). *P* values of < 0.05 were considered statistically significant.

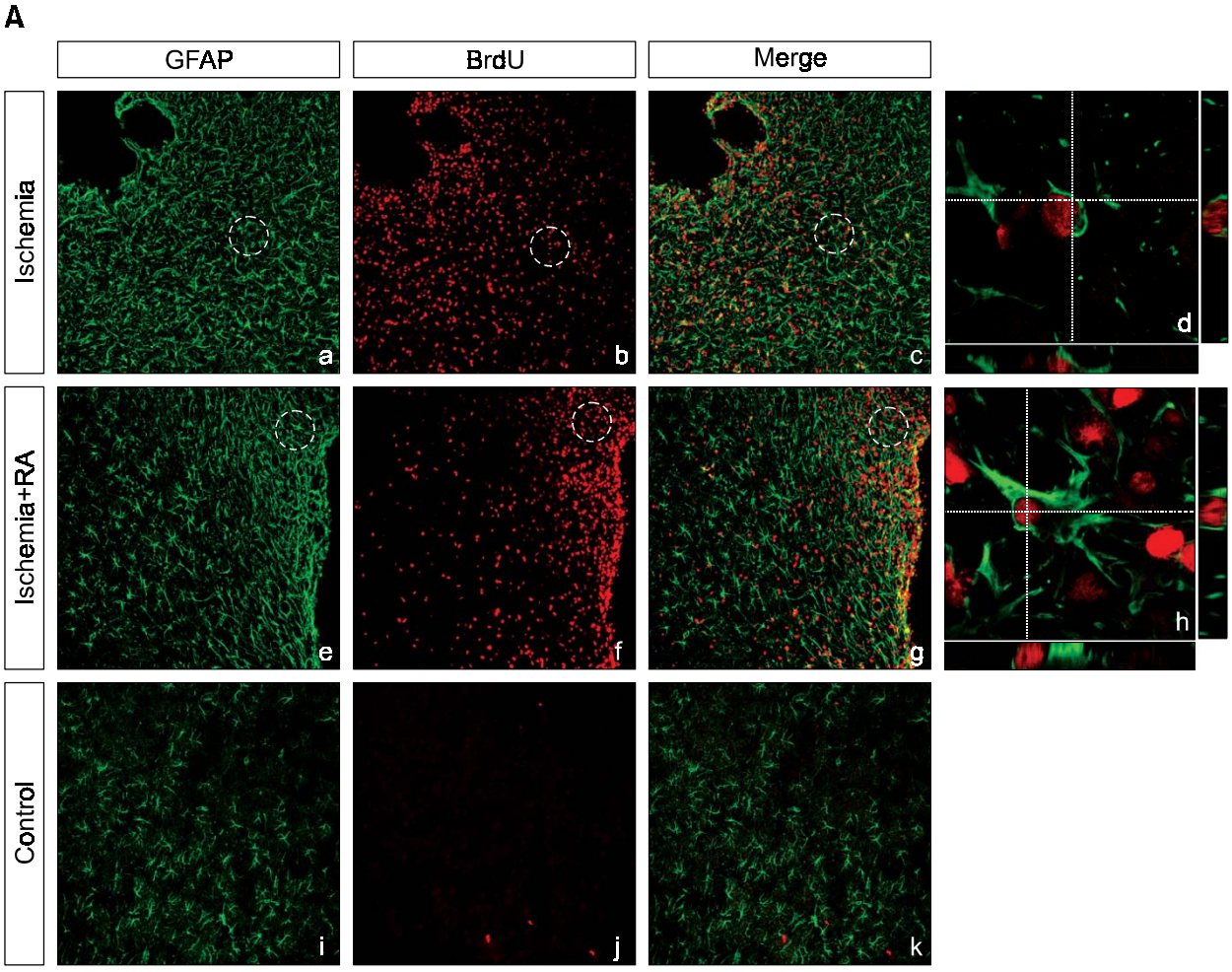

Figure 6. Retinoic acid (RA) reduced the gliosis in the penumbral region. The photothrombosis-induced cerebral ischemia increased gliosis in the penumbral region. This induction of the gliosis was reduced by RA treatment. Control rats received DMSO instead of RA. The rats were sacrificed 7 d after the ischemia. (A) The brain sections were stained with anti-BrdU (red, b, c, d, f, g, h, j, k) and anti-GFAP (green, a, c, d, e, g, h, i, k) antibodies. The doubly labeled cells were interpreted as newly generated astrocytes. Confocal 3D reconstructed images (d, h) are presented as viewed in the x-z (bottom) and y-z (right) planes. Circles indicate the magnified area in d and h. Magnification × 200 (a-c, e-g, i-k), × 1,600 (d, h). (B) The newly generated astrocytes were counted in the penumbra region. Data are mean ± SEM. *$P < 0.05$.

Results

The penumbral region is a peri-infarction region, in which cerebral blood flow is significantly reduced, but necrotic cell death is not as extensive. In the penumbral region, complex reactions, such as neurogenesis, angiogenesis, gliosis and inflammatory reaction occur.

To induce cerebral ischemia, we used the photothrombotic rat model because it gives us consis-

tent size of cerebral infarction compared to other models.

The effect of RA on neurogenesis was examined in the penumbral region where high level of GFAP was expressed. Anti-BrdU and anti-NeuN antibodies were used to check cell proliferation and to mark neurons respectively. Newly generated neurons were stained with both anti-BrdU and anti-NeuN antibodies. The cerebral ischemia increased the number of BrdU+ cells in the penumbral region. However, RA treatment significantly reduced this increase (Figure 2). The number of doubly-stained newly generated neurons after cerebral infarction was counted, and RA treatment reduced the cerebral ischemia-induced neurogenesis by 48% (Figure 2B).

In the SVZ and the SGZ neurogenesis is constitutive, and thus, we examined how cerebral ischemia affected neurogenesis in these areas. To examine the SVZ, we focused on the rostral lateral wall of the lateral ventricle, where neuroblast proliferation is most frequently observed, and for the SGZ, we focused on the dorsal hippocampus. Anti-doublecortin (DCX) and anti-BrdU antibodies were used to check for the proliferation of immature neuroblasts. The proliferation of DCX+ immature neuroblasts in the SVZ was significantly increased by cerebral ischemia, and this increase in neuroblast proliferation was reduced by RA treatment (Figure 3). The number of proliferating neuroblasts double-labeled by anti-DCX and anti-BrdU antibodies was counted. RA treatment reduced the cerebral ischemia-induced increase of neuroblast proliferation at SVZ by 43% (Figure 3B). RA treatment alone without cerebral ischemia also significantly reduced the constitutive neurogenesis at SVZ compared to the control (data not shown). The intensity of anti-DCX staining at SGZ was significantly reduced by the antigen retrieval process for BrdU staining, which was described under the Materials and Methods section, although the counting of doubly stained cells is possible. The proliferation of DCX+ immature neuroblasts in the SGZ was also significantly increased by cerebral ischemia, and this increase in neuroblast proliferation was reduced by RA treatment (Figure 4). RA treatment reduced the cerebral ischemia-induced increase of neuroblast proliferation at SGZ by 29% (Figure 4B). RA treatment alone without cerebral ischemia also significantly reduced the constitutive neurogenesis at SGZ compared to the control (data not shown).

Next, to examine effects of loss-of-function, we tested effects of disulfiram, an inhibitor of endogenous RA synthesis, on neurogenesis. Treatment of disulfiram (5 mg/kg) for 6 d significantly reduced neurogenesis at SGZ and SVZ in a physiological condition and at penumbral regions after ischemia (data not shown).

Neurogenesis has been reported to be associated with angiogenesis (Shen *et al*., 2004; Ohab *et al*., 2006), and thus, angiogenesis in the penumbral region was also examined. Lectin and anti-BrdU antibody were used to identify newly generated endothelial cells. Cerebral ischemia induced the generation of new endothelial cells and RA treatment reduced this induction of angiogenesis by 39% (Figure 5).

Astrocytes can produce trophic factors to neurons and vascular cells. Thus, we examined the effects of RA on gliosis using anti-GFAP and anti-BrdU antibodies, where doubly-labeled cells are newly generated astrocytes. Cerebral ischemia induced extensive gliosis in the penumbral region (Figure 6). RA treatment reduced this cerebral ischemia-induced gliosis by 44% (Figure 6B).

Discussion

New neurons are produced in the penumbral region after the cerebral ischemia. The underlying molecular mechanism of neurogenesis in the penumbral region is poorly understood. Because RA had been shown to increase neurogenesis during the development and *in vitro* culture, we examined effects of RA on neurogenesis after the cerebral ischemia. In contrast to our expectation, RA did not increase the neurogenesis in the penumbral region after the cerebral ischemia. Instead, RA reduced neurogenesis, angiogenesis, gliosis in the penumbral region.

Effects of RA on neurogenesis are controversial. Some results suggest that RA enhances neurogenesis. For example, in neural stem cell cultures isolated from the embryonic striatal SVZ or adult hippocampus, RA exposure stimulates neurogenesis (Wohl and Weiss, 1998; Takahashi *et al*., 1999). Moreover, in SVZ neurosphere stem cell and parasagittal brain slice cultures derived from postnatal mouse, RA exposure increased neurogenesis by enhancing the proliferation and neuronal differentiation of forebrain SVZ neuroblasts (Wang *et al*., 2005). Furthermore, prolonged oral RA administration also increases adult rat SVZ cell proliferation (Giardino *et al*., 2000). However, opposite effects of RA were also reported. Chronic exposure to 13-cis-RA reduced cell proliferation in the hippocampus and SVZ (Crandall *et al*., 2004).

Under the pathological conditions such as cerebral ischemia, effects of RA on neurogenesis have never been tested. Regulatory mechanism of neurogenesis under the pathological condition may be different from that under the physiological condition. For example, inflammatory processes under

the pathological condition can modulate neurogenesis. In addition to the regulatory mechanism, the origin of neural stem cells under the pathological condition is not clearly defined. A previous report suggests the astrocyte which originated from the radial glial cell during the developmental period acts as a cortical stem cell (Merkle *et al*., 2004). Another study showed neural stem cells from SVZ could migrate into the infarction area (Jin *et al*., 2003). In present study, we found that RA reduced ischemia-induced neurogenesis in penumbra region, SVZ and SGZ (Figure 2-4). It remains to be studied how RA regulates ischemia-induced neurogenesis.

Effects of disulfiram, an inhibitor of endogenous RA synthesis, were consistent with the previous report (Wang *et al*., 2005), although this result was against our conclusion. However many other effects of disulfiram have been reported. Disulfiram has been shown to induce cytotoxic effects *in vivo* and *in vitro* via inhibition of the proteasome activity (Chen *et al*., 2006; Wickstrom *et al*., 2007). Disulfiram can also inhibit superoxide dismutase I (Dimayuga *et al*., 2007) and dopamine β-hydroxylase (Bourdelat-Parks *et al*., 2005). So, there is possibility that these other effects of disulfiram might have affected results in the present study.

There is substantial overlap between the molecular and cellular events of angiogenesis and neural stem cell responses (Carmichael, 2003; Ohab *et al*., 2006). For example, both angiogenesis and neurogenesis are initiated by hypoxia, VEGF and EPO (Studer *et al*., 2000; Shingo *et al*., 2001; Sun *et al*., 2003). Moreover, newly formed blood vessels and neural stem cells share similar extracellular adhesion proteins (Carmeliet, 2000; Hayashi *et al*., 2003). Futhermore, neural stem cells associate with brain microvasculature in proliferative clusters (Palmer *et al*., 2000). In addition, human cerebral endothelial cells express a set of neurgenic proteins (Shusta et al., 2002). So, endothelial cells can directly induce neurogenesis from nearby neural stem cell. In present study, the cerebral ischemia induced new endothelial cells in the penumbral region. RA treatment reduced this induction (Figure 5). Regulation of angiogenesis by RA has never been reported before. In the future study, it should be defined whether this regulation by RA is direct or indirect.

Astrocytes have been shown to play both positive and negative roles after cerebral ischemia (Nedergaard and Dirnagl, 2005; Trendelenburg and Dirnagl, 2005). Astrocytes provide metabolites, shuttle ions, and water and scavenge reactive oxygen species (ROS) and reactive metals. Astrocytes are key contributors to protection against cerebral ischemia induced by many types of preconditioning. However, under certain conditions, activated astrocytes actively contribute to the demise of brain tissue after cerebral ischemia. By the repulsion of regenerating axons, reactive astrocytes and their proteoglycan products may actively impede brain reorganization. In present study, RA treatment significantly reduced gliosis in the penumbral region. This effect may be beneficial for the axonal sprouting of new neurons (Figure 6). Whether the regulation of gliosis by RA is direct or indirect remains to be dissolved.

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