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Cortical neurogenesis in adult rats after ischemic brain injury: most new neurons fail to mature

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Abstract

The present study examines the hypothesis that endogenous neural progenitor cells isolated from the neocortex of ischemic brain can differentiate into neurons or glial cells and contribute to neural regeneration. We performed middle cerebral artery occlusion to establish a model of cerebral ischemia/reperfusion injury in adult rats. Immunohistochemical staining of the cortex 1, 3, 7, 14 or 28 days after injury revealed that neural progenitor cells double-positive for nestin and sox-2 appeared in the injured cortex 1 and 3 days post-injury, and were also positive for glial fibrillary acidic protein. New neurons were labeled using bromodeoxyuridine and different stages of maturity were identified using doublecortin, microtubule-associated protein 2 and neuronal nuclei antigen immunohistochemistry. Immature new neurons coexpressing doublecortin and bromodeoxyuridine were observed in the cortex at 3 and 7 days post-injury, and semi-mature and mature new neurons double-positive for microtubule-associated protein 2 and bromodeoxyuridine were found at 14 days post-injury. A few mature new neurons coexpressing neuronal nuclei antigen and bromodeoxyuridine were observed in the injured cortex 28 days post-injury. Glial fibrillary acidic protein/bromodeoxyuridine double-positive astrocytes were also found in the injured cortex. Our findings suggest that neural progenitor cells are present in the damaged cortex of adult rats with cerebral ischemic brain injury, and that they differentiate into astrocytes and immature neurons, but most neurons fail to reach the mature stage.

***Key Words:** nerve regeneration; middle cerebral artery occlusion; brain injury; neurons; astrocytes; oligodendrocytes; neural progenitor cells; proliferation; differentiation; neurogenesis; neural regeneration*

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Introduction

Current treatment strategies for ischemic stroke focus primarily on reducing the area of ischemic injury and protecting the dying cells early after the injury occurs (Gilman, 2006; Goldstein, 2007; Sahota and Savitz, 2011). Few treatments to date have proved satisfactory in clinical trials, despite advances in stem cell transplantation and many neuroprotective compounds showing positive effects in animal models of ischemic brain injury (Dirnagl, 2006; Honmou et al., 2011; Qureshi and Mehler, 2011). The only therapy for acute ischemic stroke that has been approved by the United States Food and Drug Administration is intravenous recombinant tissue plasminogen activator. However, its use is limited by a narrow therapeutic window; it must be administered within 3–4.5 hours of symptom onset (Nakashima and Minematsu, 2009; El Amki et al., 2012). The lack of effective drugs for the repair of damaged brain tissue has motivated researchers to focus on stem cells as a potential avenue to replace cells lost to injury. Functional recovery was demonstrated by transplanting exogenous stem cells into rodent models of ischemic brain injury (Burns et al., 2009; Li et al., 2009; Lee et al., 2010; Honmou et al., 2011; Chen et al., 2014). However, many problems are yet to be addressed, for instance, an appropriate source for the stem cells, immune rejection, ethical issues and theoretical questions (Yi et al., 2013). Interest in cellular regeneration after ischemic brain injury has therefore turned toward the use of endogenous neural stem cells.

In adult mammals, endogenous neural stem cells, known under physiological conditions as neural progenitor cells, reside in the subventricular zone (Lois and Alvarez-Buylla, 1994), subgranular zone of the dentate gyrus (Kornack and Rakic, 2001), and other regions including the neocortex and hypothalamus (Whitman and Greer, 2009; Kernie and Parent, 2010). Enhanced neurogenesis has been reported in the subventricular and subgranular zones in rodent brains with ischemia (Arvidsson et al., 2002; Türeyen et al., 2004). Neurogenesis occurs in the ischemic striatum but not in the neocortex, despite a large lesion in this area (Arvidsson et al., 2002; Parent et al., 2002). However, some studies have shown that repair mechanisms may allow cortical recovery (Magavi et al., 2000; Thored et al., 2006), so cortical regeneration after ischemia remains controversial. Neural stem cells from damaged adult rat cerebral cortex and adult human neocortex have been isolated *in vitro* (Richardson et al., 2006; Itoh et al., 2013), but it is unclear whether those cells have the potential to differentiate into neurons or glia and to what extent they contribute to neurogenesis after injury.

Here, we used immunofluorescence to examine a rat model of ischemic stroke produced by temporary middle cerebral artery occlusion and reperfusion, to determine whether endogenous neurogenesis occurs in the adult rat cortex after ischemic brain injury.

Materials and Methods

Animals and focal cerebral ischemia

Sixty female specific pathogen-free Sprague-Dawley rats (Nantong University, license No. SCXK (Su) 2008-0010) weighing 220 ± 10 g were randomly and equally allocated to a model group and a sham-operated group. Animal procedures were carried out according to the NIH Guidelines for the Care and Use of Laboratory Animals and approved by the Animal Experimental Committee of Nanjing Medical University of China.

All rats were anesthetized with chlorpent (Sigma, San Francisco, CA, USA; 2 mL/kg), and those in the model group underwent left middle cerebral artery occlusion using monofilament thread, as first described by Longa et al. (1989) and revised by Ma et al. (2006). After 90 minutes of occlusion, the thread was removed and the incision was sutured. In the sham-operated group, the left common carotid artery was exposed to the same level as that in the model group, but arterial ligation was not performed. Upon recovery from anesthesia, the rats were returned to their cages with postoperative care and free access to food and water.

Bromodeoxyuridine (BrdU) injection

To label new cells, all rats were injected intraperitoneally with BrdU (50 mg/kg; Sigma, St. Louis, MO, USA), twice a day for 7 days following surgery.

Immunofluorescent labeling of cortical cells

At each experimental time point (1, 3, 7, 14 or 28 days after surgery), six model rats and six sham-operated rats were anesthetized and perfused transcardially with 0.9% NaCl followed by 4% paraformaldehyde in 0.1 M phosphate buffer. The brains were removed and post-fixed in 4% paraformaldehyde for 4 hours at room temperature. Coronal sections, 30 μm thick, were prepared using a cryostat (Leica CM1900, Munich, Germany), and blocked with 10% goat serum in 0.01 M sodium phosphate buffer containing 0.05% (v/v) Tween-20 (pH 7.4) for 1 hour at room temperature. The sections were incubated for 24 hours at room temperature with the following primary antibodies (Yi et al., 2013): mouse anti-nestin monoclonal antibody (1:2,000; Millipore, Billerica, MA, USA) and rabbit anti-sox-2 polyclonal antibody (1:1,000; Abcam, Cambridge, UK), both markers for neural stem cells; rat anti-BrdU polyclonal antibody (1:100; Abcam), a marker for new cells; guinea pig anti-doublecortin (DCX) polyclonal antibody (1:1,000; Millipore), a marker for immature neurons; mouse anti-microtubule associated protein 2 (MAP-2) monoclonal antibody (1:500; Millipore), a marker for semi-mature and mature neurons; mouse anti-NeuN monoclonal antibody (1:500; Millipore), a marker of mature neurons; mouse anti-glial fibrillary acidic protein (GFAP) monoclonal antibody (1:200; Sigma), an astrocyte marker; or mouse anti-2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNP) monoclonal antibody (1:800; Millipore), an oligodendrocyte marker. They were then incubated for 24 hours at room temperature with Alexa Fluor 568-conjugated goat anti-rabbit IgG (1:1,000; Molecular Probes, Los Angeles, USA), Alexa Fluor 488-conjugated goat anti-mouse or anti-guinea pig IgG (both 1:200; Invitrogen, Carlsbad, CA, USA), or Alexa Fluor 647-conjugated goat anti-rat IgG (1:200). Cell nuclei were counterstained with Hoechst33342 for 30 minutes at room temperature. Immunopositive cells were counted in 0.5 mm^2 of cortex in three brain sections per rat (front, middle and rear of the injured area in model rats, and the equivalent regions in sham-operated rats) by an experimenter blind to the grouping.

Statistical analysis

Data were presented as the mean ± SD. One-way analysis of variance was performed using SPSS 16.0 software (SPSS, Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

Results

Endogenous nestin/sox-2 double-labeled neural progenitor cells appeared in the injured cortex of adult rats after ischemic brain injury

Immunofluorescence staining for nestin and sox-2 (both markers for neural stem cells) was performed on days 1, 3, 7, 14 and 28 post-surgery to determine whether endogenous neural progenitor cells emerged in the adult rat cortex after ischemic brain injury. Double-labeled cells were observed in the injured cortex 1 and 3 days after surgery, after which staining was no longer observed. There were more neural progenitor cells in the injured cortex on day 3 than on day 1 ($P < 0.05$). No cells expressing nestin or sox-2 were found in sham-operated rat cortex (**Figure 1**).

Nestin/sox-2 double-labeled endogenous neural progenitor cells originated from activated astrocytes in the damaged cortex of adult rats after ischemic brain injury

Triple immunofluorescence staining for nestin, sox-2 and GFAP was carried out 1, 3, 7, 14 and 28 days after surgery to determine whether neural progenitor cells originated from astrocytes in the injured cortex of model rats. Nestin/sox-2/GFAP triple-labeled neural progenitor cells were observed in the cortex of model rats 1 and 3 days post-injury; no triple-labeled cells were found in the cortex of sham-operated rats (**Figure 2**).

New neurons in the damaged cortex of adult rats after ischemic brain injury

DCX/BrdU (**Figure 3**), MAP-2/BrdU (**Figure 4**) and NeuN/BrdU (**Figure 5**) immunofluorescence was used to identify new neurons at different stages of maturity. Immature DCX/BrdU double-labeled neurons were found in the cortex of model rats 3 and 7 days post-injury, with more double-labeled cells on day 7 than day 3 ($P < 0.05$). Some semi-ma-

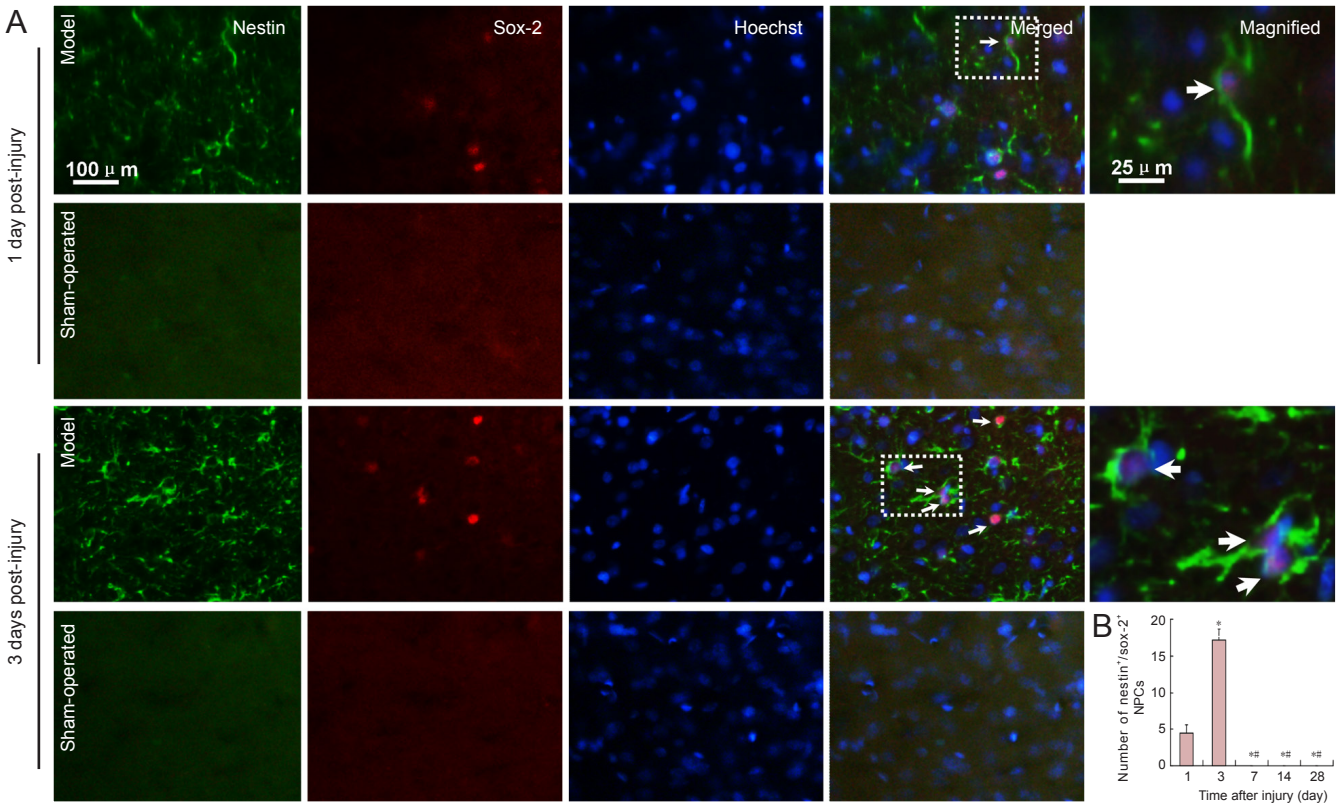

Figure 1 Endogenous neural progenitor cells (NPCs) in the damaged cortex of adult rats after ischemic brain injury.
(A) Immunofluorescence micrographs of nestin/sox-2 double-labeled endogenous NPCs in model rats; no nestin or sox-2 expression was seen in sham-operated rats. Green (Alexa Fluor 488): nestin; red (Alexa Fluor 568): sox-2; blue (Hoechst 33342): nuclei; arrows: nestin/sox-2 double-labeled cells. Scale bar: 100 μm (scale bar in magnified image: 25 μm). (B) Quantification of nestin/sox-2 double-labeled NPCs in 0.5 mm^2 of injured cortex in model rats. Data are presented as the mean ± SD. $*P < 0.05$, *vs.* day 1; $\#P < 0.05$, *vs.* day 3 (one-way analysis of variance).

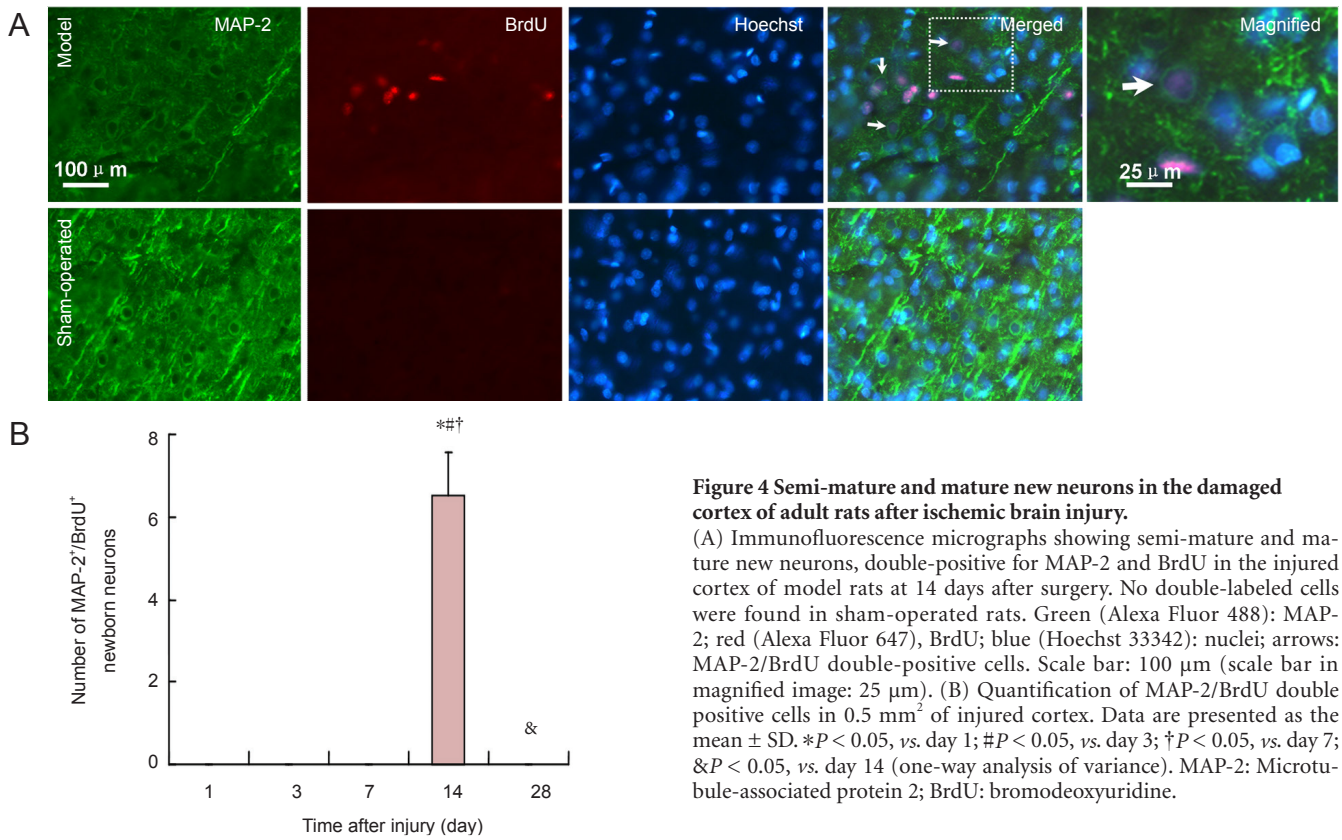

Figure 4 Semi-mature and mature new neurons in the damaged cortex of adult rats after ischemic brain injury.
(A) Immunofluorescence micrographs showing semi-mature and mature new neurons, double-positive for MAP-2 and BrdU in the injured cortex of model rats at 14 days after surgery. No double-labeled cells were found in sham-operated rats. Green (Alexa Fluor 488): MAP-2; red (Alexa Fluor 647), BrdU; blue (Hoechst 33342): nuclei; arrows: MAP-2/BrdU double-positive cells. Scale bar: 100 μm (scale bar in magnified image: 25 μm). (B) Quantification of MAP-2/BrdU double positive cells in 0.5 mm^2 of injured cortex. Data are presented as the mean ± SD. $*P < 0.05$, *vs.* day 1; $\#P < 0.05$, *vs.* day 3; $\dagger P < 0.05$, *vs.* day 7; $\&P < 0.05$, *vs.* day 14 (one-way analysis of variance). MAP-2: Microtubule-associated protein 2; BrdU: bromodeoxyuridine.

ture and mature MAP-2/BrdU double-positive cells were observed 14 days after injury, and a small number of mature NeuN/BrdU double-labeled neurons were found 28 days after injury. No BrdU-positive cells were found in sham-operated rats (**Figures 3–5**).

New astrocytes in the damaged cortex of adult rats after ischemic brain injury

New astrocytes, double-positive for GFAP/BrdU, were found in the injured cortex of model rats at 1, 3, 7, 14 and 28 days after surgery, with the number peaking at day 7 and remaining stable thereafter. No GFAP/BrdU double-labeled cells were found in the cortex of rats in the sham-operated group (**Figure 6**).

No new oligodendrocytes in adult rat cortex after ischemic brain injury

No CNP/BrdU double-labeled cells were found in rats of either group at any time point (**Figure 7**).

Quantification of neural progenitor cells, new neurons and new astrocytes in adult rat cortex after ischemic brain injury

BrdU-immunopositive cells were found in the injured cortex of ischemic brain injury rats at all time points examined after surgery (**Figures 3–6**), peaking in number at day 7 and then decreasing, indicating that some new cells subsequently died. New BrdU-positive cells in the injured cortex of rats mainly comprised neural progenitor cells, new differentiated cells and reactive glial cells. The percentage of new astrocytes (GFAP/BrdU double-positive) was greater than that of new neurons (DCX/BrdU double-positive + MAP-2/BrdU double-positive + NeuN/BrdU double-positive) ($P < 0.05$; **Figure 8**).

Discussion

In contrast with previous findings that cells lost to injury in the adult mammalian brain could not be regenerated, there is mounting evidence not only that neural stem cells exist in the brain but also that neurogenesis is an ongoing process throughout life (Johansson et al., 1999; Song et al., 2002). Neural stem cells can proliferate and divide exponentially, have the capacity to differentiate into multiple cell types, and can migrate in response to damage signals, all characteristics of a promising treatment for stroke (Abrahams et al., 2004; Dong et al., 2012).

Cortical regeneration after ischemia remains a subject of debate. Several studies have demonstrated that neurogenesis is enhanced in the subventricular and subgranular zones in adult rodent brains after focal ischemia (Arvidsson et al., 2002; Türeyen et al., 2004; Kaneko et al., 2013; Li et al., 2014; Liu et al., 2014; Sun et al., 2014). Adult brain injuries can induce NPC proliferation, an attempt at self-repair, and endogenous progenitors can generate new neurons in the ischemic striatum but not in the neocortex, despite a large injury burden in this area (Arvidsson et al., 2002; Parent et al., 2002).

However, Magavi and colleagues (Magavi et al., 2000) observed that latent neural progenitor cells in the adult cortex are activated after brain injury. Furthermore, neural stem cells isolated from damaged adult rat cerebral cortex can be identified *in vitro* 3 days after injury, corresponding to a peak in nestin expression in the injured cortex (Itoh et al., 2005, 2013). In the present study, the neural stem cell markers nestin and sox-2 were employed to investigate endogenous neural progenitor cells in the cortex after ischemic brain injury. Nestin/sox-2 double-positive neural progenitor cells were found 1 and 3 days after injury, but then disappeared. The neural progenitor cells may migrate from the subventricular zone along the corpus callosum (Leker et al., 2007; Yi et al., 2013) or originate from quiescent potential neural stem cells that already reside within the neocortex (Ziemka-Nałęcz and Zalewska, 2012; Yi et al., 2013). In our study, the nestin/sox-2 double-positive neural progenitor cells were also positive for GFAP, indicating that these cells originate from activated astrocytes in the injured cortex.

From 7 days after injury, no neural progenitor cells were found in the injured cortex. We investigated NPC differentiation by observing cells double-immunopositive for antibodies against BrdU and markers for astrocytes, oligodendrocytes, or neurons at different stages of maturity. Immature neurons (DCX/BrdU double-positive) were observed in the injured cortex at 3 and 7 days after injury; at 14 days, some semi-mature and mature neurons (MAP-2/BrdU double-positive) were found in the injured cortex; 28 days after injury, a small number of mature neurons (NeuN/BrdU double-positive) were seen in the injured cortex. These results show that neural progenitor cells emerge in the injured cortex of adult rats after ischemic stroke and differentiate into immature neurons, but most of the immature neurons fail to develop into mature neurons, possibly undergoing apoptosis before maturing (Zhang et al., 2009; Otero et al., 2012; Chen et al., 2013; Song et al., 2013). In the present study, the total number of new cells peaked at 7 days post-injury and decreased thereafter, indeed indicating that some new cells subsequently died. Further study is needed to explore ways in which to protect the new cells from apoptosis and increase successful regeneration.

Most new cells are sensitive to the pathological environment and die, and others become glial cells (Zhao and Overstreet-Wadiche, 2008; Li et al., 2010). In the present study, new astrocytes (GFAP/BrdU double-positive) were found in injured cortex at all time points studied, reaching a maximum 7 days after injury. New cells in the injured cortex comprised mainly reactive glial cells, neural progenitor cells and new differentiated cells; of these, astrocytes were more numerous than neurons, some proliferating by reactive gliosis. No oligodendrocytes (CNP/BrdU double-positive) were found. These results indicate that most neural progenitor cells differentiate into glial cells; the mechanisms remain to be determined. Neurogenesis was not found in the normal cortex.

Taken together, our results suggest that neural progenitor cells emerge in adult rat cortex after ischemia/reperfusion injury and differentiate into astrocytes and immature neurons, but few develop into mature neurons.

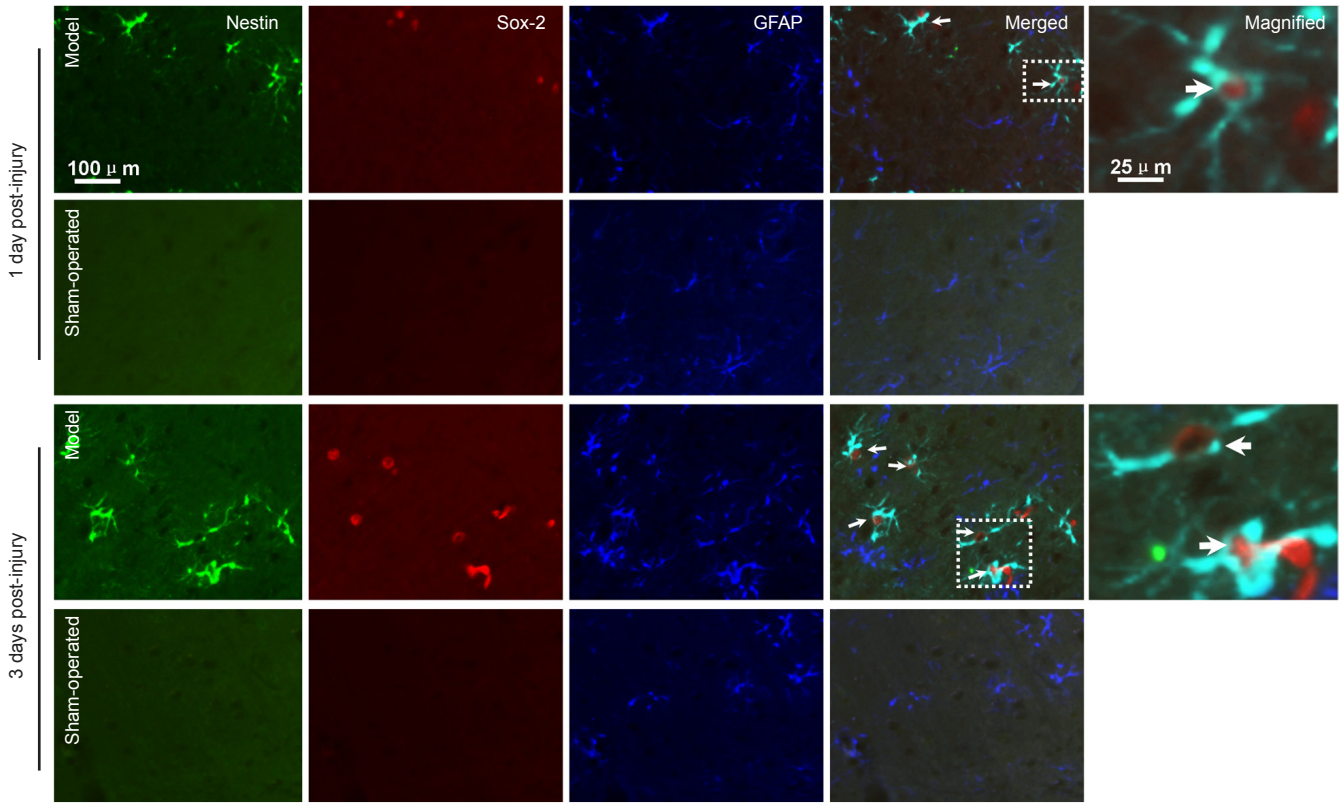

Figure 2 Nestin/sox-2/GFAP triple-labeled endogenous neural progenitor cells in the damaged cortex of adult rats after ischemic brain injury.
Green (Alexa Fluor 488): nestin; red (Alexa Fluor 568): sox-2; blue (Alexa Fluor 647): GFAP. Arrows refer to nestin/sox-2/GFAP triple-labeled cells. Scale bar: 100 μm (scale bar in magnified image: 25 μm). GFAP: Glial fibrillary acidic protein.

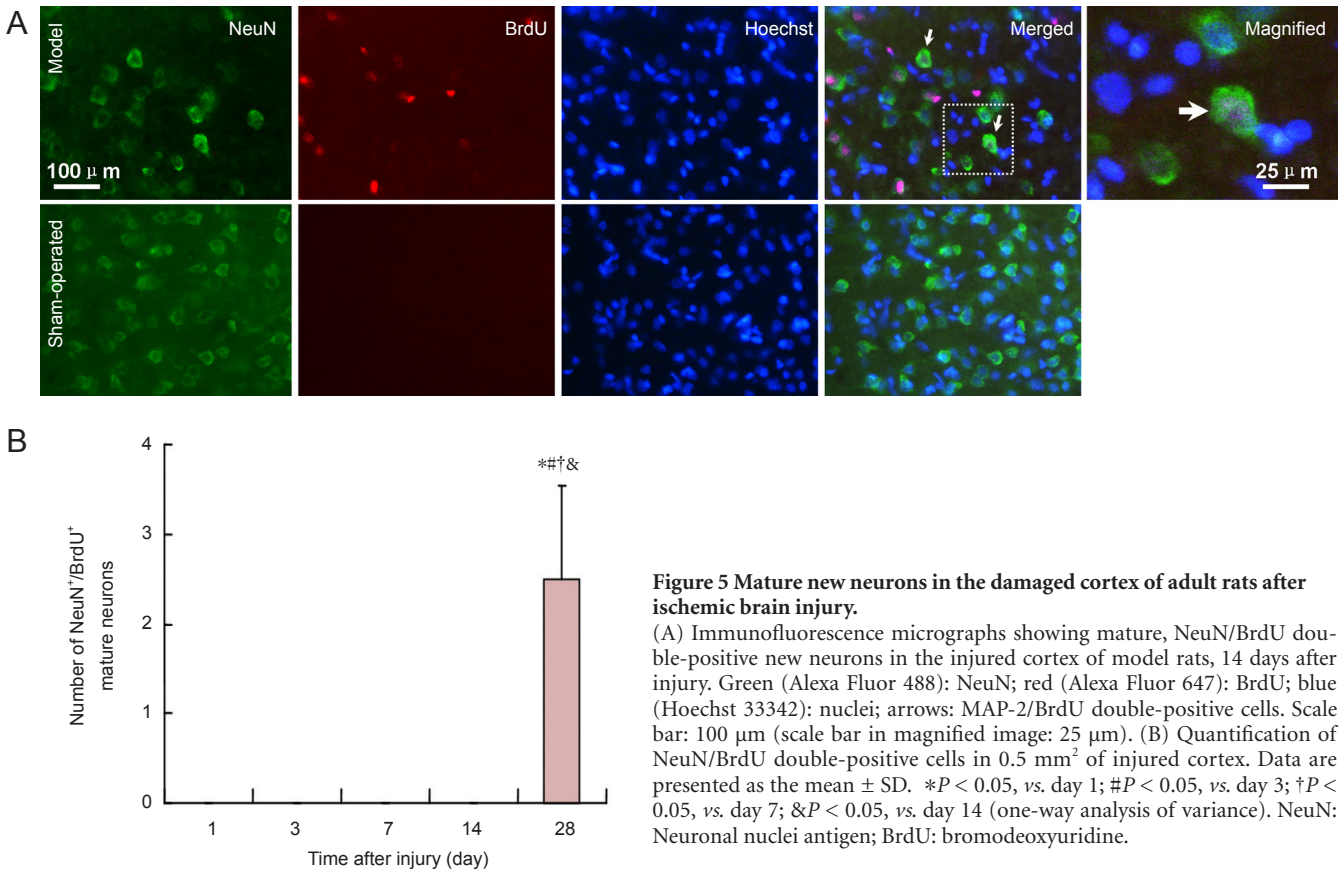

Figure 5 Mature new neurons in the damaged cortex of adult rats after ischemic brain injury.
(A) Immunofluorescence micrographs showing mature, NeuN/BrdU double-positive new neurons in the injured cortex of model rats, 14 days after injury. Green (Alexa Fluor 488): NeuN; red (Alexa Fluor 647): BrdU; blue (Hoechst 33342): nuclei; arrows: MAP-2/BrdU double-positive cells. Scale bar: 100 μm (scale bar in magnified image: 25 μm). (B) Quantification of NeuN/BrdU double-positive cells in 0.5 mm^2 of injured cortex. Data are presented as the mean ± SD. $*P < 0.05$, *vs.* day 1; $\#P < 0.05$, *vs.* day 3; $\dagger P < 0.05$, *vs.* day 7; $\&P < 0.05$, *vs.* day 14 (one-way analysis of variance). NeuN: Neuronal nuclei antigen; BrdU: bromodeoxyuridine.

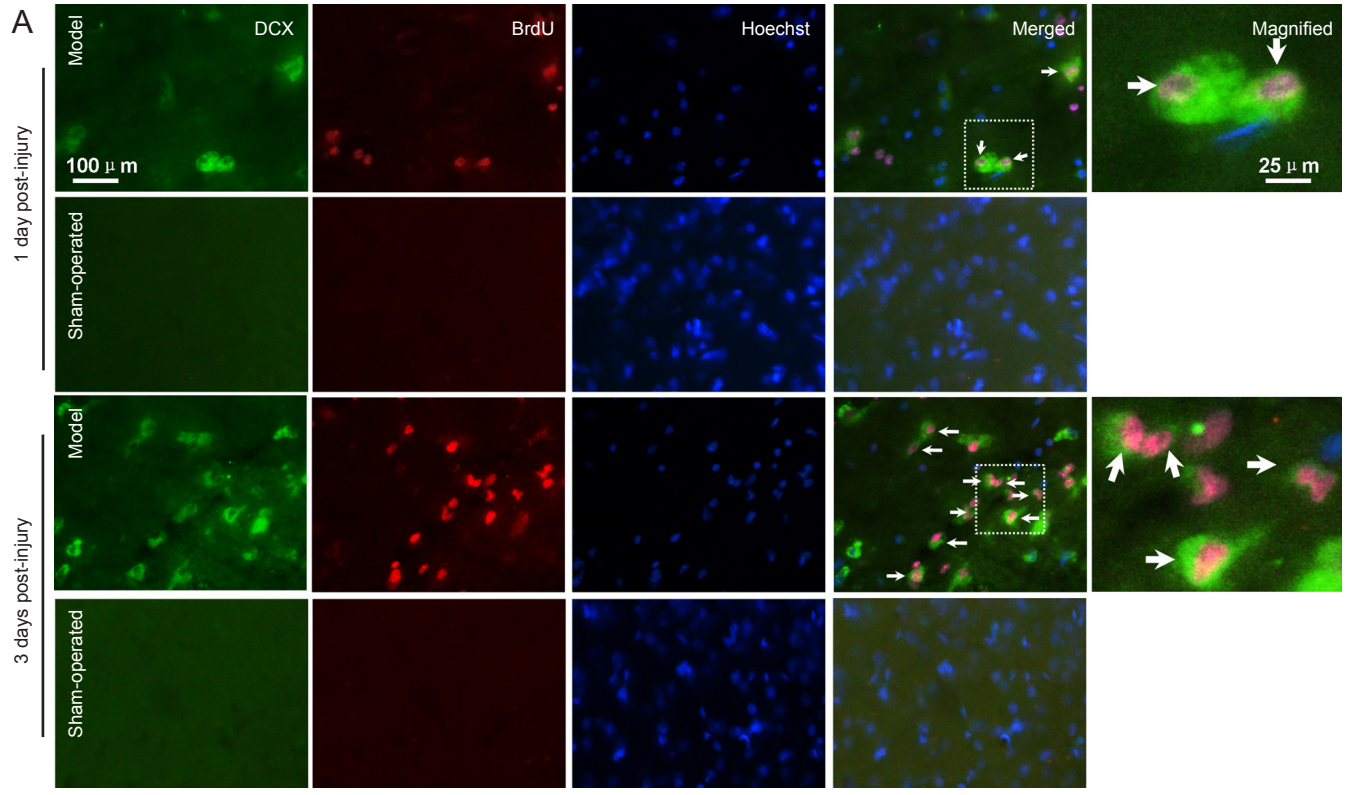

Figure 3 Immature new neurons in the damaged cortex of adult rats after ischemic brain injury.

(A) Immunofluorescence micrographs showing immature, DCX/BrdU double-positive neurons in the injured cortex of model rats. No double-labeled cells were observed in the sham-operated group. Green (Alexa Fluor 488): DCX; red (Alexa Fluor 647): BrdU; blue (Hoechst 33342): nuclei; arrows: DCX/BrdU double-positive cells. Scale bar: 100 μm (scale bar in magnified image: 25 μm). (B) Quantification of DCX/BrdU double-positive cells in 0.5 mm^2 of model rat cortex. Data are presented as the mean ± SD. $*P < 0.05$, *vs.* day 1; $\#P < 0.05$, *vs.* day 3; $\dagger P < 0.05$, *vs.* day 7 (one-way analysis of variance). DCX: Doublecortin; BrdU: bromodeoxyuridine.

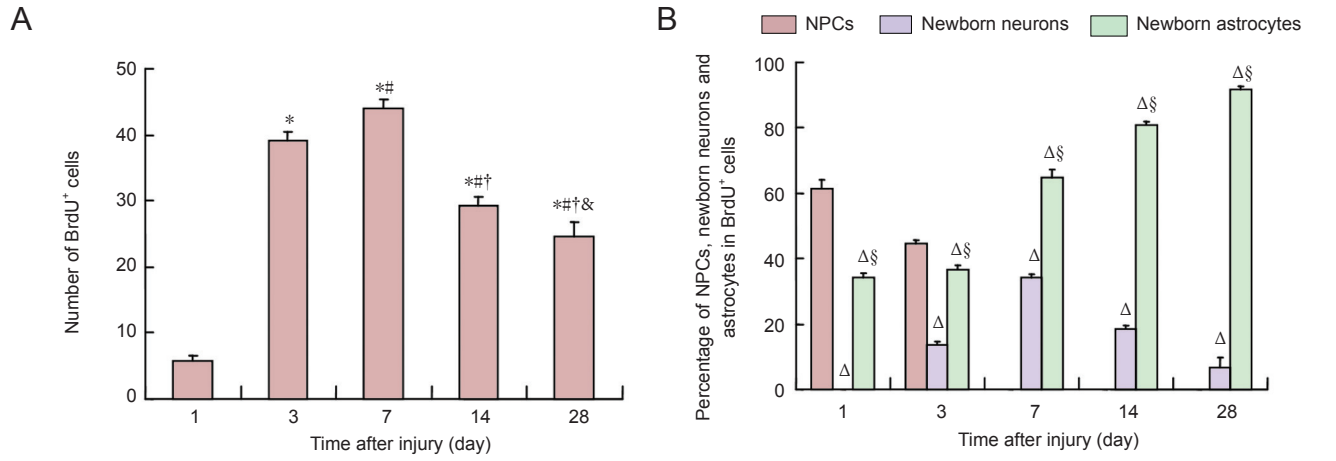

Figure 8 Quantification and classification of neural progenitor cells (NPCs), new neurons and new astrocytes in the damaged cortex of adult rats after ischemic brain injury.

(A) Total numbers of BrdU-positive cells in 0.5 mm^2 of damaged cortex at 1, 3, 7, 14 and 28 days post-injury. (B) Percentage of BrdU-positive cells that were NPCs, new neurons or new astrocytes. Data are presented as the mean ± SD. $*P < 0.05$, *vs.* day 1; $\#P < 0.05$, *vs.* day 3; $\dagger P < 0.05$, *vs.* day 7; $\&P < 0.05$, *vs.* day 14; $\Delta P < 0.05$, *vs.* NPCs; $\S P < 0.05$, *vs.* new neurons (one-way analysis of variance). BrdU: Bromodeoxyuridine.

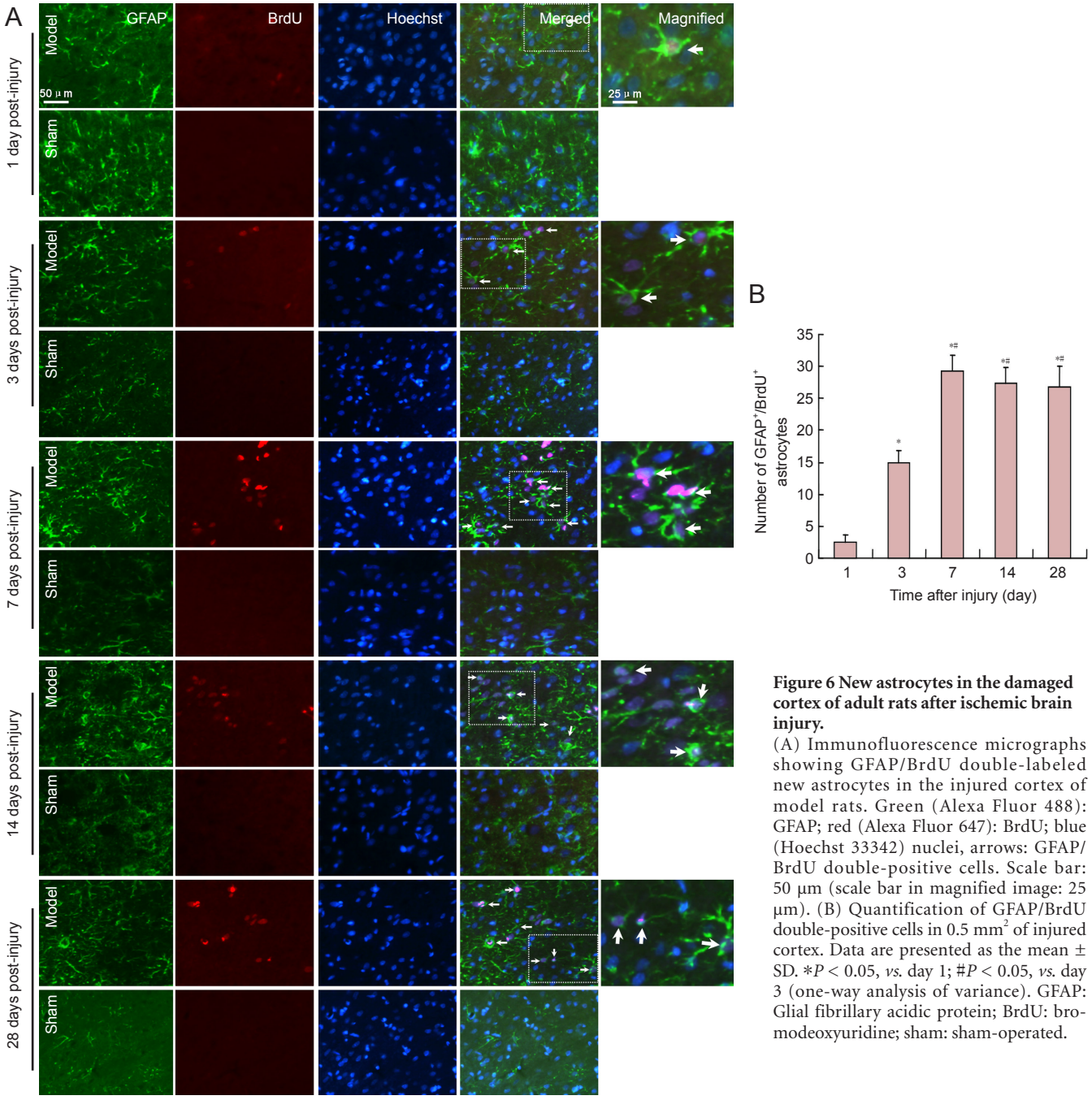

Figure 6 New astrocytes in the damaged cortex of adult rats after ischemic brain injury.

(A) Immunofluorescence micrographs showing GFAP/BrdU double-labeled new astrocytes in the injured cortex of model rats. Green (Alexa Fluor 488): GFAP; red (Alexa Fluor 647): BrdU; blue (Hoechst 33342) nuclei, arrows: GFAP/BrdU double-positive cells. Scale bar: 50 μm (scale bar in magnified image: 25 μm). (B) Quantification of GFAP/BrdU double-positive cells in 0.5 mm^2 of injured cortex. Data are presented as the mean ± SD. $*P < 0.05$, *vs.* day 1; $\#P < 0.05$, *vs.* day 3 (one-way analysis of variance). GFAP: Glial fibrillary acidic protein; BrdU: bromodeoxyuridine; sham: sham-operated.

Author contributions: *QQL and GQQ completed the majority of experiments, wrote the manuscript, and took the fluorescence images. JM and HWF performed part of the animal experiments and image acquisition and processing. YBL was responsible for the study proposal and design. All authors approved the final version of the paper.*

Conflicts of interest: *None declared.*

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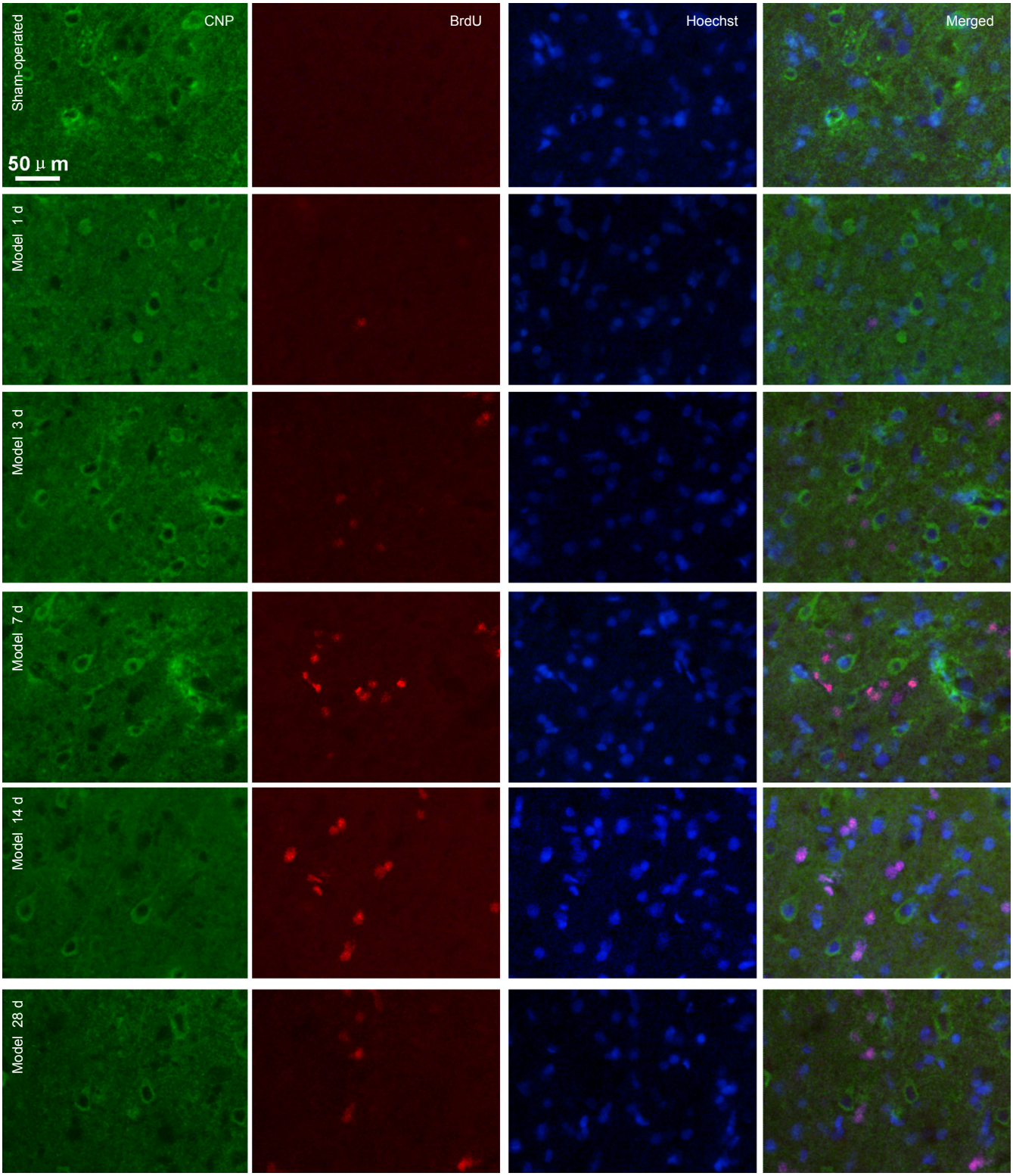

Figure 7 Micrographs of CNP/BrdU double immunofluorescence for new oligodendrocytes in the damaged cortex of adult rats after ischemic brain injury.

No cells in the damaged cortex coexpressed CNP and BrdU. Green (Alexa Fluor 488): CNP; red (Alexa Fluor 647): BrdU; blue (Hoechst 33342): nuclei. Scale bar: 50 μm. CNP: 2′,3′-Cyclic nucleotide 3′-phosphodiesterase; BrdU: bromodeoxyuridine; d: day(s).

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