

Bcl-2 enhances the formation of newborn striatal long-projection neurons in adult rat brain after a transient ischemic stroke

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Abstract: Objective It has been reported that B-cell lymphoma 2 (Bcl-2) enhances neurogenesis as well as supporting axonal growth after injury. In the present study, we investigated whether Bcl-2 overexpression plays a role in the formation of newborn striatonigral projection neurons in the adult rat brain after transient middle cerebral artery occlusion (MCAO). **Methods** We infused human Bcl-2-expressing plasmid (pBcl-2) into the lateral ventricle immediately after 30 min of MCAO, injected 5'-bromodeoxyuridine (BrdU) intraperitoneally to label proliferative cells, and microinjected fluorogold (FG) into the substantia nigra at 11 weeks of reperfusion followed by multiple immunostaining of striatonigral projection neurons at 12 weeks. **Results** We found that pBcl-2 treatment significantly increased the number of newborn neurons (BrdU⁺-NeuN⁺) in the striatum ipsilateral to the MCAO. We further detected newborn striatonigral projection neurons (BrdU⁺-FG⁺-NeuN⁺) in the ipsilateral striatum at 12 weeks. More interestingly, the number of newborn striatonigral projection neurons (BrdU⁺-FG⁺) was significantly increased by pBcl-2 treatment compared to that by pEGFP, a control plasmid. **Conclusion** Taken together, we found that Bcl-2 overexpression in the brain enhanced the generation of newborn striatonigral projection neurons. This provides a potential strategy for promoting the reestablishment of neural networks and brain repair after ischemic injury.

Keywords: cerebral ischemia; Bcl-2; striatonigral projection neuron; neurogenesis; brain repair

1 Introduction

It is well documented that neurogenesis occurs in special areas of the adult mammalian brain throughout life. These areas include the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone of the hippocampal dentate gyrus^[1-3]. Following pathological injury, such as stroke, neuroblasts in the SVZ can migrate into areas which are not normally neurogenic, e.g. the striatum and

cerebral cortex^[4], and replace injured neurons^[5]. Moreover, newly-generated striatal neurons induced by ischemia can be morphologically and functionally integrated into local neural networks with pre-existing neurons in the young adult^[6] and aged rat brain^[7]. Recently, we have interestingly found that those new striatal neurons can form long projections to the substantia nigra (one of the target regions for striatal projection neurons^[6]) in the adult brain after MCAO^[8]. These findings raise the possibility that neuronal replacement by neurons from endogenous neurogenesis could be a great help for restoring brain function after stroke. However, a major barrier blocking endogenous neurogenesis as a potential strategy for cell replacement is

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that almost 80% of newborn neurons in the adult brain injured by ischemia undergo apoptosis and only a very small fraction survive for a long time^[5,9].

To overcome the blockade, several groups have investigated growth factors and found that the anti-apoptotic factor B-cell lymphoma 2 (Bcl-2) enhances neurogenesis and prevents the apoptotic death of new neurons in the adult brain^[9–14]. Bcl-2, a member of the Bcl-2 family^[15,16], is an inhibitory regulator of apoptosis in the brain after ischemic and traumatic injury. It is highly expressed in the embryonic and developing mammalian brain^[17,18]. In normal adult brain, Bcl-2 expression is restricted to the progenitor-rich regions^[19]. Functional analysis showed that overexpression of Bcl-2 in the ischemic brain reduces the apoptosis of newborn neurons and significantly increases the number of newborn neurons that become mature in the striatum of adult rat by inhibiting BMP-4 function *via* activation of β -catenin signaling^[9,20]. In addition, Bcl-2 overexpression enhances retinal axon regeneration after transection of the optic tract^[21] and axonal growth from transplanted fetal dopaminergic neurons in the striatum of rats^[22]. In contrast, Bcl-2 deficiency attenuates axon extension from cultured sensory neurons^[23]. Collectively, it seems that Bcl-2 plays a beneficial role in neurite growth in the adult brain.

Therefore, we asked whether Bcl-2 promotes the formation of newborn striatonigral projection neurons in the adult brain following ischemic injury. In the present study, we used intraventricular injection of human Bcl-2-expressive plasmid (pBcl-2) to induce overexpression of Bcl-2 in adult rat brain after transient middle cerebral artery occlusion (MCAO), intraperitoneal injection of 5'-bromodeoxyuridine (BrdU) combined with multiple immunostaining to label newborn neurons in the striatum, and nigral microinjection of fluorogold (FG), a retrograde tracer, to identify striatonigral projection neurons.

2 Materials and methods

2.1 Animal experimental protocols Eighteen adult male Sprague-Dawley rats (220–250 g) from Shanghai Experimental Animal Center, Chinese Academy of Sciences,

were used. The study was conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, and the protocols were approved by the Medical Experimental Animal Administrative Committee of Shanghai. All efforts were made to minimize animal suffering and reduce the number of animals used.

2.1.1 Transient MCAO Rats were anesthetized with 10% chloral hydrate (360 mg/kg, *i.p.*), and arterial blood samples obtained *via* a femoral catheter were collected to measure pO_2 , pCO_2 and pH with a blood gas analyzer (AVL 990, AVL Co., Graz, Austria). Rectal temperature was maintained at $37 \pm 0.5^\circ\text{C}$ during surgery with a regulated heating lamp^[24,25]. Rats with physiological variables in the normal range were subjected to transient focal cerebral ischemia induced by left MCAO as previously described^[9,11]. Briefly, a 4-0 nylon monofilament suture was gently advanced into the internal carotid artery for ~22 mm from the common carotid artery bifurcation to occlude MCA blood flow. During the process of ischemia, a laser Doppler perfusion monitor (Periflux System 5000, Terimed AB, Sweden) was used to measure blood flow in the trunk of the MCA. A blood flow <20% of baseline denoted successful blockage of the MCA. After 30 min of occlusion, the suture was withdrawn and blood flow was restored. Rats were then subjected to plasmid injection.

2.1.2 Plasmid administration Complementary deoxyribonucleic acid (cDNA) for human *BCL-2* was inserted into the pEGFP-N1 plasmid (Clontech, Palo Alto, CA) between the constitutively active cytomegalovirus promoter and the enhanced green fluorescent protein (EGFP) reporter gene and is referred to as pBcl-2. The plasmid pEGFP-N1 was used as control (pEGFP). Following MCAO, rats were randomly divided into Bcl-2 (pEGFP-N1-Bcl-2 plasmid) and control (pEGFP-N1 empty plasmid) groups. The plasmid mixture, 5 $\mu\text{g}/2 \mu\text{L}$ plasmid and 1 μL Lipofectamine 2000 (Invitrogen, Carlsbad, CA) was stereotactically delivered into the ipsilateral lateral ventricle (coordinates from bregma: AP –0.8 mm, ML –1.4 mm, DV –3.6 mm from the pial surface)^[26]. After recovering from anesthesia, rats were returned to their cages and given *ad libitum* access to food and water.

2.1.3 Bromodeoxyuridine administration 5'-Bromodeoxyuridine (BrdU) is incorporated into DNA during the S phase of the cell cycle as well as into DNA lesion sites during repair. Considering that DNA repair mainly occurs within hours and cell proliferation mainly occurs days after stroke, we injected BrdU intraperitoneally (50 mg/kg body weight once daily, freshly prepared) (Roche Applied Science, Mannheim, Germany) 4–6 days after MCAO, based on a previously-described protocol^[5,9,11,12] to label proliferating cells. BrdU incorporation was revealed by immunohistochemical or immunofluorescent staining as described below.

2.1.4 FG administration At 11 weeks of reperfusion after MCAO or in normal controls, the rats ($n = 7$ per group) received an ipsilateral stereotaxic injection of FG (Biotium, Hayward, CA; total volume, 0.3 μ L of a 2% FG solution in 0.9% saline) into the substantia nigra (coordinates: AP –5.2 mm, ML –2.5 mm, DV –8.0 mm)^[26]. Seven days after FG injection, the animals were sacrificed and the brains were removed for sectioning. FG was directly detected under a microscope with ultra-violet fluorescence epi-illumination or revealed by further immunostaining.

2.2 Immunoblotting Tissues from the sham-operated or ischemic striatum of adult rats at 3 days after plasmid injection were homogenized in RIPA buffer (1 $\times$ PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, and 1 mmol/L sodium orthovanadate) containing a protease inhibitor cocktail (Roche, Basel, Switzerland). Protein lysates were obtained after centrifugation at 12 000 g for 30 min. Protein concentration was measured with a BCA protein assay kit according to the manufacturer's instructions (Beyotime Institute of Biotechnology, Jiangsu, China). Equal amounts of protein lysate were separated on 12% sodium dodecyl sulfate-polyacrylamide gels and electrophoretically transferred onto polyvinylidene difluoride membranes. The membranes were incubated with rabbit polyclonal anti-Bcl-2 antibody (1:500 dilution; BD Pharmingen, San Jose, CA) at 4°C overnight, and then with horseradish peroxidase-conjugated anti-rabbit IgG (1:3 000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA). The immunoreactivity was visualized with an

enhanced chemiluminescence substrate system and Kodak X-OMAT film. Normalization was achieved by stripping filters and reprobing for β -actin. The films were scanned.

2.3 Histological analysis

2.3.1 Tissue preparation Animals were deeply anesthetized and quickly transcardially perfused with freshly-prepared 0.9% saline followed by 4% paraformaldehyde in PBS (0.1 mol/L, pH 7.4). The brains were post-fixed in 4% paraformaldehyde overnight and immersed sequentially in 20% and 30% sucrose in 0.1 mol/L PBS. Then coronal sections (30 μ m) were cut on a freezing microtome (Jung Histocut, Model 820-II, Leica, Germany) and stored at –20°C in cryoprotectant for further histological analysis. Sections at 1.0–0.48 mm from bregma were used for immunostaining and sections at 1.2–0.2 mm from bregma were used for quantification.

2.3.2 Immunohistochemical staining For double-staining, two antibody combinations were applied as follows: 1) rabbit polyclonal anti-BrdU antibody (1:200; Megebase, Brussels, Belgium) and mouse monoclonal anti-NeuN antibody (1:1000; Abcam, Cambridge, UK); and (2) mouse monoclonal anti-BrdU antibody (1:200; Roche Applied Science, Mannheim, Germany) and rabbit polyclonal anti-FG antibody (1:4 000; Chemicon, Temecula, CA). Free-floating sections were first incubated with 50% formamide/2 $\times$ SSC (0.3 mol/L NaCl, 0.03 mol/L sodium citrate) and 2N HCl for DNA denaturation^[9,27] and then rinsed with Tris buffer (0.1 mol/L, pH 7.6) and treated with 1% H₂O₂ for 1 h. After washing with buffer A (0.1 mol/L Tris buffer/0.1% Triton X-100) and buffer B (0.1 mol/L Tris buffer/0.1% Triton X-100/0.05% BSA) for 15 min each, sections were placed in 10% goat serum for 1 h at 37°C and incubated with rabbit or mouse anti-BrdU antibody (1:200) at 4°C overnight. After washing, sections were incubated with biotinylated anti-rabbit or -mouse IgG (1:200) for 1 h. Then signals were amplified using avidin-biotin-alkaline phosphatase complex (1:200) for 1 h at 37°C and visualized by Vector® Blue. After rinsing, sections were treated again with mouse monoclonal anti-NeuN antibody (1:1 000) or rabbit polyclonal anti-FG antibody (1:4 000) overnight at 4°C, then incubated with the corresponding biotinylated secondary

antibodies and avidin–biotin–peroxidase. The immunoreactivity was visualized by 0.05% diaminobenzidine as the chromogen. Controls received identical treatment except that the primary antibodies were omitted; they showed no specific staining.

2.3.3 Immunohistochemistry and confocal microscopy For double fluorescent staining, three combinations were used as follows: (1) mouse monoclonal anti-NeuN (1:1 000; Abcam) and rabbit polyclonal anti-FG (1:4 000; Chemicon); (2) goat polyclonal anti-EGFP (1:500; Abcam) and mouse monoclonal anti-nestin (1:1 000; NeoMarker, Fremont, CA); and (3) mouse monoclonal anti-BrdU (1:200; Roche Applied Science) and rabbit polyclonal anti-FG (1:4 000; Chemicon). Sections were incubated with primary antibody overnight at 4°C. After washing, sections were incubated with anti-mouse/goat IgG-FITC (1:50; Santa Cruz Biotechnology) or anti-mouse IgG-rhodamine (1:50; Roche Applied Science). After rinsing twice for 15 min, sections were treated again with the second primary antibody overnight at 4°C. Then anti-rabbit IgG-Cy5 (1:150; Amersham Pharmacia Biotechnology, Buckinghamshire, UK), anti-rabbit IgG-FITC (1:50; Santa Cruz Biotechnology) or anti-mouse IgG-rhodamine (1:50; Roche Applied Science) was added for 1 h at 37°C. FG fluorescence was directly detected under a fluorescence microscope at 380 nm excitation and 530 nm emission. Therefore, for triple fluorescent labeling of BrdU-NeuN-FG and BrdU-GFAP-FG, sections were incubated with rabbit polyclonal anti-BrdU (1:200; Megebase) primary antibody overnight at 4°C. After rinsing in PBS, sections were incubated with anti-rabbit IgG-rhodamine (1:50; Santa Cruz Biotechnology) for 1 h at 37°C and then incubated with mouse monoclonal anti-NeuN antibody (1:1 000) or anti-GFAP antibody (1:200; NeoMarker) overnight at 4°C. After rinsing, sections were incubated with anti-mouse FITC (1:50) for 1 h at 37°C. Finally, sections were mounted on slides and coverslipped with fluorescence mounting medium (Vector Laboratories, Burlingame, CA). The fluorescent signals were detected at 535 nm excitation and 565 nm emission (rhodamine), 490 nm and 525 nm (FITC), 650 nm and 670 nm (Cy5) by confocal laser scan-

ning microscopy (TCS SP5, Leica, Germany).

2.4 Quantification and statistical analysis For quantification of BrdU⁺-NeuN⁺ and BrdU⁺-FG⁺ cells, four sections (12 sections between each; from bregma 1.2–0.2 mm following double immunohistochemical staining) chosen from each animal were taken for stereological counting. Total numbers of double-labeled cells were counted in the ipsilateral striatum of the four sections and striatal volume was calculated by Stereo Investigator 6.5 (MicroBrightField Inc., Williston, VT). Then the final results were expressed as cells/mm³ ($n = 4$ in sham group; $n = 7$ in MCAO+pEGFP and MCAO+pBcl-2 groups)^[8,11,12]. All values are expressed as mean $\pm$ standard error of the mean (SEM). One-way ANOVA followed by Student's *t*-test was used for each evaluation. Statistically significant difference was set at $P < 0.05$.

3 Results

3.1 Formation of newly-generated striatonigral projection neurons in adult rat brain after transient MCAO

One week after nigral microinjection (Fig. 1A), we detected strong FG signals in the substantia nigra and striatum as well as the pyramidal layer of the cerebral cortex in the hemisphere ipsilateral to the injection, but not in the contralateral hemisphere (Fig. 1B–F). These FG-positive (FG⁺) cells were co-stained with NeuN (FG⁺-NeuN⁺) (Fig. 1G–H), but not with GFAP (Fig. 2C). These results clearly confirmed that nigral injection of FG can be used to successfully trace striatonigral projection neurons. Then, we applied triple labeling with BrdU-FG-NeuN to identify newborn striatonigral projection neurons in adult rat brains after MCAO (Fig. 2A).

As we showed previously, at 2 weeks of reperfusion after MCAO, only newborn neurons and pre-existing striatonigral projection neurons were detectable in the ipsilateral striatum^[8]. However, we interestingly observed that newborn neurons were further co-stained with FG (BrdU⁺-NeuN⁺-FG⁺ cells; Fig. 2A, B) at 12 weeks after MCAO, in accordance with our previous report^[8]. Meanwhile, we also detected cells double-labeled for BrdU with NeuN (BrdU⁺-NeuN⁺) or GFAP (BrdU⁺-GFAP⁺) in the striatum. It should

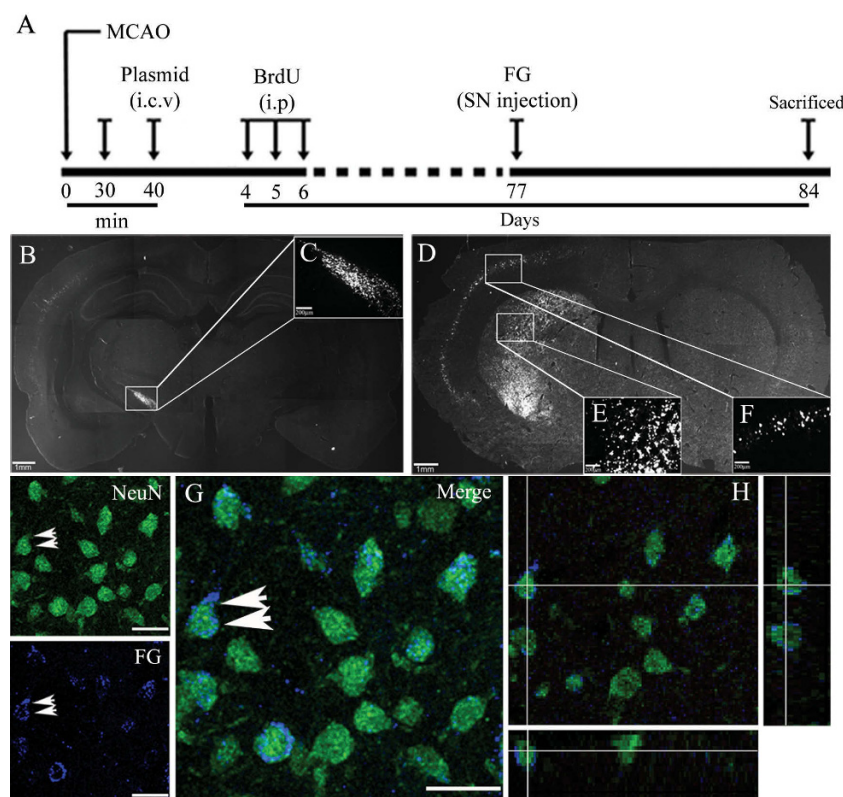


Fig. 1. Experimental protocol (A) and identification of fluorogold (FG) in the injection site (B–C) and retrogradely transported (D–F). **A:** Animals were subjected to middle cerebral artery occlusion (MCAO) followed by plasmid injection, then 5'-bromo-2'-deoxyuridine (BrdU) injection during days 4–6 and FG injection in the substantia nigra (SN) on day 77, and finally sacrificed on day 84. **B–C:** Photomicrographs of the FG injection site in the SN. **C** shows the zoomed area in **B**. **D–F:** One week after FG injection, retrograde FG signals were detected in the striatum and pyramidal neurons of the cerebral cortex (**D**). **E** and **F** show the zoomed areas in **D**. **G** and **H:** Photomicrographs showing immunofluorescent double-staining with FG-NeuN in the striatum (**G**). **H** shows Z-scanned image corresponding to **G**. Cell double-stained with NeuN and FG is indicated by two arrows. Scale bars in **B** and **D**, 1 mm; **C**, **E**, and **F**, 200 μm; **G**, 20 μm.

also be noted that FG was detected only in the newborn (BrdU⁺-NeuN⁺) or pre-existing neurons (NeuN⁺) but not in astrocytes (Fig. 2C), and all FG⁺ cells were labeled with neuronal markers at 12 weeks after ischemic injury. Therefore, in the following experiments, striatal BrdU⁺-FG⁺ cells were considered to represent newly-generated striatonigral projection neurons in the adult brain after transient MCAO.

3.2 Enhancement of newly-generated striatonigral projection neurons by Bcl-2 overexpression in adult rats after ischemia Bcl-2-expressing plasmid was injected into the lateral ventricle and immunoblotting was used to detect the expression of Bcl-2 protein in the striatum of rats with ischemic injury at 3 days after injection. We detected endogenous Bcl-2 (26-kD band) and exogenously expressed

Bcl-2 (EGFP-Bcl-2 fusion protein, 53-kD band) in the pBcl-2 group. However, in the pEGFP control group, only endogenous Bcl-2 was detected (Fig. 3A). Furthermore, immunostaining showed that the EGFP-positive cells were located along the wall of the lateral ventricle mainly dorso-laterally near the SVZ (Fig. 3C). However, the EGFP-positive cells were discontinued facing the striatum (Fig. 3D). In addition, most of the nestin-positive cells demonstrated a neural stem cell-like pattern in the SVZ away from the ischemic striatum (Fig. 3C), while astrocyte-like morphology appeared in the periphery of the ischemic striatum (Fig. 3D), consistent with previous results that nestin is also expressed in active astrocytes in regions close to the ischemic striatum^[28]. Most importantly, either nestin- (a

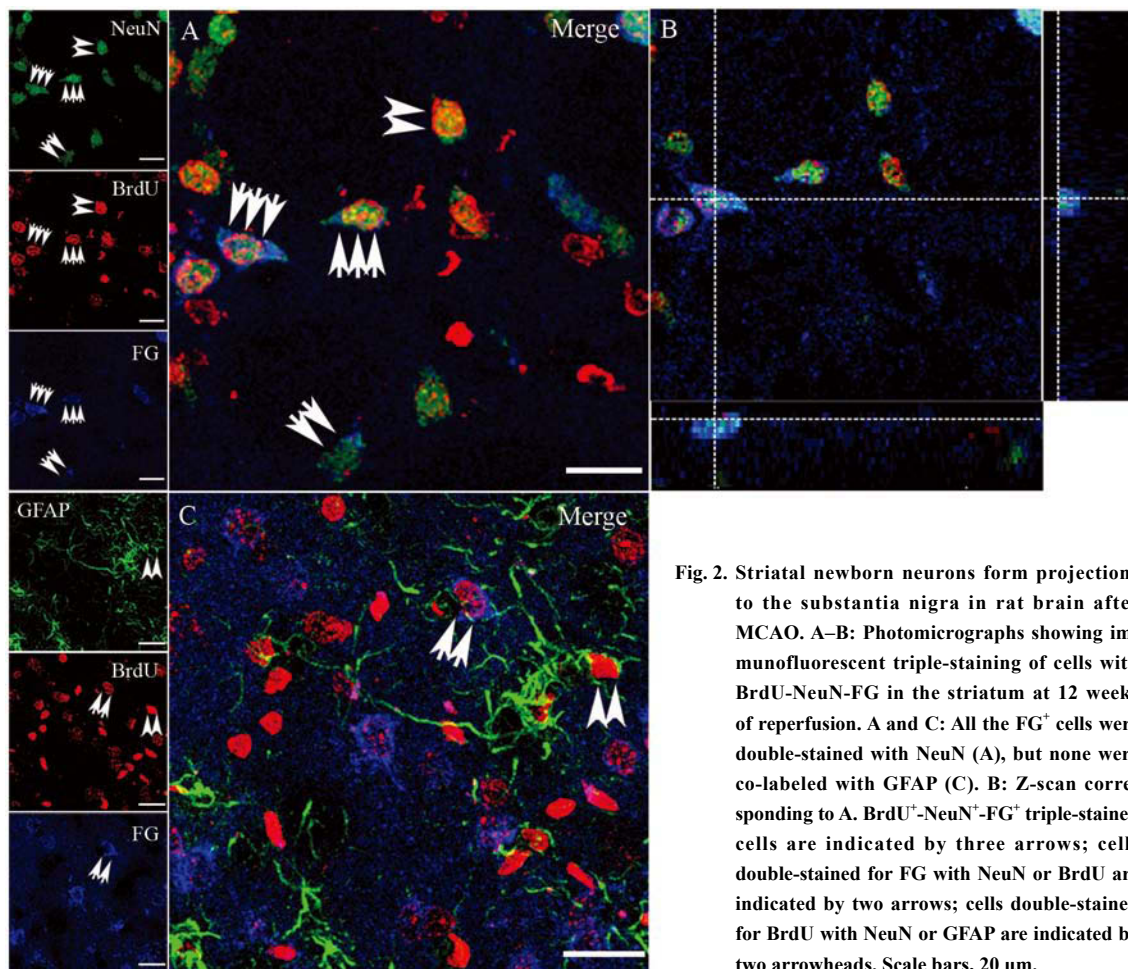


Fig. 2. Striatal newborn neurons form projections to the substantia nigra in rat brain after MCAO. A–B: Photomicrographs showing immunofluorescent triple-staining of cells with BrdU-NeuN-FG in the striatum at 12 weeks of reperfusion. A and C: All the FG⁺ cells were double-stained with NeuN (A), but none were co-labeled with GFAP (C). B: Z-scan corresponding to A. BrdU⁺-NeuN⁺-FG⁺ triple-stained cells are indicated by three arrows; cells double-stained for FG with NeuN or BrdU are indicated by two arrows; cells double-stained for BrdU with NeuN or GFAP are indicated by two arrowheads. Scale bars, 20 μ m.

marker for neural stem/progenitor cells) or EGFP-positive cells were single-stained. Meanwhile, cells double-stained with EGFP and nestin were detected in the SVZ or striatum close to the infarct core away from the lateral ventricle (Fig. 3C, D). Our previous study demonstrated that Bcl-2 accelerates the differentiation and maturation of newborn neurons in the ischemic striatum at an early stage of reperfusion after MCAO (2–4 weeks)^[9]. In the present study we found that, at 12 weeks after MCAO, the number of BrdU⁺-NeuN⁺ cells was significantly increased after pBcl-2 treatment, from 335.70 ± 102.50 cells/mm³ in the pEGFP group to 655.70 ± 66.77 cells/mm³ in the pBcl-2 group (Fig. 3E–G). These results indicate that exogenous overexpression of Bcl-2 still up-regulates striatal neurogenesis in rat brain even 3 months after MCAO.

As noted above, the FG⁺ cells only co-labeled with neuronal markers although BrdU-labeled cells could be neurons or astrocytes. Therefore, we assumed that BrdU⁺-FG⁺ cells were newborn striatonigral projection neurons and found that the BrdU signal was located in the nuclei of FG⁺ neurons in the striatum of rat brain at 12 weeks after MCAO (Fig. 4A, C–G). To quantify the number of newborn striatonigral projection neurons, we next performed immunohistochemical double-staining with BrdU and FG and counted the number of BrdU-FG double-stained cells (Fig. 4C–G). We found that the number of BrdU⁺-FG⁺ cells increased remarkably in the pBcl-2-treated rats (118.40 ± 28.88 cells/mm³, $n = 7$) compared with that in pEGFP-treated rats (21.79 ± 4.28 cells/mm³, $n = 7$) (Fig. 4B). These results suggested that Bcl-2 overexpression not only enhanced

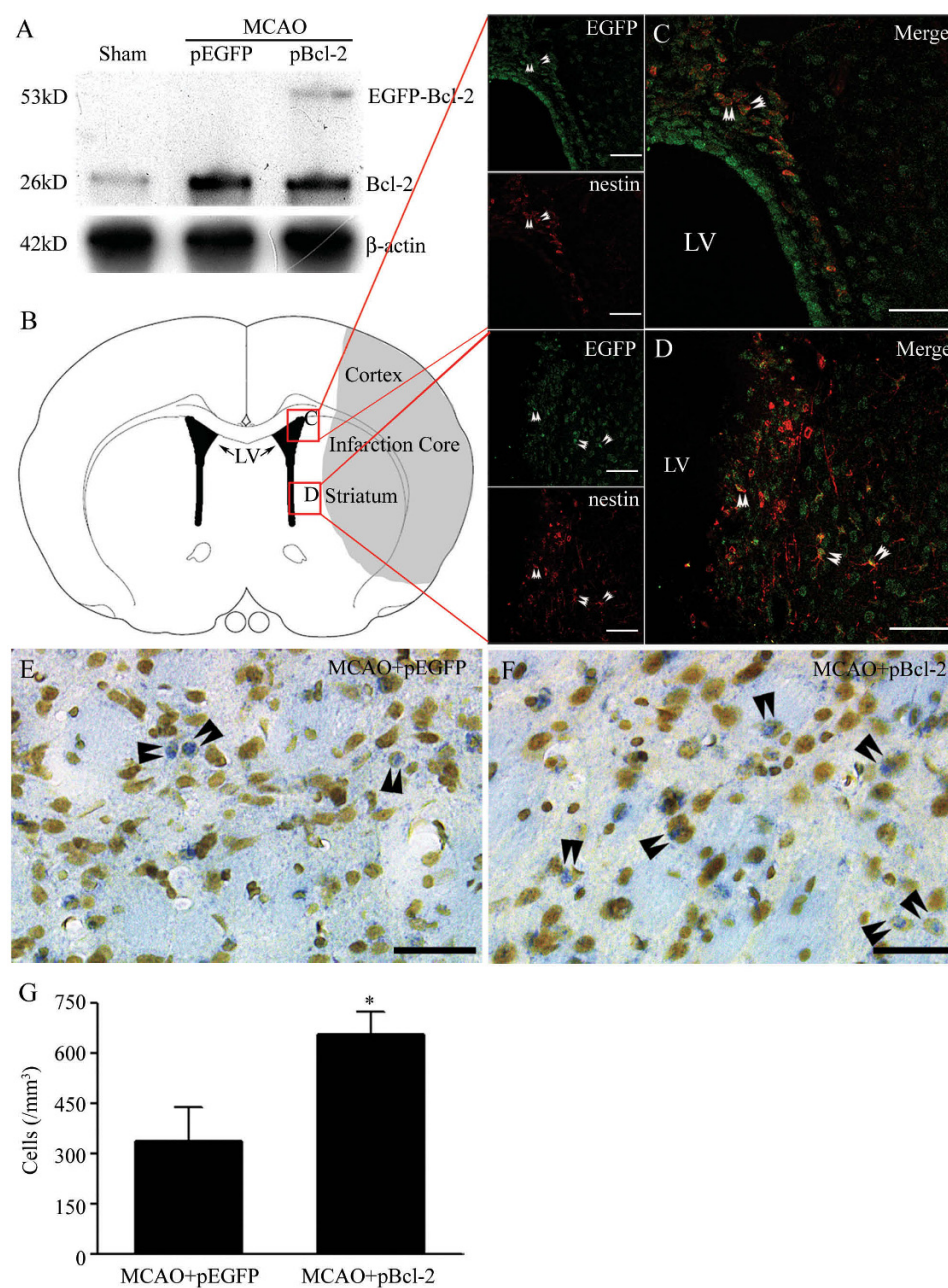


Fig. 3. Bcl-2 overexpression increased newborn striatal neurons in the rat brain at 12 weeks after middle cerebral artery occlusion (MCAO). **A:** Western blots showing Bcl-2 expression in rat brain 3 days after plasmid injection. Endogenous Bcl-2 (26 kD) was expressed in all three groups but exogenous EGFP-Bcl-2 (53 kD) was only seen in the pBcl-2 group. **B:** Cartoon indicates the area of the ischemic infarct core across striatum and cortex (shaded), and the red squares indicate the locations of confocal scanning for C and D. Double-staining for EGFP and nestin showing exogenous Bcl-2 expression in neural stem/progenitor cells in the ipsilateral SVZ and striatum away from the lateral ventricle (LV) close to infarct core 3 days of reperfusion after MCAO in the pBcl-2 group. EGFP⁺-nestin⁺ cells are indicated by two white arrows. **E–F:** Double-staining for BrdU and NeuN to identify newborn neurons that had matured in the ipsilateral striatum. BrdU⁺-NeuN⁺ cells are indicated by two black arrowheads. **G:** Bcl-2 treatment significantly increased the number of BrdU⁺-NeuN⁺ cells. Data are expressed as mean $\pm$ SEM ($n = 7$ in each of the MCAO+pEGFP and MCAO+pBcl-2 groups). * $P < 0.05$. Scale bars in C and D, 50 μ m; E and F, 40 μ m.

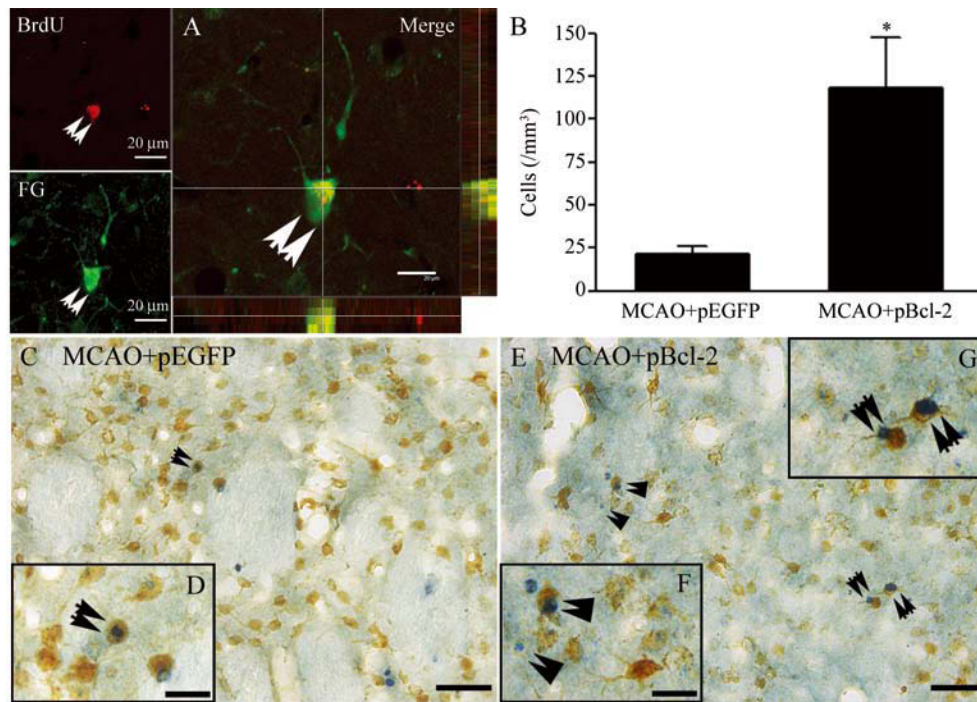


Fig. 4. Bcl-2 overexpression increased newborn striatonigral projection neurons in rat striatum injured by ischemia. Newborn striatonigral projection neurons were identified by BrdU⁺-FG⁺ immunofluorescence (A) and immunohistochemical staining (C–G) in the striatum ipsilateral to middle cerebral artery occlusion (MCAO). BrdU plus FG-stained cells are indicated by two arrows or arrowheads (A, C–G). D is high power view of BrdU⁺-FG⁺ indicated by two arrows in C; F and G are high power views of BrdU⁺-FG⁺ indicated by two arrowheads and arrows, respectively in E. B: Bcl-2 treatment significantly increased the number of BrdU⁺-FG⁺ cells in the ipsilateral striatum. **P* < 0.05. Data are expressed as mean ± SEM (*n* = 7 in each of the MCAO+pEGFP and MCAO+pBcl-2 groups). Scale bars in A, D, F and G, 20 μm; C and E, 50 μm.

neurogenesis but also promoted the generation of newborn striatonigral projection neurons in brains injured by ischemia.

4 Discussion

In our study, a rat MCAO stroke model combined with microinjection of pBcl-2 into the lateral ventricle and FG into the substantia nigra was used to reveal the effects of Bcl-2 overexpression on the formation of long striatonigral projection neurons in brains injured by ischemia. On that basis, we found that pBcl-2 treatment increased the number of newborn neurons that were mature at 12 weeks of reperfusion. Importantly, our data indicated that overexpression of Bcl-2 further promoted the formation of long projections from newborn striatal neurons to the substantia nigra. The present results provided evidence for the first time that Bcl-2 can enhance the generation of new stria-

tonigral projection neurons in the injured striatum of the adult rat brain after ischemic insult. The results provide a potential target of therapeutic intervention for recovery in stroke patients.

FG, a retrograde tracer, is taken up presynaptically and transported retrogradely to the neuronal cell bodies. We therefore injected FG into the ipsilateral substantia nigra to trace its input projection neurons. Consistent with present knowledge^[8], FG signals were detected in the cerebral cortex and striatum ipsilateral to the nigral injection (Fig. 1D) and were present in neurons as indicated by double-labeling with FG and NeuN (Fig. 1G and H). Moreover, we also noted that the FG signals were not detected in astrocytes, since no FG was found in GFAP-positive cells (Fig. 2C), confirming that nigral injection of FG traces the corticonigral and striatonigral projection neurons.

BrdU is incorporated into DNA during the S phase

of the cell cycle and into DNA lesion sites during repair. Because DNA repair occurs mainly within hours and cell proliferation in days after stroke, we injected BrdU at 4–6 days after MCAO to label proliferating cells since it is metabolized in hours after i.p. injection ($t_{1/2} \leq 2$ h)^[5,9,11,12]. Consistent with previous results^[11], we detected newborn/proliferating neurons and astrocytes in the ischemic striatum as indicated by double-staining of BrdU with NeuN or GFAP (Fig. 2). Consistent with our previous report^[8], we also found some FG⁺-NeuN⁺ cells with BrdU⁺ signals in the ipsilateral striatum at 12 weeks of reperfusion after MCAO (Fig. 2A–B), but not at 2 weeks^[8], suggesting that new striatal neurons form projections to the substantia nigra. It should be noted that FG was only detected in neurons, not astrocytes (Figs. 1 and 2). Taken together, BrdU⁺-FG⁺ cells represented newborn striatonigral projection neurons.

Neurogenesis occurs in the striatum after ischemic injury caused by MCAO^[11]. Although the majority of newborn striatal neurons in the adult brain are dead two months after stroke^[5], our previous research demonstrated that the living newborn striatal neurons develop neurites with a time-dependent increase in dendrite length and branch number, and further become functionally integrated into local neural networks in the ischemia-injured striatum of adult rat brain^[11,29]. In the present study, we further supported our previous notion^[8] that newborn striatal neurons can form striatonigral projections and integrate into the neural circuitry between the nuclei as indicated by cells triple-stained with BrdU⁺-NeuN⁺-FG⁺ or double-stained with BrdU⁺-FG⁺ (Figs. 2 and 4).

Our previous study showed co-localization of Bcl-2 with nestin in the MCAO rat brain and the presence of EGFP-positive cells along the wall of the ventricle as well as in the ipsilateral striatum after injection of plasmids^[9]. In the present study, we further confirmed expression of EGFP-Bcl-2 in the ischemic striatum (Fig. 3A). We found that exogenous Bcl-2 was efficiently expressed in neural stem/progenitor cells in the SVZ as well as in the injured striatum (Fig. 3C, D). Although we still did not know whether the Bcl-2-expressing neural stem cells could further differentiate into new striatal neurons, we interestingly

found that exogenous overexpression of Bcl-2 increased the development of newborn striatonigral projection neurons, since Bcl-2 overexpression remarkably increased the number of BrdU⁺-NeuN⁺ as well as BrdU⁺-FG⁺ cells (Figs. 3G, 4B). The exact mechanism of the Bcl-2-enhanced neurogenic effects in the ischemic brain is still unknown, but it seems that Bcl-2 plays neurogenic roles through several pathways. Bcl-2 increases the number of newborn neurons *via* its protection of newborn and immature neurons against apoptosis^[9] and the activation of Wnt/ β -catenin signaling pathways^[20]. Theoretically, keeping newborn neurons alive is the key step for the further formation of long projections. Besides, in *in vitro* assays, it has been shown that Bcl-2 accelerates axon growth by increasing neuritogenesis and neural regeneration, which might be associated with activation of intracellular Ca²⁺ signaling^[30] and increased synthesis of neurofilament proteins^[31]. In the present study, we provided the first evidence that overproduction of Bcl-2 in the brain could be beneficial for the development of new striatonigral projections although we still do not know the mechanism.

The substantia nigra and striatum are main components of the basal ganglia, which participate in voluntary motor control, procedural learning, and cognitive and emotional functions. Within the basal ganglia, several neural projection pathways connect the nuclei, including the striatopallidal, striatonigral and nigrostriatal pathways. In previous studies^[6,8], we found that newborn striatal neurons can generate projections to the nigra, a target region, and express dopamine and glutamate receptors on their membrane. These results provided morphological evidence that newborn striatal neurons can regenerate striatonigral projections and are ready to receive dopaminergic inputs from the nigra and glutamatergic inputs from the cerebral cortex, which are fundamentally important for establishing new neural networks within the basal ganglia of the adult brain after stroke. In the present study, we interestingly found that Bcl-2 overexpression enhanced the generation of new striatonigral projection neurons (Fig. 4) as well as increasing the maturation of newborn striatal neurons (Fig. 3). These results will help to develop therapeutic strategies to

enhance brain repair after stroke by augmenting adult neurogenesis and neuritogenesis.

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