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ScienceDirect
Biochemical and Biophysical Research Communications 367 (2008) 109–115
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Sustained up-regulation of Semaphorin 3A, Neuropilin1, and Doublecortin expression in ischemic mouse brain during long-term recovery

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Received 12 December 2007
Available online 26 December 2007

Abstract

Strategies to provide neuroprotection and to promote regenerative axonal outgrowth in the injured brain are thwarted by the plethora of axon growth inhibitors and the ligand promiscuity of some of their receptors. Especially, new neurons derived from ischemia-stimulated neurogenesis must integrate this multitude of inhibitory molecular cues, generated as a result of cortical damage, into a functional response. More often than not the response is one of growth cone collapse, axonal retraction and neuronal death. Therefore, characterization of the expression of inhibitory molecules in long-term surviving ischemic brains following stroke is important for designing selective therapeutics. Here, we describe a long-term recovery mouse model for cerebral ischemia in which a brief transient occlusion of the middle cerebral artery (30 min) was followed by up to 30 days of long-term reperfusion. Significantly decreased grip strength motor function and increased expression of one of the major repulsive guidance cues, Semaphorin 3A (Sema3A) and its receptor Neuropilin1 (NRP1) occurred in brains of these mice. Interestingly, increased Doublecortin (DCX) expression occurred only in the lateral ventricular wall zone, but not in the dentate gyrus granule cell layer on the ischemic side of the brain. Importantly, no DCX positive cells were detected in the infarct core region after 30 d ischemic recovery. Collectively, these studies demonstrated the sustained elevation of Sema3A/NRP1 expression in the ischemic territory, which may contribute to the inhibitory microenvironment responsible for preventing new neurons from entering the infarct area. This model will be of use as a platform for testing anti-inhibitory therapies to stroke.

Keywords: Stroke; Semaphorin 3A; Neuropilin; Doublecortin; MAP2; Double-immunostaining; Long-term stroke recovery

Chemorepulsive guidance molecules are not only important for the proper wiring of developing neurons, but are also increasingly implicated in axonal regeneration inhibition in the adult brain. In particular, Sema/NRP interaction has been regarded as a major inhibitor of axonal regeneration and a potent inducer for neuronal death *in vitro* [1–4].

Semaphorin family members are classified as either transmembrane, GPI-linked, or secreted and are currently designated into eight subclasses expressed in both vertebrates and invertebrates (Semaphorin Nomenclature Committee [5]). Sema3A, a prototypical class 3, is a secreted chemorepulsive molecule which consists of an N-terminal signal peptide followed by the Sema domain and an IgG domain of 70 amino acids [7]. A basic domain is present at the carboxyl end of the molecule. Sema3A plays a key role in axonal guidance during development through induction of growth cone collapse [6,7]. The process occurs at the tip of the growth cone and is manifested by depolymerization and loss of F-actin. The downstream pathways

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0006-291X/$ - see front matter
doi:10.1016/j.bbrc.2007.12.103

by which semaphorins exert their actions are known to involve NRPs and plexin receptors as well as intracellular G-proteins and collapsin response mediator proteins [8–12]. The biological activities of repulsive axon guidance molecule, Sema3A, are known to be responsible for the elimination of neurons during development where axons are still too far away from reaching the target [1,2]. The cellular targets of Sema3A also appear to be selective since Sema3A inhibits the outgrowth of a specific set of neurons such as spinal motor neurons and neurons in the embryonic dorsal root ganglion and sympathetic ganglion [13,1].

Cellular receptors for Sema3A are NRPs [14–16]. NRP contains two family members: NRP1 and NRP2. Both of them comprise an extracellular domain of two CUB motifs, next to two domains with homology to coagulation factors V and VIII, a MAM domain, a single transmembrane domain, and a short intracellular domain of 39 amino acids lacking any known signaling motifs [17]. NRPs are non-tyrosine kinase transmembrane proteins. Their short intracellular segments lack cytoplasmic signal transduction domains. Therefore, NRPs participate in signal transduction as co-receptors with plexins and vascular endothelial growth factor receptors. NRP1 is a cell surface glycoprotein expressed on axons [1], and functions as a receptor for axon guidance factors such as Sema3A during the process of axonal pathfinding [18]. NRP1 binds to all classes of Sema3, whereas NRP2 binds selectively to the secreted semaphorins with the exception of Sema3A.

Because of the inhibitory roles of Sema3A during development, it is not surprising to see increased implications of semaphorins in a number of neurodegenerative diseases including Alzheimer's, motor neuron degeneration and injuries caused by cerebral ischemia (as reviewed by De Winter in Ref. [1]). Changes in Sema3A expression has been described in both neurons and the component of the scar tissues such as glial cells in the ischemia injured adult brains [19–21]. However, so far no long term studies on the expression of these inhibitory molecules, in particular, their association with the expression of neurogenesis protein DCX have been reported.

In the present study, we described the establishment of a long-term recovery mouse model of cerebral ischemia and the quantitative analysis of the expression of a battery of inhibitory proteins, including Sema3A, NRP1, NPR2, GFAP, and their association with decreased motor functions. These studies lay the foundation for further mechanistic studies and will be useful for testing therapeutic intervention against inhibitory molecules during long-term stroke recovery.

Materials and methods

Cerebral ischemia produced by middle cerebral artery occlusion (MCAO). All procedures using animals were approved by the local Animal Care Committee (Protocol 2004.13). C57BL/6 mice (20–23 g) were obtained from Charles River (St. Foie, PQ). Under temporary isofluorane anesthesia, mice were subjected to MCAO using an intraluminal filament as previously described [22,23]. After 30 min of MCAO, the filament was withdrawn, blood flow restored to normal by laser Doppler flowmetry and wounds sutured. Animals were sacrificed after 7 or 30 d of reperfusion. Consistency in brain infarctions was evaluated by cutting brains into four 2 mm thick coronal slices through forebrain which were stained with 5 ml of 2% TTC for 90 min at 37 °C. The tissue was rinsed with saline and the formazan product solubilized in ethanol/dimethylsulfoxide (1:1). After

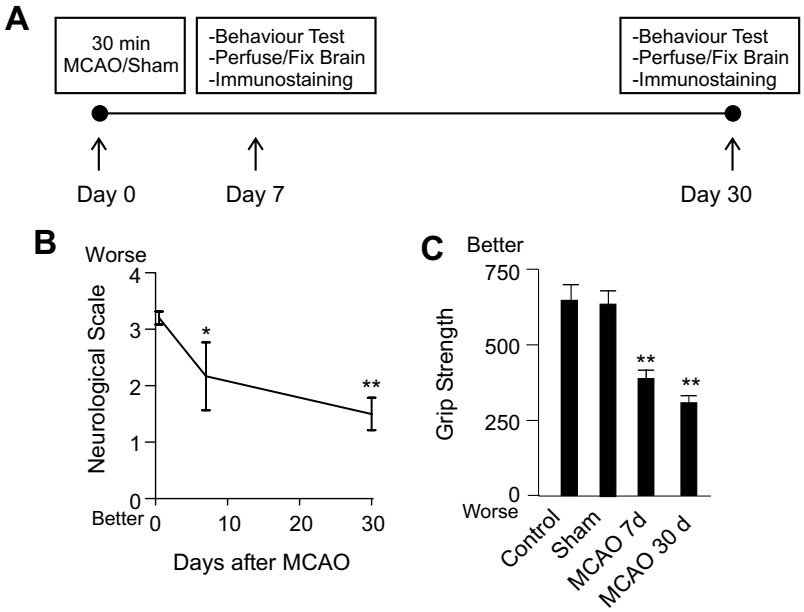

Fig. 1. Schematic diagram of the long-term recovery MCAO mouse model (A) and behavioral assessments (B and C). Adult mice were subjected to 30 min transient MCAO and 7 or 30 d reperfusion. Behavioral tests were performed (both forelimb grip strength test and 6-point turning behavioral test). Animal was killed and brain collected for frozen serial sectioning (A). The behavioral test scores for turning behavior and grip strength test were presented in B and C, respectively. One-way ANOVA was performed with Tukey's *post hoc* test to identity significant groups. **Indicate statistical significant at $p < 0.01$. Error bars represent STDEV.

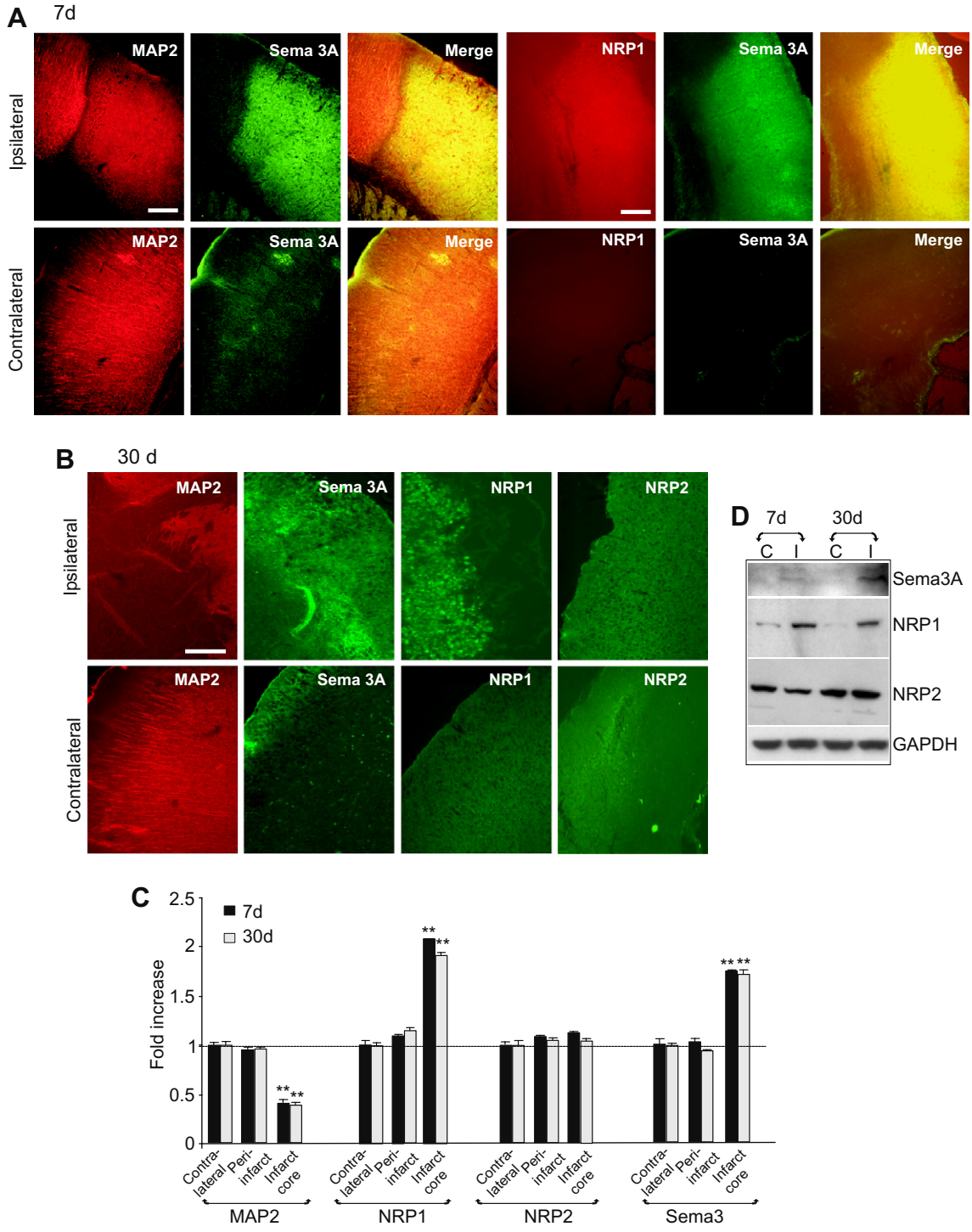

Fig. 2. Increased expression of Sema3A, NRP1, and GFAP in the ischemic brains. Mice brains were subjected to frozen serial sectioning and double-immunostaining to co-localize protein expressions. As shown in (A and B), 7 and 30 d after reperfusion, MAP2 expression (red) was reduced in the infarct core, while the expression of Sema3A and NRP1 increased in areas lacking MAP2 expression. The contralateral side was used as a control to illustrate the basal level of expression of these proteins and the quantification was presented in (C). The intensity of the protein expression on digitized imaged was measured and quantified (C). A fold of changes in image intensity was present. One-way ANOVA was performed with Tukey's *post hoc* test to identity significant groups. **Indicate statistical significant at $p < 0.01$. Error bars represent STDEV. Scale bars, 200 μm. Protein extractions from these mice were subjected to protein isolation and Western blotting as shown in (D). Clear induction of NRP1 and Sema3A occurred in the ischemic side of the brain. GAPDH was used an internal control for protein loading.

24 h incubation in the dark, the red solvent extracts were diluted 1:20 with fresh ethanol/DMSO solvent in three tubes and placed in cuvettes. The absorbance was measured at 485 nm in a spectrophotometer and the values were averaged. Percentage decrease in brain TTC staining in the ischemic side of the brain was compared to the contralateral side of the brain of the same animal using the following equation: %

Decrease = [1 − (absorbance of ischemic hemisphere/absorbance of contralateral hemisphere)] × 100.

Neurological scores. Both a six-point scale assessment and forelimb grip strength test were performed. (a) An expanded six-point scale turning behavior test was used exactly as described previously [22,23]. Briefly, behavioral assessments were made at 7 and 30 d after reperfusion by an individual blinded to the treatment of the mice. The neurological deficits were scored as follows: 0, normal; 1, mild turning behavior with or without inconsistent curling when picked up by tail, 50% attempts to curl to the contralateral side; 2, mild consistent curling, 50% attempts to curl to contralateral side; 3, strong and immediate consistent curling, mouse holds curled position for more than 1–2 s, the nose of the mouse almost reaches the tail; 4, severe curling progressing into barrelling, loss of walking or righting reflex; 5, comatose or moribund. At least eight mice per group were evaluated and scores were averaged for statistical analysis. (b) Forelimb grip strength test was performed at 0.5 h, 7 and 30 d reperfusion using the Grip Strength Meter from Columbus Instruments (MyNeurolab, St. Louis, MO) which measures muscle strength and neuromuscular integration relating to the grasping reflex in the forepaws. The peak preamplifier automatically stores the peak pull force and shows it on a liquid crystal display. For each animal, at least 10 measurements were taken at a specific time point and the mean and standard error were calculated.

Double-immunofluorescent staining. The mouse brain was perfused with 4% formalin and post-fixed for 16 h in 4% formalin, followed by incubation in 30% sucrose (in phosphate saline buffer) overnight at 4° C. The brain tissue was then mounted in OCT and kept at −80° C before cutting into 10 μm thick frozen serial sections using a cryostat. The procedures for double-fluorescent immunocytochemistry were exactly as described previously [24,25]. The primary antibodies against Sem3A, NRP1, NRP2, were purchased from R&D Systems (Cat. No. AF1250; Minneapolis, MN, USA), Calbiochem (Cat. No. PC343; EMD Chemicals, Inc. San Diego, CA, USA) and Upstate Biotech (Cat. No. AB9160; Lake Placid, NY, USA), respectively. The optimal concentrations of these primary antibodies were determined empirically and used at 1:100. The concentrations of the secondary antibody used were also determined empirically and were at 1:1000. Brain sections were mounted in Hoechst33588-spiked antifading mounting medium and examined immediately under a fluorescent microscope (Carl Zeiss, AX10 vert 200 M).

Western blotting. Proteins at 10 μg was electrophoresed in a 10% SDS mini gel and then electroblotted onto a nitrocellulose membrane in transfer buffer exactly as described previously [30,31]. The membrane was then probed with a primary antibody at dilutions ranging between 1:100 and 1:200 at 4 °C overnight. After washing with TBST (10 mM Tris–HCl, pH 7.8; 150 mM NaCl and 0.1% Tween 20), horseradish peroxidase-conjugated secondary antibody was applied to the membrane for 1 h at room temperature. Enhanced chemiluminescence detection of the target protein was performed using a LumiGlo substrate kit (Amersham Biosciences, Piscataway, NJ, USA) and X-ray film.

Image analysis and statistics. To quantify the expression levels of NRPs, Sema3A, and MAP2, images of immunostaining of these proteins were taken under a fluorescent microscope using identical fluorescent intensity settings. Identical areas of the brain were selected on digitized images with resolutions of 1300 × 1030 pixels. Fluorescent intensity was measured using NIH Image J software (http://rsb.info.nih.gov/ij/). At least four measurements were taken from non-overlapping areas of each region of interest on the brain. The average intensity and standard deviations were calculated using Prism 3.0 software. Data from ischemic core of the MCAO brain was normalized against the fluorescent intensity of the sham-operated brain, the contralateral side and the peri-infarct area.

Data analysis. All data was analyzed by one way ANOVA and further *post hoc* tests for significant groups using Tukey's test.

Results and discussion

Adult mice were subjected to 30 min transient MCAO and following by reperfusion. Sham-operated mice were also kept for up to 30 d as controls (Fig. 1A). Changes in motor functions were measured using our previously described 6-point scale turning behavior test (Fig. 1B) [22] and forelimb grip strength test (Fig. 1C). Significant improvement in turning behavior test occurred after 7 and 30 d recovery (Fig. 1B). The improvement of turning behavior may be caused by compensatory learning during the recovery period [26] since the overall grip strength test scores has decreased significantly over the testing period of 7 and 30 d after MCAO indicating sustained deterioration of motor functions after cerebral ischemia.

Brains were collected and perfusion fixed at 7 and 30 d MCAO with sham-operated brains as controls. Serial coronal sections were cut and double-immunostained using antibodies to MAP2/Sema3A, NRP1/Sema3A, NRP2/Sem3A, MAP2/NRP1, and MAP2/NRP2 (Fig. 2). MAP2 expression decreased significantly (more than 50%) on the ipsilateral side of the ischemic brain compared to those on the sham and contralateral side of the brain at 7 and 30 d. The loss of MAP2 expression correlated with the loss of NeuN positive cells (not shown), suggesting the loss of neurons through neuronal death. The area of reduced MAP2 staining therefore represents the ischemic territory. Importantly, the level of MAP2 staining remained low in both 7 and 30 d MCAO ischemic brains (Fig. 2C) indicating that no significant re-growth of neurons occurred in the ischemic territory during the long-term recovery.

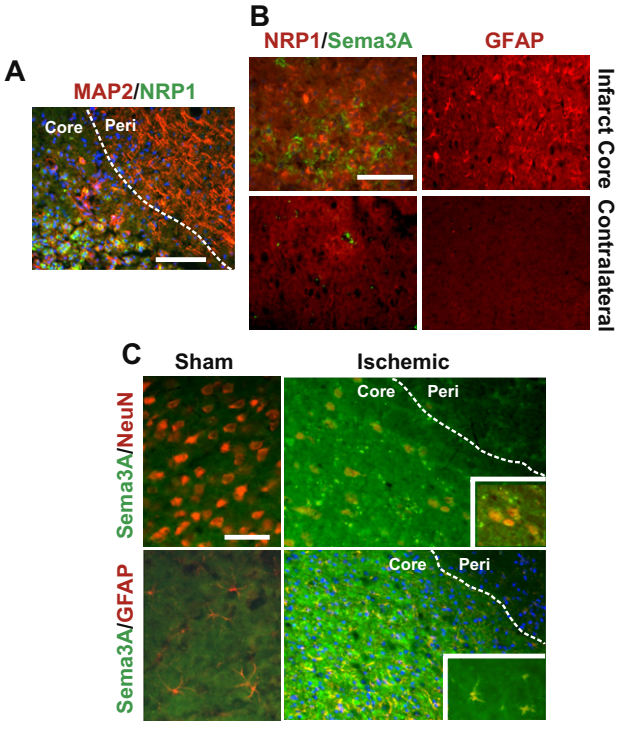

Fig. 3. Double-labelling detection of NRP1/Sema3A in ischemic brains. Higher magnification images are shown in (A–C) to highlight the cell-type specific expression of MAP2, NRP1, and Sema3A. NRP1 is expressed in the ischemic core (green color, A), while Sema3A is expressed in GFAP positive (red color in B and C) astrocytes. Insets in C show a higher magnification. Scale bars, 200 μm.

Increased expression of NRP1 and Sema3A also occurred in the ischemic side of the brain, which co-localized with the area lacking MAP2 staining (Fig. 2A). The level of Sema3A and NRP1 all remained significantly high in both 7 and 30 d reperfusion mouse brain as determined by quantification of immunostaining (Fig. 2C) and Western blotting (Fig. 2D). The intensities of immunostainings were measured on digitized images as described in the Methods section and shown in Fig. 2(A and B). In comparison with contralateral and peri-infarct areas, MAP2 expression was reduced more than 50% in the infarct core, while NRP1 and Sema3A expression increased more than a fold, respectively (Fig. 2C). NRP2 expression did not change significantly. Importantly, no obvious changes in these genes were present between 7 and 30 d ischemic brains.

At a higher magnification, NRP1 was found to be expressed on neurons (green color in Fig. 3A) only in the area lacking of MAP2 staining (red color in Fig. 3A). The increased expression of Sema3A mostly localized diffusely in the ischemic territory in spaces among NRP1 positive neurons (Fig. 3B). In the brain of 30 d reperfusion mice, there also occurred a drastic increase in the GFAP immunostaining indicating astrocytosis in the ischemic core (Fig. 3B–C). Interestingly, Sema3A expression also occurred in GFAP positive astrocytes, but in NeuN positive neurons, as shown by double-immunolabelling (Fig. 3C).

Although neurogenesis has been reported to occur in ischemic brains, it is not yet known whether this also occurs during the long-term recovery phase of the injured brains. Using immunostaining on serial sections we determined the expression of DCX, a maker for migrating immature neurons. As shown in Fig. 4, increased expression of DCX occurred in the lateral ventricle wall zone in the ischemic side of the brain (green color in Fig. 4A–C and G–I), but not in the dentate gyrus granule cell layer (Fig. 4D–F and J–L). DCX immunostaining mostly appeared in the cytoplasmic part of the cell. These cells were not positive for NeuN staining (red color in Fig. 4) suggesting that they are immature neurons. In the sham-operated mice brain, DCX staining was found mostly in the lateral ventricular wall lining and among the dentate granule cell layer (Fig. 3). These cells are often arranged in single or a multiple layer fashion with cellular projec-

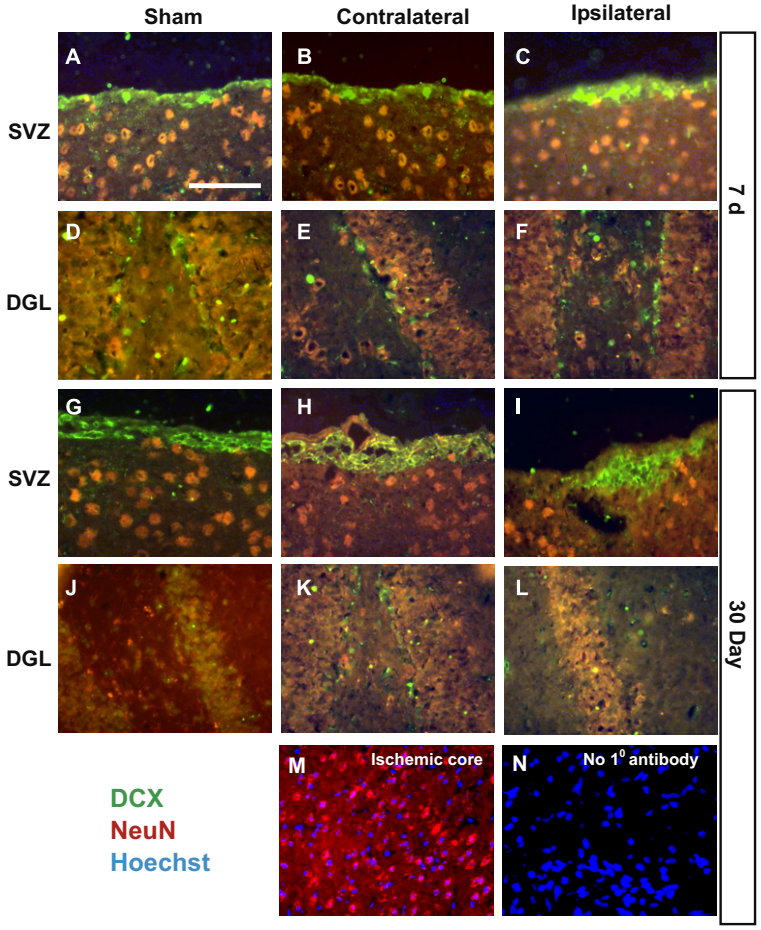

Fig. 4. Increased DCX expression in the lateral ventricle zone in the ischemic mice brain. Brains after 7 or 30 d reperfusion were collected and frozen serial sections were cut and double-immunostained for DCX (green color) and NeuN (red color). Images were taken from sham-operated brains, contralateral side and ipsilateral side of the ischemic brains. The level of DCX expression only appeared to have increased marginally in the ischemic side of the brain within the lateral ventricular zone (C and I). Scale bar, 200 μm.

tions towards the parenchyma. In the ischemic brain, only a marginal increase in the level of DCX expression occurred in cells located on the lateral ventricle zone, but no increase in DCX staining occurred in the dentate gyrus granule cell layer. Most importantly, immunostaining did not detect any DCX positive cells in the infarct core area (Fig. 4M and N) indicating that the inhibitory environment may have prevented the large scale migration of newly generated neurons into the damaged area. Future studies using this model are warranted.

Cerebral ischemia-induced axonal damage, neuronal loss, and regeneration failure may result in permanent deficits in visual, sensory, language, and motor capabilities. The molecular and biochemical mechanisms involved in the retraction and collapse of the axonal network during neuronal death following cerebral ischemia are still unclear. In studies of white matter damage, axonal injury in response to ischemia is associated with increased axonal membrane permeability with excess Na^+ and/or Ca^{2+} influx into the axon [27]. This imbalanced Ca^{2+} influx activates deleterious cascades involving activation of intracellular proteases and subsequent breakdown of cytoskeletons and disturbance of axon transport leading to degeneration. However, novel degradative pathways must exist, since inhibition of the well known protease calpain is necessary but not sufficient for white matter protection, emphasizing the existence of novel pathways activated following injury [27]. Indeed, several studies have shown that increased expression of inhibitory molecules is an integral part of the brain response to ischemic injury which repels regenerating axons, and perhaps even new neurons, from entering the damaged territory and ultimately causing neuronal death [1,28]. Blocking inhibitory molecules in the brain has been shown to have profound neuroprotective and regenerative effects. For example, blocking Sema3A/NRP1 interaction is potently neuroprotective in spinal cord injury [29]. Our recent studies also showed that NRPs are important mediators of axonal retraction/collapse through its cytosolic effectors, such as collapsin response mediator protein-3 during neuronal death induced by cerebral ischemia [30] and that the role of NRP1 is regulated by the pro-apoptotic transcription factor E2F1 [31]. The current studies allowed us to establish a model system to evaluate the long-term effect of Sema3A/NRP during the recovery phase of cerebral ischemia. Studies are underway to investigate the therapeutic effect of blocking Sema3A/NRP1 interaction in enhancing neuronal survival/regeneration in ischemic brains.

Acknowledgments

We thank the IBS Animal Facility for the timely supply of experimental animals. This work was supported by grant-in-aid from the Heart and Stroke Foundation of Ontario (#NA5393 and #T5760) to S.H.T.

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