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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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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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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

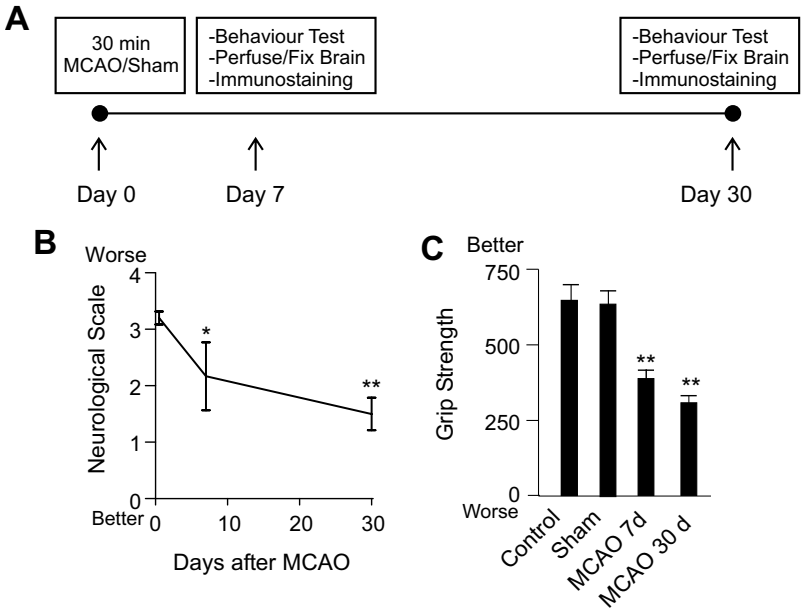

Fig. 1. Schematic diagram of the long-term recovery MCAO mouse model (A) and behavioral assessments (B and C). Adult mice were subjected to 30 min transient MCAO and 7 or 30 d reperfusion. Behavioral tests were performed (both forelimb grip strength test and 6-point turning behavioral test). Animal was 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.