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Research Paper

Sox9 knockout mice have improved recovery following stroke

Xiaoyun Xu[a], Bethany Bass[a,b], William M. McKillop[a,b], Janina Mailloux[a], Tony Liu[a], Nicole M. Geremia[a], Todd Hryciw[a], Arthur Brown[a,b,*]

[a] Robarts Research Institute, University of Western Ontario, London, Canada
[b] Department of Anatomy and Cell Biology, University of Western Ontario, London, Canada

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ABSTRACT

The partial recovery that can occur after a stroke has been attributed to structural and functional plasticity that compensate for damage and lost functions. This plasticity is thought to be limited in part by the presence of growth inhibitors in the central nervous system. Blocking or reducing signals from inhibitors of axonal sprouting such as Nogo and chondroitin sulfate proteoglycans (CSPGs) increases post-stroke axonal sprouting and improves recovery. We previously identified the transcription factor SOX9 as a key up-regulator of CSPG production and demonstrated that conditional *Sox9* ablation leads to increased axonal sprouting and improved recovery after spinal cord injury. In the present study we evaluate the effect of conditional *Sox9* ablation in a transient middle cerebral artery occlusion (MCAO) model of stroke. We demonstrate that conditional *Sox9* ablation leads to reduced CSPG levels, increased tissue sparing and improved post-stroke neurological recovery. Anterograde tract tracing studies demonstrate that in the *Sox9* conditional knockout mice corticorubral and corticospinal projections from the contralateral, uninjured cortex increase projections to targets in the midbrain and spinal cord denervated by the injury. These results suggest that targeting SOX9 is a viable strategy to promote reparative axonal sprouting, neuroprotection and recovery after stroke.

1. Introduction

An emerging therapeutic strategy for the treatment of stroke is to improve outcomes by increasing neuroplasticity. Indeed, increased neuroplasticity is thought to underpin spontaneous recovery or recovery attributed to rehabilitation after stroke. For example motor recovery in stoke patients has been linked to the re-organization of cortical pathways so that undamaged neurons are recruited to new functions (Feydy et al., 2002; Fujii and Nakada, 2003; Pineiro et al., 2001; Schaechter et al., 2002; Small et al., 2002; Zemke et al., 2003). Similar reorganization of cortical activity has also been demonstrated in the stroke-injured rat (Dijkhuizen et al., 2003). The fact that increased capacity for neuroplasticity decreases with developmental age and myelination led to the hypothesis that myelin associated inhibitors of axon growth may be responsible for the loss of plasticity in the adult brain (Kapfhammer and Schwab, 1994). One of the best characterized myelin inhibitors of axonal growth is Nogo-A (Chen et al., 2000; GrandPre et al., 2000; Prinjha et al., 2000). Using genetic models of reduced Nogo-A activity (Nogo-A knockouts and Nogo-receptor knockouts) as well as pharmacological blockade of Nogo signaling, researchers have demonstrated that reducing Nogo-A activity increases neuroplasticity and improves recovery in mouse and rat models of stroke (Lee et al., 2004; Seymour et al., 2005; Wiessner et al., 2003).

Chondroitin sulfate proteoglycans (CSPGs) are also well-described inhibitors of axonal growth (Asher et al., 2001; Galtrey and Fawcett, 2007) that limit neuroplasticity in the developing nervous system. CSPGs are critical components of perineuronal nets (PNNs), highly condensed matrix that surrounds the cell bodies and dendrites of many classes of neurons. One suggested function of the CSPGs in PNNs is to stabilize synapses by preventing axonal sprouting onto inappropriate targets after appropriate connections have been made (Galtrey and Fawcett, 2007). The role of CSPGs in limiting synaptic plasticity is well illustrated in the development of ocular dominance columns (Berardi et al., 2003; Pizzorusso et al., 2002; Pizzorusso et al., 2006). Furthermore, perilesional, intracerebral (Gherardini et al., 2015) or intraspinal (Soleman et al., 2012) administration of chondroitinase to rats after a stroke injury has been shown to reduce CSPG levels, increase neuroplasticity and improve functional recovery.

We previously identified SOX9 as a transcription factor that up-regulates the expression of CSPGs in astrocyte cultures (Gris et al., 2007) and have shown reduced expression of a battery of genes in spinal cord-injured *Sox9* conditional knockout mice including: XT-I (xylosyltransferase-I), GFAP and three CSPG core proteins (aggrecan, brevican and neurocan) (Gris et al., 2007; McKillop et al., 2013). The

* Corresponding author at: Robarts Research Institute, Schulich School of Medicine, University of Western Ontario, 100 Perth Drive, London, Ontario N6A 5K8, Canada.
E-mail address: abrown@robarts.ca (A. Brown).

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reduction in mRNA expression of these genes was accompanied by reductions in CSPG protein levels in the glial scar and in PNNs distant from the injury (McKillop et al., 2013). The *Sox9* conditional knockout mice demonstrated improved recovery of hind limb function that was attributed to increased axonal sprouting below the level of the injury (McKillop et al., 2016). These results led us to the prediction that conditional *Sox9* ablation would have similar beneficial effects on recovery in a mouse model of stroke. We herein report that after MCAO *Sox9* conditional knock out mice demonstrate decreased CSPG and PNN matrix levels, improved neurological recovery, and increased reparative sprouting from the contralateral undamaged cortex onto denervated targets in the midbrain and spinal cord.

2. Materials and methods

2.1. Experimental design

All protocols for these experiments were approved by the University of Western Ontario Animal Care Committee in accordance with the policies established in the Guide to Care and Use of Experimental Animals prepared by the Canadian Council on Animal Care. Mice were randomly assigned a number code without regard to genotype by a person otherwise not involved in the study. Animals were randomly selected for MCAO surgery. Four sets of both *Sox9* knockout and control mice were used for specific analyses with different endpoints as set out in Fig. 1. The numbered code was not broken until all analyses were complete.

2.2. Mouse breeding and Sox9 conditional knockouts

We bred a mouse strain (kindly provided by Dr. Andreas Schedl) carrying floxed *Sox9* alleles (exons 2 and 3 of *Sox9* surrounded by loxP sites) (Akiyama et al., 2002) with a transgenic mouse line ubiquitously expressing Cre-recombinase fused to the mutated ligand binding domain of the human estrogen receptor (ER) under the control of a chimeric cytomegalovirus (CMV) immediate-early enhancer/chicken ß-actin promoter (B6.Cg-Tg (CAG-*Cre/Esr1*)*5Amc/J*) (Jackson Laboratories, Bar Harbor, ME). The two populations of resulting offspring served as tamoxifen-inducible *Sox9* knockout mice (*Sox9*$^{flox/flox}$;*CAGGCreER*, here after referred to as *Sox9*$^{flox/flox}$;*Cre*), and control mice with normal expression levels of *Sox9* (*Sox9*$^{flox/flox}$). Both *Sox9*$^{flox/flox}$;*Cre* and *Sox9*$^{flox/flox}$ mice were administered tamoxifen (Sigma Aldrich, St. Louis, MO) by oral gavage at a dose of 3 mg/20 g mouse to generate *Sox9* conditional knockout and control mice, respectively. Tamoxifen was administered daily for 7 days to all mice, followed by a 7 day period without treatment. Mice were housed in pairs at 22 °C with food and water available ad libitum.

2.3. Surgery

Male mice weighing 25–30 g (3–4 months of age) were anesthetized with 4% isofluorane and maintained at 1.5% isofluorane and 30% O_2. Cerebral ischemia was induced using an intraluminal filament technique (Rupadevi et al., 2011). A 5–0 nylon monofilament coated with 0.1% poly-L-lysine was introduced through a small incision into the common carotid artery and advanced to the carotid bifurcation for MCAO. Reperfusion was initiated 30 min later by monofilament withdrawal. In sham-operated animals, the monofilament was placed into the MCA and withdrawn immediately.

Laser Doppler Flowmetry (Oxford Optronix, Oxford, UK) was used to measure cerebral blood flow. A midline incision was made and a burr-hole (0.7 mm in diameter) was drilled 1 mm posterior and 4 mm lateral to the sagittal suture on the left side of the skull. The probe was held in a micromanipulator and stereotaxically advanced to gently touch the dura. Warm saline (37 °C) was slowly rinsed around the probe and maintained a clear medium between the probe and the dura during the measurement. Stable baseline laser doppler flowmetry readings were obtained prior to MCAO surgery. The changes of regional CBF were recorded 5 min into the occlusion of the middle cerebral artery. A sharp drop over 85% (85.8 ± 2.5%) of baseline was noted, which remained stable throughout the 30 minute occlusion. Sample size estimation was based on the effect sizes and variability as other studies of MCAO in mice(Chen et al., 2014; Hill et al., 2012; Lindau et al., 2014; Reitmeir et al., 2011; Seymour et al., 2005; Soleman et al., 2012). Thus we planned to conduct our studies with approximately 12 mice per group for behavioral studies, 4–6 mice per group for biochemical and lesion analyses, and 8–10 mice per group for tract tracing analyses. Anticipating a mortality rate in the first few days after MCAO at ~20% we performed MCAO on 40 controls and 42 *Sox9* conditional knockout mice. Seven control and ten *Sox9* knockout mice died after the MCAO surgery. Thus leaving us with 33 control and 32 *Sox9* conditional knockout mice for our studies. No mice required early euthanasia for health reasons during the study.

Day -14	Day -7	Day 0	Day 1	Day 14		Day 3 - 28			Day 42		
Tamoxifen Administration (7 days)	Tamoxifen Clearance (7 days)	Surgery	TTC Staining	CV Stain	Protein & RNA	Corner Test	Cylinder Test	Grip strength	BDA Fiber	BDA Puncta	WFA
									(number analyzed out of 4 BDA-injected mice)		
		Sham controls (n=8)	-	-	4	-	-	-	3	3	4
		Sham KOs (n=8)	-	-	4	-	-	-	3	3	4
						(number out of 15 that met performance criteria)			(number out of 9 BDA-injected mice that met labeling criteria)		
		MCAO Controls (n=33)	7	7	4	12	11	9	6	6	6
		MCAO KOs (n=32)	6	7	4	14	11	8	8	6	6

Fig. 1. Outline of the experimental design. All mice were treated with tamoxifen once a day for 7 days followed by one week of rest to allow for tamoxifen clearance. MCAO or sham surgeries were then performed on day 0. All control mice were genotypically *Sox9*$^{flox/flox}$ and the Sox9 knockout mice were *Sox9*$^{flox/flox}$;*Cre*. Protein and RNA levels in the sham control and *Sox9* knockout mice were measured to reveal the effect of MCAO on RNA and protein levels studied. In both sham control and sham *Sox9* knockout mice 4 animals were used for protein (Western blot) and RNA (Q-PCR) analyses. Sham control and Sox9 knockout mice (n = 4 per group) underwent BDA injections to evaluate the frequency of contralateral corticorubral and ipsilateral corticospinal projections in these uninjured mice. Three of the 4 shams in each group has sufficient labeling to allow analyses. Sections from all 4 control and Sox9 knockout mice were used to assess WFA staining. Of the 33 MCAO control mice 7 were used for TTC staining one day post-MCAO, 7 for cresyl violet staining 2 weeks post MCAO, 4 for protein and RNA analyses, and 15 for behavioral analyses. Of the 32 MCAO *Sox9* knockout mice 6 were used for TTC staining one day post-MCAO, 7 for cresyl violet staining at 2 weeks post MCAO, 4 for protein and RNA analyses, and 15 for behavioral analyses. Reductions in the number of mice analyzed in the individual behavioral tests is due to the exclusion of mice that failed to reach performance criteria (too weak to perform the cylinder test, insufficient frequency of rearing for the corner test or failure to grasp the bar in the grip strength test). Nine out of the 15 control and *Sox9* knockout mice used for behavioral testing underwent BDA injections and anterograde labeling analyses. Six of the 9 BDA-injected mice had sufficient BDA-labeling to allow for analyses and sections from these mice were also used to assess WFA staining.