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Overexpression of Sox1 1 Promotes Corticospinal Tract Regeneration after Spinal Injury While Interfering with Functional Recovery

Zimei Wang
Marquette University

Ashley Reynolds
Marquette University

Adam Kirry
Marquette University

Christopher Nienhaus
Marquette University

Murray G. Blackmore
Marquette University, murray.blackmore@marquette.edu

Overexpression of Sox11 Promotes Corticospinal Tract Regeneration after Spinal Injury While Interfering with Functional Recovery

Zimei Wang, Ashley Reynolds, Adam Kirry, Christopher Nienhaus, and Murray G. Blackmore

Department of Biomedical Sciences, Marquette University, Milwaukee, Wisconsin 53201

Embryonic neurons, peripheral neurons, and CNS neurons in zebrafish respond to axon injury by initiating pro-regenerative transcriptional programs that enable axons to extend, locate appropriate targets, and ultimately contribute to behavioral recovery. In contrast, many long-distance projection neurons in the adult mammalian CNS, notably corticospinal tract (CST) neurons, display a much lower regenerative capacity. To promote CNS repair, a long-standing goal has been to activate pro-regenerative mechanisms that are normally missing from injured CNS neurons. Sox11 is a transcription factor whose expression is common to a many types of regenerating neurons, but it is unknown whether suboptimal Sox11 expression contributes to low regenerative capacity in the adult mammalian CNS. Here we show in adult mice that dorsal root ganglion neurons (DRGs) and CST neurons fail to upregulate Sox11 after spinal axon injury. Furthermore, forced viral expression of Sox11 reduces axonal dieback of DRG axons, and promotes CST sprouting and regenerative axon growth in both acute and chronic injury paradigms. In tests of forelimb dexterity, however, Sox11 overexpression in the cortex caused a modest but consistent behavioral impairment. These data identify Sox11 as a key transcription factor that can confer an elevated innate regenerative capacity to CNS neurons. The results also demonstrate an unexpected dissociation between axon growth and behavioral outcome, highlighting the need for additional strategies to optimize the functional output of stimulated neurons.

Key words: axon regeneration; gene therapy; Sox11; spinal cord injury; transcription factor

Introduction

Recovery from axon injury in the CNS is constrained by the intrinsic inability of many neurons to mount an effective regenerative response (Liu et al., 2011; Blackmore, 2012). In contrast, embryonic CNS neurons, peripherally injured PNS neurons, and CNS neurons in anamniotes like zebrafish are capable of re-extending severed axons, which then integrate into functional circuitry and enable remarkable behavioral recovery (Blackmore, 2012; Becker and Becker, 2014). Central to this regenerative success is the initiation of pro-regenerative transcriptional programs by injured neurons, likely orchestrated by key transcription factors (Smith and Skene, 1997; Veldman et al., 2007). Identifying and recapitulating these pro-regenerative mechanisms is a long-standing strategy to enhance the regenerative abilities of mammalian CNS neurons. Indeed, forced activation of transcription factors such as KLF7, STAT3, and SMADs has improved axon growth in injured CNS neurons (Smith et al., 2009; Parikh et al., 2011; Blackmore et al., 2012). The evoked regeneration, however,

falls short of that seen in embryonic, peripheral, or zebrafish neurons, and the functional contribution of the stimulated growth remains unclear. Thus, additional factors await discovery and functional testing.

Sox11, a transcriptional activator, is widely expressed in the embryonic CNS and PNS during periods of axon growth, and then is developmentally downregulated (Penzo-Méndez, 2010). Regeneration-competent neurons such as dorsal root ganglion (DRG) neurons and zebrafish retinal ganglion cells rapidly upregulate Sox11 in response to axotomy (Tanabe et al., 2003; Jankowski et al., 2009). Moreover, knockout of Sox11 interferes with developmental and regenerative PNS axon growth (Jankowski et al., 2009; Lin et al., 2011; Shim et al., 2012a). It remains unclear, however, whether forced Sox11 expression might be sufficient to promote axon growth and functional recovery after CNS injury. Here we show that virus-mediated expression of Sox11 reduces retraction in centrally injured DRG axons, and in corticospinal tract (CST) neurons it promotes both compensatory sprouting and the growth of injured axons in the spinal cord. We find, however, that recovery of forelimb dexterity is impaired in Sox11-treated animals. These data show that CNS axon regeneration is limited in part by insufficient activation of the Sox11 transcriptional program, but also provide an illustrative example hinting that reinitiation of this program, in the absence of additional mechanisms to properly sculpt axon function, can lead to functional impairment.

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Correspondence should be addressed to Murray G. Blackmore, Department of Biomedical Sciences, Marquette University, Milwaukee, WI 53201. E-mail: murray.blackmore@marquette.edu.

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Materials and Methods

Cloning and virus construction

Adeno-associated virus pseudotype 8 (AAV8)-enhanced blue fluorescent protein (EBFP)-2A-mCherry and AAV8-EGFP have been previously described (Blackmore et al., 2012). To generate AAV8-Sox11-2A-mCherry, PCR-amplified Sox11 (BC078643; OpenBiosystems) replaced EBFP in the AAV-2A-mCherry plasmid using Kpn1 and BamHI sites. Viral packaging was performed at the Miami Project Viral Vector Core. All titers were brought to 5×10^{13} /ml. Sox11 expression was confirmed via immunohistochemistry on free-floating, paraformaldehyde-fixed tissue sections using goat polyclonal anti-Sox11 (C-20, 1:300; Santa Cruz Biotechnology).

Viral injections and injuries

All animal procedures were performed on 8- to 10-week-old female C57BL/6 mice (Harlan) and approved by the Marquette University Institutional Animal Care and Use Committee. DRG neurons were transduced by intrathecal injection of $1 \mu\text{l}$ of AAV (Parikh et al., 2011; Schuster et al., 2013). Cortical neurons were transduced by $0.5 \mu\text{l}$ of AAV stereotactically injected to sensorimotor cortex (1.5 mm lateral, 0.5 mm anterior, 0.5 mm depth), as described in the study by Blackmore et al. (2012). For pyramidotomies, a ventral midline incision was made, the occipital bone was exposed, and the ventrocaudal part was removed using fine rongeurs, the dura was punctured, and the right pyramid was cut completely using a micro-feather scalpel. For spinal injuries, animals were mounted into a custom spine stabilizer, the dorsal surface of the spinal cord was exposed between C4/C5, and a lesion was made to a depth of 0.85 mm, extending 1 mm to the left of the midline and beyond the right edge of the spinal cord, using a Vibraknife (Ping Zheng and Christopher Shields, Norton Neurologicals, Louisville, KY) as reported previously (Blackmore et al., 2012). Viral injections occurred 1 week after injury in the pyramidotomy experiment, 1 week before injury in the first spinal injury experiment, and 8 weeks postinjury in the chronic injury experiment. To retrogradely label CST neurons, $1 \mu\text{l}$ of Cholera toxin B (CTB)-Alexa Fluor 647 was injected stereotactically via Hamilton syringe into C4/C5 spinal cord, and to label ascending DRG axons, Dextran 488 tracer (Invitrogen) was injected into the sciatic nerve 7 d before the mice were killed.

Histological assessment

Sagittal (spinal injury experiments) or transverse (pyramidotomy) sections of spinal cord were cut via vibratome, and immunohistochemistry analysis for PKC γ (C-19, 1:500; Santa Cruz Biotechnology) or GFAP (DAKO) was performed. Exclusion criteria for pyramidotomy experiments was a $<80\%$ decrease in PKC γ levels in the affected CST; for spinal injuries, exclusion criteria were as follows: (1) lesion depth $<800 \mu\text{m}$; (2) axons with straight white matter trajectory distal to the lesion; and

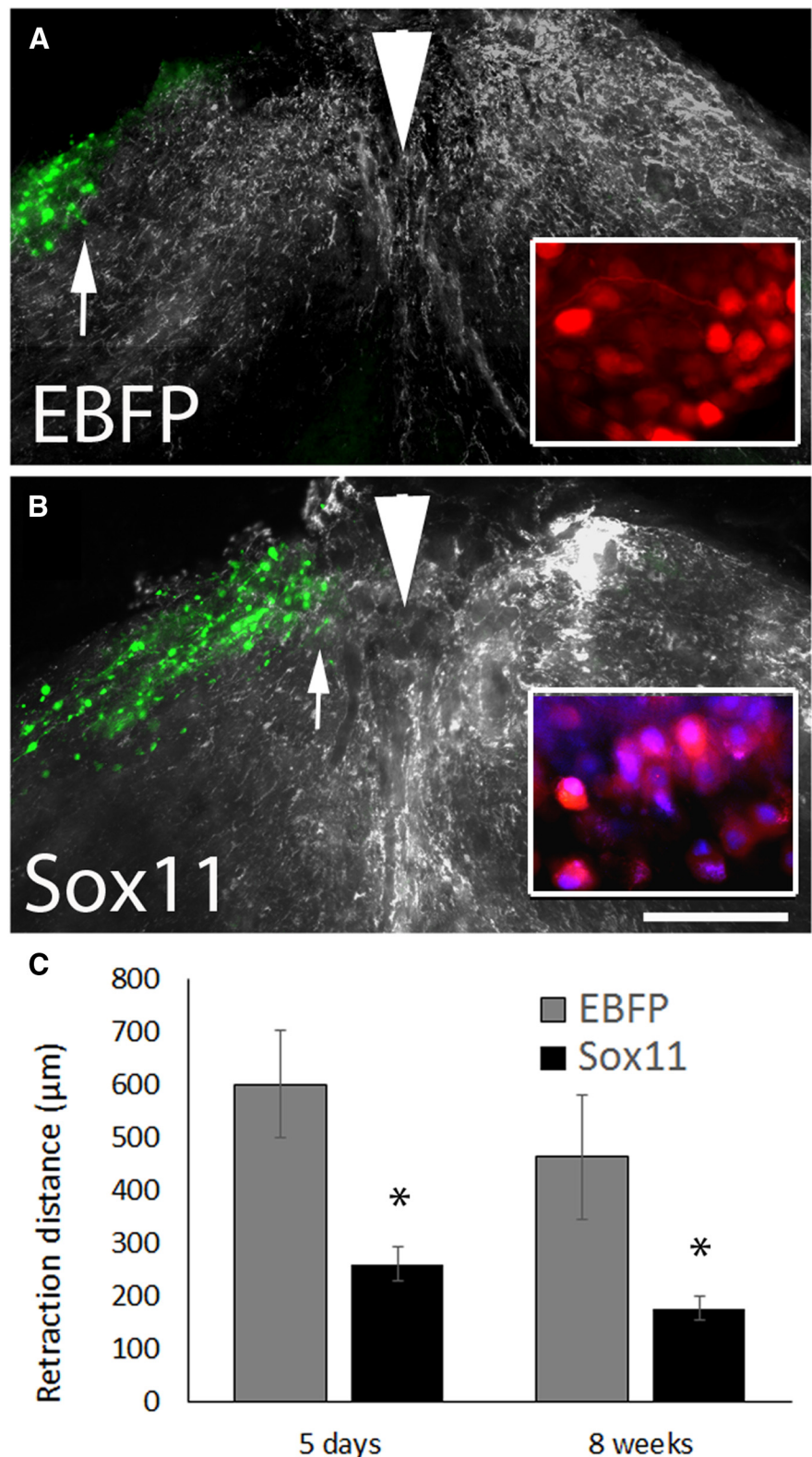


Figure 1. Sox11 overexpression reduces net retraction in centrally injured DRG axons. **A, B**, Cervical spinal cords 8 weeks after dorsal injury (arrowheads) and 9 weeks after intrathecal delivery of AAV8-EBFP-2A-mCherry control (**A**) or AAV8-Sox11-2A-mCherry (**B**). Viral transduction is confirmed by mCherry expression in DRG cell bodies (red, insets), and Sox11 expression is confirmed by immunohistochemistry (blue, insets). Ascending DRG axons were labeled by dextran-Alexa Fluor 488 (green) to visualize the position of injured axons (arrows) relative to the lesion center (arrowhead), outlined by GFAP immunoreactivity (white). **C**, Quantification shows a significant reduction in the distance between the lesion center and the tip of the nearest injured axon at both 5 d and 8 weeks postinjury in Sox11-treated animals. Scale bars: **A, B**, 200 μm ; insets, 50 μm . $N = 8$ animals per group, $*p < 0.05$, paired t test. Error bars show SEM.

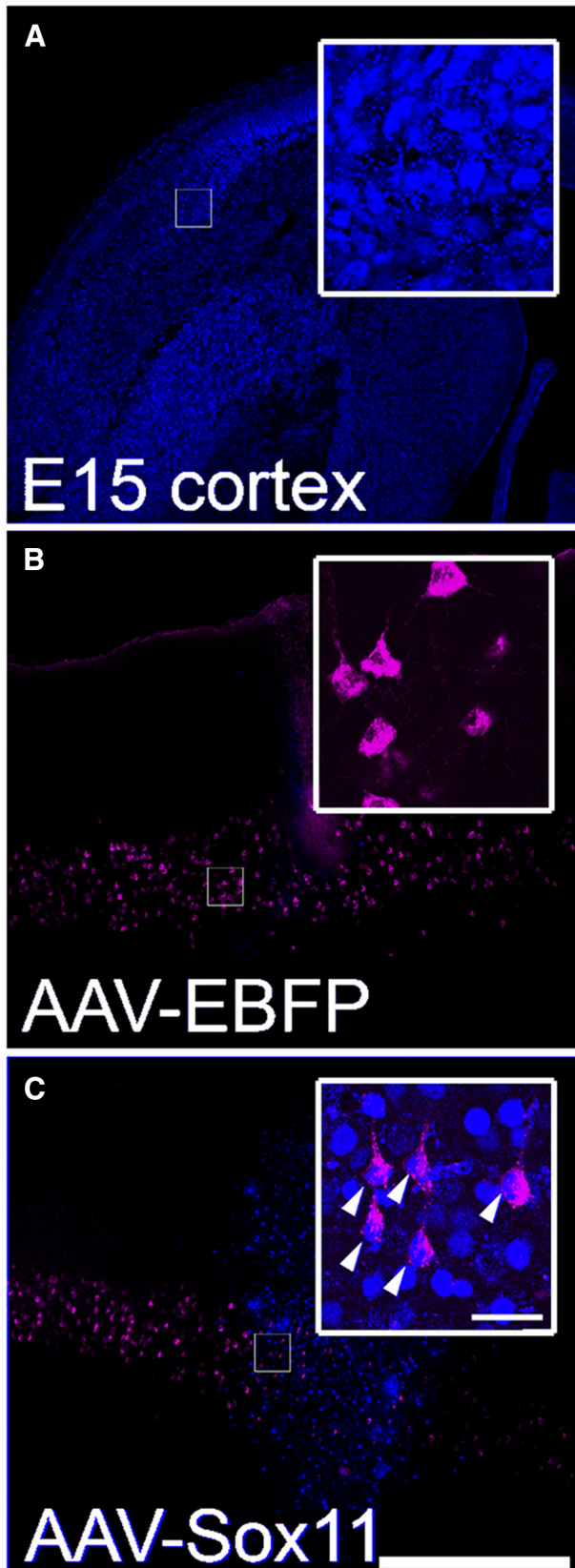


Figure 2. Sox11 is developmentally downregulated and is not re-expressed by spinally injured CST neurons. *A*, Sox11 (blue) is readily detected by immunohistochemistry in the embryonic cortex. *B*, *C*, Adult cortex 3 weeks after cortical injection of AAV8-EBFP (*B*) or AAV-Sox11 (*C*), and 2 weeks after cervical spinal injury and injection of retrograde CTB-647 (purple). Sox11 is not detected in injured cortex that received control virus (*B*) but is detected in the vicinity of the AAV8-Sox11 injection (*C*). Scale bars: *A–C*, 500 μm ; insets, 20 μm .

(3) EGFP⁺ axons in thoracic cord (too far for regenerative growth). EGFP⁺ profiles that intersected virtual lines at set distances from the center of the injury or the midline of the spinal cord were quantified by a blinded observer on an Olympus IX81 microscope, then were normalized to total axons in transverse medullary sections (Blackmore et al., 2012). TUNEL staining was performed on ethanol/acetic acid-treated cryostat sections of cortical tissue, 1 or 4 weeks postinjury and injection with virus, or 2 d after administration of a staurosporine control (1 μM , 1 mM; Sigma) via In Situ Cell Death Kit (Roche) according to the instructions of the manufacturer (Theus et al., 2014).

Behavioral assessment

Pellet retrieval. Animals were pretrained daily for 2 weeks to grasp and retrieve a food pellet using the right forelimb through a displaced opening in a transparent cage (Farr and Whishaw, 2002). Inclusion criteria were an average of 50% success (clear grasping and withdrawal, not sweeping). Animals were tested postinjury in sessions consisting of 15 pellets or 10 min maximum; retrievals/attempts were quantified by a blinded observer via video analysis.

Horizontal ladder. Animals were pretrained in two four-run sessions on a horizontal ladder (30 cm long, 1 cm rung spacing), then were tested after treatment in four-crossing sessions with the percentage of error quantified on the last three runs. On the final test date, rung spacing was made unequal by randomly removing rungs. Errors were defined as missteps or slips by the right forelimb.

Results

Sox11 is upregulated in DRG neurons after peripheral injury, but not central injury, and overexpression of Sox11 accelerates peripheral nerve growth (Jing et al., 2012). A potential role for Sox11 in CNS regeneration remains unexplored. We therefore tested whether forced upregulation of Sox11 promotes regeneration in the central branch of DRG axons after spinal injury. Sox11 or EBFP control was delivered to DRG neurons *in vivo* by intrathecal AAV injection, with mCherry coexpressed using a 2A peptide bridge, as described previously (Parikh et al., 2011; Blackmore et al., 2012). Two weeks later, mCherry expression confirmed a mean (\pm SEM) transduction of $51.9 \pm 4.6\%$ of lumbar DRG neurons (Fig. 1*A, B*). Sox11 was not detected by immunohistochemistry in DRG neurons transduced with AAV-EBFP, which is consistent with previous reports that central injury to DRG neurons does not induce Sox11 expression (Fig. 1*A*; Jankowski et al., 2009). In contrast, immunohistochemistry in Sox11-treated DRGs showed strong expression of Sox11 protein (Fig. 1*A, B*), on par with its detection in embryonic neurons (Fig. 2*A*), demonstrating effective viral overexpression. Eight weeks after C5 dorsal hemisection, neither control nor Sox11-treated DRG neurons showed regeneration of axons distal to the injury site (Fig. 1). Sox11-treated DRG axons, however, were significantly closer to the lesion center (EBFP, $463.9 \pm 117 \mu\text{m}$; Sox11, $177.7 \pm 21.5 \mu\text{m}$; $p = 0.017$, paired *t* test). A second experiment showed similar results at 5 d postinjury (EBFP, $601 \pm 100.9 \mu\text{m}$; Sox11, $260.5 \pm 21.5 \mu\text{m}$; $p = 0.027$, paired *t* test), suggesting that Sox11 reduced the initial axonal retraction as opposed to facilitating regeneration. These data indicate that forced expression of Sox11 in centrally injured DRG neurons alters their response to injury in a manner that reduces net axonal dieback.

We next examined the role of Sox11 in the injury response of CNS neurons, in which Sox11 is known to be expressed developmentally during periods of initial axon growth but is downregulated with age (Penzo-Méndez, 2010). We delivered AAV8-Sox11-2A-mCherry or AAV8-EBFP-2A-mCherry control to adult cortical neurons by direct injection, and then performed C5 cervical hemisections (Blackmore et al., 2012). The tracer CTB-Alexa Fluor 647 was injected into the injury site to retrogradely identify CST

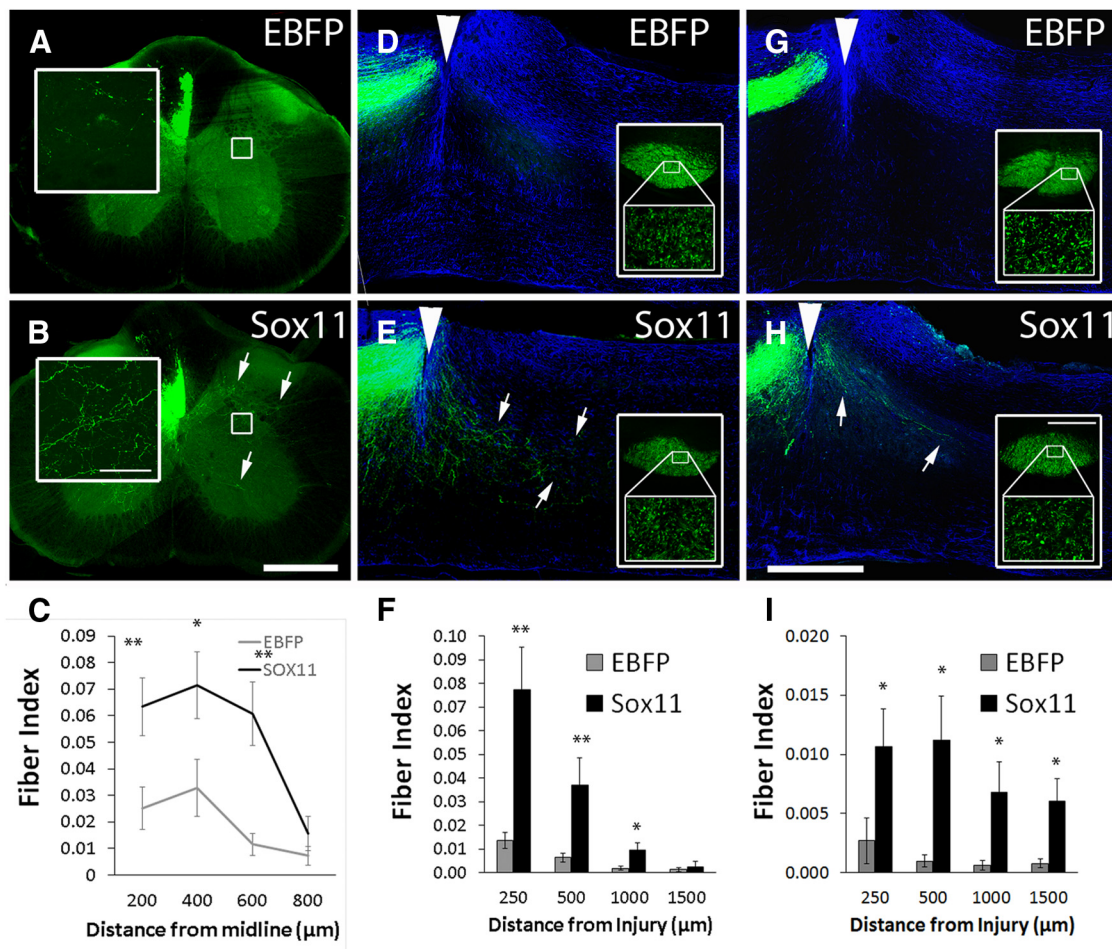


Figure 3. Viral overexpression of Sox11 promotes the growth of injured CST axons. **A–C**, Transverse sections of cervical spinal cord 8 weeks after left pyramidotomy and right unilateral cortical AAV injection. Sprouting across the midline (insets) is significantly elevated in Sox11-treated animals. **D–F**, Sagittal sections of cervical spinal cord 8 weeks postinjury and 9 weeks after cortical AAV injection. GFAP reactivity (blue) indicates the injury site (arrowheads) and transduced CST axons are green. The number of EGFP⁺ CST axons distal to the injury, normalized to the total number of transduced CST axons quantified in transverse sections of the medullary pyramid (insets), is significantly elevated in Sox11-treated animals (arrows, **E**, and quantified in **F**). **G–I** are similar to **D** and **E** except viral treatment was delayed until 8 weeks postinjury. $N \geq 8$ animals per group. * $p < 0.05$, ** $p < 0.01$, two-way repeated-measures ANOVA with Bonferroni's *post hoc* correction. Scale bars: **A–I**, 500 μm ; insets, 50 μm . Error bars show SEM.

neurons. Two weeks later, immunohistochemistry detected Sox11 expression in transduced (mCherry⁺), retrogradely identified CST neurons (CTB-647⁺) treated with AAV-Sox11, but not control AAV-EBFP (Fig. 2C,D). Sox11 was readily detectable in embryonic cortex (Fig. 2A,B). These data confirm a developmental downregulation of Sox11, show a lack of re-expression in injured CST neurons, and demonstrate successful viral expression of Sox11 in CST neurons.

Is Sox11 expression sufficient to promote axonal growth in injured CST neurons? To assess the effects on axon sprouting, we performed a left pyramidotomy in adult mice and then injected AAV-Sox11-2A-mCherry or EBFP-2A-mCherry into the right sensorimotor cortex, along with AAV-EGFP as an axonal tracer (Blackmore et al., 2012). This paradigm deprives the right half of the spinal cord of CST input and assesses the ability of uninjured left CST axons to extend collateral branches across the midline into denervated territory. Successful pyramidotomy was confirmed by a more than fivefold reduction in PKC γ immunoreactivity in the right CST (Materials and Methods; Liu et al., 2010). Eight weeks after pyramidotomy, transverse sections of cervical spinal cord were prepared. Sox11-treated animals showed significantly more transduced (EGFP⁺) CST fibers crossing up to 600 μm into the left spinal cord. (Fig.

3A–C). Thus, forced expression of Sox11 promotes compensatory sprouting by CST axons.

Next, we tested the effect of Sox11 on the growth of injured CST axons. Sox11 or control virus was coinjected with EGFP tracer to the left sensorimotor cortex, and right cervical dorsal hemisections were performed. Eight weeks postinjury, Sox11-injected animals showed a significantly greater number of EGFP⁺ CST axons up to 1 mm distal to the injury site (Fig. 3D–F). In a second experiment, viral treatments were delayed 8 weeks after spinal injury, and then axon growth was quantified after an additional 8 weeks. Again, although the effect appeared reduced compared with the acute paradigm (Fig. 3F), Sox11-treated animals showed a significant elevation in CST axon growth distal to the injury site compared with control animals (Fig. 3G–I). In both experiments, axons were observed circumventing the injury site but did not appear to grow across the injury gap. In addition, the dystrophic end bulbs at the tips of the main CST axons appeared unaffected by Sox11 treatment, and the elevated growth appeared to arise from collateral branches of CST axons. Combined, these data indicate that the overexpression of Sox11 in CST neurons promotes axon growth in the injured spinal cord, both in the short term and when applied 8 weeks postinjury.

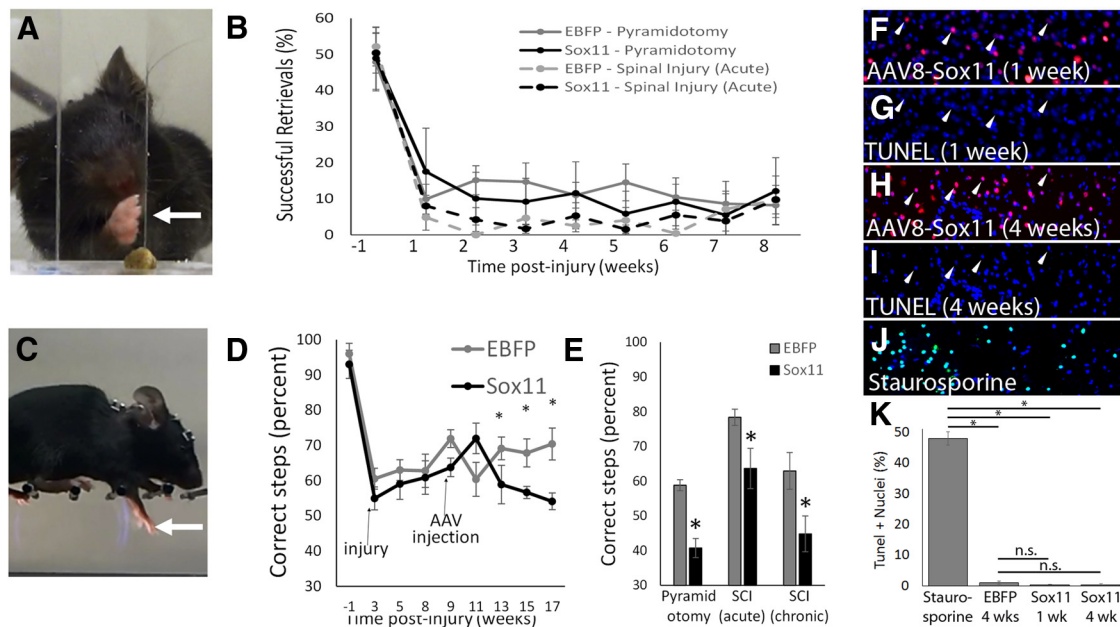


Figure 4. Viral overexpression of Sox11 in the cortex interferes with forelimb function after injury without affecting cell survival. **A, B**, Adult mice were subjected to pyramidotomy or spinal injury and were cortically injected with AAV-EBFP control or AAV-Sox11, and then were tested weekly on a pellet retrieval task. Injury caused a persistent reduction in successful retrievals that was unaffected by viral treatment ($p > 0.05$, two-way repeated-measures ANOVA with Bonferroni's *post hoc* correction). **C, D**, Mice received spinal injury, and 8 weeks later received cortical injection of AAV-EBFP control or AAV-Sox11. Quantification of footfall errors by the right forelimb on a horizontal task revealed a significant reduction in accuracy in Sox11-treated animals that emerged 5 weeks after viral injection. $*p < 0.05$, two-way repeated-measures ANOVA with Bonferroni's *post hoc* correction. **E**, Animals were tested 8 weeks after viral treatment on a horizontal ladder with unevenly spaced rungs. Sox11-treated animals showed significantly reduced accuracy of forelimb placement in all three injury paradigms. $N \geq 8$ animals per group. $*p < 0.05$, paired *t* test. **F–K**, Sox11-transduced cortical cells (**F, H**, red) display no TUNEL reactivity either 1 week (**F, G**) or 4 weeks (**H, I**) after viral injection and cervical injury (**G, I**, arrows). **J**, As a control, staurosporine toxin causes robust TUNEL signal (green). **I**, Quantification reveals no difference in TUNEL signal between EBFP- or Sox11-injected animals. $N = 3$ animals per group. $*p < 0.05$, ANOVA with Tukey's HSD test. Error bars show SEM.

The elevated axon growth observed in Sox11-expressing CST neurons raised the question of whether these anatomical changes are paralleled by improvements in forelimb function. Mice were tested on a pellet retrieval task and a horizontal ladder task, both of which reliably detect deficits in forelimb function after pyramidotomy or cervical spinal injury (Starkey et al., 2005; Shim et al., 2012b). As expected, both pyramidotomy and spinal injury dramatically reduced the rate of successful pellet retrieval (Fig. 4A, B). In the 8 weeks following viral injection, performance in both injury paradigms was similar in AAV-EBFP-treated and AAV-Sox11-treated animals, suggesting that Sox11-induced CST growth did not affect forelimb dexterity in this task. In the horizontal ladder task, injury produced a reliable increase in footslips in the right forelimb (Fig. 4C–E). Unexpectedly, Sox11 treatment resulted in an increased error rate compared with control animals. In the chronic injury experiment, the deficits in the Sox11-treated animals emerged 4 weeks after viral injection (12 weeks postinjury) and persisted for the duration of the experiment (Fig. 4D). Similarly, in the acute treatment paradigm and in the pyramidotomy experiment, Sox11-treated animals showed a significant elevation in footslips compared with controls when confronted with unevenly spaced rungs (Fig. 4E). Importantly, TUNEL assays in Sox11-treated cortex performed at 1 week postinjection (the onset of viral expression) or at 4 weeks postinjection (the onset of behavioral deficits; Fig. 4D) showed no evidence for apoptosis (Fig. 4F–K). Moreover, the average number of transduced CST axons detected at 8 weeks postinjection in the medullary pyramids (Fig. 3D, E, G, H, insets) was not reduced in Sox11-treated animals [Sox11 vs EBFP: acute spinal cord injury (SCI), 1949 ± 298 vs 1943 ± 343 ; chronic SCI, 2470 ± 711 vs 2577 ± 452 ; pyramidotomy, 2289 ± 209 vs 2532 ± 345]. These

data demonstrate that the behavioral deficits were not secondary to a reduction in cell survival or overt loss of CST axons. Combined with the histological data, these data show that the overexpression of Sox11 in CST neurons reliably promotes axon growth across a range of injury paradigms, while also producing deficits in forelimb dexterity.

Discussion

We found that Sox11, a transcription factor that is widely associated with examples of successful regeneration, is not upregulated by DRG or CST neurons after spinal injury, and that forced expression is sufficient to enhance CNS axon growth. The effects in DRGs were limited to a reduction in net axonal retraction, but effects in CST neurons were more pronounced. Indeed, although comparisons between experiments must be made cautiously, the magnitude of the CST response, when normalized to the total number of transduced CST axons, appears to be similar to that reported previously for PTEN (phosphatase and tensin homolog) knockout and overexpression of VP16-KLF7 (Liu et al., 2010; Blackmore et al., 2012). AAV8-driven protein expression commences within a week of injection (Blackmore et al., 2012), so in the first CST regeneration experiment exogenous Sox11 protein was likely already present at the time of injury. Importantly, we also show that Sox11 promotes CST growth even when delivered 2 months postinjury, showing that it can act independently of intracellular and extracellular conditions that might be unique to the acute injury response.

By what molecular mechanism might Sox11 be acting to promote axon growth? Although Sox11 is known to affect cell survival during early embryogenesis (Thein et al., 2010), we detected almost no apoptosis in the cortex of spinally injured animals

treated with either EBFP control or Sox11 virus (Fig. 4; Nielson et al., 2011). It is therefore unlikely that cell survival effects can explain the enhanced sprouting in Sox11-treated animals. On the other hand, regeneration-associated genes, including *Sprr1a* (small proline-rich protein 1a), BDNF, and TANK (TRAF family member-associated nuclear factor- κ B activator), have been identified as direct transcriptional targets of Sox11 in regenerating DRG neurons (Jing et al., 2012; Salerno et al., 2012, 2013). These data favor the possibility that Sox11 may similarly promote axon growth in CST neurons by activating pro-regenerative genes. Identifying genes that act downstream of Sox11 to promote CST axon growth represents an important direction for future research.

A striking finding of this work is that the overexpression of Sox11 promotes CST axon growth while also reducing forelimb dexterity. CST axons are known to participate in postinjury plasticity in the spinal cord by elongating collateral branches, engaging interneurons and propriospinal neurons, and ultimately participating in novel circuitry (Bareyre et al., 2004; Carmel et al., 2010; Hunanyan et al., 2013). This innate CST plasticity appears to be behaviorally beneficial, as subsequent lesioning of CST axons abrogates endogenous recovery (Bareyre et al., 2004). In other cases, however, plasticity in the CNS can be maladaptive. For instance, endogenous remodeling of local spinal circuitry after spinal injury contributes to neuronal dysfunction, and species that are capable of axon regeneration in the optic system can remain functionally blind due to errors in topographic targeting (Beazley et al., 2003; Beauparlant et al., 2013). In one intriguing example of dysfunctional regeneration, axon growth across a cellular bridge in a transected spinal cord led to suppressed locomotor activity that was improved by retranssection of the bridge (Takeoka et al., 2011).

These examples illustrate a growing awareness that although enhancing the regrowth or sprouting of axons after injury remains a major goal, the potential exists for both endogenous and treatment-induced growth to be maladaptive, perhaps due to errors in targeting. For instance, appropriate targeting of regenerated fibers is emerging as an important challenge in the optic system (Luo et al., 2013; Pernet and Schwab, 2014). Interestingly, previous interventions (Carmel et al., 2010; Wang et al., 2011) to augment CST sprouting after injury such as cortical stimulation or combined rehabilitative training and degradation of growth-inhibiting chondroitin sulfate proteoglycans, have reported positive correlations between increased CST sprouting and behavioral recovery. It remains unclear why Sox11-treated animals showed reduced dexterity, but one speculative possibility is that Sox11-stimulated CST axons may form or maintain erroneous connections in the absence of activity-dependent mechanisms to sculpt connectivity. Regardless, these findings highlight the potential of a new gene-based manipulation to augment CST growth after injury, while also highlighting the need for additional strategies to optimize not only the growth but the function of regenerated axons.

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