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A novel and robust conditioning lesion induced by ethidium bromide

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ABSTRACT

Molecular and cellular mechanisms underlying the peripheral conditioning lesion remain unsolved. We show here that injection of a chemical demyelinating agent, ethidium bromide, into the sciatic nerve induces a similar set of regeneration-associated genes and promotes a 2.7-fold greater extent of sensory axon regeneration in the spinal cord than sciatic nerve crush. We found that more severe peripheral demyelination correlates with more severe functional and electrophysiological deficits, but more robust central regeneration. Ethidium bromide injection does not activate macrophages at the demyelinated sciatic nerve site, as observed after nerve crush, but briefly activates macrophages in the dorsal root ganglion. This study provides a new method for investigating the underlying mechanisms of the conditioning response and suggests that loss of the peripheral myelin may be a major signal to change the intrinsic growth state of adult sensory neurons and promote regeneration.

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Introduction

Peripheral conditioning lesion refers to the phenomenon that an initial peripheral nerve injury induces an intrinsic growth state in dorsal root ganglia (DRG) sensory neurons. This growth state can increase the rate of regeneration after a subsequent peripheral injury (McQuarrie and Grafstein, 1973), and, incredibly, drive regeneration of the normally quiescent central branch within the central nervous system (CNS) (Richardson and Issa, 1984). The expression of many genes undergo changes caused by peripheral nerve injury, some of which may be responsible for the acquired capacity for regeneration (Hoffman and Cleveland, 1988; Tsujino et al., 2000; Costigan et al., 2002; Seijffers et al., 2007; Stam et al., 2007). While some identified cues can increase regeneration, none have been shown to fully mimic the growth-promoting effects of the nerve crush (Qiu et al., 2002; Seijffers et al., 2007; Parikh et al., 2011).

Sciatic nerve crush leads to axon damage and loss of myelin (Gupta et al., 2004). Peripheral demyelination results in clearance of myelin debris by macrophages, Schwann cell dedifferentiation and proliferation, followed in time by eventual remyelination of spared or regenerated peripheral axons (Hall, 1973; Riet-Correa et al., 2002). After chemical demyelination, peripheral axons sprout small branches that associate with proliferating Schwann cells (Hall, 1973). We propose that this sprouting is a correlate of axon regeneration and asked whether peripheral injection of demyelinating agents results in regeneration after spinal cord injury. We hypothesize that demyelination may be a major component of the conditioning lesion effect that drives the increase of intrinsic growth potential. We tested this hypothesis utilizing two distinct demyelinating agents, the intercalating agent ethidium bromide (EtBr) and the detergent lysolecithin (lysophosphatidylcholine, LPC) that both result in the breakdown of the myelin sheath through progressive vesiculation (Allt et al., 1988; Riet-Correa et al., 2002). We show here that peripheral injection of EtBr produces a much greater conditioning response than nerve crush, resulting in dramatically increased spinal cord regeneration. Additionally, injection of EtBr or LPC, in the absence of concurrent axotomy, induces gene changes in the DRG characteristic of peripheral conditioning. Unlike nerve crush, EtBr injection does not induce macrophage activity in the demyelinated region of the sciatic nerve over the period of observed peripheral conditioning. Nor does it induce a sustained macrophage activation in the DRG compared to nerve crush. This suggests that either inflammation is an early component of peripheral conditioning lesion, or a divergence in the mechanisms of EtBr-mediated and nerve crush-mediated peripheral conditioning.

Materials and methods

Surgical procedures

Sciatic nerve crush

All animal work in this research was approved by the University of California, San Diego Institutional Animal Care and Use Committee.

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Unilateral or bilateral sciatic nerve crush was performed on adult female Fischer 344 rats (120–135 g) unresponsive to toe or tail pinch under isoflurane anesthetic. An area over the hindlimb was shaved and cleaned with povidone–iodine before incision caudal and parallel to the femur. The sciatic nerve was exposed and crushed for 10 s with a pair of fine (#55) forceps. After crush, the skin was closed with surgical staples.

Sciatic nerve injection

Unilateral or bilateral sciatic nerve injection was performed on adult female Fischer 344 rats (120–135 g) unresponsive to toe or tail pinch under isoflurane anesthetic. The sciatic nerve was exposed as above and injected with 4 μl (2 μl/branch) 1% wt/vol LPC in PBS with 10% vol/vol DMSO, 2 μl (1 μl/branch) of 0.01%, 0.05% or 0.1% wt/vol EtBr in PBS or PBS alone. Injections were made longitudinally towards the DRG, with a 36 ga NanoFil needle (World Precision Instruments Inc., Sarasota, FL) at a rate of 2 μl/min and the needle was held in place for an additional 10 s following injection. After injection, the skin was closed with surgical staples.

C4 dorsal column injury

Animals were deeply anesthetized with 2 ml/kg of ketamine cocktail (25 mg/ml ketamine, 1.3 mg/ml xylazine and 0.25 mg/ml acepromazine). Spinal level C4 was exposed by laminectomy and the dura was punctured over the dorsal horn, approximately 1.2 mm lateral to midline. A Scouten wire-knife (David Kopf Instruments, Tujunga, CA) was lowered to a depth of 1 mm from the surface of the spinal cord and extruded. The dorsal columns were lesioned with two passes of the wire-knife and the intact dura was pressed against the wire edge of the lifted wire-knife to ensure complete transection of ascending dorsal column sensory axons. The lesion cavity was filled with approximately 200,000 syngeneic bone marrow stromal cells (BMSCs) suspended in PBS [100,000 cells/μl], injected using glass micropipettes connected to a picospritzer (General Valve, Fairfield, NJ) as previously described (Hollis and Zou, 2012). Primary BMSCs were isolated from adult female Fischer 344 rats by flushing cells out with DMEM with 10% FBS and Pen/Strep/Glu, cells were cultured in the same media and passaged at 80% confluence. The dorsal musculature was sutured with 4–0 silk sutures and the skin was closed with surgical staples. Three days prior to sacrifice, animals were injected bilaterally into the sciatic nerve with a 1% wt/vol solution of the transganglionic tracer cholera toxin B (CTB; List Biological Laboratories, Campbell, CA) in dH$_2$O using a 36 ga NanoFil syringe. The sciatic nerve was exposed as described above and 1 μl was injected into each of the tibial and common peroneal branches bilaterally (4 μl total).

Sacrifice and tissue processing

Animals were deeply anesthetized with ketamine cocktail and transcardially perfused with ice-cold PBS followed by 4% wt/vol paraformaldehyde in PBS. Spinal columns, sciatic nerves, DRGs and *tibialis anterior* muscles were post-fixed overnight at 4 °C in 4% wt/vol paraformaldehyde. Tissue was transferred to 30% wt/vol sucrose in PBS for cryoprotection and sectioned on a cryostat (Leica, Buffalo Grove, IL) at 20 μm (DRGs, sciatic nerves) or 30 μm (muscle) and mounted directly on Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA). Grafted spinal cords were sectioned sagittally at 40 μm thick and collected as free-floating sections. Sections were washed three times with PBS, blocked for one hour at room temperature (RT) in PBS with 0.25% triton-X100 (PBST) and 5% donkey serum, then incubated overnight (except where noted below) at 4 °C with primary antibodies in PBST plus 5% donkey serum. On the second day of staining, sections were washed three times, incubated with Alexa Fluor conjugated secondary antibodies (Life Technologies, Grand Island, NY; Jackson ImmunoResearch, West Grove, PA) for 2.5 h at RT, counterstained with DAPI [1 g/ml] (Sigma-Aldrich, St. Louis, MO) and washed three

final times in PBS. For Frizzled2 immunohistochemistry, antigen retrieval with ice-cold acetone for 10 min prior to staining was utilized. Antibodies used for fluorescent immunohistochemistry were: goat anti-CTB [1:10,000; 3-day incubation] (List Biological Laboratories, Cat# 703, RRID: AB_10013220), monoclonal N52 anti-NF200 [1:500] (Sigma-Aldrich, Cat# N0142, RRID: AB_477257), rat anti-Frizzled2 [1:50] (R&D Systems, Minneapolis, MN, Cat# MAB1307-050, RRID: AB_2109221), rabbit anti-GFAP [1:750] (Dako, Carpinteria, CA, Cat# Z0334, RRID: AB_10013382), rabbit anti-cJun [1:100] (Cat# 9165, RRID: AB_2130165) and rabbit anti-pSmad [1:100] (Cell Signaling, Danvers, MA, Cat# 9516S, RRID: AB_491015), rabbit anti-ATF3 [1:200] (Cat# sc-188, RRID: AB_2258513), goat anti-arginase I [1:200] (Cat# sc-18354, RRID: AB_2227469) and rabbit anti-synaptophysin [1:100] (Santa Cruz Biotechnology, Santa Cruz, CA, Cat# sc-9116, RRID: AB_2199007), rabbit anti-Iba1 [1:1500] (Wako Chemicals USA, Inc., Richmond, VA, Cat# 019-19741, RRID: AB_839504), monoclonal ED1 anti-CD68 [1:500] (AbD Serotec, Raleigh, NC, Cat# MCA341R, RRID: AB_2291300), monoclonal 24F anti-CD86 [1:100] (BD Biosciences, San Jose, CA, Cat# 555016, RRID: AB_395648), Isolectin B$_4$ biotin conjugated [1:100] (Sigma-Aldrich, Cat# L2140), tetramethylrhodamine-conjugated α-bungarotoxin [1:1500] (Life Technologies, Cat# T-1175).

Myelin staining

Myelin staining was performed as described (Schmued, 1990). Briefly, sections washed twice in dH$_2$O then twice in 0.025 M PB with 0.9% NaCl (working solution) were incubated for 1 h at RT in 0.2% wt/vol gold (III) chloride solution with 0.0075% vol/vol H$_2$O$_2$ in working solution. Sections were washed twice more in working solution, then incubated for 5 min in 5% sodium thiosulfate at room temperature before three final washes in dH$_2$O.

DRG neuron culture

Rats were deeply anesthetized with ketamine cocktail, L4 and L5 DRGs were surgically removed, and the animals were decapitated. DRGs were collected in ice-cold L15 medium then digested for 1 h at 37 °C in collagenase type XI (1:1, DMEM/F-12:0.5% collagenase XI in L15 medium; Worthington Biochemical Corp., Lakewood, NJ) with gentle agitation every 15 min. Cells were centrifuged 2 min at 3000 rpm, collagenase solution was removed and gently replaced with DMEM/F-12 + 10% FBS so as not to disturb the cell pellet. Cells were then washed twice by gently applying DRG culture medium (DMEM/F-12 with B-27 supplement and Pen/Strep/Glu) so as not to disturb the pellet. Cells were resuspended in DRG culture medium, allowed to settle for 45 s then the top cell suspension was added to DRG culture medium, leaving behind the sedimented tissue fragments. DRG cell suspension was then plated on poly-D-lysine coated cell culture dishes. 16 h after plating, cells were fixed with 4% PFA in PBS for 30 min at RT, washed 3 times in PBS and stained with monoclonal N52 anti-NF200 as described above for free-floating sections.

Image acquisition and analysis

Images were acquired on an inverted Zeiss LSM510 confocal microscope with LSM acquisition software (Carl Zeiss Microscopy, LLC, Thornwood, NY). Ethidium bromide fluorescence emission at 590 nm was imaged on a Zeiss LSM510. An Axiovert 40 CFL with an AxioCam MRm and AxioVision software (Carl Zeiss Microscopy, LLC) was used to image gold (III) chloride myelin staining of sciatic nerves. For neurite length quantification images of NF200-immunoreactive DRG neurons were acquired with PictureFrame software for a MicroFire digital camera (Optronics, Goleta, CA) mounted on an upright fluorescent microscope (Olympus, Center Valley, PA). The NeuronJ plugin for ImageJ (NIH, Bethesda, MD) was used for longest neurite tracing and quantification on acquired images (Meijering et al., 2004). Image density

quantification was done on thresholded images using ImageJ (NIH, Bethesda, MD). ImageJ was used to measure myelin density along a 100 μm thick tracing of thresholded panoramic images stitched together in Photoshop CS5 (Adobe, San Jose, CA). NF200 ratios were calculated by comparing ImageJ density measurements of thresholded and skeletonized (linearized) NF200 images 1.5 mm distal and proximal to the sciatic injury site. For neuromuscular junction (NMJ) innervation, 100 tetramethylrhodamine-conjugated α-bungarotoxin labeled NMJs per animal were examined, in *tibialis anterior* muscles, for apposition by synaptophysin-labeled pre-synaptic terminals. Statistical tests indicated in main text were performed using JMP 9 software (SAS Institute, Cary, NC). An investigator blinded to the experimental group performed all analyses.

Behavioral testing

Animals were trained on accelerating rotarod (5 rpm initial, 45 rpm max, 300 s max; Stoelting, Wood Dale, IL) and beam crossing (1 in. diameter, 60 in. span, 3 passages per trial) for two weeks prior to bilateral sciatic injection of 0.1% wt/vol EtBr, 1% wt/vol LPC, or sciatic nerve crush. For toe spread analysis, hind paw plantar surfaces were dipped in povidone-iodine solution and animals walked on paper in a narrow path between two barriers. The mean distance between the first and fifth toes from three prints was averaged bilaterally. Animals were tested prior to sciatic injury and weekly for 16 weeks after spinal cord injury by investigators blind to the experimental groups.

Paw 50% withdrawal threshold (PWT) was assessed bilaterally using a series of calibrated von Frey filaments (Kom Kare, Middletown, OH, USA) using the up–down method exactly as described elsewhere (Chaplan et al., 1994; Calcutt, 2004). Thermal withdrawal latency was assessed bilaterally using a Hargreaves apparatus (UARDG, Department of Anesthesiology, University of California, San Diego; La Jolla, CA). Prior to testing, rats were acclimated in individual acrylic chambers on a glass surface that was maintained at 30 °C. After an initial acclimation measurement, the median of three subsequent measurements tested at a heating rate of 1 °C/s with a maximal cut off at 20 s was taken as the thermal response latency for that paw, and the mean of values of both paws was used to represent each animal.

Electrophysiology

For measurement of motor nerve conduction velocity (MNCV), rats were anesthetized with isoflurane and nerve temperature maintained at 37 °C using a heating lamp and near-nerve thermistor. The sciatic nerve was stimulated (single 5 V, 0.05 ms square wave pulse) by fine needle electrodes placed at the sciatic notch and Achilles tendon and the evoked electromyograms (EMGs) recorded from the interosseus muscles of the ipsilateral foot via two fine needle electrodes. MNCV was calculated by dividing distance between the two stimulation sites by the time difference between their associated M wave peaks.

Results

Peripheral injection of the chemical demyelinating agent ethidium bromide promotes more robust axon regeneration than nerve crush

In this study, we tested the peripheral conditioning effects of two known demyelinating agents that do not result in peripheral axon degeneration (Hall, 1973; Riet-Correa et al., 2002): ethidium bromide (EtBr), and lysophosphatidylcholine (lysolecithin, LPC). Animals received a sham operation, bilateral sciatic nerve crush, bilateral PBS injection, bilateral LPC, or bilateral EtBr injection in the sciatic nerve. One week later, we lesioned the dorsal columns at cervical level 4 (C4) and grafted naïve bone marrow stromal cells (BMSCs). One month later, bilateral sciatic injection of cholera toxin B subunit (CTB)

was used to assess regeneration into BMSC grafts. BMSC grafts fill the lesion cavity, preventing cystic cavitation, and allow for clear determination of regenerated axons as those that have grown into the graft. Peripheral nerve crush resulted in a nearly 3.7-fold increase in the number of regenerated axon profiles (axon segments counted in every seventh sagittal section and normalized to total CTB axons below the injury) within BMSC grafts compared to sham operated animals (Fig. 1). Demyelination with EtBr augmented axon regeneration much more robustly, with a 7.1-fold increase over PBS injected and 9.9-fold increase over sham operated animals (Fig. 1). The regenerative response induced by EtBr was 2.7-fold greater than the response to sciatic nerve crush (ANOVA P < 0.0001, Bonferroni corrected post-hoc t-test P < 0.0001). The number of regenerated axon profiles in LPC injected animals was not significantly higher than PBS injected or sham controls.

One, 7 and 28 days after sciatic nerves were treated with EtBr, L4 and L5 DRG neurons were dissociated and grown in vitro. Large-diameter, NF200-immunoreactive neurons extended significantly longer neurites when isolated from DRGs ipsilateral to demyelinated sciatic nerve, compared to contralateral crushed sciatic nerve (Fig. 1; paired t-test, P < 0.05).

The extent of demyelination differs between peripheral injection of LPC or EtBr and nerve crush

Injection of the demyelinating agents LPC [1% wt/vol] or EtBr [0.1% wt/vol] longitudinally along the sciatic nerve reduced myelin staining with gold (III) chloride as did nerve crush (Fig. 2). The most severe demyelination occurred proximal to injection of 0.1% EtBr and at the site of nerve crush, however EtBr or LPC disrupted sciatic myelin over significantly longer distances (3.52 ± 0.83 mm and 3.41 ± 0.81 mm, respectively) than nerve crush (1.52 ± 0.06 mm), at 3 days post-injury (ANOVA P < 0.001, Bonferroni corrected post-hoc t-test P < 0.05). Importantly, nerve crush, and not demyelination, resulted in axotomy marked by the loss of axons 1.5 mm distal to the lesion at 3 days (Fig. 3). Demyelination continued to progress after EtBr injection with extensive demyelination apparent at two weeks after injection, a time at which LPC injected animals were showing some recovery of myelin (Fig. 2).

We examined the integrity of the sensory and motor circuitry of the sciatic nerve after nerve crush or demyelinating agent injection. Images of large-diameter, heavy-chain neurofilament (NF200) immunoreactive axons in the sciatic nerve proximal to the injury or demyelinated region and 1.5 mm distal to the crush or injection site were skeletonized (linearized) and the ratio of distal to proximal pixel density showed a dramatic reduction in axons 3 days after nerve crush (Fig. 3). Additionally, nerve crush resulted in a total loss of innervation of the *tibialis anterior* at one week after injury, while LPC and EtBr injected animals showed pre-synaptic terminals at every neuromuscular junction (NMJ) examined (Fig. 3).

Peripheral injection of chemical demyelinating agents induces a similar set of regeneration-associated genes (RAGs) as nerve crush

Peripheral nerve crush and transection result in the induction of many RAGs in sensory neurons, with two of the most robust being ATF-3 and c-Jun (Jenkins and Hunt, 1991; Tsujino et al., 2000; Boeshore et al., 2004; Seijffers et al., 2007; Stam et al., 2007). Additionally, chemical demyelination with LPC induces expression of the RAG ATF-3 in DRG neurons in vitro (Kiryu-Seo et al., 2010). We examined whether RAG induction after injection of peripheral demyelinating agents was similar to RAG induction after nerve crush in vivo. ATF-3 and c-Jun were dramatically induced in DRG neurons three days after unilateral sciatic injection of EtBr (0.05% or 0.1% wt/vol) in adult rats, similar to sciatic nerve crush (Fig. 4). A lower concentration of EtBr [0.01% wt/vol] or 1% wt/vol LPC induced expression of c-Jun and ATF-3
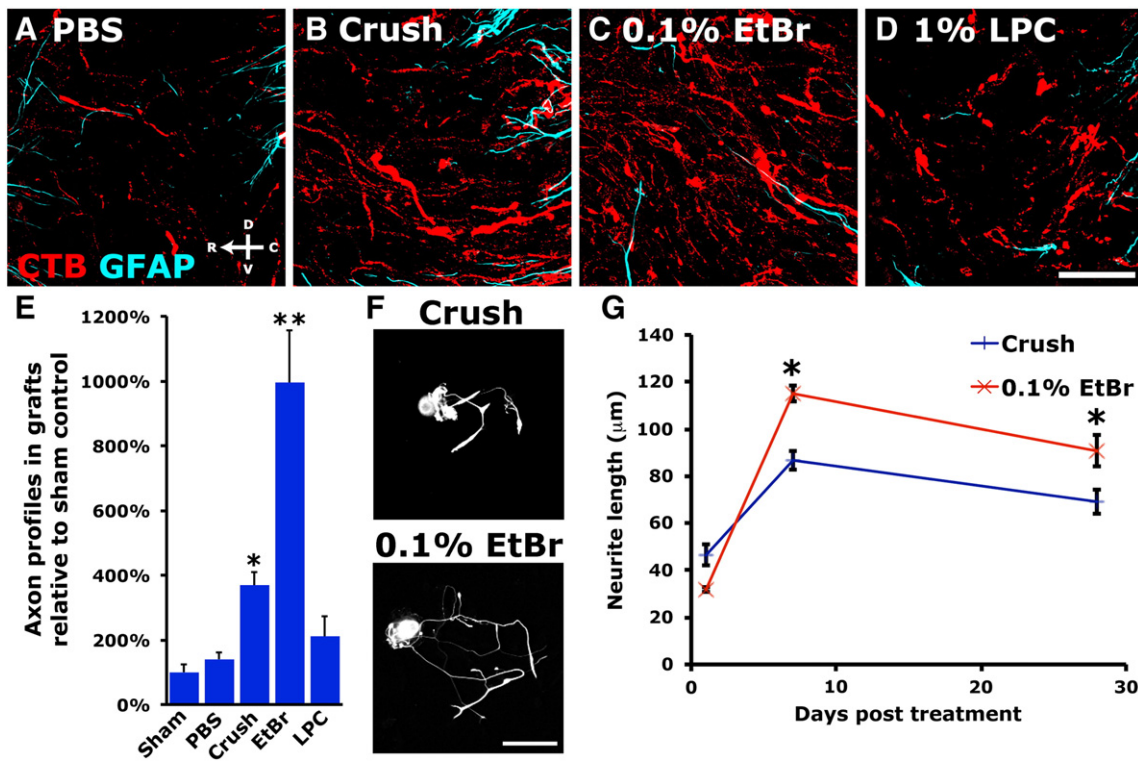
Fig. 1. EtBr injection promotes robust regeneration after spinal cord injury. Regeneration of CTB-labeled DRG axons into BMSC grafts one month after C4 lesion. Control, sham operated animals or those injected in the sciatic nerve with PBS (A) show little regeneration of ascending dorsal column sensory axons. The significant regeneration observed after peripheral nerve crush (B) is dwarfed by the regeneration induced by 0.1% wt/vol EtBr injection (C). (D) Injection of 1% wt/vol LPC has a more limited effect than EtBr on sensory axon regeneration. (E) Quantification of CTB-labeled regenerated axon profiles shows a 7.1-fold increase in regeneration of EtBr treated neurons over PBS controls, 2.7-fold more regenerated axons than observed after peripheral nerve crush (ANOVA $P < 0.0001$, Bonferroni corrected post-hoc t-test *$P < 0.05$, **$P < 0.0001$; n = 5 (Sham, LPC), 6 (PBS, EtBr, Crush)). (F, G) 0.1% wt/vol EtBr sciatic injection promotes greater neurite outgrowth than contralateral sciatic nerve crush when NF200$^+$ L4 and L5 DRG neurons are dissociated and cultured at 7 or 28 days after injury (paired two-tailed t-test *$P < 0.05$; n = 3 animals/time point, >100 neurons per data point). Data presented as mean ± s.e.m. Scale bars represent 50 μm (A–D) and 100 μm (F).

at lower levels than nerve crush (Fig. 4). ATF-3 and c-Jun were induced in NF200-immunoreactive proprioceptive neurons, the axons of which constitute the ascending dorsal funiculi. c-Jun and ATF-3 expression persisted over two weeks in NF200-immunoreactive neurons in animals after 0.1% EtBr and nerve crush at levels significantly higher than those induced by LPC (Fig. 4). Across all treatment groups and time points measured, the levels of c-Jun and ATF-3 induction in NF200-immunoreactive DRG neurons correlated tightly with the extent of remaining myelin (Spearman's $\rho = -0.730$ (ATF-3 vs. average myelin density) $P < 0.0001$, Spearman's $\rho = -0.571$ (c-Jun vs. average myelin density) $P < 0.0001$, Fig. 4). Specifically, in animals injected with different concentrations of EtBr (examined at day 3), the extent of remaining myelin correlated tightly with the induction of c-Jun and ATF-3 (Spearman's $\rho = -0.741$ (ATF-3 vs. average myelin density) $P < 0.01$, Spearman's $\rho = -0.641$ (c-Jun vs. average myelin density) $P < 0.05$, Fig. S1).

In addition to ATF-3 and c-Jun, injection of peripheral demyelinating agents induced expression of arginase-1, and components of the morphogen signaling pathways of bone morphogenic protein and Wnt in the DRG, similar to peripheral nerve crush (Fig. 4) (Boeshore et al., 2004; Zou et al., 2009; Hollis and Zou, 2012). We found that the highest concentration of EtBr tested, 0.1% wt/vol in PBS, most closely mimicked sciatic nerve crush in terms of RAG gene expression. It should be noted that EtBr and LPC at these concentrations induce Schwann cell myelin rejection but not Schwann cell death (Hall and Gregson, 1971; Allt et al., 1988; Riet-Correa et al., 2002). EtBr was not detected in axons or DRGs, by confocal microscopy, and is unable to enter the nuclei of living cells (Beletsky and Umansky, 1990). Control injections of PBS affected neither gene expression in the DRG, nor myelin density in the sciatic nerve, similar to sham operated controls.

EtBr induces macrophage activity on a different time scale than nerve crush

Infiltrating non-resident macrophages clear myelin debris in damaged peripheral nerves (Hall and Gregson, 1971; Griffin et al., 1993; Brück, 1997). Additionally, inflammatory agents induce a form of peripheral conditioning (Lu and Richardson, 1991; Steinmetz et al., 2005) while macrophages pre-incubated with injured sciatic nerve have been shown to promote regenerative growth (Lazarov-Spiegler et al., 1996). Within the DRG, Iba1-immunoreactive macrophages are activated following sensory axon injury, surrounding large-diameter NF200 immunoreactive neurons (Ton et al., 2013). Recently, it has been proposed that activation or increase of DRG macrophages is required for conditioning-mediated pro-regenerative effects (Kwon et al., 2013; Niemi et al., 2013).

We tested whether the elevation of RAGs induced by EtBr or LPC injection is correlated with activation of resident or infiltrating macrophages. Following nerve crush, the density of Iba1 immunoreactive macrophages peaked at one week post-crush in both the DRG and the injured sciatic nerve, while ED1 reactivity of phagocytic CD68$^+$ macrophages (Damoiseaux et al., 1994) in the injured sciatic nerve decreased from 3 to 14 days post-crush (Fig. 5). In contrast, activation of Iba1 macrophages in the DRG was highest at 3 days post-injection of EtBr before dropping precipitously to control levels by 7 days (Fig. 5). Additionally, despite clear differences in RAG induction, nerve crush, control PBS, or LPC injection mildly increased macrophage staining at 3 days, though not significantly compared to sham control (Dunnett's test: crush $P = 0.78$, PBS $P = 0.31$, LPC $P = 0.15$; Fig. 5). High levels of DRG macrophage activation were present in both EtBr and nerve crushed animals, however macrophage activation after nerve crush peaked at 7 days, later than the 3 days after EtBr injection.
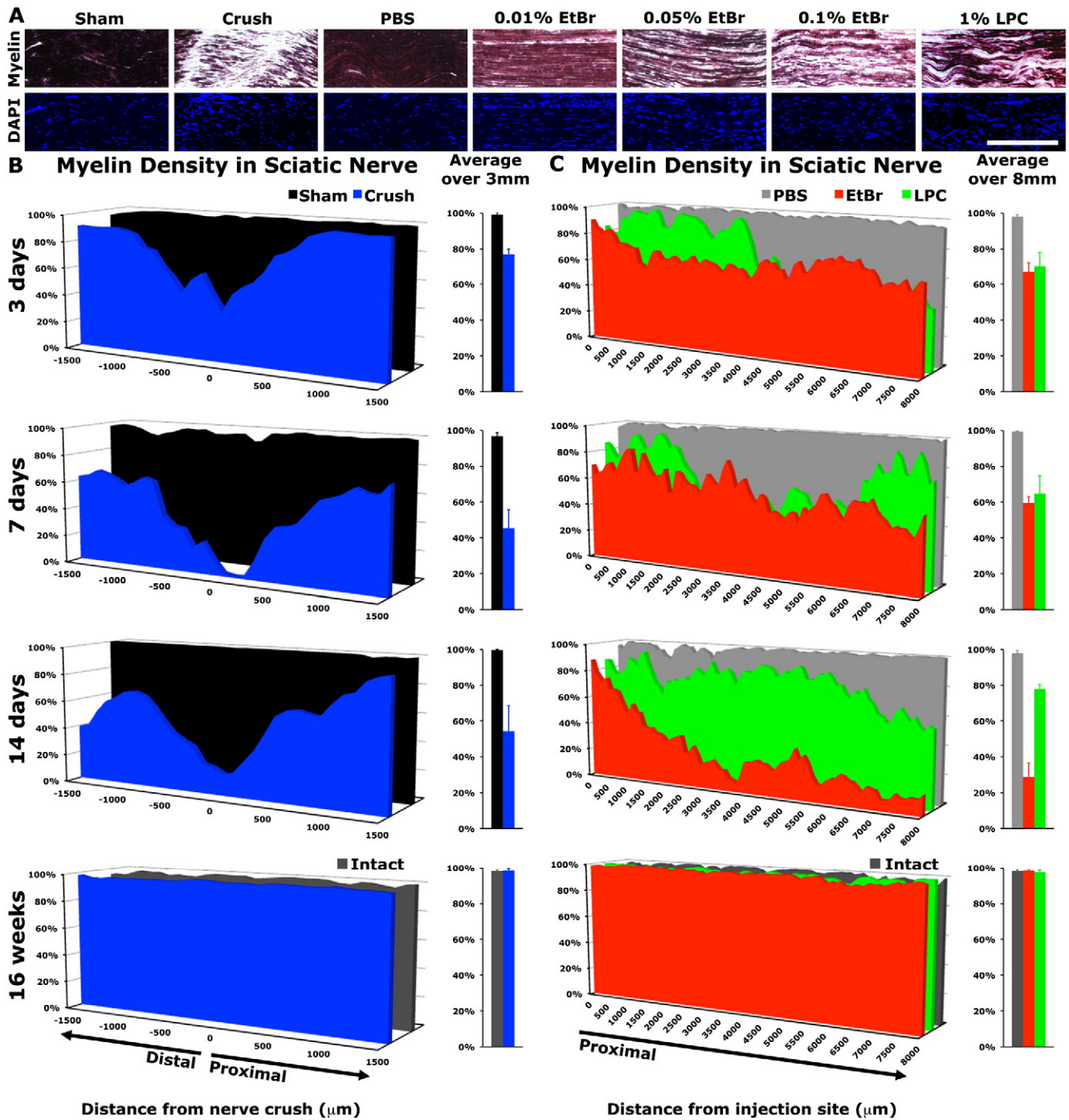
Fig. 2. Demyelination within the sciatic nerve. (A) Gold (III) chloride staining of longitudinal sciatic nerve sections demonstrates loss of myelin staining three days after nerve crush or injection of demyelinating agents (dark purple indicates myelin staining) (B) At the lesion epicenter, myelin density is lowest at the site of sciatic nerve crush, with considerable demyelination at 14 days distal to the injury, along the region of Wallerian degeneration (two-tailed t-test: 3 days P < 0.005, 7 days P < 0.001, 14 days P < 0.05). (C) EtBr [0.1% wt/vol] and LPC [1% wt/vol] injected to the sciatic nerve resulted in demyelination over several millimeters proximal to the injection site (ANOVA: 3, 7 days P < 0.05, 14 days P < 0.0001). Myelin density recovered in all groups by 16 weeks post injury. Data presented as mean (n = 3/group). Scale bar represents 250 μm.

In the center of the demyelinated sciatic nerve, the density of phagocytic ED1 stained macrophages was elevated over the first 14 days after LPC injection, but was noticeably lower after EtBr injection (Fig. 5). Only limited ED1 staining was present after EtBr injection, and only at 3 days post-injection (Dunnett's P = 0.22). As expected there was observable inflammation along the needle tracts at the site of injection and epineuria disruption where PBS, EtBr and LPC resulted in similarly elevated levels of phagocytic macrophage infiltration shortly after injection (Fig. 5). Crushed sciatic nerves showed robust elevation in glial fibrillary

acid protein (GFAP) positive reactive Schwann cells, and DAPI stained nuclei by 3 days post injury (ANOVA P < 0.0001 DAPI, P < 0.05 GFAP, Bonferroni corrected post-hoc t-tests, Fig. S2). Chemical demyelination resulted in the elevation of GFAP and DAPI staining over a much longer time course, with elevation through 14 days post-injection, by which time EtBr-damaged myelin had been cleared from the sciatic nerve (Fig. S2). While only limited phagocytic macrophages stained with ED1 were detected during the clearance of EtBr-damaged myelin, Schwann cells may be able to drive the early clearance of myelin after
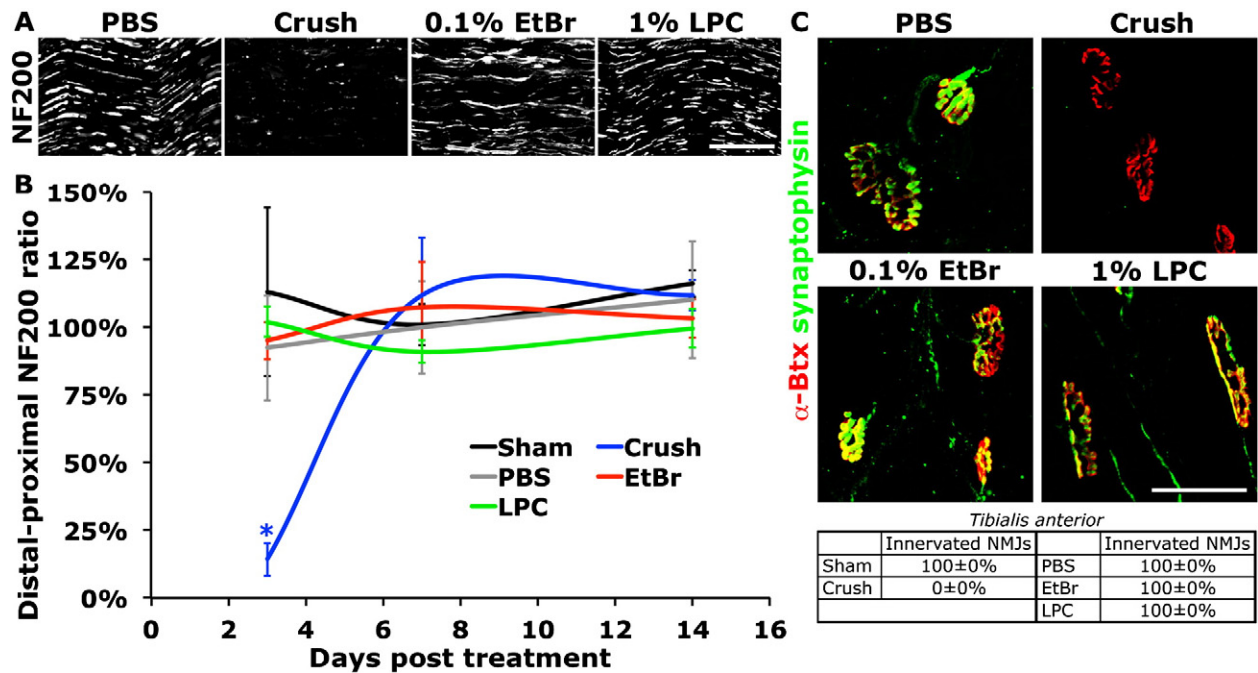
Fig. 3. Only nerve crush interrupted innervation distal to sciatic injury. (A) NF200 immunostaining at day 3 demonstrates the disruption of sciatic axons 1.5 mm distal to the nerve crush or injection site. (B) The distal:proximal ratio of skeletonized NF200 immunoreactive axons illustrates that axons distal to the injury remained intact following demyelination (ANOVA P < 0.05, Dunnett's t-test *P < 0.05). (C) All neuromuscular junctions (stained with tetramethylrhodamine-conjugated α-bungarotoxin, α-Btx) in *tibialis anterior* lacked pre-synaptic synaptophysin apposition at 7 days after sciatic nerve crush, but not after demyelination. Data presented as mean ± s.e.m. Scale bars represent 100 μm (A) and 50 μm (C).

peripheral injury in the absence of infiltrating macrophages (Perry et al., 1995). The alternatively-activated M2 macrophage marker arginase-1 was found to be substantially elevated only in crushed sciatic nerves (Fig. S2) (Kigerl et al., 2009).

Within the injured sciatic nerve, we observed no correlation between activation of macrophages, or GFAP induction by reactive Schwann cells and the expression of RAGs (Figs. 5, S2). In the DRG, both nerve crush and EtBr activated Iba1-immunoreactive macrophages robustly, but on disparate time courses (Fig. 5).

EtBr causes more severe functional deficits than LPC or nerve crush

In the study of regeneration, the recovery of behavioral function is a key outcome measure after injury. After LPC injection, it has been shown that remyelination and recovery of electrophysiological function is completed between 2 weeks and 2 months post injection (Hall, 1973). Due to the remarkable increase in regeneration found following EtBr injection, we sought to determine the consequences of EtBr-induced demyelination on sciatic nerve function and whether it was significantly different from LPC-induced demyelination. Animals were trained to perform behavioral tasks for two weeks prior to bilateral sciatic injection with LPC or EtBr, or bilateral sciatic nerve crush. Recovery of sensory and motor function was then assessed weekly on an accelerating rotarod, beam crossing, and hindlimb toe spread tasks. Animals injected with EtBr exhibited the slowest recovery of function on the rotarod (repeated measures ANOVA P < 0.05) and beam crossing task (repeated measures ANOVA P < 0.005) and also failed to completely recover hindlimb toe spread (repeated measures ANOVA P < 0.0001; Fig. 6). Animals subject to bilateral nerve crush or LPC injection recovered to pre-lesion and age-matched control levels on rotarod, beam crossing, and hindlimb toe spread tasks. In addition to the recovery of positive behavioral function, we tested animals for adverse reactions characterized by mechanical allodynia and thermal hyperalgesia as peripheral nerve crush has been demonstrated to induce neuropathic pain-like states, characterized by mechanical allodynia and increased temperature sensitivity (Lancelotta et al., 2003; Vogelaar et al., 2004). Von Frey filaments were used to test mechanical sensation and thermal

sensitivity was tested using a Hargreaves apparatus. No treatment group exhibited more severe adverse reactions than any other (Fig. 6).

Nerve conduction velocity (NCV) was measured at five time points after sciatic injury, starting at two weeks. The nerve was stimulated at the sciatic notch and Achilles tendon and the evoked electromyogram recorded from the ipsilateral interosseous muscles. Animals injected with LPC exhibited full recovery of electrophysiological function within 2 to 8 weeks (Fig. 6). After nerve crush, recovery of NCV peaked between 8 and 12 weeks at significantly reduced levels relative to pre-injury measurements or age-matched controls. Consistent with observed behavioral deficits, EtBr injected animals exhibited a delayed and attenuated recovery of NCV (repeated measures P < 0.0001, EtBr vs. Crush P < 0.005; Fig. 6).

Discussion

We report here the novel finding that peripheral injection of the demyelinating agent EtBr induces a more robust axon regeneration response than peripheral nerve crush and provides a second method of peripheral conditioning to increase the intrinsic growth state of sensory neurons. Our key finding is that axotomy is not a required component of the peripheral conditioning effect and that injection of a chemical demyelinating agent alone can promote regeneration of the central branch in the spinal cord. This alternative peripheral conditioning model will provide a new tool for studying the mechanisms underlying the induction of regeneration by peripheral conditioning.

We also observed that the more severe treatments resulting in a longer period to recover function caused better regeneration of the central branch. While EtBr-injected animals were delayed in their peripheral functional recovery, both EtBr-injected and nerve crushed animals recovered to similar levels on most behavioral measures, with the only exception being the measure of total toe spread, a component of the sciatic functional index (de Medinaceli et al., 1982). The induction of mechanical allodynia by the injection of chemical demyelinating agents followed a similar time course as after sciatic nerve crush, peaking at 4 weeks, similar to previous work on nerve crush (Vogelaar et al., 2004).
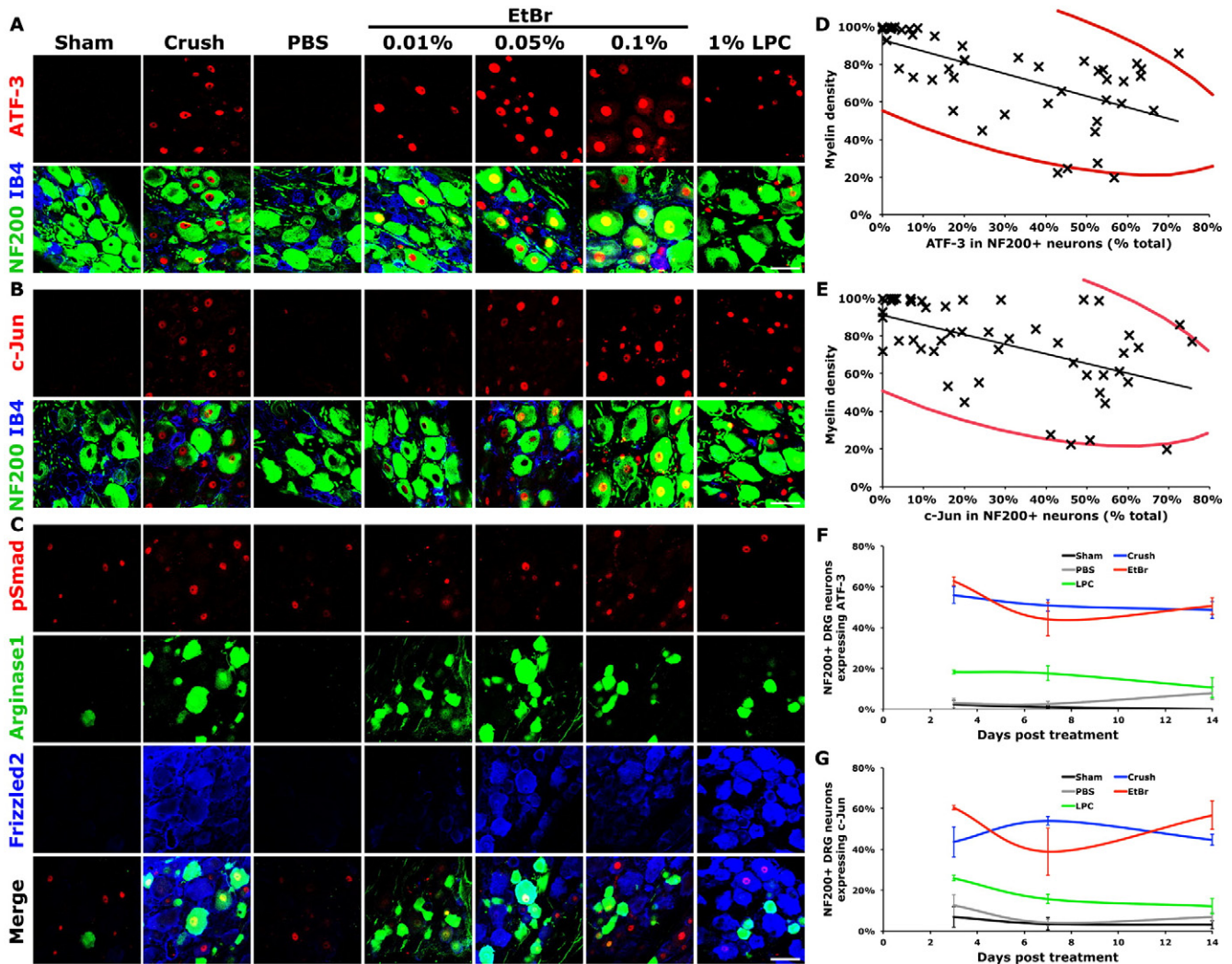
Fig. 4. Chemical demyelinating agents induced expression of regeneration-associated genes. At three days after treatment, numerous RAGs are induced by nerve crush or peripheral injection of either EtBr or LPC. EtBr (0.01%, 0.05% and 0.1% wt/vol in PBS) and 1% wt/vol LPC in PBS with 10% DMSO induce expression of (A) ATF-3, (B) c-Jun, (C) phosphorylated-Smad1/5, arginase-1 and Frizzled-2. (D, E) Decreased myelin density, independent of treatment or time post-injury, correlates strongly with the induction of (D) ATF-3 or (E) c-Jun in NF200$^+$ DRG neurons (ATF-4: Spearman's $\rho = -0.730$ P < 0.0001, c-Jun: Spearman's $\rho = -0.571$ P < 0.0001, red ellipses represent 95% bivariate normal density). (F) ATF-3 and (G) c-Jun expression in NF200$^+$ neurons after 0.1% wt/vol EtBr injection was similar to that after sciatic crush (ATF-3: ANOVA at 3, 7 and 14 days P < 0.0001, c-Jun: ANOVA at 3, 7 and 14 days P < 0.0005). Data presented as mean ± s.e.m. Scale bars represent 50 μm, n = 3/group.

After sciatic nerve crush, clearance of distal myelin has been proposed to be required for normal peripheral regeneration (Brown et al., 1994). This is based on observations in the slow Wallerian degeneration mouse (Wld^s), in which recruitment of hematogenous macrophages is limited, de-afferented axons remain, demyelination proceeds slowly, and peripheral conditioning by nerve crush is attenuated (Brown et al., 1994; Niemi et al., 2013). It has been shown that induced immunological demyelination distal to a crush site significantly enhances the rate of peripheral regeneration (Kosins et al., 2011). These studies are consistent with our finding that removal of myelin correlates with enhanced regeneration. In our studies, EtBr augmented the growth potential of large diameter DRG neurons over nerve-crushed neurons at individual time points (Fig. 1), not just over a longer period; consistent with the idea that peripheral myelin actively inhibits axon regeneration (Brown et al., 1994). Therefore, lack of peripheral myelin per se could be a major signal for a conditioning lesion effect. Post-mortem analysis of patients with the demyelinating disease multiple sclerosis has shown increased expression of another RAG, growth-associated protein-43 (GAP43) (Schirmer et al., 2012). This suggests that myelin loss in the central nervous system may also be a growth-promoting signal for axons in the central nervous system.

Following the primary insult to axons and myelin, inflammation occurs through the activation of resident microglia or macrophages and the infiltration of blood-derived macrophages. Macrophage infiltration of the CNS correlates with secondary degeneration of neurons and glia after injury, as depletion of peripheral macrophages results in fewer infiltrating macrophages and reduced secondary damage after spinal cord contusion injury (Popovich et al., 1999). Macrophage infiltration in the injured sciatic nerve that occurs along with Schwann cell proliferation has been proposed to promote regeneration (Hall, 1989). These pro-regenerative effects may be due in part to macrophage clearance of debris, but could potentially be through anti-inflammatory pathways similar to the alternatively-activated M2 macrophages found after CNS injury (Kigerl et al., 2009). In contrast to sciatic nerve crush, we did not observe a correlation between macrophage activity, alternative or inflammatory, in the sciatic nerve with the induction of peripheral conditioning through the injection of chemical demyelinating agents. Instead, we did observe strong macrophage activation in the DRG following both nerve crush and EtBr injection, indicating that macrophage activity proximal to the cell body may have a role in mediating the conditioning response to injury, consistent with previous studies (Kwon et al., 2013; Niemi et al., 2013). However, we found that EtBr induced
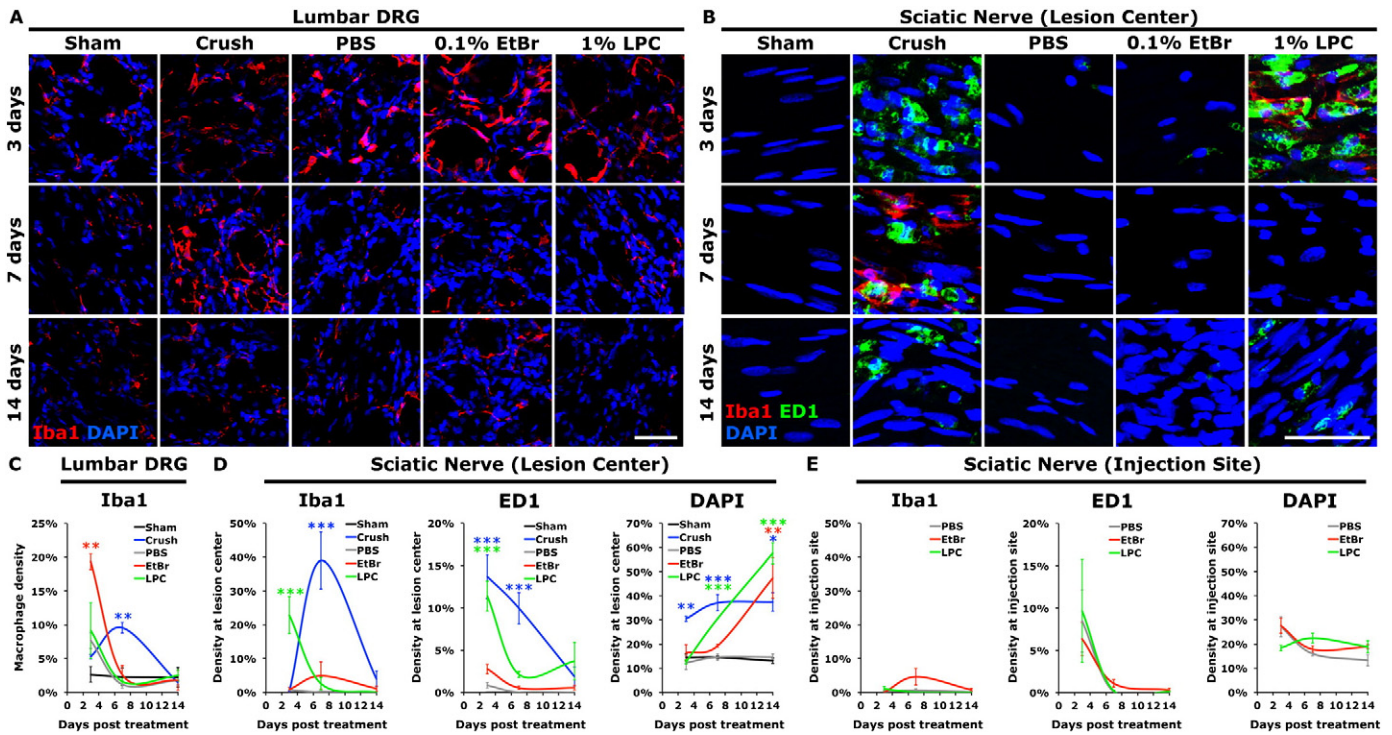
Fig. 5. The inflammatory response after demyelination differs from that after nerve crush. (A, C) Macrophage (Iba1) activation in DRGs was induced by nerve crush and by injection of EtBr into the sciatic nerve (ANOVA at 3 and 7d P < 0.005). (B) Longitudinal sections of sciatic nerve show CD68-immunoreactive (ED1) macrophages were present at the site of nerve crush and LPC-mediated demyelination, but not significantly elevated at the epicenter of EtBr intoxication. (D) Quantification of macrophage density at the lesion epicenter show that macrophage activity was limited in EtBr injected animals relative to animals after nerve crush or LPC injection (ANOVA: Iba1 at 3 and 7 days P < 0.0005; ED1 at 3 and 7 days P < 0.0001; DAPI 3 days P < 0.001, 7 and 14 days P < 0.0001). (E) Inflammation at the injection site was similar in LPC, EtBr and control PBS injected sciatic nerves. Data presented as mean ± s.e.m. Scale bar represents 50 μm. Dunnett's test *P < 0.05, **P < 0.005, ***P < 0.0005.

a robust macrophage response in the DRG at an earlier time point than nerve crush and that unlike in nerve transection experiments (Kwon et al., 2013), the macrophage response to EtBr and nerve crush was transient and reduced within a few days. The difference in the timing of the macrophage response to sciatic crush compared to transection may be relevant in light of the distinct differences in initiation and rate of regeneration after peripheral injury. Animals that undergo nerve crush injury show faster onset and rate of regeneration than animals after nerve transection (Gutmann et al., 1942; Forman et al., 1979). It is perhaps more likely then that macrophage activation is an early event in peripheral conditioning and not necessary for the maintenance of the conditioning response. Alternatively, nerve crush and EtBr could promote RAG expression through different mechanisms.

In addition to its role as a demyelinating agent, chronic EtBr exposure over multiple weeks in vitro can inhibit mitochondrial RNA synthesis and cell growth in some eukaryotic cells (von Wurmb-Schwark et al., 2006; Kao et al., 2012); however, it should be noted that it is unlikely that EtBr will have such chronic effects in our experiments. EtBr was cleared from Schwann cell myelin quickly (Fig. S2), completely undetectable by confocal microscopy by day 14, and we observed extensive proliferation or infiltration of cells in the sciatic nerve after demyelination with either LPC or EtBr (Fig. 5). This potential proliferation is consistent with previous reports of Schwann cell proliferation following experimental demyelination of the sciatic by LPC, EtBr or anti-galactocerebroside (Hall, 1973; Saida and Saida, 1986; Griffin et al., 1990; Riet-Correa et al., 2002).

We observed no evidence of sciatic axon disruption by injection of the chemical demyelinating agents LPC or EtBr, indicating that axotomy was not present in our novel model of peripheral conditioning. This is consistent with previous reports of treatment of the sciatic nerve as well as the caudal cerebellar peduncle that found no demonstrable induction of axonal damage by demyelinating agents (Hall and Gregson, 1971; Woodruff and Franklin, 1999; Riet-Correa et al., 2002). It is

possible that EtBr injection affected other aspects of axonal morphology and function not evident from analysis of neurofilament staining, despite evidence that LPC-mediated demyelination showed no evidence of morphological changes of sciatic axons by electron microscopy (Hall and Gregson, 1971). Functionally, however, chemical demyelination of sensory neuron cultures has demonstrated an increase in the rate of mitochondrial transport (Kiryu-Seo et al., 2010).

We hypothesize that the abnormal distribution, trafficking and function of mitochondria caused by demyelination is a candidate signal for triggering the conditioning growth state. 1) Both chemical demyelination (with LPC) and sciatic nerve transection increase mitochondrial transport in injured sensory neurons (Kiryu-Seo et al., 2010; Mar et al., 2014). Chemical demyelination, both in vitro and in vivo (with either LPC or EtBr, respectively), results in increased axonal mitochondrial content and mitochondrial hypertrophy (Kiryu-Seo et al., 2010; Zambonin et al., 2011), similar to mitochondrial hypertrophy seen after axotomy (Dimova and Markov, 1976), or to the hypertrophy observed in axons with impaired Node of Ranvier formation (Einheber et al., 2006). This may be due to a higher energy demand for nerve conduction in incompletely remyelinated or dysmyelinated axons (Liu et al., 2001). 2) Knocking down the RAG ATF-3 in sensory neuron cultures blocks chemical demyelination-induced increases in mitochondrial transport velocity and increases a marker of oxidative stress in the axon (Kiryu-Seo et al., 2010). 3) Activity of axonal mitochondria is required for local protein synthesis and branch formation (Spillane et al., 2013). Therefore, local sprouting of axon branches associated with supernumerary Schwann cells after chemical demyelination (Hall, 1973) may be indicative of the convergence on a mitochondrial response to stress pathways induced by either nerve crush or chemical demyelination.

In summary, we found that axotomy is not required for peripheral conditioning. Our results suggest that loss of myelin in the periphery can trigger a growth state of adult dorsal root ganglion axons in the
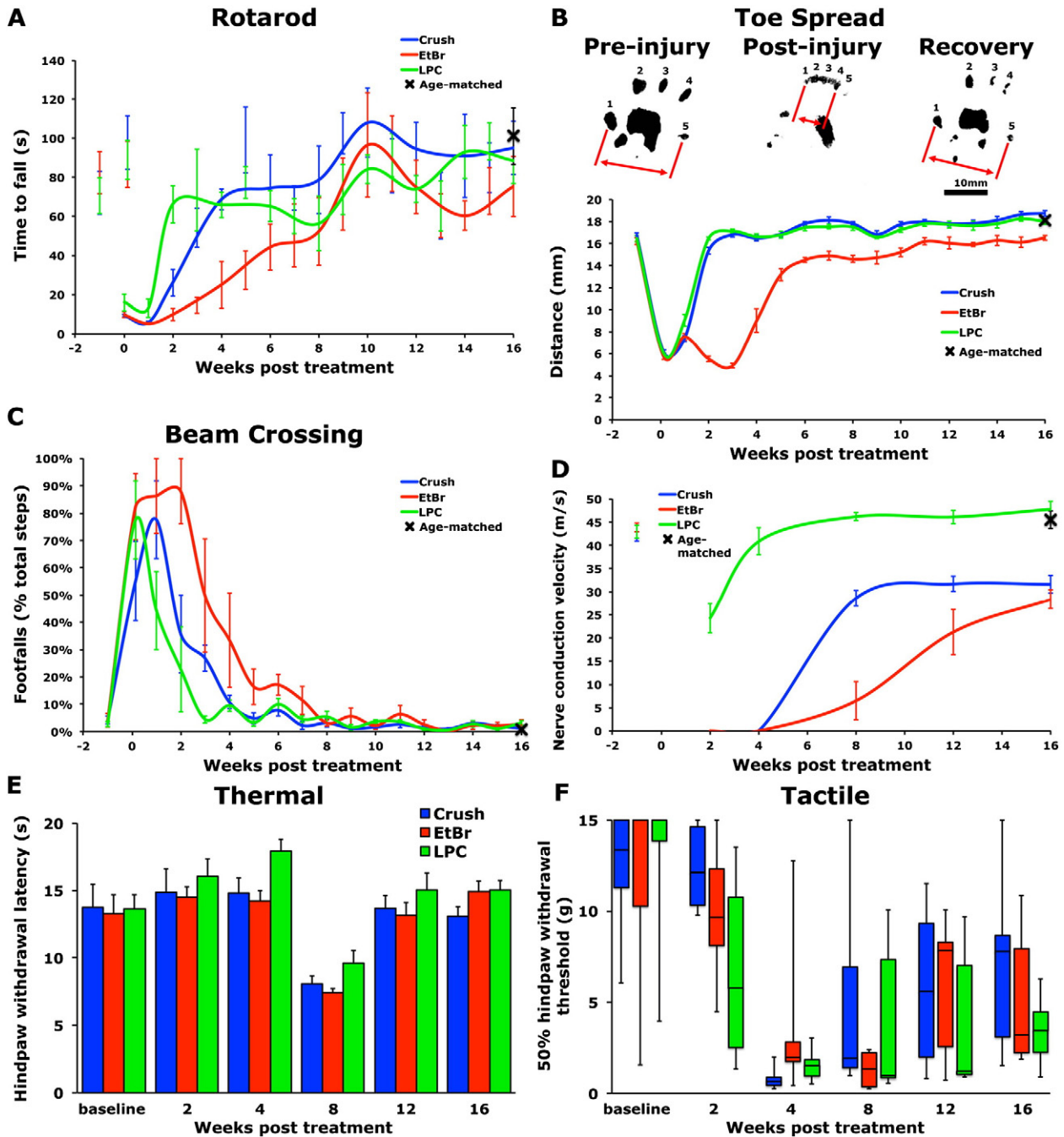
Fig. 6. EtBr injection dramatically impairs recovery of behavioral performance. 0.1% wt/vol EtBr treated animals exhibit slower recovery on (A) accelerating rotarod (repeated measures ANOVA P < 0.05) and (C) beam crossing (repeated measures ANOVA P < 0.005). (B) EtBr treatment resulted in reduced recovery of hindpaw toe spread (repeated measures ANOVA P < 0.0001). (D) EtBr treatment and nerve crush reduced recovery of sciatic nerve conduction velocity (repeated measures ANOVA P < 0.0001). No differences were seen in thermal responses (E) with hindpaw withdrawal latencies measured by Hargreaves apparatus or mechanical responses (F) measured with Von Frey filaments. Data presented as mean ± s.e.m., except tactile data presented as interquartile-range. n = 6/group.

central nervous system. This new model of peripheral conditioning provides new opportunities to study the mechanisms underlying the growth promoting effects. By determining the common underlying mechanisms between these two models, we can hope to gain a fundamental understanding of how to reprogram the adult nervous system to respond differently to injury.

Appendix A. Supplementary material

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.expneurol.2014.12.004.

References

Allt, G., Ghabriel, M.N., Sikri, K., 1988. Lysophosphatidyl choline-induced demyelination. Acta Neuropathol. 75, 456–464.

Beletsky, I.P., Umansky, S.R., 1990. A new assay for cell death. J. Immunol. Methods 134, 201–205.

Boeshore, K.L., Schreiber, R.C., Vaccariello, S.A., Sachs, H.H., Salazar, R., Lee, J., Ratan, R.R., Leahy, P., Zigmond, R.E., 2004. Novel changes in gene expression following axotomy of a sympathetic ganglion: a microarray analysis. J. Neurobiol. 59, 216–235.

Brown, M.C., Perry, V.H., Hunt, S.P., Lapper, S.R., 1994. Further studies on motor and sensory nerve regeneration in mice with delayed Wallerian degeneration. Eur. J. Neurosci. 6, 420–428.

Brück, W., 1997. The role of macrophages in Wallerian degeneration. Brain Pathol. 7, 741–752.

Calcutt, N.A., 2004. Modeling diabetic sensory neuropathy in rats. In: Luo, Z.D. (Ed.), Pain Research. Humana Press, pp. 55–65.

Chaplan, S.R., Bach, F.W., Pogrel, J.W., Chung, J.M., Yaksh, T.L., 1994. Quantitative assessment of tactile allodynia in the rat paw. J. Neurosci. Methods 53, 55–63.

Costigan, M., Befort, K., Karchewski, L., Griffin, R.S., D'Urso, D., Allchorne, A., Sitarski, J., Mannion, J.W., Pratt, R.E., Woolf, C.J., 2002. Replicate high-density rat genome oligonucleotide microarrays reveal hundreds of regulated genes in the dorsal root ganglion after peripheral nerve injury. BMC Neurosci. 3, 16.

Damoiseaux, J.G., Dopp, E.A., Calame, W., Chao, D., MacPherson, G.G., Dijkstra, C.D., 1994. Rat macrophage lysosomal membrane antigen recognized by monoclonal antibody ED1. Immunology 83, 140–147.

de Medinaceli, L., Freed, W.J., Wyatt, R.J., 1982. An index of the functional condition of rat sciatic nerve based on measurements made from walking tracks. Exp. Neurol. 77, 634–643.

Dimova, R., Markov, D., 1976. Changes in the mitochondria in the initial part of the axon during regeneration. Acta Neuropathol. 36, 235–242.

Einheber, S., Bhat, M.A., Salzer, J.L., 2006. Disrupted axo-glial junctions result in accumulation of abnormal mitochondria at nodes of ranvier. Neuron Glia Biol. 2, 165–174.

Forman, D.S., Wood, D.K., DeSilva, S., 1979. Rate of regeneration of sensory axons in transected rat sciatic nerve repaired with epineurial sutures. J. Neurol. Sci. 44, 55–59.

Griffin, J.W., Stocks, E.A., Fahnestock, K., Praagh, A., Trapp, B.D., 1990. Schwann cell proliferation following lysolecithin-induced demyelination. J. Neurocytol. 19, 367–384.

Griffin, J.W., George, R., Ho, T., 1993. Macrophage systems in peripheral nerves. A review. J. Neuropathol. Exp. Neurol. 52, 553–560.

Gupta, R., Rowshan, K., Chao, T., Mozaffar, T., Steward, O., 2004. Chronic nerve compression induces local demyelination and remyelination in a rat model of carpal tunnel syndrome. Exp. Neurol. 187, 500–508.

Gutmann, E., Guttmann, L., Medawar, P.B., Young, J.Z., 1942. The rate of regeneration of nerve. J. Exp. Biol. 19, 14–44.

Hall, S.M., 1973. Some aspects of remyelination after demyelination produced by the intraneural injection of lysophosphatidyl choline. J. Cell Sci. 13, 461–477.

Hall, S.M., 1989. Regeneration in the peripheral nervous system. Neuropathol. Appl. Neurobiol. 15, 513–529.

Hall, S.M., Gregson, N.A., 1971. The in vivo and ultrastructural effects of injection of lysophosphatidyl choline into myelinated peripheral nerve fibres of the adult mouse. J. Cell Sci. 9, 769–789.

Hoffman, P.N., Cleveland, D.W., 1988. Neurofilament and tubulin expression recapitulates the developmental program during axonal regeneration: induction of a specific beta-tubulin isotype. Proc. Natl. Acad. Sci. 85, 4530–4533.

Hollis II, E.R., Zou, Y., 2012. Reinduced Wnt signaling limits regenerative potential of sensory axons in the spinal cord following conditioning lesion. Proc. Natl. Acad. Sci. 109, 14663–14668.

Jenkins, R., Hunt, S.P., 1991. Long-term increase in the levels of c-jun mRNA and jun protein-like immunoreactivity in motor and sensory neurons following axon damage. Neurosci. Lett. 129, 107–110.

Kao, L.-P., Ovchinnikov, D., Wolvetang, E., 2012. The effect of ethidium bromide and chloramphenicol on mitochondrial biogenesis in primary human fibroblasts. Toxicol. Appl. Pharmacol. 261, 42–49.

Kigerl, K.A., Gensel, J.C., Ankeny, D.P., Alexander, J.K., Donnelly, D.J., Popovich, P.G., 2009. Identification of two distinct macrophage subsets with divergent effects causing either neurotoxicity or regeneration in the injured mouse spinal cord. J. Neurosci. 29, 13435–13444.

Kiryu-Seo, S., Ohno, N., Kidd, G.J., Komuro, H., Trapp, B.D., 2010. Demyelination increases axonal stationary mitochondrial size and the speed of axonal mitochondrial transport. J. Neurosci. 30, 6658–6666.

Kosins, A.M., Scholz, T., Mendoza, C., Lin, P., Shepard, B., Evans, G.R., Keirstead, H.S., 2011. Improvement of peripheral nerve regeneration following immunological demyelination in vivo. Plast. Reconstr. Surg. 127, 1813–1819.

Kwon, M.J., Kim, J., Shin, H., Jeong, S.R., Kang, Y.M., Choi, J.Y., Hwang, D.H., Kim, B.G., 2013. Contribution of macrophages to enhanced regenerative capacity of dorsal root ganglia sensory neurons by conditioning injury. J. Neurosci. 33, 15095–15108.

Lancelotta, M.P., Sheth, R.N., Meyer, R.A., Belzberg, A.J., Griffin, J.W., Campbell, J.N., 2003. Severity and duration of hyperalgesia in rat varies with type of nerve lesion. Neurosurgery 53, 1200–1209.

Lazarov-Spiegler, O., Solomon, A.S., Zeev-Brann, A.B., Hirschberg, D.L., Lavie, V., Schwartz, M., 1996. Transplantation of activated macrophages overcomes central nervous system regrowth failure. FASEB J. 10, 1296–1302.

Liu, Y.Y., Wong-Riley, M.T., Liu, H.L., Jia, Y., Jiao, X.Y., Wang, C.T., You, S.W., Ju, G., 2001. Increase in cytochrome oxidase activity in regenerating nerve fibers of hemitransected spinal cord in the rat. Neuroreport 12, 3239–3242.

Lu, X., Richardson, P.M., 1991. Inflammation near the nerve cell body enhances axonal regeneration. J. Neurosci. 11, 972–978.

Mar, F.M., Simões, A.R., Leite, S., Morgado, M.M., Santos, T.E., Rodrigo, I.S., Teixeira, C.A., Misgeld, T., Sousa, M.M., 2014. CNS axons globally increase axonal transport after peripheral conditioning. J. Neurosci. 34, 5965–5970.

McQuarrie, I.G., Grafstein, B., 1973. Axon outgrowth enhanced by a previous nerve injury. Arch. Neurol. 29, 53–55.

Meijering, E., Jacob, M., Sarria, J.C., Steiner, P., Hirling, H., Unser, M., 2004. Design and validation of a tool for neurite tracing and analysis in fluorescence microscopy images. Cytometry A 58, 167–176.

Niemi, J.P., DeFrancesco-Lisowitz, A., Roldán-Hernández, L., Lindborg, J.A., Mandell, D., Zigmond, R.E., 2013. A critical role for macrophages near axotomized neuronal cell bodies in stimulating nerve regeneration. J. Neurosci. 33, 16236–16248.

Parikh, P., Hao, Y., Hosseinkhani, M., Patil, S.B., Huntley, G.W., Tessier-Lavigne, M., Zou, H., 2011. Regeneration of axons in injured spinal cord by activation of bone morphogenetic protein/Smad1 signaling pathway in adult neurons. Proc. Natl. Acad. Sci. 108, E99–E107.

Perry, V.H., Tsao, J.W., Feam, S., Brown, M.C., 1995. Radiation-induced reductions in macrophage recruitment have only slight effects on myelin degeneration in sectioned peripheral nerves of mice. Eur. J. Neurosci. 7, 271–280.

Popovich, P.G., Guan, Z., Wei, P., Huitinga, I., van Rooijen, N., Stokes, B.T., 1999. Depletion of hematogenous macrophages promotes partial hindlimb recovery and neuroanatomical repair after experimental spinal cord injury. Exp. Neurol. 158, 351–365.

Qiu, J., Cai, D., Dai, H., McAtee, M., Hoffman, P.N., Bregman, B.S., Filbin, M.T., 2002. Spinal axon regeneration induced by elevation of cyclic AMP. Neuron 34, 895–903.

Richardson, P.M., Issa, V.M., 1984. Peripheral injury enhances central regeneration of primary sensory neurones. Nature 309, 791–793.

Riet-Correa, G., Fernandes, C.G., Pereira, L.A.V., Graça, D.L., 2002. Ethidium bromide-induced demyelination of the sciatic nerve of adult Wistar rats. Braz. J. Med. Biol. Res. 35, 99–104.

Saida, K., Saida, T., 1986. Proliferation of Schwann cells in demyelinated rat sciatic nerve. Acta Neuropathol. 71, 251–258.

Schirmer, L., Merkler, D., König, F.B., Brück, W., Stadelmann, C., 2012. Neuroaxonal regeneration is more pronounced in early multiple sclerosis than in traumatic brain injury lesions. Brain Pathol. 23, 2–12.

Schmued, L.C., 1990. A rapid, sensitive histochemical stain for myelin in frozen brain sections. J. Histochem. Cytochem. 38, 717–720.

Seijffers, R., Mills, C.D., Woolf, C.J., 2007. ATF3 increases the intrinsic growth state of DRG neurons to enhance peripheral nerve regeneration. J. Neurosci. 27, 7911–7920.

Spillane, M., Ketschek, A., Merianda, T., Twiss, J., Gallo, G., 2013. Mitochondria coordinate sites of axon branching through localized intra-axonal protein synthesis. Cell Rep. 5, 1564–1575.

Stam, F.J., MacGillavry, H.D., Armstrong, N.J., de Gunst, M.C., Zhang, Y., van Kesteren, R.E., Smit, A.B., Verhaagen, J., 2007. Identification of candidate transcriptional modulators involved in successful regeneration after nerve injury. Eur. J. Neurosci. 25, 3629–3637.

Steinmetz, M.P., Horn, K.P., Tom, V.J., Miller, J.H., Busch, S.A., Nair, D., Silver, D.J., Silver, J., 2005. Chronic enhancement of the intrinsic growth capacity of sensory neurons combined with the degradation of inhibitory proteoglycans allows functional regeneration of sensory axons through the dorsal root entry zone in the mammalian spinal cord. J. Neurosci. 25, 8066–8076.

Ton, B.-H.T., Chen, Q., Gaina, G., Tucureanu, C., Georgescu, A., Strungaru, C., Flonta, M.-L., Sah, D., Ristoiu, V., 2013. Activation profile of dorsal root ganglia Iba-1 (+) macrophages varies with the type of lesion in rats. Acta Histochem. 115, 840–850.

Tsujino, H., Kondo, E., Fukuoka, T., Dai, Y., Tokunaga, A., Miki, K., Yonenobu, K., Ochi, T., Noguchi, K., 2000. Activating transcription factor 3 (ATF3) induction by axotomy in sensory and motoneurons: a novel neuronal marker of nerve injury. Mol. Cell. Neurosci. 15, 170–182.

Vogelaar, C.F., Vrinten, D.H., Hoekman, M.F.M., Brakkee, J.H., Burbach, J.P.H., Hamers, F.P.T., 2004. Sciatic nerve regeneration in mice and rats: recovery of sensory innervation is followed by a slowly retreating neuropathic pain-like syndrome. Brain Res. 1027, 67–72.

von Wurmb-Schwark, N., Cavelier, L., Cortopassi, G.A., 2006. A low dose of ethidium bromide leads to an increase of total mitochondrial DNA while higher concentrations induce the mtDNA 4997 deletion in a human neuronal cell line. Mutat. Res. Fundam. Mol. Mech. Mutagen. 596, 57–63.

Woodruff, R.H., Franklin, R.J.M., 1999. Demyelination and remyelination of the caudal cerebellar peduncle of adult rats following stereotaxic injections of lysolecithin, ethidium bromide, and complement/anti-galactocerebroside: a comparative study. Glia 25, 216–228.

Zambonin, J.L., Zhao, C., Ohno, N., Campbell, G.R., Engeham, S., Ziabreva, I., Schwarz, N., Lee, S.E., Frischer, J.M., Turnbull, D.M., Trapp, B.D., Lassmann, H., Franklin, R.J.M., Mahad, D.J., 2011. Increased mitochondrial content in remyelinated axons: implications for multiple sclerosis. Brain 134, 1901–1913.

Zou, H., Ho, C., Wong, K., Tessier-Lavigne, M., 2009. Axotomy-induced Smad1 activation promotes axonal growth in adult sensory neurons. J. Neurosci. 29, 7116–7123.