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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.

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_2O 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_2O 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_2O_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_2O.

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