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An in vitro assay to study induction of the regenerative state in sensory neurons

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ABSTRACT

After injury, peripheral neurons activate a pro-regenerative program that facilitates axon regeneration. While many regeneration-associated genes have been identified, the mechanism by which injury activates this program is less well understood. Furthermore, identifying pharmacological methods to induce a pro-regenerative state could lead to novel treatments to repair the injured nervous system. Therefore, we have developed an in vitro assay to study induction of the pro-regenerative state following injury or pharmacological treatment. First, we took advantage of the observation that dissociating and culturing sensory neurons from dorsal root ganglia activates a pro-regenerative program. We show that cultured neurons activate transcription factors and upregulate regeneration-associated genes common to the pro-regenerative program within the first hours after dissection. In a paradigm similar to pre-conditioning, neurons injured by dissociation display enhanced neurite outgrowth when replated as early as 12 h after being removed from the animal. Furthermore, stimulation of the pro-regenerative state improves growth on inhibitory substrates and requires DLK/JNK signaling, both hallmarks of the pro-regeneration response in vivo. Finally, we modified this assay in order to identify new methods to activate the pro-regenerative state in an effort to mimic the pre-conditioning effect. We report that after several days in culture, neurons down-regulate many molecular hallmarks of injury and no longer display enhanced neurite outgrowth after replating. Hence, these neurons are functionally naïve and are a useful tool for identifying methods to induce the pro-regenerative state. We show that both injury and pre-treatment with forskolin reactivate the pro-regenerative state in this paradigm. Hence, this assay is useful for identifying pharmacological agents that induce the pro-regenerative state in the absence of injury.

Introduction

Axon injury is a consequence of many conditions including trauma, toxic insults, and neurodegenerative and genetic disorders (Coleman, 2005; Wang et al., 2012). In order to restore function, axons must regenerate and reinnervate their targets. However, many factors affect how well regeneration occurs and thus limit functional recovery. In the central nervous system (CNS) injured axons fail to regenerate due to a combination of low intrinsic growth potential and environmental factors (Bradke et al., 2012). In the peripheral nervous system (PNS), injured neurons can have robust regeneration, but in many cases this regeneration is slow and the target cell degenerates or becomes non-functional before reinnervation eventually occurs (Gordon et al., 2011). Furthermore, the regenerative potential of peripheral neurons can be hindered by aging, toxic insults, and neurodegenerative disorders (Kennedy and Zochodne, 2005; Pan et al., 2003; Verdú et al., 2000). Thus there is a need to better understand regeneration mechanisms and exploit these mechanisms to promote axon regeneration in both the central and peripheral nervous systems.

In the peripheral nervous system, injury triggers a sequence of events that promotes axon regeneration. After axon injury, local responses promote resealing of the injured axon and reorganization of the cytoskeleton to allow for growth cone formation (Bradke et al., 2012; Rishal and Fainzilber, 2014). Meanwhile, local signaling pathways activate and drive retrograde transport of injury signals to the nucleus which then triggers a pro-regenerative transcriptional program (Rishal and Fainzilber, 2014; Tedeschi, 2011). This reprograms neurons into a regenerative state and efficient axon regeneration is delayed until this state has been induced (Smith and Skene, 1997). Prior activation of the pro-regenerative program by a conditioning lesion removes this delay and accelerates axon regeneration after a secondary test lesion (McQuarrie and Grafstein, 1973; McQuarrie et al., 1977; Smith and Skene, 1997). This is termed the "pre-conditioning effect" and results from activation of the pro-regenerative program by the conditioning

Abbreviations: DRG, dorsal root ganglion; CNS, central nervous system; PNS, peripheral nervous system; JNK, cJun N-terminal kinase; DLK, dual leucine zipper kinase; CSPG, chondroitin sulfate proteoglycan; SCG10, superior cervical ganglion 10.

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injury. Consequently, at the time of the second injury axons are already in a regenerative state and thus are primed for growth. Furthermore, a peripheral conditioning lesion also enhances the regenerative capacity of central axons such that they are able to overcome the inhibitory CNS growth environment following spinal cord injury (Neumann and Woolf, 1999; Richardson and Issa, 1984). Understanding the mechanisms responsible for inducing the pro-regenerative program and/or identifying alternative methods to induce the pro-regenerative state could have important consequences for axon regeneration in a variety of neurological disorders.

Historically, the pro-regenerative response has been studied in vivo, either by a conditioning lesion or drug application into the dorsal root ganglion (DRG) or sciatic nerve followed by an assay of axonal growth either in vivo or in culture (Kilmer and Carlsen, 1984; McQuarrie and Grafstein, 1973; Neumann et al., 2002; Qiu et al., 2002; Smith and Skene, 1997). More recently, it has been recognized that axotomy of DRG neurons by dissociating and then culturing them also triggers activation of injury-activated transcription factors and upregulation of regeneration-associated genes, two features of the pro-regenerative program (Saijilafu et al., 2013; Zou et al., 2009). Furthermore, this injury enhances neurite outgrowth in a manner similar to in vivo conditioning lesions and is blocked by transcription and translation inhibitors (Saijilafu et al., 2013; Zou et al., 2009).

Since stimulating the pro-regenerative state has important consequences for axon regeneration, we characterized the induction of the pro-regenerative response after dissociation. We show that molecular and functional features of the pro-regenerative state are active within the first 24 h of culture. In addition, we show that the pro-regenerative program does not persist in vitro. Indeed, we found that between 4 and 7 days in culture, neurons have down-regulated the molecular hallmarks of the growth program and no longer display enhanced neurite outgrowth after replating. Thus after several days in culture, neurons injured by dissociation revert to a "functionally naïve" phenotype. Using these "functionally naïve" neurons, we show that both injury and pharmacological stimulation of a known regeneration pathway re-induce the pro-regenerative state in vitro. Thus, we have assessed the induction of the pro-regenerative response following injury and show that it maintains many of the features of in vivo injury and the pre-conditioning paradigm. Furthermore, we have developed a novel assay to induce the pro-regenerative state using pharmacological methods in the absence of injury in order to identify drugs that mimic the pre-conditioning effect. This new assay can be used to identify new candidate therapeutics that promote regeneration in vitro and that can then be further explored in vivo.

Materials and methods

Animals

Adult C57BL6 mice were purchased from Charles River. DLK conditional knockout (KO) mice ($DLK^{f/f}$; Adv-$CRE^{+/-}$) were generated by crossing floxed DLK ($DLK^{f/f}$) mice (Miller et al., 2009) with Advillin-Cre mice (Zurborg et al., 2011). Control and DLK conditional KO mice were age and sex-matched. Mouse husbandry was performed under the supervision of Division of Comparative Medicine at Washington University.

Primary DRG neuron culture

Neurons from cervical, thoracic, and lumbar dorsal root ganglia (DRG) display enhanced growth after replating, indicating that DRG from all levels activate a pro-regenerative response when dissociated. Thus, all DRG were dissected from adult mice and pooled. DRG were incubated in an enzyme solution containing 0.35 mg/mL Liberase Blendzyme (Roche), 10 mg/mL bovine serum albumin (Sigma), and 0.6 mg/mL DNase (Sigma) for 15 min at 37 °C. After 15 min, the enzyme solution was removed and DRG were digested with 0.05% Trypsin-EDTA (Invitrogen) for 15 min at 37 °C. Trypsin was removed and replaced with DMEM (Invitrogen) culture media containing 10% FBS (Invitrogen), 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen). DRG were triturated with a plastic pipet tip and diluted in culture media. Cells were plated at a density of 0.7–1.4 DRG/mL.

Lab-Tek Permanox chamber slides (Fisher Scientific) or plastic tissue culture plates were coated with 10 mg/mL PDL (Sigma) overnight. Then the slides/dishes were washed and coated with 10 mg/mL laminin (Sigma) for at least 2 h before cells were plated. As indicated, some slides were coated with 1 μg/mL chondroitin sulfate proteoglycans (CSPGs, Millipore) at the time of laminin coating (Hur et al., 2011). CSPGs were a mixture of neurocan, aggregcan, versican, and phosphacan isolated from embryonic chicken brain. To ensure that any growth inhibiting effects were due directly to the CSPGs, neurons were cultured on PDL/laminin/CSPG coated slides that were pre-treated with 0.5 U/mL chondroitinase ABC (Sigma) for 3 h. Digestion of the chondroitin sulfate glycosaminoglycans rescued neurite formation and growth (data not shown). Neurons were maintained at 37°C with 5% CO_2. Media was changed on DIV1, 4, and 7 and 10 nM AraC (Sigma) was added.

Replating assay and neurite length analysis

For the replating assay, media was removed from cultured neurons and replaced with 0.025% Trypsin-EDTA (Invitrogen). Neurons were incubated for 5 min at 37°C with 5% CO_2. Then trypsin was removed and replaced with fresh media. Cells were washed and gently pipetted to detach cell bodies and these were transferred to PDL and laminin coated Lab-Tek Permanox slides for neurite length analysis. After replating, neurons were cultured for 18 h then fixed in 4% paraformaldehyde (Electron Microscopy Services) for 20 min at room temperature. Cells were washed with phosphate-buffered saline (PBS) containing 0.1% Triton and blocked with PBS containing 10% goat serum and 0.2% Triton. Next, cells were incubated in mouse anti-Tuj1 primary antibody (Covance, 1:500) with or without rabbit NF200 primary antibody (Sigma, 1:1000) overnight at 4°C. Then cells were washed in PBS containing 0.1% Triton followed by incubation with goat anti-mouse AF488 (Life Technologies, 1:1000) for at least 1 h. Goat anti-rabbit Cy3 (Jackson Immuno, 1:1000) was used to detect NF200. Finally, cells were washed in 0.1% Triton PBS, mounted with Vectashield (Vector Laboratories), and sealed with clear nail polish. Neurons were imaged using a Nikon Eclipse 80i microscope using a 10× air objective. Approximately 100 neurons were imaged per condition for each experiment and the length of the longest neurite was measured using the Neurite Tracer Plugin for ImageJ (Pool et al., 2008). At least 3 independent experiments were used. Representative images were obtained using a Nikon D-Eclipse C1 confocal microscope with 20× air objective.

Pharmacology

JNK inhibitor (SP600125, Sigma) was applied to neurons immediately after dissociation at a final concentration of 15 μM. Twenty-four hours after treatment, vehicle (DMSO) and JNK inhibitor were removed by washing with DMEM. Neurons were then replated as described above. Forskolin (Sigma) was used at a final concentration of 30 μM. Vehicle (DMSO) or forskolin were applied 24 h before replating (pre-treatment and pre/post treatment). Immediately preceding replating, neurons were washed with DMEM to remove drugs before trypsinization. After replating, vehicle or forskolin were added back to neurons in the pre/post- and post-treatment groups.

Immunocytochemistry

Neurons were fixed in 4% paraformaldehyde (Electron Microscopy Services) for 20 min at room temperature and then washed with