Published in final edited form as:

Exp Neurol. 2015 January ; 263: 350–363. doi:10.1016/j.expneurol.2014.10.012.

An *In Vitro* Assay to Study Induction of the Regenerative State in Sensory Neurons

E. Frey^a, V. Valakh^a, S. Karney-Grobe^a, Y. Shi^a, J. Milbrandt^b, and A. DiAntonio^{a,*}

^aDepartment of Developmental Biology, Hope Center for Neurological Disorders, Washington University School of Medicine, St. Louis, MO 63110, USA

^bDepartment of Genetics, Hope Center for Neurological Disorders, Washington University School of Medicine, St. Louis, MO 63110, USA

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 hours 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

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*Correspondence should be addressed to Dr. Aaron DiAntonio, Department of Developmental Biology, Washington University School of Medicine, Campus Box 8103, 660 S. Euclid, St. Louis, MO 63110. dian Antonio@wustl.edu.. erin.frey@wustl.edu (E.F.), vvalakh@wustl.edu (V.V.), karneygrobe@wustl.edu (S.K.G.), popstone07@gmail.com (Y.S.), jmilbrandt@wustl.edu (J.M.)

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AUTHOR CONTRIBUTIONS

A.D., J.M., and E.F. designed experiments. E.F, V.V., S.K.G., and Y.S. performed experiments. E.F. analyzed data. A.D. and E.F. wrote the manuscript.

CONFLICT OF INTEREST

The authors declare no competing financial interests.

assay is useful for identifying pharmacological agents that induce the pro-regenerative state in the absence of injury.

Keywords

axon regeneration; replating assay; dorsal root ganglion (DRG); dual leucine zipper kinase (DLK); cJun N-terminal kinase (JNK); superior cervical ganglion 10 (SCG10)

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 IG and Grafstein B, 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 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 IG and Grafstein B, 1973, 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 hours of culture. In addition, we show that the pro-regenerative program does not persist *in vitro*. Indeed, we found that between 4-7 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 minutes at 37°C. After 15 minutes, the enzyme solution was removed and DRG were digested with 0.05% Trypsin-EDTA (Invitrogen) for 15 minutes 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 hours before cells were plated. As indicated, some slides were coated with 1 µg/mL chondroitin sulfate proteoglycans (CSPG, Millipore) at the time of laminin coating (Hur et al., 2011). CSPGs were a mixture of neurocan, aggrecan, 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 hours. Digestion of the chondroitin sulfate glycosaminoglycans rescued neurite formation and growth (data not shown). Neurons were maintained at 37°C with 5% CO₂. 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 minutes at 37°C with 5% CO₂. 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 hours then fixed in 4% paraformaldehyde (Electron Microscopy Services) for 20 minutes 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 hour. 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 10x 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 Image J (Pool et al., 2008). At least 3 independent experiments were used. Representative images were obtained using Nikon D-Eclipse C1 confocal microscope with 20X 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 hours 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 minutes at room temperature and then washed with phosphate buffered saline (PBS) containing 0.1% Triton and blocked with PBS containing 10% goat serum and 0.2% Triton. Cells were then incubated in mouse anti-Tuj1 (Covance, 1:500) and rabbit anti-phospho-cJun (p-cJun, Cell Signaling, 1:300) or rabbit anti-SCG10 ((Shin et al., 2014), 1:5000), or rabbit anti-GAP43 (Millipore, 1:500) overnight at 4°C. SCG10 antibody was purified from Anti-SCG10 rabbit antiserum (Novus Biologicals) using the SulfoLink Kit (Thermo). Then cells were washed in PBS containing 0.1% Triton followed by incubation with goat anti-mouse AF488 (Life Technologies, 1:1000) and goat anti-rabbit Cy3 (Jackson Immuno, 1:1000) secondary antibodies for at least 1 hour. Finally cells were washed in 0.1% Triton, mounted with Vectashield (Vector Laboratories), and sealed with clear nail polish. For intensity measurements of p-cJun, SCG10, and GAP43 staining, at least 50 cells per condition for each experiment were imaged using a Nikon D-Eclipse C1 confocal microscope with 40X oil immersion objective. All groups within an experiment were imaged with the same gain. Gain was set based on signal in the 24 hr/DIV1 condition such that the signal was not saturated. Nuclear p-cJun and somal SCG10 and GAP43 intensities were measured using ImageJ. β III-tubulin was used as a counter stain to identify neurons. At least 3 independent experiments were used. Representative images were obtained using a 60X oil immersion objective.

Quantitative Real-Time PCR

Neurons were cultured for the indicated duration before RNA was extracted using a standard trizol protocol. Briefly, neurons were treated with Trizol (Ambion), followed by chloroform and centrifuged for 15 minutes at 12,000 g (4°C). The aqueous fraction was collected and treated with glycogen (Roche). Then the aqueous fraction was washed with isopropanol and incubated for 10 minutes at room temperature before centrifugation for 10 minutes at 12,000 g (4°C). The isopropanol wash was removed and the pellet was washed with 75% EtOH and centrifuged for 5 minutes at 7,400 g (4°C). After centrifugation the EtOH was removed and the pellet was air-dried for 5-10 minutes before being resuspended in RNase-free water. RNA samples were treated with the RQ1 RNase-Free DNase (Promega) for 30 minutes at 37°C to remove any contaminating genomic DNA. The DNase reaction was terminated using the RQ1 stop solution (Promega) and incubating at 65°C for 10 minutes. First-strand cDNA synthesis was performed using Quanta Biosciences qScript cDNA synthesis kit according to manufacturer's instructions. The resulting cDNA was diluted for quantitative RT-PCR and an equivalent amount of DNase-treated RNA was used to confirm the absence of genomic DNA. Quanta Biosciences PerfeCTa SYBR Green FastMix with Rox was used for quantitative RT-PCR. Primers included: Galanin (Forward: 5'-GCCACATGCCATTGACAAC-3', Reverse: 5'-CGGACAATGTTGCTCTCAGG-3'), Sprr1a (Forward: 5'-AGAGAACCTGCTCTTCTCTGAGT-3', Reverse: 5'-CTGGTGCAGCTGAGGAGGTA-3'), and GAPDH (Forward: 5'-

TGTGAACGGATTTGGCCGTA-3', Reverse: 5' - ACTGTGCCGTTGAATTTGCC-3') (Finelli et al., 2013). Standard cDNA curves were used to validate primer sets and melt curve analysis ensured single PCR products. Data was analyzed using the CT Method (Schmittgen and Livak, 2008). Data from each time point was normalized to GAPDH from the same time point and then to the 24 hr/DIV1 timepoint.

Statistics

Students t-test, 1-way ANOVA, and 2-way ANOVA were used to determine statistical significance as indicated. Tukey or Bonferroni post hoc tests were performed for mean comparisons following 1-way or 2-way ANOVA, respectively. For experiments described in Figure 9, repeated measures ANOVAs were used. P-values < 0.05 were considered significant.

RESULTS

Dissociation and culture of DRG neurons activates the pro-regenerative program and induces a regenerative state

Axon injury initiates a retrograde signaling cascade to activate transcription and translation of regeneration-associated genes (Rishal and Fainzilber, 2014; Tedeschi, 2011). This pro-regenerative program is essential for effective axon regeneration (Raivich et al., 2004; Saijilafu et al., 2013; Shin et al., 2012; Zou et al., 2009). This can be measured in two ways: (1) molecularly using markers of the pro-regenerative program and (2) functionally by assessing the regenerative state. To determine how early the pro-regenerative program is activated, we assessed three molecular markers: phosphorylated cJun (p-cJun), superior cervical ganglion 10 (SCG10, also known as stathmin 2), and growth associated protein 43 (GAP43). Phosphorylated c-Jun (p-cJun) is an injury-activated transcription factor that is required for regeneration (Raivich et al., 2004). SCG10 and GAP43 are molecular markers for regenerating and injured axons, respectively (Shin et al., 2014; Van der Zee et al., 1989). These three molecular markers were assessed via immunocytochemistry after 1, 4, 8, and 24 hours *in vitro* (**Figure 1**). p-cJun is modestly upregulated after 4 hrs in culture and levels are further elevated by 8 and 24 hrs (**Figure 1A, 1B**). SCG10 is nearly undetectable after 1 hr in culture, but increases steadily from 4 to 24 hrs (**Figure 1A, 1C**). Upregulation of GAP43 is slightly delayed and is not significantly elevated until 8 hrs *in vitro* (**Figure 1A, 1D**). However, by 24 hrs there is robust GAP43 staining in the DRG cell bodies (**Figure 1A, 1D**). As an independent test of injury-induced molecular changes *in vitro*, we used quantitative RT-PCR to assess the expression of the regeneration-associated genes Galanin and Sprr1a (**Table 1**). Galanin is expressed at low levels 1 hour after dissociation and increases by 24 hours in culture ($p < 0.001$, 1-way ANOVA). Sprr1a is undetectable 1 hour after dissociation. By 8 hours, Sprr1a is detectable but is not abundantly expressed. Like Galanin, Sprr1a mRNA is robustly up-regulated by 24 hours after dissociation ($p < 0.001$, 1-way ANOVA). These results demonstrate that molecular changes associated with the pro-regenerative program begin to turn on as soon as four hours after DRG are dissociated and continue to increase within the first 24 hours.

Next, we assessed how early in culture the pro-regenerative program can induce a functional regenerative state. For this experiment, neurite length was measured after an 18 hour growth phase for all groups. “Naïve” neurons were dissociated and cultured for 18 hours (**Figure 2A**). Naïve neurons display very little outgrowth in the first 18 hours of culture (**Figure 2, Naïve**). Indeed only ~25% of naïve neurons extend neurites longer than 75 μm (**Figure 2A, 2C, 2D**). Replating cultured neurons can mimic the pre-conditioning effect and show improved neurite outgrowth if neurons have activated a regenerative program at the time of replating (Zou 2009, Saijilafu 2013). In this paradigm removing DRG from the mouse and dissociating them acts as the conditioning lesion and replating them acts as the test lesion. We replated neurons after 1, 4, 8, 12, or 24 hours in culture to remove any existing neurites. After replating, neurons were cultured for 18 hours (the same growth duration as Naïve neurons) and neurite length was assessed (**Figure 2A**). Replating DRG neurons after 1 and 4 hours in culture did not reveal any significantly improved axon regeneration (**Figure 2**). The majority of these neurons extended very short neurites (< 75 microns) and displayed similar distributions and mean neurite lengths as Naïve neurons (**Figure 2**). This is in contrast to molecular changes that occur as early as 4 hours in culture (**Figure 1**) and suggests that even though these neurons are beginning to activate molecular markers of the pro-regenerative program there has not been sufficient time to functionally promote neurite outgrowth. In contrast, replating neurons after 8 hours in culture shows an increased proportion of neurons with neurites greater than 75 μm , indicating an improvement in neurite outgrowth (**Figure 2B-D**, $p < 0.001$, 2-way ANOVA). However, this improvement is modest (**Figure 2E**). Replating after 12 hours also shows an increase in the number of neurons with neurites longer than 75 μm compared to Naïve (**Figure 2B-D**, $p < 0.001$, 2-way ANOVA) and the mean neurite length is increased by ~2.6 fold (**Figure 2E**). Replating neurons after 24 hours in culture shows a further increase in the proportion of neurons with neurites greater than 75 μm (**Figure 2B-D**, $p < 0.001$ compared to Naïve, $p < 0.01$ compared to 12 hrs, 2-way ANOVA). Nearly 20% of neurons extend neurites to lengths of 400 μm or greater (**Figure 2B-D**). Furthermore, this corresponds to an increase in mean neurite length of ~4.6 fold over Naïve neurons (**Figure 2E**). This is consistent with the robust activation of p-cJun and upregulation of SCG10, GAP43, Galanin, and Sprr1a at 24 hours in culture. Together, these results show that removing DRG from adult mice activates a pro-regenerative program that can be assayed molecularly via immunostaining and qRT-PCR and triggers a regenerative state that can be assessed functionally by measuring neurite outgrowth after replating. This paradigm recapitulates several features characteristic of *in vivo* nerve injury and the pre-conditioning effect.

Previous reports have shown that small nociceptive neurons have impaired regeneration (Kalous and Keast, 2010; Leclerc et al., 2007). In order to test whether different neuronal subtypes activate the pro-regenerative state after dissociation, we classified neurons as being NF200 positive (large diameter mechano- and proprioceptors) or NF200 negative (enriched for small diameter nociceptors) and assessed neurite length (**Figure S1**). Naïve NF200 positive and negative neurons displayed similar neurite outgrowth ($p > 0.05$). Both subpopulations also displayed equally enhanced neurite outgrowth after replating (**Figure S1A, S1B**). The proportion of large and small diameter neurons was not significantly different in naïve or replated conditions (**Figure S1C**). These results indicate that in this

model system, large and small diameter neurons regenerate equally well and replating neurons does not cause a selective loss of either major neuron type.

Activation of the pro-regenerative response promotes growth on inhibitory substrates *in vitro*

In vivo pre-conditioning lesions of the peripheral nerve promote axon regeneration following spinal cord injury and enhance neurite outgrowth on inhibitory substrates *in vitro* (Neumann and Woolf, 1999; Neumann et al., 2002; Richardson and Issa, 1984). Therefore, we tested whether activation of the pro-regenerative response by dissection and dissociation is sufficient to promote neurite outgrowth on the inhibitory substrates chondroitin sulfate proteoglycans (CSPGs). Naïve neurons were cultured on control or CSPG substrates and growth was assessed after 18 hours. Consistent with the literature, CSPGs inhibited neurite outgrowth of Naïve neurons (**Figure 3**). Only ~2% of Naïve neurons cultured on CSPGs extended neurites beyond 75 μ m compared to ~17% cultured on control substrates (**Figure 3B**, $p < 0.001$, 2-way ANOVA). For replating, neurons were first cultured on control substrate for 24 hours and then replated onto either control or CSPG substrates. After replating, ~36% of neurons were able to extend neurites further than 75 μ m on CSPG substrate (**Figure 3A-C**, $p < 0.001$, 2-way ANOVA). Furthermore, replating neurons on CSPGs significantly improved mean neurite length (**Figure 3D**). While neurons replated on CSPGs displayed less absolute growth than neurons replated onto control substrate (**Figure 3D**, $p < 0.001$, 1-way ANOVA), the fold-increase in replating-induced growth was similar between control and CSPG substrates (Control: 7 ± 1 , CSPG: 9 ± 2 fold change, $p = 0.3$, unpaired Students t-test). Together, these data show that activation of the pro-regenerative program is not only sufficient to promote neurite outgrowth on permissive substrates, but also improves neurite outgrowth on inhibitory substrates similarly to *in vivo* pre-conditioning.

DLK and JNK are necessary for robust activation of the pro-regenerative program.

In vivo pre-conditioning requires the dual leucine zipper kinase (DLK, MAP3K12) for activation and retrograde transport of injury signaling components (Shin et al., 2012). To test if this *in vitro* model also requires the DLK signaling pathway, we used adult DRG neurons from control (DLK^{f/f};Adv-CRE^{-/-}) and conditional DLK knockout mice (DLK KO, DLK^{f/f};Adv-CRE^{+/-}) that lack DLK in sensory neurons. Naïve neurons from control and DLK KO mice showed no significant change in neurite outgrowth in the first 18 hours in culture (**Figure 4A-D**). However, when neurons are replated and allowed to grow for 18 hours, DLK KO neurons have significantly less neurite outgrowth compared to replated control neurons (**Figure 4A-D**). After replating only ~30% of DLK KO neurons extend neurites beyond 75 μ m compared to ~60% of replated control neurons (**Figure 4B**, $p < 0.001$, 2-way ANOVA). Furthermore, replated DLK KO neurons display significantly shorter neurites compared to control neurons (**Figure 4D**). While the regenerative response of DLK KO neurons is strongly impaired in this assay, there is still a modest increase in axon growth following replating of the mutant neurons. This is in contrast to the complete loss of the pre-conditioning effect in DLK KO mice *in vivo* (Shin et al., 2012).

JNK is a stress kinase that is downstream of DLK and promotes axon regeneration through activation of the transcription factor cJun (Kenney and Kocsis, 1998; Lindwall et al., 2004; Raivich et al., 2004). Since dissection and plating of DRG neurons upregulates p-cJun (**Figure 1A, 1B**), we tested the requirement of JNK in this *in vitro* assay using the pharmacological inhibitor (SP600125) (Bennett et al., 2001; Miller et al., 2009). Wildtype DRG neurons were treated with SP600125 immediately after dissection and dissociation. After 24 hours, neurons were fixed and p-cJun levels were assessed. Treatment with the JNK inhibitor (JNKi) blocked upregulation of p-cJun (**Figure 4E**) indicating that JNK contributes to the activation of the pro-regenerative program. To test the functional consequence, we pre-treated neurons with DMSO or JNKi for 24 hours. Following treatment, drugs were washed out and neurons were replated and then grown for 18 hours in the absence of drug. Neurons replated after DMSO pre-treatment showed significantly improved neurite outgrowth compared to Naïve neurons (**Figure 4F-I**). JNKi pre-treated neurons did show some improvement in neurite outgrowth after replating compared to Naïve (**Figure 4G, 4H**, $p < 0.001$ % Neurons with neurites $< 75 \mu\text{m}$), however this was quite modest compared to DMSO pre-treated neurons. Indeed ~20% of replated neurons pre-treated with DMSO extended neurites greater than $400 \mu\text{m}$, while only ~1.5% of JNKi pre-treated neurons were able to reach these lengths (**Figure 4G, 4H**, $p < 0.05$). While DMSO-treated neurons had an ~7 fold increase in mean neurite length, JNKi-treated neurons did not significantly improve mean neurite length compared to naïve neurons (**Figure 4I**). Hence, both DLK and JNK contribute to activation of the pro-regenerative program in this *in vitro* model.

In the replating paradigm described above, the pro-regenerative program is induced by the injury of dissecting the neurons from the animal. Hence, this system is useful for identifying factors necessary for the injury-induced activation of the regeneration program, but not for identifying mechanisms that are sufficient to activate the program. It might be possible to identify such activating mechanisms if the pro-regenerative program were to “turn off” sometime after dissection *in vitro*. To explore this possibility, we assessed molecular and functional readouts of the pro-regenerative program over time.

The pro-regenerative program decays in vitro

In order to determine if the pro-regenerative program induced by DRG dissection decays *in vitro*, we assessed p-cJun, SCG10, and GAP43 on days *in vitro* (DIV) 1, 3, and 7. p-cJun is highly expressed at DIV1 (24 hours in culture) but is down-regulated by DIV3 (**Figure 5A, 5B**). Although p-cJun decreases after several days in culture, it still remains slightly elevated over the levels observed at 1 hr after dissociation (**Figure 1**). SCG10 is also robustly expressed at DIV1 but decreases dramatically by DIV3 and further decreases by DIV7 (**Figure 5A, 5C**). In fact SCG10 intensity is similar at 1 hr and 7 days after dissection and dissociation (**Figure 1**). GAP43 remains highly expressed in DRG cell bodies at DIV3 at levels similar to DIV1 (**Figure 5A, 5D**). However, GAP43 is down regulated by DIV7 (**Figure 5A, 5D**) and reaches similar levels to those observed 1 hr after dissociation (**Figure 1**). Quantitative RT-PCR was used to independently assess the relative expression of Galanin and Sprr1a. By DIV3 both regeneration-associated genes have become down-regulated to ~40% of the maximal levels achieved 24 hours after dissociation (**Table 1**, $p <$

0.001 for both, 1-way ANOVA). However, mRNA levels for these genes plateau and do not further decrease by DIV7. For Galanin, the relative amount of mRNA on DIV7 is similar to levels observed 1 hour after dissociation ($p > 0.05$). However, relative *Sprr1a* mRNA levels are still elevated compared to the 1 hour time point, when *Sprr1a* was undetectable ($p < 0.05$). This indicates that some injury pathways and regeneration-associated genes become inactivated to a similar level as “naïve” neurons, while other pathways remain active, although to a lesser extent in culture. Together, these results demonstrate that molecular characteristics of the pro-regenerative program decay over time. Therefore, we hypothesized that after DIV1 neurons begin to revert to a more “naïve-like” phenotype. Thus, we predicted that neurons replated after DIV1 would have reduced neurite outgrowth compared to neurons replated on DIV1. We tested this hypothesis by replating neurons on DIV1, DIV2, DIV3, DIV4, and DIV7. We found that ~65% of neurons replated on DIV1 extended neurites greater than 75 μm , while only ~45% of neurons replated on DIV2 (**Figure 6A-C**, $p < 0.01$) and ~33% of neurons replated on DIV3 (**Figure 6A-C**, $p < 0.001$) developed neurites greater than 75 μm . However, neurons replated on DIV2 and DIV3 still had improved neurite outgrowth, as only 20% of naïve neurons extend neurites beyond 75 μm (**Figure 6A-C**, $p < 0.01$ and 0.05 , respectively). Replating neurons on DIV2 and DIV3 also showed an increase in mean neurite length of ~4 fold and ~3 fold over naïve growth, respectively (**Figure 6D**). However this was a significant reduction compared to the ~6.3 fold change achieved by neurons replated on DIV1. Hence, the pro-regenerative program is still able to promote regeneration at DIV 2 and 3, albeit not as robustly as on DIV1. This is in contrast to neurons replated at later timepoints. Only ~14% of neurons replated on either DIV4 or DIV7 had neurites that extended beyond 75 μm , which was not significantly different than Naïve neurons (**Figure 6A-C**). Furthermore, there was no significant increase in mean neurite length of neurons replated on DIV4 or DIV7 compared to naïve neurons (**Figure 6D**). This suggests that DRG neurons cultured for 4-7 days are “functionally naïve,” with a similar axon growth potential as bona fide naïve neurons.

To determine whether neurons replated on DIV7 had delayed growth or a complete inability to extend neurites, we cultured neurons for 18 or 72 hours after replating on DIV7. After 18 hr of growth only 20% of neurons developed neurites (**Figure 6E, 6F**). However, after 72 hours of growth nearly 90% of neurons developed neurites (**Figure 6E, 6F**). This demonstrates that the lack of growth observed 18 hours after replating on DIV7 is not due to unhealthy neurons that are unable to regenerate.

The pro-regenerative program can be reactivated in vitro

Since neurons cultured for seven days have down-regulated the pro-regenerative program and display less neurite outgrowth 18 hours after replating (**Figure 5 and 6**), we hypothesized that replating neurons on DIV7 may act like a conditioning lesion and reactivate the pro-regenerative program. If this were true, we would expect replating on DIV7 to re-induce the pro-regenerative program. To test this, we cultured neurons for 7 days and either fixed them (“DIV7”) or replated them (“Replated DIV7”, **Figure 7A**). Replated neurons were cultured for 24 hours before fixing. Using immunocytochemistry, we assessed p-cJun, SCG10, and GAP43 to determine if the molecular characteristics of the pro-regenerative program had been re-induced. Indeed, replating neurons on DIV7 upregulated

p-cJun (~2.7 fold), SCG10 (~4 fold), and GAP43 (~1.8 fold) compared to non-replated neurons (**Figure 7B-D**). Since the pro-regenerative program was re-induced 24 hours after replating on DIV7, we hypothesized that neurons replated on DIV7 and again on DIV8 would display enhanced neurite outgrowth. To test this, neurons were replated at DIV7 and cultured for 24 hours (in order to re-activate the decayed pro-regenerative program). To determine if the regenerative state had been successfully induced, neurons were replated again on DIV8 and cultured for 18 hours to assay neurite outgrowth (**Figure 7E**). Similar to our previous findings, neurons only replated on DIV7 displayed very little growth in 18 hours, and only ~17% of these neurons extended neurites beyond 75 μm (**Figure 7F-H**). In contrast, neurons replated on DIV7 and again on DIV8 had much greater neurite outgrowth with ~52% of neurons extending neurites beyond 75 μm and ~22% of those extended neurites beyond 400 μm (**Figure 7F-H**, $p < 0.001$ and $p < 0.05$ respectively). Furthermore, replating neurons on DIV7 and again on DIV8 improved mean neurite length by ~3.6 fold over neurons that were only replated on DIV7 (**Figure 7I**). These experiments show that even after the pro-regenerative program decays *in vitro* it can be reactivated with replating.

Having shown that injury can reactivate the pro-regenerative program in these functionally naïve neurons, we next tested whether a pharmacological agent could re-induce the pro-regenerative program in the absence of injury. Forskolin, a stimulator of the cAMP/PKA pathway, enhances axon regeneration *in vivo* and neurite outgrowth *in vitro* (Kilmer and Carlsen, 1984; Neumann et al., 2002). Furthermore, stimulating the cAMP/PKA pathway *in vivo* mimics the pre-conditioning effect (Kilmer and Carlsen, 1984; Neumann et al., 2002; Qiu et al., 2002). With our new replating assay we now have the ability to temporally control when neurons are exposed to pharmacological agents (i.e.: before replating, during the growth phase after replating, or both; **Figure 8A**) and to assess if drugs can mimic the pre-conditioning effect *in vitro*. Since neurons cultured for 4 days and replated show similar growth phenotypes to naïve neurons, DMSO or forskolin were applied on DIV4 for 24 hours (**Figure 8A**). After treatment, drugs were washed away and neurons were replated to assess neurite outgrowth in the absence of drug (Pre-Tx). In addition, some neurons were treated with DMSO or forskolin only after replating; therefore drugs were present during the growth phase only (Post-Tx). Finally, some neurons received a combination treatment and were treated with drugs before and after replating (Pre/Post-Tx). All neurons were grown for 18 hours after replating and neurite outgrowth was assessed. Neurons exposed to forskolin before replating displayed improved neurite outgrowth with ~36% of neurons extending neurites beyond 75 μm compared to only ~19% of DMSO treated neurons (**Figure 8C**, Pre-Tx, $p < 0.01$), resulting in a ~1.7 fold increase in mean neurite length (**Figure 8D**). Hence, forskolin can re-induce the pro-regenerative response to promote neurite outgrowth even when it is not present while the neurons are actively growing. Neurons treated with forskolin only during the growth phase also displayed an improvement in neurite outgrowth as ~34% of neurons extended neurites beyond 75 μm compared to only ~21% of DMSO treated neurons (**Figure 8C**, Post-Tx, $p < 0.01$) and increased mean neurite length by ~1.5 fold (**Figure 8D**). This observation is consistent with previous reports where culturing neurons in the presence of forskolin enhanced neurite outgrowth (Neumann et al., 2002). Finally, treating neurons with forskolin before and after replating improved neurite outgrowth with ~43% of neurons extending neurites beyond 75 μm compared to ~18% of DMSO treated

neurons (**Figure 8C**, Pre/Post-Tx, $p < 0.001$). The combination treatment increased mean neurite length by ~2.3 fold and was an improvement over Post-Tx alone (**Figure 8D**, $p < 0.05$ compared to Post-Tx forskolin). Thus, this assay is amenable to identifying pharmacological agents that enhance neurite outgrowth by inducing the pro-regenerative state (i.e.: pre-treatment).

DISCUSSION

Axon regeneration is critical for restoring neuronal function after axon injury. Thus, there is much interest in understanding how axon regeneration occurs and identifying novel therapeutics to promote regeneration. The pre-conditioning effect highlights the ability of neurons to respond to injury and enhance regeneration in both the central and peripheral nervous systems by inducing a pro-regenerative transcriptional program that induces a regenerative state (McQuarrie IG and Grafstein B, 1973, 1973; Neumann and Woolf, 1999; Richardson and Issa, 1984; Rishal and Fainzilber, 2014; Smith and Skene, 1997; Tedeschi, 2011). Being able to induce a pro-regenerative state may have important consequences for axon regeneration in a variety of contexts. Studies in animal models show that some regeneration pathways become inactivated as early as 7 days after injury, well before reinnervation is complete (Abe et al., 2010; Devor et al., 1979; Magill et al., 2007; Saijilafu et al., 2013; Sun et al., 2013). In addition, chronic denervation results in decreased regenerative capacity of peripheral neurons further limiting recovery (Fu and Gordon, 1995). Thus maintaining or enhancing activation of these pathways may accelerate regeneration resulting in improved functional recovery after injury. In fact constitutive activation of regeneration pathways speeds regeneration after injury and more rapid regeneration aids functional recovery in the peripheral nervous system (Gobrecht et al., 2014; Gordon et al., 2011; Seijffers et al., 2007). Other factors such as aging and neurodegenerative disorders also limit effective axon regeneration (Kennedy and Zochodne, 2005; Pan et al., 2003; Verdú et al., 2000). For example, in peripheral neuropathies and ALS, axon degeneration is balanced by compensatory regeneration (Dadon-Nachum et al., 2011). Overtime, the regenerative processes become overwhelmed and are no longer able to compete with axon degeneration. Drug treatments that foster axon regeneration in these cases may be helpful for treating diseases and prolonging neuronal function. In the central nervous system, injury does not activate the pro-regenerative program, and neurons fail to regenerate. However, inducing the pro-regenerative program with peripheral nerve injury promotes axon growth into the CNS after spinal cord injury (Neumann and Woolf, 1999; Richardson and Issa, 1984). Excitingly, this effect is not restricted to activating the pro-regenerative program before spinal cord injury. Injuring the peripheral nerve even weeks after spinal cord injury improves regeneration (Kadoya et al., 2009; Richardson and Issa, 1984). This indicates a broader therapeutic window where the pro-regenerative program could be activated to promote repair. Clearly, there is a need to understand how the pro-regenerative program is induced following injury as well as to identify compounds that can induce a pro-regenerative state in the absence of a prior nerve injury. Towards this end, we have developed and characterized a novel *in vitro* assay to study induction of the pro-regenerative state.

We show that dissociating adult DRG neurons and culturing them induces the pro-regenerative program and enhances neurite outgrowth after replating, and this is consistent

with previous reports (Saijilafu et al., 2013; Zou et al., 2009). In addition, here we show that activation of the pro-regenerative program via dissociation recapitulates several features of the *in vivo* pre-conditioning response. First, activation of the pro-regenerative state was able to overcome CSPG-inhibited growth. The fold change in neurite outgrowth was the same regardless of substrates, but the absolute growth was still not as robust as neurons replated onto control substrates. Thus, the reduced absolute growth on CSPGs is not likely a result of impaired activation or inactivation of the pro-regenerative program, but instead may be due to activation of distinct pathways to inhibit neurite outgrowth (Beller et al., 2013; Dickendesher et al., 2012; Fisher et al., 2011; Hur et al., 2011; Monnier et al., 2003; Shen et al., 2009; Sivasankaran et al., 2004). This highlights the important need to target both intrinsic and extrinsic mechanisms to achieve effective regeneration. Second, initial regeneration does not require DLK, but complete activation of the pro-regenerative response requires the DLK/JNK pathway. This is consistent with our previous findings that control and DLK KO mice show identical regeneration in the first 24 hours after peripheral nerve injury and confirms that DLK does not play a role in the immediate axon outgrowth response but is required for the pre-conditioning response (Shin et al., 2012). However, unlike *in vivo* studies, DLK KO neurons maintained some injury-induced capacity to facilitate growth after replating. Following nerve crush the pre-conditioning response is completely absent in mice lacking DLK in sensory neurons (Shin et al., 2012). This difference implies that shearing axons from the cell body (i.e.: dissociation) activates additional injury signals that do not require DLK. DLK is required for the retrograde transport of injury signals following axonal injury *in vivo*, and so severing the axon at the cell body may have less of a need for such retrograde transport. Furthermore, inhibition of JNK prevented the upregulation of p-cJun, however this only partially blocked neurite regeneration following replating. This is consistent with previous reports that show additional pathways contribute to axon regeneration *in vitro* (Saijilafu et al., 2013; Zou et al., 2009).

Other studies have examined the ability of different neuronal subtypes to activate a pro-regenerative response after injury (Christie et al., 2010; Kalous and Keast, 2010; Leclere et al., 2007). In these studies small diameter nociceptive neurons fail to activate a regenerative response in culture after an *in vivo* pre-conditioning injury (Kalous and Keast, 2010; Leclere et al., 2007). More recently, it has been shown that a subpopulation of small diameter neurons have increased PTEN expression and this may counteract the regenerative response after a pre-conditioning injury (Christie et al., 2010). Interestingly, we found that different neuronal populations (NF200 positive/negative) were equally able to activate the pro-regenerative response (Figure S1). This suggests that some of the subtleties of the *in vivo* regenerative response are lost in this *in vitro* system. For example, if large and small diameter neurons differ in their ability to retrogradely transport injury signals from the axon, such differences would be lost following removal of axons at the cell body during dissection. In addition, the influence of glia and other non-neuronal cells that contribute to the axonal environment *in vivo* will not be captured in this assay.

Several studies have screened small molecules to identify drugs/pathways that regulate neurite outgrowth (Gao et al., 2010; Koprivica et al., 2005; Sivasankaran et al., 2004; Usher

et al., 2010). In these screens, neurons were dissociated and cultured in the presence of drugs and neurite outgrowth was assessed. These studies have successfully identified components that regulate neuronal outgrowth by affecting growth cone formation/stability and growth dynamics. However, because the drug is present during neurite outgrowth, it is difficult to disentangle any potential role in inducing the pro-regenerative program from direct effects on growth cone dynamics. Furthermore, since dissociation of neurons is an injury, endogenous injury signaling may occlude drug effects, thereby hindering efforts to identify drugs that induce the pro-regenerative program. By developing an approach where drugs can be applied to functionally naïve neurons before or during the growth phase, we hope to identify inducers of the pro-regenerative state. Activating this pro-regenerative transcriptional program should induce a suit of genes that promote axonal re-growth and should complement efforts to identify agents that directly manipulate growth cone dynamics.

Towards this end, we adapted this *in vitro* assay to study induction of the pro-regenerative response in the absence of injury. We first set out to determine how long the pro-regenerative program remains active *in vitro*. *In vivo* studies indicate that the pre-conditioning effect is maintained for several weeks to months (McQuarrie IG and Grafstein B, 1973; Neumann and Woolf, 1999; Richardson and Issa, 1984; Smith and Skene, 1997). However, at least some regenerative pathways are diminished by one week after *in vivo* injury indicating that activation of the pro-regenerative program dissipates with time (Abe et al., 2010; Saijilafu et al., 2013; Sun et al., 2013). We found that molecular and functional features of the pro-regenerative program begin to decay shortly after 24 hours in culture. Culturing neurons for 4 days results in “functionally naïve” neurons that no longer display enhanced neurite outgrowth following replating compared to true naïve neurons. The pro-regenerative program can be re-induced by injury or by stimulating a regenerative pathway. Indeed replating neurons on DIV7 reactivated molecular and functional characteristics of the pro-regenerative program in a manner similar to dissociating neurons from the animal. This suggests that the delayed growth observed 72 hours after replating on DIV7 is likely due to the time it takes for neurons to activate the pro-regenerative state. Furthermore, we show that forskolin improves neurite outgrowth when it is present before or after replating. This is consistent with forskolin inducing a pro-regenerative state (pre-treatment) and directly regulating axon elongation (post-treatment). These findings are consistent with previous *in vivo* findings with cAMP or forskolin treatment (Kilmer and Carlsen, 1984; Neumann et al., 2002; Qiu et al., 2002).

While this assay recapitulates several features of *in vivo* injury, there are also differences. These differences could be due to a number of factors. For example, while our culture system includes some non-neuronal cells that could affect axon growth in this model, the influence of cell-cell interactions with glia and macrophages that occurs *in vivo* (Arthur-Farraj et al., 2012; Chen and Strickland, 2003; Kwon et al., 2013; Napoli et al., 2012; Niemi et al., 2013) will not be replicated *in vitro*. Differences could also arise from the location of the injury, which is at the somal/axon junction *in vitro* but axonal *in vivo*. Finally, there are limitations associated with all *in vitro* assays. Culturing neurons at low density is the most effective way to assay neurite length. However, sparsely cultured do not behave identically

to densely cultured neurons (Smith and Skene, 1997) and are clearly different from neurons *in vivo*. For example, *in vivo*, DRG neurons are pseudounipolar with central and peripheral branches, and only the peripheral branch typically displays robust regeneration after injury. In culture, DRG neurons lose this complexity. Hence, while this assay is a useful tool for interrogating the induction of the pro-regenerative program, ultimately new findings derived from this assay will need to be validated *in vivo*.

Conclusion

We have developed a novel *in vitro* assay that can identify drugs that activate the pro-regenerative state. We show that pre-treating “functionally naïve” neurons with forskolin in the absence of a concurrent injury promotes neurite outgrowth. Thus, this assay is capable of selectively identifying drugs that induce a pro-regenerative state, rather than directly modulate neurite extension. This had not been possible with previous *in vitro* assays. Future studies will utilize this new assay in combination with high-throughput methods to identify novel activators of the pro-regenerative program. Work from our lab has already discovered two pharmacological agents that activate the pro-regenerative program by stimulating the DLK pathway *in vitro*, and one of these drugs mimics the pre-conditioning effect when applied *in vivo* (Valakh et al., in revision). Thus, this assay promises to be a valuable new tool for the axon regeneration community.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

This work was supported by funding from the Neilson Foundation, a Hope Center for Neurological Disorders Grant, and National Institutes of Health Grants NIH NS087562 and NIH NS065053 to A.D. and J.M., and the Philip and Sima Needleman Student Fellowship in Regenerative Medicine to S.K.G. We thank members of the DiAntonio and Milbrandt laboratories for helpful discussions.

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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Highlights

- Dissection and dissociation of DRG neurons activates a pro-regenerative response.
- The regenerative response fades in culture resulting in functionally naïve neurons.
- Injury or Forskolin re-induce the regenerative response of functionally naïve neurons.
- Assay can identify drugs that induce regenerative response in the absence of injury.

Figure 1. Molecular markers of the pro-regenerative program are upregulated *in vitro*
 Neurons were fixed at the indicated times after dissection and dissociation to assess molecular markers of the pro-regenerative program. **A.** Representative images of p-cJun, SCG10, and GAP43 at the indicated times after dissociation. Scale bar = 10 μ m. **B-D.** Quantification of mean intensity normalized to the mean intensity at 24 hours. Data represent mean $\pm$ SEM. One-way ANOVA and Tukey posthoc test were used to determine statistical significance. $p < 0.01$ and 0.001 compared to “1 hr” are indicated by “**” and “***”, respectively. “n.s.” indicates no significant difference. 200-258 total cells were analyzed from 4-5 independent experiments.

Figure 2. Neurons are re-programmed into a pro-regenerative state *in vitro*

To test whether activation of the pro-regenerative program (Figure 1) induces a pro-regenerative state, neurons were replated and neurite outgrowth was assessed. **A.** Model of assay. Neurons were dissected from adult mice and dissociated for culture. Naïve neurons were cultured for 18 hours to assess baseline growth. For replating experiments, neurons were cultured for 1-24 hours before replating. After replating, neurons were grown for 18 hours to assess any improvement in neurite outgrowth compared to Naïve. Representative images are shown in **B** (scale bar = 100 μm). Binned neurite distribution (**C**) and representative cumulative frequency plot (**D**) show changes in the distribution of neurite lengths. **E.** Quantification of mean neurite length. Data represent mean ± SEM. One-way ANOVA and Tukey posthoc test were used to determine statistical significance. $p < 0.01$ and 0.001 are indicated by “***” and “****”, respectively. Unless otherwise noted, p -values reflect significance compared to Naïve. Data are from 3 independent experiments.

Figure 3. The pro-regenerative response improves neurite outgrowth on inhibitory substrates

To test whether the pro-regenerative state was sufficient to promote growth on inhibitory substrates, neurons were originally plated on control (PDL/laminin) substrate. Twenty-four hours later, they were replated onto either control or CSPG (PDL/laminin/CSPG) substrates and neurite outgrowth was compared to naïve neurons. **A.** Representative images of naïve and replated neurons grown on control or CSPG substrates. Scale bar = 100 μm . Neurite length distributions are shown as binned distributions (**B**) and with a representative cumulative frequency plot (**C**). **D.** Quantification of mean neurite length. Data represent mean $\pm$ SEM. One-way ANOVA and Tukey posthoc test were used to determine statistical significance. $p < 0.01$ and 0.001 compared to Naïve groups are indicated by “***” and “****”, respectively. Data are from 5 independent experiments.

Figure 4. The DLK/JNK pathway contributes to the pro-regenerative state

The requirement of the DLK/JNK pathway in re-programming neurons into a pro-regenerative state was assessed genetically with DLK conditional knockout (KO) neurons and pharmacologically with the JNK inhibitor SP600125. **A**. Representative images of Control or DLK KO naïve and replated neurons. Scale bar = 100 μm . Neurons were replated 24 hours after dissection and dissociation. Neurite length distributions are shown as binned distributions (**B**) and with a representative cumulative frequency plot (**C**). **D**. Quantification of mean neurite length. Data are from 3 independent experiments. In **E-I**, neurons were

treated with DMSO or 15 μ M SP600125 to inhibit JNK. Drugs were applied immediately after neurons were dissociated and plated for culture. After 24 hours, neurons were either fixed to assess p-cJun intensity (**E**) or washed and replated (**F-I**). After replating, neurons were grown for 18 hours in the absence of drug. **E**. Representative images and quantification of mean p-cJun intensity ($n = 151-153$ neurons from 3 independent experiments). Scale bar = 10 μ m. Data were normalized to mean intensity of DMSO treated neurons. Statistical significance was determined by Students t-test. **F**. Representative images of Naïve neurons grown for 18 hours and DMSO/JNKi pre-treated neurons. Drugs were not present during neurite outgrowth. Scale bar = 100 μ m. Neurite length distributions are displayed as binned distributions (**G**) and a representative cumulative frequency plot (**H**). **I**. Quantification of mean neurite length. Data are from 5 independent experiments. All data represent mean $\pm$ SEM. For neurite growth experiments, one-way ANOVA and Tukey posthoc test were used to determine statistical significance. $p < 0.01$ and 0.001 are indicated by “**” and “***”, respectively.

Figure 5. Molecular markers of the pro-regenerative program are down-regulated after several days *in vitro*

Neurons were fixed at the indicated times after dissection and dissociation to assess molecular markers of the pro-regenerative program. **A.** Representative images of p-cJun, SCG10, and GAP43 at the indicated times. Scale bar = 10 μm. **B-D.** Quantification of mean intensities normalized to the mean intensity at 24 hours. Data represent mean ± SEM. One-way ANOVA and Tukey posthoc test were used to determine statistical significance. $p < 0.01$ and 0.001 are indicated by “***” and “****”, respectively. “n.s.” indicates no significant difference. 140-180 total cells were analyzed from 3 independent experiments.

Figure 6. The pro-regenerative state decays with time

Neurons were dissected from adult mice and dissociated for culture. Naïve neurons were cultured for 18 hours to assess baseline growth. For replating experiments, neurons were cultured for 1-7 days before replating. After replating, neurons were grown for 18 hours to assess any improvement in neurite outgrowth compared to Naïve. Representative images are shown in **A**. Binned neurite distribution (**B**) and representative cumulative frequency plot (**C**) show changes in the distribution of neurite lengths. **D**. Quantification of mean neurite length. **E-F**. Neurons were cultured for 7 days, replated, and grown for either 18 or 72 hours. Representative images show that neurons replated on DIV7 are capable of extending very long neurites if given enough time in culture (**E**). The percentage of neurons bearing neurites is quantified (**F**). “Neurons with Neurites” was defined as neurons bearing neurites that were longer in length than the diameter of the cell body. Scale bars = 100 μm . Data represent mean $\pm$ SEM. One-way ANOVA with Tukey posthoc test and Students t-test were used to determine statistical significance for graphs in **D** and **F**, respectively. $p < 0.05$, 0.01 and

0.001 are indicated by ”*”, ”**” and ”***”, respectively. Data are from 3-5 independent experiments.

Figure 7. Axon injury reactivates the pro-regenerative program and induces a regenerative state
 Axons were re-injured to determine if the pro-regenerative program and/or the regenerative state could be re-induced *in vitro*. **A**. Experimental design to test the molecular activation of the pro-regenerative program. Neurons were cultured for 7 days and either fixed (DIV7) or replated (Replated DIV7). After replating neurons were cultured for 24 hours and molecular markers of the pro-regenerative program were assessed. Representative images and quantification for p-cJun (**B**), SCG10 (**C**), and GAP43 (**D**) are shown. Scale bar = 10 μ m. Data are from 151-161 total neurons over 3 independent experiments. Data are normalized

to DIV7. **E.** Experimental design to functionally test the re-induction of the pro-regenerative state. Neurons were cultured for 7 days and then replated. After replating on DIV7, half of the neurons were fixed after 18 hours (DIV7). The other neurons were cultured for 24 hours and replated again on DIV8. After replating on DIV8, neurons were cultured for 18 hours and fixed (DIV7+1). Representative images are shown in **F**. Scale bar = 100 μm . Binned neurite distribution (**G**) and representative cumulative frequency plot (**H**) show changes in the distribution of neurite lengths. **I.** Quantification of mean neurite length. Data are from 3 independent experiments. Students t-test was used to determine significance. $p < 0.05$ or 0.001 are indicated by “*” or “***”, respectively.

Figure 8. Forskolin reactivates the pro-regenerative response

We tested the ability of forskolin, a drug that mimics the pre-conditioning effect when administered *in vivo*, to re-induce the pro-regenerative state *in vitro*. **A**. Experimental design. Neurons were cultured for 4 days and then split into “Pre-Tx”, “Post-Tx”, or “Pre/Post-Tx” groups. On DIV4 “Pre” and “Pre/Post” groups were treated with DMSO or forskolin (30 μ M). Twenty-four hours later, media containing the drug was completely removed and neurons from all groups were replated. Only “Post” and “Pre/Post” groups received drugs after replating. All neurons were grown for 18 hours after replating and then fixed to assess neurite outgrowth. Representative images are shown in **B**. Scale bar = 100 μ m. Neurite outgrowth was evaluated by quantifying binned distributions (**C**) and mean neurite length (**D**). Data are from 9 independent experiments. Since each condition was tested in each individual experiment, a repeated measures one-way ANOVA and Tukey posthoc test was used to determine statistical significance. $p < 0.05$ or 0.001 are indicated by “*” or “***”, respectively.

Table 1Transcriptional changes of RAGs in culture^a

Time	Galanin	Sprr1a
1 hr	0.17 ± 0.07 (4)	Undetectable
4 hr	0.23 ± 0.06 (4)	Undetectable
8 hr	0.32 ± 0.09 (4)	0.05 ± 0.03 (3)
24 hr/DIV1	1.01 ± 0.01 (4)	1.04 ± 0.02 (4)
DIV3	0.43 ± 0.07 (4)	0.36 ± 0.09 (4)
DIV7	0.44 ± 0.09 (4)	0.31 ± 0.2 (3)

Data are Mean ± SEM (n)

^aRelative fold changes of mRNA levels compared to 24 hr/DIV1 timepoint.