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Schwann Cells Regulate Sensory Neuron Gene Expression Before and After Peripheral Nerve Injury

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Abstract

Sensory neurons in the PNS demonstrate substantial capacity for regeneration following injury. Recent studies have identified changes in the transcriptome of sensory neurons, which are instrumental for axon regeneration. The role of Schwann cells (SCs) in mediating these changes remains undefined. We tested the hypothesis that SCs regulate expression of genes in sensory neurons before and after PNS injury by comparing mice in which LDL Receptor-related Protein-1 (*LRP1*) is deleted in SCs (scLRP1$^{-/-}$ mice) with wild-type (scLRP1$^{+/+}$) littermates. LRP1 is an endocytic and cell-signaling receptor that is necessary for normal SC function and the SC response to nerve injury. scLRP1$^{-/-}$ mice represent a characterized model in which the SC response to nerve injury is abnormal. Adult DRG neurons, isolated from scLRP1$^{-/-}$ mice, with or without a conditioning nerve lesion, demonstrated increased neurite outgrowth when cultured *ex vivo*, compared with neurons from wild-type mice. Following sciatic nerve crush injury, nerve regeneration was accelerated *in vivo* in scLRP1$^{-/-}$ mice. These results were explained by transcriptional activation of RAGs in DRG neurons in scLRP1$^{-/-}$ mice prior to nerve injury. Although the presence of abnormal SCs in scLRP1$^{-/-}$ mice primed DRG neurons for repair, nerve regeneration in scLRP1$^{-/-}$ mice resulted in abnormalities in ultrastructure, principally in Remak bundles, and with the onset of neuropathic pain. These results demonstrate the importance of SCs in controlling RAG expression by neurons and the potential for this process to cause chronic pain when abnormal. The SC may represent an important target for preventing pain following PNS injury.

Graphical Abstract

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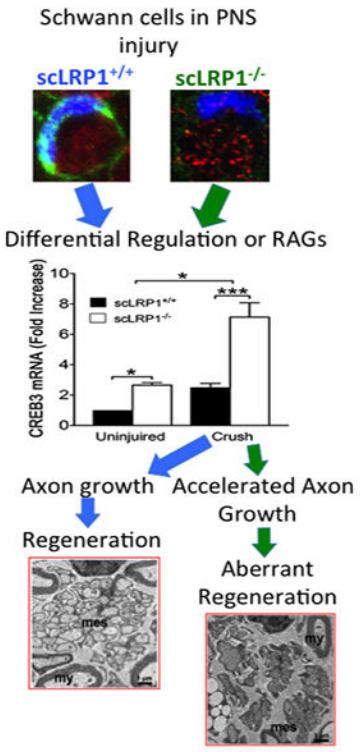

Keywords

Schwann cell; peripheral nerve; LRP1; regeneration associated genes (RAGs); DRG; axonal growth; pain

Introduction

Neurons in the PNS have a greater capacity for regeneration compared with neurons in the CNS (Aguayo et al., 1981; David and Aguayo, 1981; Richardson and Issa, 1984). However, PNS regeneration is often imperfect; impaired axonal growth rates may prevent successful re-innervation of target tissues and subsequent functional recovery (Singh et al., 2012; Kennedy and Zochodne, 2005). Thus, one strategy to facilitate PNS regeneration may involve increasing the intrinsic growth capacity of PNS neurons by over-expression of regeneration-associated genes (RAGs) (Seijffers et al., 2007; Ma et al., 2011; Sung et al., 2006; Dulin et al., 2015). Identified RAGs in sensory neurons include: activated transcription factor 3 (ATF3), small proline rich protein 1a (SPRR1A), signal transducers and activators of transcriptome-3 (STAT3), lumna/cAMP response element binding protein 3 (CREB3), cytoskeletal associated protein (CAP-23) and growth associated protein (GAP-43) (Aigner et al., 1995; Strittmatter et al., 1995; Caroni et al., 1998; Bomze et al., 2001; Bonilla et al., 2002; Williams, 2006; Chandran et al., 2016). Unfortunately, to date, axonal RAGs have not been individually or collectively shown to enhance functional recovery in the injured PNS.

In the PNS, Schwann cells (SCs) are intimately associated with axons and necessary for axonal viability and integrity (Chen et al., 2007; Viader et al., 2011; Viader et al., 2013; Campana, 2007; Lehman and Hoke, 2010). Some SCs provide myelination to large caliber axons (Svaren and Meijer, 2008), while others, in Remak bundles, do not generate myelin but still provide essential metabolic and trophic support. Remak bundle SCs ensheathe small diameter axons (C-fibers) involved in pain transmission (Griffin and Thompson, 2008). In PNS injury, SCs serve as first responders (Jessen and Mirsky, 2005), de-differentiating and reprogramming to support nerve repair (Arthur-Farraj et al., 2012). These changes in SC transcription and physiology occur simultaneously with activation of RAGs in damaged sensory neurons (Abe and Cavalli, 2008). In rodents, aged SCs fail to appropriately activate the SC repair program in response to injury, which reduces the growth capacity of regenerating peripheral axons (Painter et al., 2014).

We identified the LDL Receptor-related Protein (LRP1) as a SC receptor that orchestrates many of the changes in cell physiology associated with activation of the SC repair program (Fluetsch et al., 2016; Campana et al., 2006a; Mantuano et al., 2011; Mantuano et al., 2008; Mantuano et al., 2010; Orita et al., 2013). When LRP1 is genetically deleted in SCs in scLRP1$^{-/-}$ mice, or antagonized with receptor-associated protein (RAP), SC survival and function are compromised (Campana et al., 2006a; Orita et al., 2013). This compromise is associated with an increased tendency for development and maintenance of chronic pain states following nerve injury (Orita et al., 2013). Although LRP1 is expressed at lower levels in SCs prior to nerve injury, abnormalities in resting Remak bundles are observed in scLRP1$^{-/-}$ mice (Orita et al., 2013). The significance of these changes remains unexplained.

Herein, we studied scLRP1$^{-/-}$ mice as a model system in which SC physiology is abnormal and examined the effects of SCs on sensory neuron regeneration. We show that even prior to nerve injury, RAGs are activated in sensory neurons in scLRP1$^{-/-}$ mice. This change in the sensory neuron transcriptome is accompanied by an increased capacity for axonal outgrowth when neurons are cultured *ex vivo*. Following sciatic nerve injury, accelerated axonal growth was observed, accompanied by accelerated recovery of sensory function; however, at later time points, evidence of neuropathic pain was observed. Although the development of chronic pain correlated with accelerated regeneration, causation was not established because of evidence of ultrastructural changes in regenerated Remak bundles, which may be a result of the inherent abnormality in SCs due to *LRP1* gene deletion. Our results demonstrate that changes in the physiology of resting SCs, even prior to nerve injury, may regulate sensory neuron gene expression.

Materials and Methods

Animals

scLRP1$^{-/-}$ and scLRP1$^{+/+}$ mice in the C57BL/6 background are previously described (Orita et al., 2013). In scLRP1$^{+/+}$ mice, both *LRP1* genes are partially flanked by loxP sites, as in scLRP1$^{-/-}$ mice; however, scLRP1$^{+/+}$ mice are P0-Cre recombinase-negative. Experiments comparing scLRP1$^{-/-}$ and scLRP1$^{+/+}$ mice were performed using 3–5-month old female littermates. Breeding procedures and animal experiments were performed according to protocols approved by the University of California, San Diego Committee on Animal

Research, and conform to NIH Guidelines for Animal Use. All mice were housed with a 12 h:12 h light:dark cycle with ad libitum access to food and water.

Mouse surgeries

In crush injury experiments, mice were anesthetized with 3% isoflurane (IsoSol; VedCo, St. Joseph MO) and maintained with 2% isoflurane. An incision was made along the long axis of the femur. The sciatic nerve was exposed at mid-thigh level by separating the biceps femoris and the gluteus superficialis and then carefully cleared of surrounding connective tissue. The sciatic nerve was crushed twice for 30 sec with flat forceps (Azzouz et al., 1996). The site of crush injury was marked with a suture on the muscle surface. The muscle and skin layers were closed using 6.0 silk sutures. Sciatic nerve crush injury is a validated method for nerve regeneration studies (Griffin et al., 2010).

Cutaneous re-innervation

Mice were habituated (30 min) on wire mesh cages and baseline tested three times (over one week) prior to sciatic nerve crush injury. An Austerlitz insect pin (size 000; FST) was gently applied to the plantar surface of the paw without moving the paw or penetrating the skin. The pin was applied to two areas, the most lateral toe and the mid-lateral area of the paw on the surgically altered limb, with at least a 5-minute interval. Each area was tested twice. A response was considered positive when the animal briskly removed its paw, and the test was graded 1 for this area. If the application did not elicit a positive response, the grade was 0. The saphenous territory (medial paw) of the same paw was tested as a positive control, which always elicited a positive response. Subsequent to injury, mice were tested on postoperative days as indicated. Scoring was done blinded to the genotype.

Toe spreading motor test

To assess motor recovery after sciatic nerve crush injury, toe movement was evaluated. The toe spreading motor test is more sensitive than analysis of gait to detect return of fine motor function after sciatic nerve injury (Bozkurt et al., 2011; Ma et al., 2011). Mice were lifted by the tail so that hind paws could be visualized clearly. Under this condition, the digits separate and maximize space between them (the toe spreading reflex). The reappearance of this reflex results from re-innervation of the small muscles of the foot and was scored as previously described (Ma et al., 2011), with modifications: 0, no spreading; 1, slight spreading with two toes; 3, spreading with four toes; and 4, full spreading. Full spreading was defined as a complete, wide, and sustained (at least 2 seconds) spreading of the toes. Contralateral toes were completely spread when tested. Mice were assessed twice in each experimental session with at least a 45-minute interval in between tests. Scoring was done blinded to the genotype.

Thermal hyperalgesia

Female mice were randomized and acclimated to the test chamber for 1 h prior to testing. Thermal withdrawal latency was evaluated using a Hargreaves apparatus (Hargreaves et al., 1998) that delivers radiant heat to the plantar surface of the paw with a 20 second cut off as we described previously and modified for mice (Campana et al., 2006b; Myers et al., 1996).

Baseline withdrawal thresholds were obtained prior to surgery and averaged 5.5 seconds ± 0.4 seconds in mice (n=11). Each paw was tested four times, alternating between paws with an interval of at least 1 minute between tests. The interval between two trials on the same paw is at least 5 minutes. A significant decrease in the PWL is defined as thermal hyperalgesia. Scoring was done blinded to the genotype

Tactile Allodynia

Female mice were randomized, acclimated, and tested to establish baselines prior to surgeries. Tactile withdrawal thresholds were determined by testing mice in plexiglass chambers placed on a smooth stainless steel grid platform, allowing access from underneath. The 50% paw withdrawal thresholds were determined using a series of calibrated von Frey filaments (Stoelting, Wood Dale, IL USA) applied to the center of the hindpaw before surgery (baseline) and at regular intervals following crush injury. The von Frey filaments were calibrated to exert a force of 0.008 to 6 grams. Data are collected using the up-down method and further modified in mice (Chaplan et al., 1994; Orita et al., 2013).

Immunoblot analysis

Immunoblot analysis was performed as described previously (Campana et al., 2006a; Inoue et al., 2010). Briefly, extracts of sciatic nerve tissue distal to the crush injury site were prepared in RIPA buffer. The protein content of each extract was determined by bicinchoninic acid (BCA) assay. An equivalent amount of protein (20–40 μg per lane) was subjected to 10% SDS-PAGE and electro-transferred to nitrocellulose membranes. Blots were blocked with 5% nonfat dry milk and subsequently incubated with primary polyclonal antibodies to GAP43 (1:1000; Sigma), STMN2 (1:1000; Novus Biologicals), total ERK1/2 (t-ERK) (1:1000; Cell Signaling), GAPDH (1:2000; Sigma), NF200 (1:800; Sigma), $p75^{NTR}$ (1:5000; Millipore) or βIII neuronal tubulin (Tuji; 1:10,000; BioLegend) overnight at 4° C. Antibody-binding was detected by HRP-conjugated species-specific secondary antibodies (1:2000; Cell Signaling) and enhanced chemiluminescence (GE Healthcare). Blots were scanned (Canoscan) and densitometry was performed using Image J software.

Neurite outgrowth in primary cultures of adult DRG neurons

Primary adult DRG neurons were cultured as previously described (Yoon et al., 2013; Blesch et al., 2012). Briefly, 24-well plates were pre-coated with Poly-D-lysine hydrobromide (PDL, 20 μg/ml in water, Sigma-Aldrich, St. Louis, MO). Adult DRGs were isolated from $scLRP1^{+/+}$ and $scLRP1^{-/-}$ mice that were naïve or 72 h after receiving a conditioning sciatic nerve crush injury. The DRGs were stripped of their roots and collected in Hanks Buffered Salt Solution (HBSS) on ice. DRGs were enzymatically digested and 1000 DRG neurons were plated in each well of 24-well tissue culture plates (Thermo Scientific, Logan, UT). All DRG neurons were cultured at 37°C in 5% CO_2 for 24 hours. The DRG neurons were then fixed in 4% formaldehyde, permeabilized and blocked with PBS + 0.25% Triton X-100 and 5% donkey serum for 1 h at room temperature. Cells were subsequently incubated with the primary antibody, mouse anti-βIII-tubulin (1:2000, Promega, Fitchburg, WI), and then with Alexa Fluor-488 anti-mouse antibody (1:1000, Life Technologies, Carlsbad, CA). DRG neurons were imaged at 4× with ImageXpress (Molecular Devices, Sunnyvale, CA). For all neurite outgrowth measurements, at least 3

individual experiments were performed (n≥3 biological replicates). Each experiment had at least 3 wells per condition (n≥3 technical replicates). Technical replicates were averaged to compute values for each biological replicate. Neurite outgrowth was automatically quantified using the MetaXpress neurite outgrowth module (Molecular Devices, Sunnyvale, CA). Individual biological replicates were normalized to neurite outgrowth on PDL to account for variability between experiments. All graphs show mean values of biological replicates ± SEM.

RNA Isolation and qPCR

Adult scLRP1$^{+/+}$ and scLRP1$^{-/-}$ mice were euthanized three days after sciatic nerve crush injury. Ipsilateral and contralateral L4-L5 DRGs were removed, stripped of their roots, pooled, and collected in RNA lysis buffer on ice. Two mice (n=4 DRGs) were used for one replicate. DRGs were homogenized in lysis buffer and total RNA was extracted using the NucleoSpin® RNA kit (Macherey-Nagel). RNA was reverse-transcribed using the iScript cDNA synthesis kit (Bio-Rad). qPCR was performed using TaqMan® gene expression products and an AB Step one Plus Real-Time PCR System (Applied Biosystems), as previously described (Inoue et al., 2010; Mantuano et al., 2017). The mRNAs analyzed included: ATF3, Spirr1A, STAT3, and CREB3. The relative change in mRNA expression was calculated using the $2^{\Delta\Delta Ct}$ method and GAPDH mRNA as an internal normalizer. Forty amplification cycles were used. Control PCR reactions were performed using samples that were not exposed to reverse transcriptase to verify the absence of genomic DNA contamination.

Immunofluorescence microscopy and immunohistochemistry

Mice were anesthetized with 3% isoflurane (IsoSol; VedCo, St. Joseph MO) and subjected to intracardiac perfusion with fresh 4% paraformaldehyde in 0.1 M sodium phosphate buffer. Distal sciatic nerve was cryoprotected and cut into transverse 10–20 μm sections. Four sections per animal were analyzed. Nonspecific binding sites were blocked with 10% goat or horse-serum in 0.1% Triton X-100 and PBS for 60 min at room temperature, and then incubated with primary antibodies diluted in blocking solution overnight at 4°C. The following primary antibodies were used: anti-NF200 (1:500; Sigma) and anti-STMN2 rabbit antibody (SCG10; 1:500; Millipore). Tissue was washed in PBS and incubated with the appropriate fluorescent secondary antibodies (Alexa Fluor 488- or 594-conjugated antibodies, 1:1000; Molecular Probes) for 1 h at room temperature. For dual-labeling studies, a second set of primary and secondary antibodies was added. Preparations were mounted on slides using Pro-long Gold with DAPI for nuclear labeling (Invitrogen). Images were captured on an Olympus FluoView1000 confocal microscope.

Lumbar (L5) DRGs were cryosectioned. Twenty micrometer serial sections were labeled with NeuroTrace™ fluorescent Nissl green stain (1:200 dilution) to identify large diameter neurons and Dapi to identify nuclei. Every fifth section was analyzed by systematic random sampling that was applied to ensure unbiased estimation of neurons numbers. Large and small neurons were identified by size and morphological criteria as described (Tandrup et al. 2000; Arthur Farraj et al. 2012). Images (10x and 40x) were collected on a Zeiss LSM 880 confocal with FAST Airyscan or a Zeiss Widefield Microscope with Apotome for

Correlative Array Tomography. The fluorescence intensity was measured in both ipsilateral and contralateral DRGs.

Transmission electron microscopy

Sciatic nerves distal to crush injury sites from scLRP1$^{-/-}$ and littermate control mice were processed for plastic embedding as described previously (Orita et al., 2013). Briefly, glutaraldehyde (2.5%)/paraformaldehyde (2%)/0.1 M sodium phosphate, 150 mM NaCl, pH 7.4 (Fixation Buffer) was applied directly onto mouse sciatic nerves prior to removal. Resected tissue was further incubated in Fixation Buffer for an additional 72 h at 4°C, washed, chilled in 0.1 M cacodylate Buffer, pH 7.4, secondarily fixed in 1% osmium tetroxide/CB for 30 min, dehydrated in serially increasing concentrations of ethanol and embedded in Durcupan resin (Sigma-Aldrich). Semi-thin sections (50–60 nm) were applied to copper grids (300 mesh). The grids were viewed using a Tecnai G^2 Spirit BioTWIN transmission electron microscope (TEM) equipped with an Eagle 4k HS digital camera (FEI, Hilsboro, OR) or with a JOEL FX1200 Transmission Electron Microscope. Quantitative TEM image analysis was performed. C-fibers in Remak bundles (number of Remak bundles, SC cytoplasmic abnormalities) and myelinated fibers (fiber number, wide incisures, lamellae, myelin outfoldings, myelin infoldings) were counted in a 500 μm^2 surface area of scLRP1$^{-/-}$ and scLRP1$^{+/+}$ mice (3–5 sections per individual mouse). Nerves were imaged at 1900X and analysed using OpenLab software (Improvision, Conventry, UK).

Statistical analysis

Data are presented as the mean ± SEM. For cutaneous innervation (pinprick), toe spread, thermal hyperalgesia, and tactile allodynia studies, results were analyzed by repeated-measures ANOVA and Scheffe's *post hoc* test. For quantitative immunohistochemistry, TEM, immunoblotting and qPCR, a one-way ANOVA was performed and followed by a Tukey's of Neuman Keuls *post hoc* test when more than two groups of results were compared. A student's t-test was used when two groups were compared.

Results

Sensory recovery is accelerated in scLRP1$^{-/-}$ mice

To determine whether SC LRP1 regulates functional axonal regeneration, scLRP1$^{-/-}$ and scLRP1$^{+/+}$ mice were subjected to sciatic nerve crush injury. Recovery of nociceptive cutaneous sensitivity was assessed by applying a pinprick to the distal skin territory of the injured sciatic nerve (lateral hind paw) in mice. Typically, young adult mice completely recover sensory function within 15 days after crush injury (Ma et al., 2011). Pinprick analysis confirmed this timeline of recovery in control scLRP1$^{+/+}$ mice, as some positive responses were observed within 9 days after injury (Fig. 1A). By contrast, scLRP1$^{-/-}$ mice showed significant and accelerated sensory recovery by day 4, and continued to have a greater extent and magnitude of recovery until day 15, when both scLRP1$^{-/-}$ and scLRP1$^{+/+}$ mice achieved maximal recovery (Fig. 1A; *p<0.05; **p<0.01).

To assess whether accelerated recovery is unique to sensory fibers, we tested whether scLRP1$^{-/-}$ mice also demonstrate accelerated recovery of motor function following sciatic

nerve crush injury. We assessed fine motor function in the toes by measuring the toe-spreading reflex, which requires plantar muscle function and gait. Recovery of the toe spreading reflex was not significantly different between the two genotypes (Fig. 1B). Both groups showed relatively little recovery until 10 days after crush injury and full recovery by 3 weeks, as anticipated (Ma et al., 2011) (Fig. 1B).

Axonal regeneration after crush injury is accelerated in scLRP1$^{-/-}$ mice

Next, axonal growth was examined following sciatic nerve crush injury in scLRP1$^{-/-}$ and scLRP1$^{+/+}$ mice. Longitudinal nerve sections were collected 72 hours post injury and immunolabeled for fluorescence microscopy with the neuronal growth cone marker, stathmin-like 2 (STMN2), to identify regenerating axons. STMN2 is specific for neuronal growth cones (Chauvin and Sobel, 2015) and unlike GAP-43 is not immunoreactive with activated SCs. In scLRP1$^{-/-}$ mice, STMN2 immunoreactivity was observed in sciatic nerve tissue harvested 5–10 mm distal from the crush site 72 hours after nerve injury, whereas in scLRP1$^{+/+}$ mice, STMN2 immunoreactivity was observed only at 0–5 mm (Fig. 2A). Axon growth in scLRP1$^{-/-}$ mice appeared more highly branched. These findings suggested that axonal growth following sciatic nerve injury is accelerated in scLRP1$^{-/-}$ mice.

To confirm this result, we collected distal nerve segments from scLRP1$^{-/-}$ and scLRP1$^{+/+}$ mice 72 hours after sciatic nerve crush injury, 5–10 mm from the crush injury site, and subjected this tissue to immunoblot analysis to detect GAP-43 and STMN2. tERK1/2 and GAPDH were studied as loading controls (Fig. 2B and Suppl. Fig. 1). GAP-43 and STMN2 were detected mainly in nerve tissue harvested from scLRP1$^{-/-}$ mice (N=3, *$p<0.05$; **$p<0.01$), suggesting that growth cones had reached this distance only in these mice (Figs. 2B, C).

Next, we performed studies to detect Neurofilament-200 (NF200) in sciatic nerves downstream from crush injury sites one week after surgery. NF200 is a specific marker of neurites emerging from NT-3-responsive sensory neurons (Lawson and Waddell, 1991; McMahon et al., 1994). After nerve injury, NF200 immunopositivity indicates either residual axonal survival, which is unlikely one week after crush injury, or new axonal growth. NF200 immunoreactivity was increased in nerve sections isolated from scLRP1$^{-/-}$ mice, 5–10 mm distal from the crush injury site, compared with nerves from scLRP1$^{+/+}$ mice (Figs. 3A, B; **$p<0.01$). Immunoblot analysis, using equivalent specimens, showed that other neuronal cytoskeletal proteins were significantly increased in nerve isolates from scLRP1$^{-/-}$ mice, including neurofilament-M (NFM) and neuronal class III β-tubulin (Figs. 3C,D, *$p<0.05$). p75^{NTR}, a marker for unmyelinated SCs and SCs that adopt a repair phenotype, was significantly decreased in distal nerve from scLRP1$^{-/-}$ mice, 1 week after injury (Figs. 3C,D, ***$p<0.005$). However, GFAP levels, which serve as a biomarker of injured and unmyelinated SCs, were similar in extracts from scLRP1$^{-/-}$ and scLRP1$^{+/+}$ mice. This suggests that although *LRP1* deficiency in SCs may be associated with an increase in SC death (Campana et al., 2006), the majority of the SCs still survive following nerve injury in these mice. It is also possible that increased SC proliferation offsets cell death in scLRP1$^{-/-}$ mice.

Neurite outgrowth *ex vivo* is increased in cultured adult DRG neurons from scLRP1$^{-/-}$ mice

To study the effects of SCs on the regenerative capacity of DRG neurons, we isolated DRG neurons from naïve and injured scLRP1$^{-/-}$ and scLRP1$^{+/+}$ mice and cultured the neurons for 24 hours *ex vivo*. Mice in the "injured cohort" received a conditioning sciatic nerve lesion 72 hours prior to tissue harvesting; a timeframe that has been shown to activate axonal growth programs and to significantly increase DRG axon regeneration *in vitro* (Van der Zee et al., 2008; Hanz et al., 2003; Jenkins and Hunt, 1991). Primary DRG neurons harvested from uninjured scLRP1$^{+/+}$ mice exhibited modest neurite outgrowth. DRG neurons isolated from uninjured scLRP1$^{-/-}$ mice demonstrated significantly increased baseline sprouting compared with wild-type mice (*$p<0.05$; Fig. 4A left panel, and B).

When harvested from mice that received a conditioning injury, DRG neurons from scLRP1$^{+/+}$ mice demonstrated a significant increase in neurite outgrowth, as anticipated (Figs. 4A, right panel and C). However, the extent of neurite outgrowth remained significantly increased in neurons from scLRP1$^{-/-}$ mice. Collectively, these results demonstrate that DRG sensory neurons in scLRP1$^{-/-}$ mice are primed for accelerated regeneration even prior to sciatic nerve injury. Since the DRG neurons were cultured in isolation from SCs, we hypothesize that abnormal SCs in scLRP1$^{-/-}$ mice induce changes in DRG neuron gene expression prior to neuron isolation that are sustained.

RAG expression is increased in DRG neurons from scLRP1$^{-/-}$ mice

Next, we compared expression of genes known to function as RAGs in DRGs harvested from uninjured scLRP1$^{-/-}$ and scLRP1$^{+/+}$ mice. The mRNAs for ATF3, SPRR1A, STAT3 and CREB3 were significantly increased prior to injury in DRGs from scLRP1$^{-/-}$ mice versus scLRP1$^{+/+}$ mice, as determined by RT-qPCR (Fig. 5A; *$p<0.05$, **$p<0.01$). We also measured neuronal βIII-tubulin protein in harvested DRG tissue by immunoblot analysis; this protein is known to be associated with RAG expression in sensory neurons (Peeva et al., 2006). Accordingly, scLRP1$^{-/-}$ mice had increased levels of class βIII-tubulin prior to injury compared with scLRP1$^{+/+}$ mice (Fig. 5B; **$p<0.01$). These results support the results of our *ex vivo* neurite outgrowth studies and demonstrate that SC dysfunction in scLRP1$^{-/-}$ mice preconditions associated sensory neurons for increased axon regeneration.

To determine whether RAG expression in DRGs is further regulated after injury, we performed sciatic nerve crush injury experiments and harvested DRGs on day 3. Of the RAGs examined, only CREB3 mRNA was significantly up regulated in scLRP1$^{-/-}$ mice, compared with scLRP1$^{+/+}$ mice (Fig. 5C, *$p<0.05$; ***$p<0.001$). CREB3 is a known injury signal that is transported retrograde from the injury site (Ying et al., 2014). ATF3, a well-described RAG, was significantly up-regulated in scLRP1$^{+/+}$ mice, as anticipated (Sejiffers et al., 2007; Ma et al., 2011), however, only a trend towards increased ATF3 was observed in neurons from scLRP1$^{-/-}$ mice ($p<0.1$, data not shown). These data indicate that DRG neurons from scLRP1$^{-/-}$ mice are in a heightened growth state due to altered axonal-glia signaling prior to injury. They further indicate that altered axonal-glia signaling in scLRP1$^{-/-}$ mice leads to additional conditioning in response to injury.

Abnormal Remak bundles form during sciatic nerve regeneration in scLRP1$^{-/-}$ mice

During development, SCs in scLRP1$^{-/-}$ mice form abnormal Remak bundles (Orita et al., 2013). For the first time, we examined formation of new Remak bundles following complete sciatic nerve crush injury after 3 weeks. Transmission electron microscopy (TEM) demonstrated significantly altered Remak bundle morphology in regenerated nerves (Fig. 6A). Abnormalities in the Remak bundle included: 1) abundant Remak bundles containing only 1 axon (Fig. 6B; *p<0.05; **p<0.01 compared to scLRP1$^{+/+}$ mice); 2) fewer Remak bundles with >2 and >11 axons (Fig. 6B; *p<0.05); 3) abnormal SC cytoplasm; and 4) the presence of axonal sprouts without an intervening layer of SC cytoplasm (asterisk in Fig. 6A; Fried et al., 1993).

Overall, the total number of unmyelinated axons in scLRP1$^{-/-}$ mice was significantly decreased compared with scLRP1$^{+/+}$ mice (Fig. 6C; *p<0.05). The percentage of myelinated axons in regenerated sciatic nerves in scLRP1$^{-/-}$ mice was decreased by 30% compared with regenerated nerves in scLRP1$^{+/+}$ mice (Fig. 6D; **p<0.01). However, genotype-specific abnormalities in myelin structure (wide incisures, lamellae, myelin outfoldings, myelin infoldings) were not observed. In addition, there were no changes in the number of DRG neurons between genotypes (Fig. 6E). Collectively, these results suggest that in this mouse model system, the abnormalities in SC function resulting from *LRP1* deficiency predominantly affect unmyelinated C-fibers during nerve regeneration.

Nerve regeneration in scLRP1$^{-/-}$ mice is associated with development of neuropathic pain

Next, we tested two pain modalities: thermal hyperalgesia and tactile allodynia. Thermal hyperalgesia is mediated mostly by heat-nociceptive C-fibers whereas tactile allodynia is mediated mostly by myelinated fibers after peripheral nerve injury (Shir and Seltzer, 1990). Immediately sciatic nerve after crush injury, proprioception is entirely absent (Fig. 1A; Ma et al., 2011); therefore we began testing at day 7. In studies to identify thermal hyperalgesia, we applied a focal heat source to the ipsilateral hindpaw of scLRP1$^{+/+}$ and scLRP1$^{-/-}$ mice. Prior to surgery, baseline withdrawal values were approximately 5.5 seconds for both genotypes (Fig. 7A). One-week post-crush injury, scLRP1$^{+/+}$ and scLRP1$^{-/-}$ mice both responded to the heat source although the mean withdrawal thresholds remained higher (less responsiveness) compared with baseline measurements. By three weeks, we observed reduced thermal withdrawal latencies in scLRP1$^{-/-}$ mice, and by four weeks, scLRP1−/− mice demonstrated significantly increased thermal hyperalgesia, compared with wild-type mice that were allowed to recover from crush injury for four weeks (Fig. 7A; *p<0.05).

To evaluate tactile allodynia, we used von Frey filaments. Baseline withdrawal thresholds were approximately 2 grams in both genotypes. One week after crush injury, all scLRP1$^{+/+}$ mice exhibited loss of tactile sensation compared with baseline values (thresholds > 6 gms cut off; Fig. 7B), consistent with results presented in Fig. 1A. scLRP1$^{-/-}$ mice also exhibited a loss of tactile sensation compared with baseline values; however, these mice were significantly more responsive than wild-type mice (thresholds <4 gms; **p<0.01), probably reflecting a faster sensory neuron regeneration, consistent with the data presented in Figure 1A. At later time points, both genotypes demonstrated tactile allodynia (withdrawal thresholds significantly lower than in baseline testing); however, the two genotypes were not

significantly different. Reduced tactile withdrawal thresholds have been previously reported in wild type mice following crush injury (Vogelaar et al., 2004).

Discussion

It is now apparent that in response to nerve injury, a system of genes is activated in sensory neurons; these genes control the nerve regeneration process (Chandran et al., 2016). In the present study, we provide evidence that SCs in peripheral nerves specifically regulate gene expression in sensory neurons in DRGs. We further show that when SCs are abnormal, gene expression by DRG neurons may be altered. In scLRP1$^{-/-}$ mice, conditional deletion of *LRP1* in SCs is associated with activation of genes in sensory neurons that have previously been described as RAGs. Even prior to nerve injury, sensory neurons in scLRP1$^{-/-}$ mice appear to be primed for regeneration. This result may be interpreted as indicating that abnormalities in SCs in scLRP1$^{-/-}$ mice are recognized by sensory neurons as a form of injury to the peripheral nerve, even prior to surgical intervention.

LRP1 is expressed at increased levels in SCs after nerve injury. For this reason, we have focused most of our energy on identifying how this receptor regulates the SC response to injury in scLRP1$^{-/-}$ mice. However, even prior to nerve injury, scLRP1$^{-/-}$ mice demonstrate abnormal Remak bundle architecture, which may be responsible, at least in part, for the changes in sensory nerve gene expression described here. When sensory neurons are cultured *ex vivo*, neurite outgrowth is increased even without sciatic nerve injury. Accordingly, the level of neuronal βIII-tubulin protein is increased in extracts of uninjured DRGs isolated from scLRP1$^{-/-}$ mice. Neuronal βIII-tubulin is expressed selectively by neurons and is observed in increased quantities when sensory neurons are primed for regeneration, for example after the sciatic nerve is subjected to a conditioning nerve (Han et al., 2004).

RAGs function in a cooperative manner to regulate PNS regeneration (Chandran et al., 2016) and may be viewed as a system. Individual RAGs include: *1.* ATF3, which when overexpressed in neurons in mice causes increased axonal growth and enhanced functional motor recovery following nerve injury (Ma et al., 2011); *2.* STAT3, which is an early phase axonal growth activator, known to prime injured axons (Bareyre et al., 2011) and induce GAP-43 expression (Qiu et al., 2005); and *3.* CREB3, which is required for axonal outgrowth of DRG neurons *in vitro* and *in vivo* (Ying et al., 2015). We hypothesize that increased expression of RAGs in sensory neurons prior to nerve injury may be a result of a changed SC-axon environment. LRP1 is a robust endocytic and cell signaling receptor (Gonias and Campana, 2014). In the absence of LRP1, the magnitude and extent of mediators is likely altered, thus contributing to an environment that may mimic an “injured” milieu. Enhanced RAG expression prior to injury may be responsible for the increased rate of axonal regeneration observed in scLRP1$^{-/-}$ mice following sciatic nerve injury.

Three days after sciatic nerve injury, CREB3 expression was significantly increased in DRG sensory neurons and the increase in CREB3 was significantly greater in DRGs from scLRP1$^{-/-}$ mice. CREB3 is a RAG that localizes to the endoplasmic reticulum of axons and, in response to injury, translocates in a retrograde manner to the sensory neuron nucleus

(Ying et al., 2014). Once in the nucleus, CREB3 supports axonal outgrowth (Ying et al., 2015). Increased CREB3 mRNA expression in sensory neurons in response to sciatic nerve injury in wild-type mice is previously reported. Our results demonstrating a significantly more robust increase in CREB3 mRNA in scLRP1$^{-/-}$ mice suggests that this transcription factor may contribute to the mechanism by which neuronal regeneration is accelerated in these mice. The initial location of CREB3 in axons places it in an intriguing position to sense changes in axonal-SC interactions. Also, CREB activation has been closely associated with the development of thermal hyperalgesia (Miletic et al., 2002) and is consistent with our findings herein.

The more rapid regeneration of sensory neurons in scLRP1$^{-/-}$ mice was associated with accelerated return of proprioception, which may be viewed as an advantage when considering human injuries. However, the increased rate of regeneration of sensory neurons in scLRP1$^{-/-}$ mice also was associated with development of chronic neuropathic pain. Possible explanations for the chronic pain include: *1)* The rate of regeneration. If this is the case, our results suggest that re-programming the timeline of events that occur following PNS injury and Wallerian degeneration may not be possible; *2)* Chronic pain that develops following nerve injury may be related to the intrinsic genetic abnormality in SCs that is inherent to the model system. We demonstrated abnormalities in the ultrastructure of reformed Remak bundles following nerve regeneration by transmission electron microscopy. In these reformed Remak bundles, adjacent nerve fibers were not consistently isolated by intervening SC cytoplasm or basal lamina. Instead, a small fraction of the axons abutted one another over part or all adjacent surfaces. These structural changes may provide a mechanism for cross-excitation that allows passage of depolarizing current directly from one axon to the next, manifesting as pain (Fried et al., 1993). Furthermore, close abutting axons are potential structural substrates for electrical discharge in injured nerves and are prevalent in pain states (Devor, 1993); *3)* An overall loss of myelinated and unmyelinated fibers and a reduction of axon numbers in Remak bundles without sensory neuron death. Prior to injury, scLRP1$^{-/-}$ mice show thinner myelin in Aδ-fibers which may contribute structurally to aberrant regeneration and eventual fiber loss (Orita et al., 2013). In Remak bundles, often only a single non-myelinated axon or small group of axons <1 μm was identified. Distal axonal degeneration, rather than sensory neuronal death, has been linked to clinically relevant small fiber neuropathies that are painful, and include those associated with diabetes and human immunodeficiency virus (HIV) (Lee-Kubli et al, 2014; Cashman and Hoke, 2015; Zochodne et al., 2001; Melli, et al., 2006). Overall, the relationship between abnormalities in SCs and development of pain states is not well characterized; however, conditional deletion of GABA-B receptors in SCs is associated with alterations in thermal withdrawal latencies indicative of hyperalgesia (Faroni et al., 2014).

We used response to pinprick to assess recovery of proprioception following sciatic nerve crush injury. It is thus important to mention that recovery of this response may be achieved by regeneration of sciatic nerve fibers or by collateral sprouting of the uninjured saphenous nerve (Devor et al., 1979; Inbal et al., 1987; Navarro et al., 2007). In our study, it is more likely that sensory recovery was due to accelerated regeneration of sciatic nerve fibers in scLRP1$^{-/-}$ mice, given our complementary data showing accelerated axon growth *in vivo* and neurite outgrowth *ex vivo*. Furthermore, within 2 weeks after crush injury, we did not

observe increased vocalization, suggesting that the saphenous nerve is not functionally expanded (Attal et al., 1994). However, it is important to note that the accelerated pinprick responses in scLRP1$^{-/-}$ mice may be associated with reduced nociceptive thresholds in structurally aberrant nerves.

In previous studies, we have examined the function of LRP1 *in vivo* and *in vitro* in cultured SCs to understand how LRP1 deficiency in SCs may affect sciatic nerve function in scLRP1$^{-/-}$ mice. LRP1 is an endocytic receptor and also a ligand-specific cell-signaling receptor (Gonias and Campana, 2014). By regulating ERK1/2, the PI3K-Akt pathway and the unfolded protein response, LRP1 supports SC survival under challenging conditions (Campana et al., 2006a; Mantuano et al., 2008; Mantuano et al., 2011). By regulating ERK1/2, Rac1, and RhoA, LRP1 promotes SC migration (Mantuano et al., 2008; Mantuano et al., 2010). Furthermore, by selectively inducing expression of CCL2/MCP-1, LRP1 may function in the pathway by which SCs in the injured PNS attract inflammatory cells (Shi et al., 2009). LRP1 also mediates phagocytosis of myelin debris (Gaultier et al., 2009; Fernandez-Casteneda et al., 2013). These are key elements of the SC Repair Program, in which the transcription factor, c-Jun, plays an important role (Arthur-Farraj et al., 2012; Jessen and Mirsky, 2016). c-Jun activation in SCs protects against loss of sensory axons in inherited neuropathy (Hantke et al., 2014). We have demonstrated phosphorylation of c-Jun directly downstream of activated LRP1 in SCs, further indicating a role of LRP1 in regulating the SC repair program (Fluetsch et al., 2016). The abnormalities in Remak bundle architecture in regenerated sciatic nerves described here are highly reminiscent of those observed in uninjured cells. This result suggests that the pathways by which LRP1 regulates Remak bundle formation by SCs in development are recapitulated in injury and potentially contribute to chronic pain states.

Our principal conclusion that the rate of axonal regeneration following PNS injury may be influenced by changes in SC function is supported by prior work by Painter et al. (2014). These investigators demonstrated changes in PNS repair in association with aging. They further showed that the intrinsic ability of axons to grow is not dependent on axonal age, but on the age of the SCs. Molecular changes in SCs, either as a result of genetic alterations or aging, clearly deserve further attention as key factors that may determine whether recovery from PNS injury is functional or not.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Main Points

Expression of regeneration-associated genes (RAGs) by sensory neurons is increased in PNS injury. We show that Schwann cells regulate neuronal RAG expression. Abnormal regulation results in ultrastructural abnormalities in the nerve and pain states.

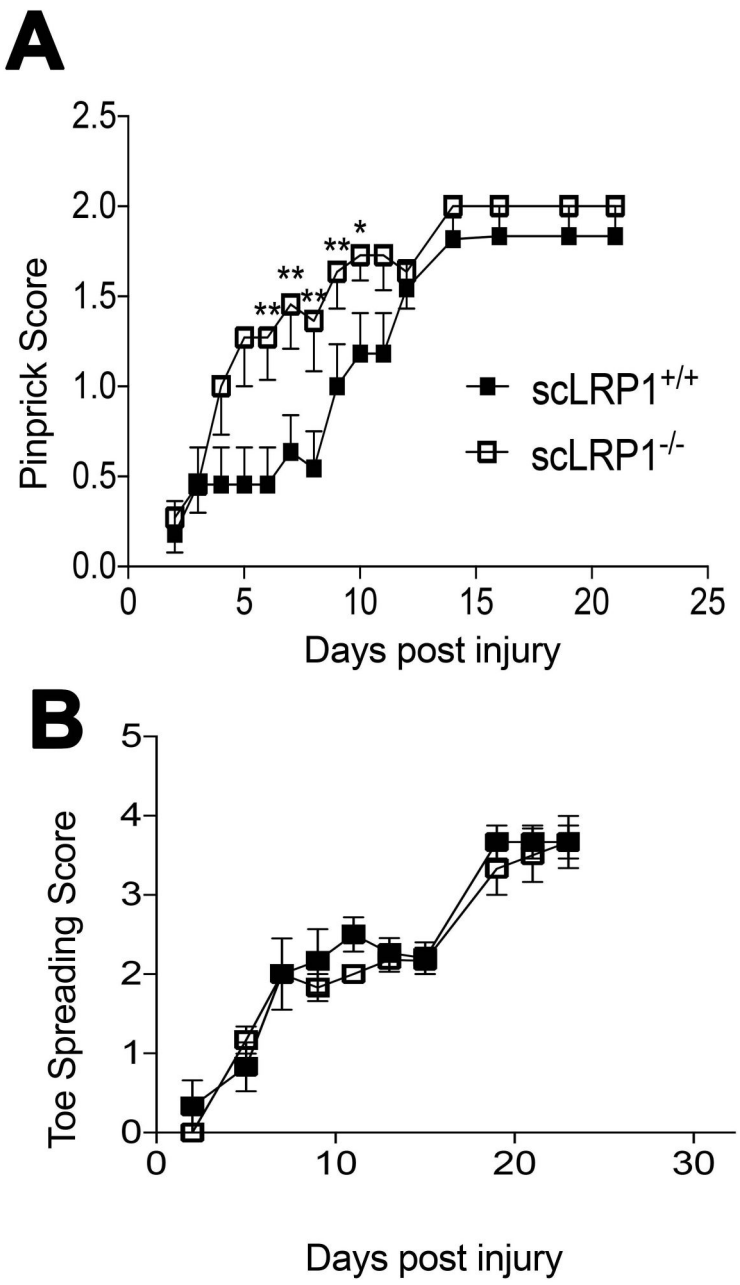

Figure 1. Genetic deletion of LRP1 in Schwann cells accelerates functional sensory recovery

(**A**) Cutaneous innervation was assessed by pinprick scores of right hind paw post sciatic nerve crush from scLRP1$^{+/+}$ and scLRP1$^{-/-}$ mice. (**B**) No difference in toe spreading reflex was evident between scLRP1$^{+/+}$ and scLRP1$^{-/-}$ mice. Scores were measured up to 23 days post crush. Data are mean±SEM (n=6–11 mice per genotype, scLRP1$^{+/+}$ and scLRP1$^{-/-}$). *p<0.05, **p<0.01 repeated measures ANOVA and Schefe *post hoc* test.

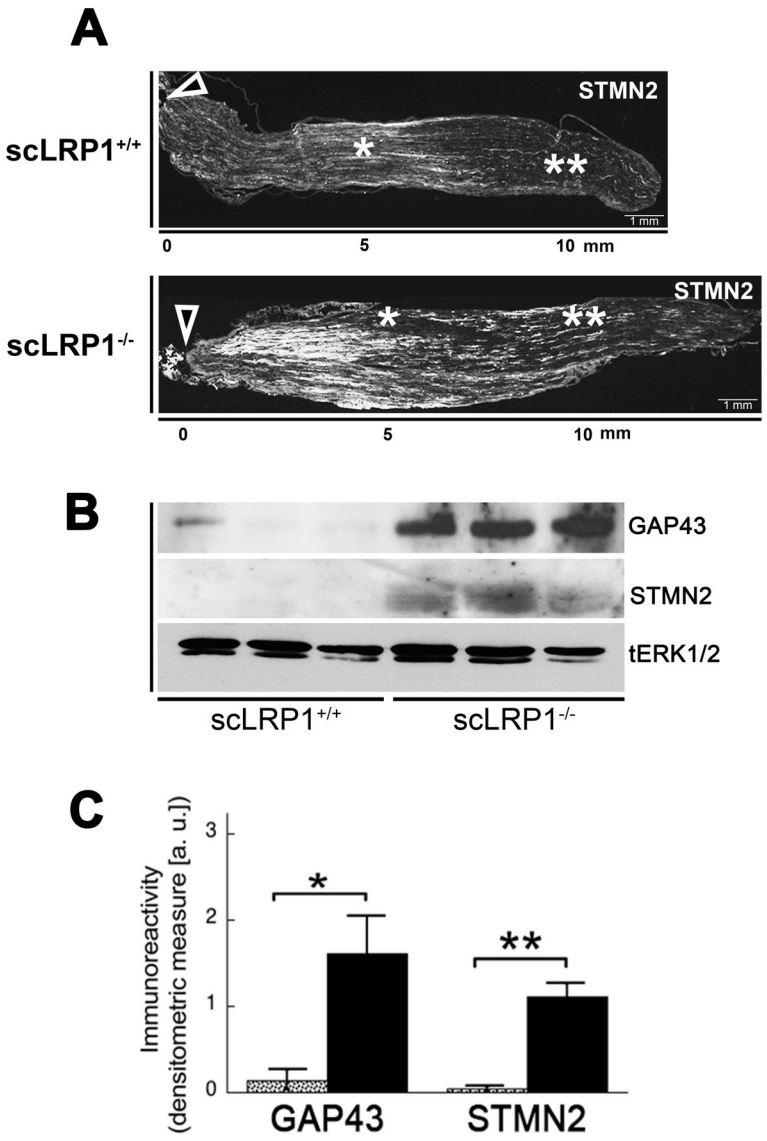

Figure 2. Conditional deletion of LRP1 in Schwann cells enhances early axonal growth after crush injury *in vivo*

(**A**) STMN2 (SCG10) immunoreactivity in longitudinal sections of sciatic nerve 72 h after crush injury in scLRP1$^{+/+}$ and scLRP1$^{-/-}$ mice. Distance from the nerve injury site (arrow) at 5 mm (*) and 10 mm (**). Scale bar: 1 mm. (**B**) Immunoblot of growth cone markers, GAP43 and STMN2 and loading control, (tERK1/2) in nerve sections 5–10mm from the crush sites after 72 h. (**C**) Quantification of growth cone markers. Data represent mean ±SEM (n=3 individual mice; *p<0.05; **p≤0.01).

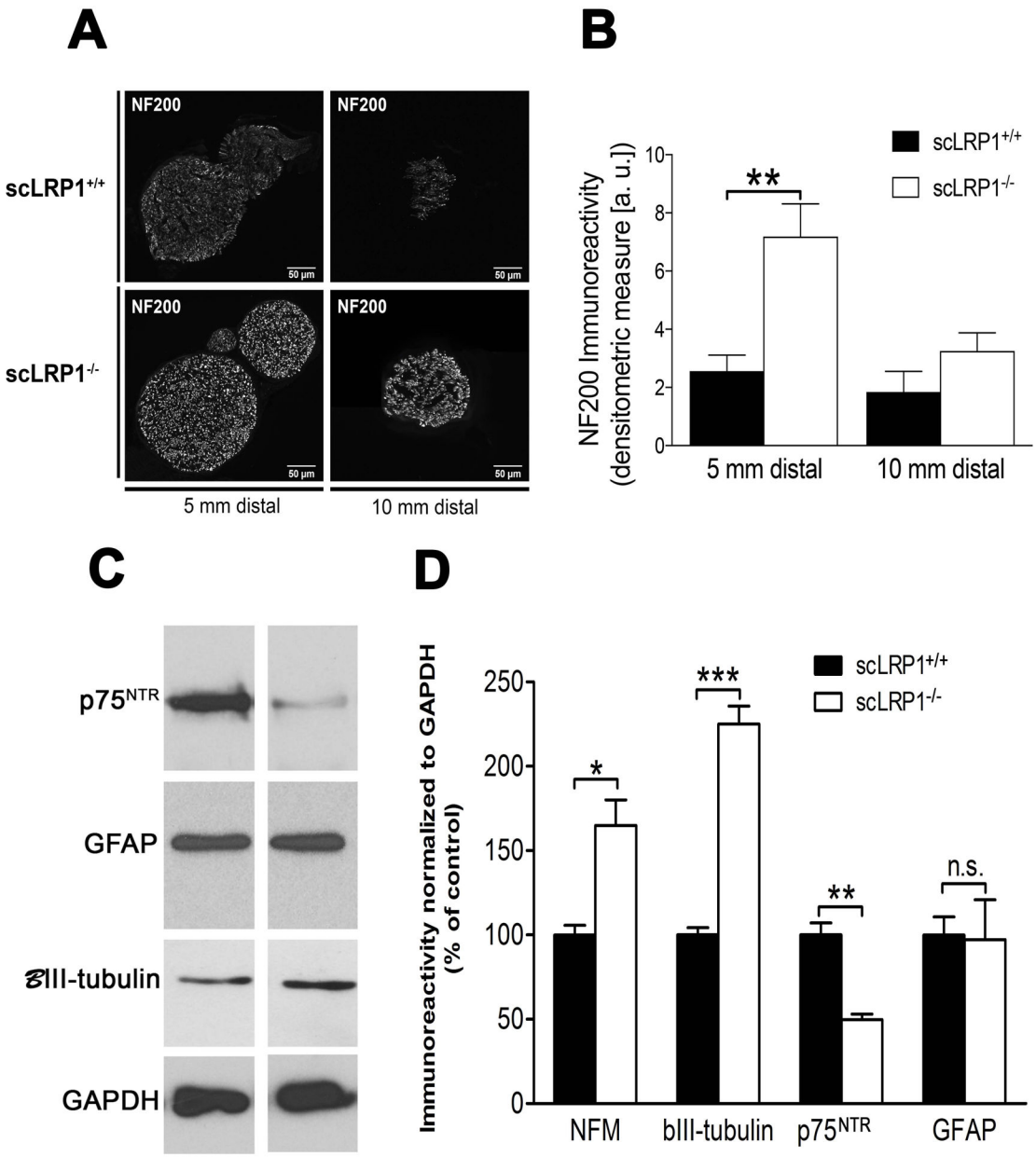

Figure 3. Neuronal cytoskeletal components, Neurofilament 200 (NF200) and βIII neuronal tubulin (Tuji), are increased, yet p75NTR, a SC nerve repair marker, is decreased after crush injury in scLRP1$^{-/-}$ mice

(**A**) NF200 immunoreactivity in transverse sections of distal sciatic nerve (5 and10 mm) one week after crush. Scale bar: 50 µM. (**B**) Quantitation of NF200-immunoreactive axons in 5-µm-thick transverse sciatic nerve sections (n=8/group; **p<0.01). (**C**) Representative immunoblot analysis of neuronal βIII-tubulin, NFM, p75^{NTR} and GFAP one week post injury. GAPDH is used as a loading control. (**D**) Densitometric measurement of immunoblot in (**C**) βIII-tubulin, NFM, p75^{NTR} and GFAP normalized to GAPDH one week post injury. Data represent mean±SEM (n=4 mice per genotype; *p<0.05; *** p<0.001).

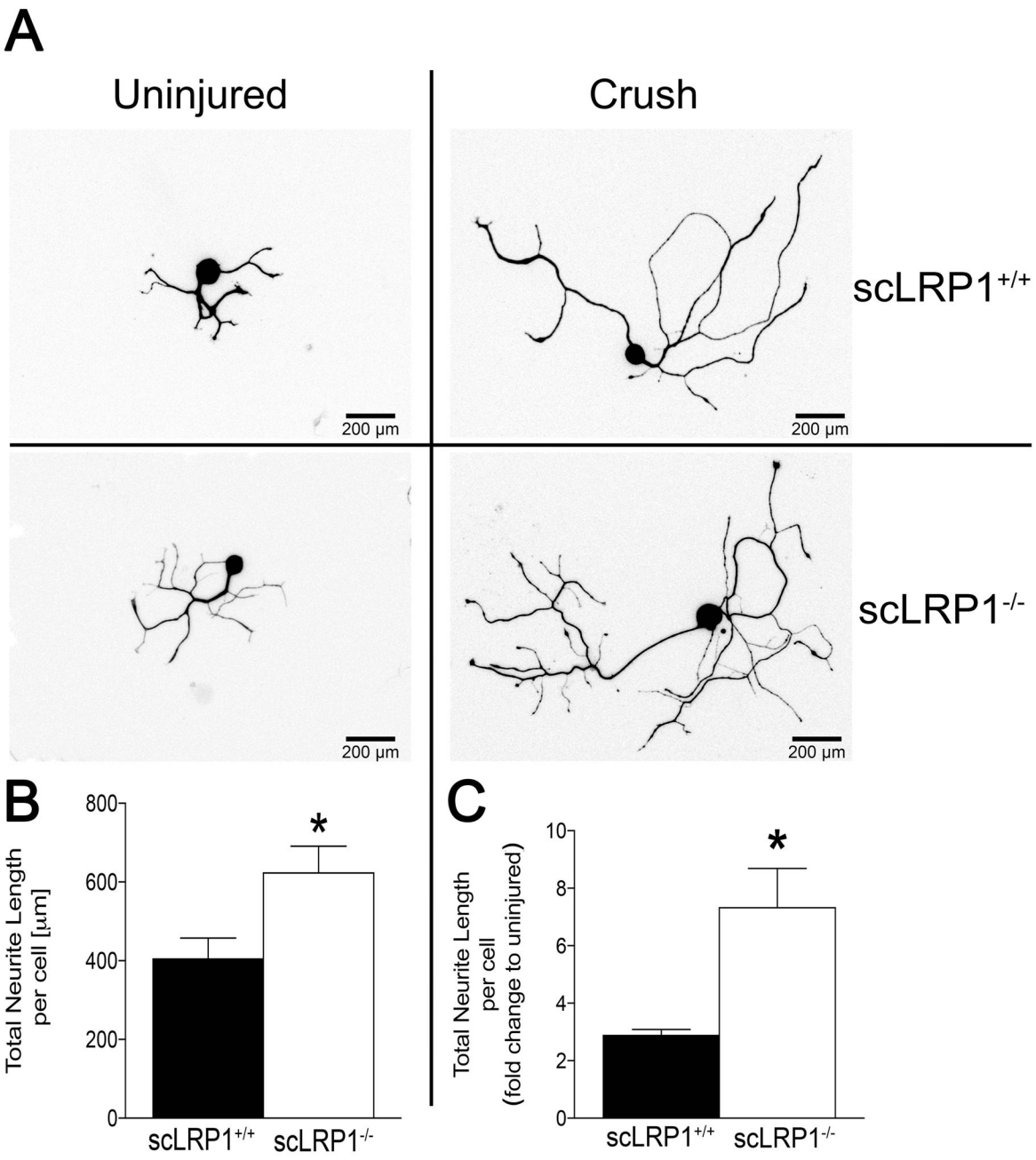

Figure 4. scLRP1$^{-/-}$ DRG neurons demonstrate increased neurite outgrowth *in vitro*

Adult DRG neurons were cultured for 24 hours from either scLRP1$^{+/+}$ or scLRP1$^{-/-}$ mice that were uninjured or injured (sciatic nerve crush) 3 days prior to plating. (**A**) Representative images of DRG neurons from uninjured scLRP1$^{+/+}$ mice (left, upper panel) and scLRP1$^{-/-}$ mice (left, lower panel) and after preconditioning injury, scLRP1$^{+/+}$ mice (right, upper panel) and scLRP1$^{-/-}$ mice (right, lower panel). Scale bar 200 µm. Quantification of βIII-tubulin immunopositivity shows total neurite length per cell in (**B**) uninjured and (**C**) fold increase in injured DRG neurons from scLRP1$^{+/+}$ and scLRP1$^{-/-}$

mice after 24 hours *in vitro.* Data represent mean±SEM (n=2–8 mice per genotype and 3 wells per condition; *p<0.05 two tailed T-test).

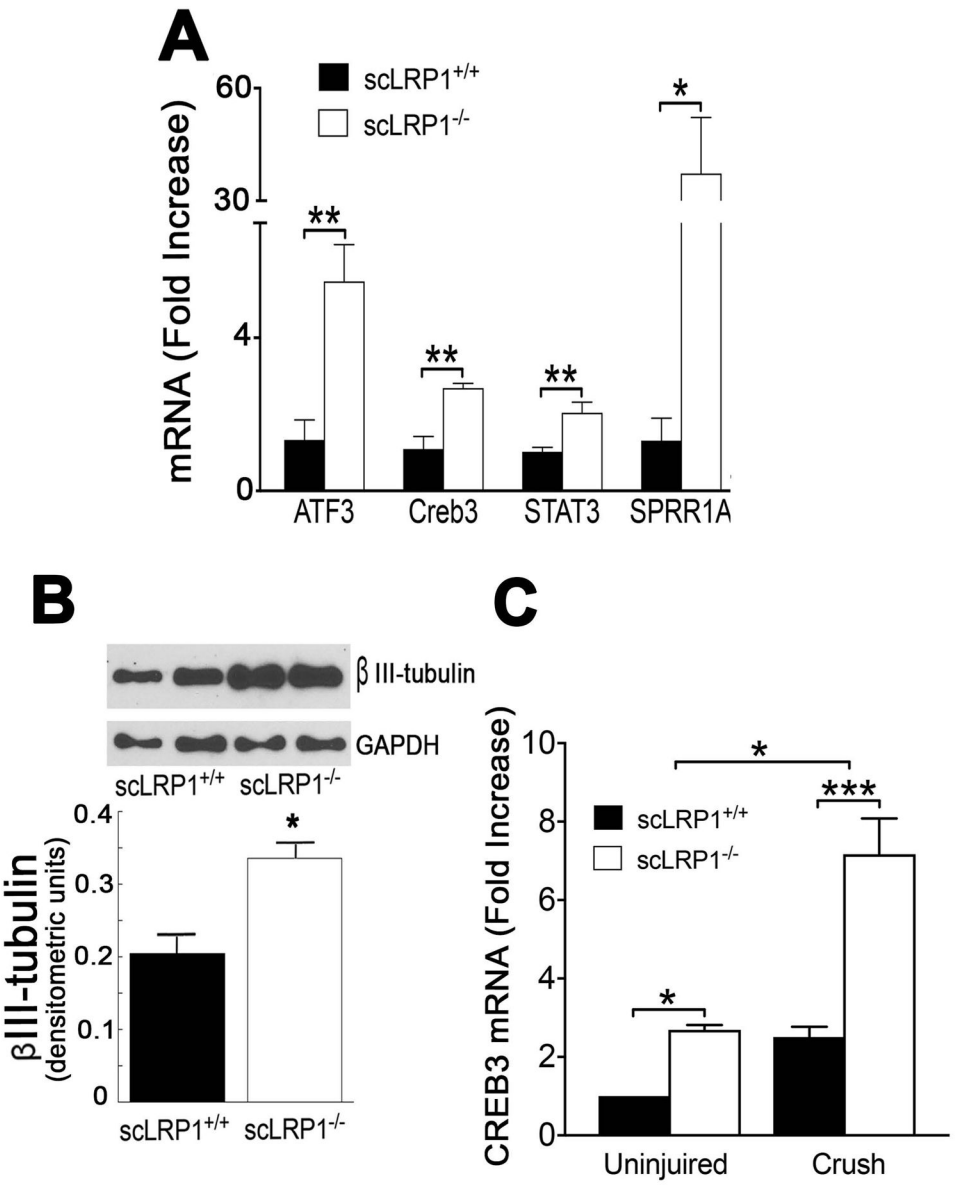

Figure 5. qPCR analysis of dorsal root ganglions (DRGs) from scLRP1$^{-/-}$ and scLRP1$^{+/+}$ mice show upregulation of regeneration associated genes (RAGs) before and after injury

Taqman qPCR analysis of (**A**) uninjured DRGs showing CREB3, ATF3, STAT3 and SPRR1A in scLRP1$^{-/-}$ mice (n=3–5 mice per genotype; *p<0.05; **p<0.01, two tailed t-test). (**B**) Immunoblot showing increased βIII neuronal tubulin in scLRP1$^{-/-}$ mice. GAPDH is used as a loading control. Quantification of increased βIII neuronal tubulin in scLRP1$^{-/-}$ mice (n=2–3 mice per genotype; **p<0.01, two tailed t-test). (**C**) Fold change of CREB3 mRNA in DRGs before and 3 days post sciatic nerve crush injury. mRNA expression in scLRP1$^{-/-}$ and scLRP1$^{+/+}$ DRGs is normalized to uninjured scLRP1$^{+/+}$ expression. Data represent mean±SEM. (n=3–5 mice per genotype; *p<0.05, ***p<0.001 one-way ANOVA with a Neuman Keuls *post hoc* analysis).

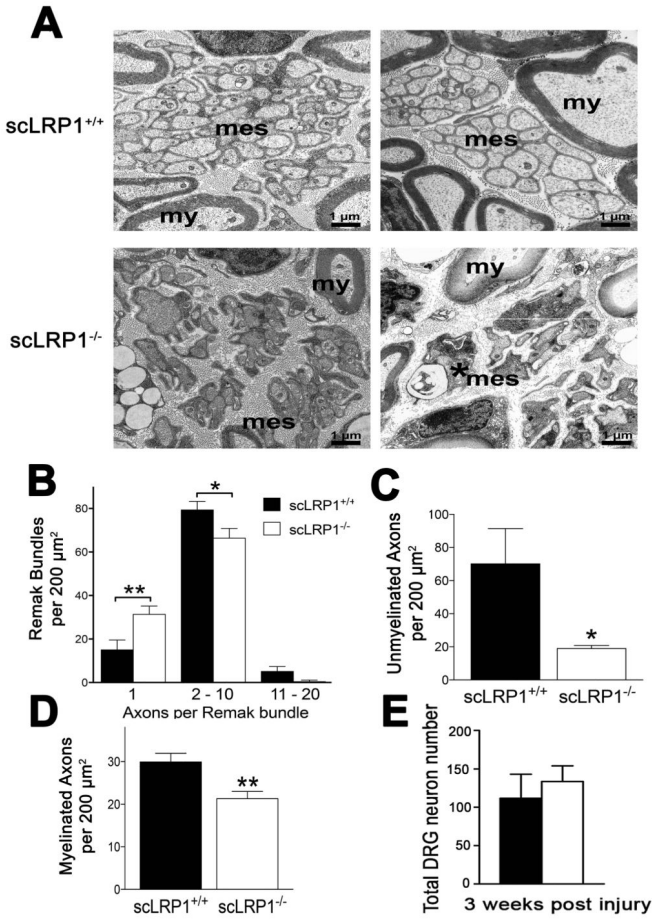

Figure 6. Aberrant Remak bundle formation in scLRP1$^{-/-}$ distal nerve after sciatic nerve crush injury

Transverse section of nerve showing unmyelinated axons (Remak bundle) three weeks after crush injury. Each of the individual axons in this bundle is compartmentalized by a mesaxonal process (mes) provided by the central Schwann cells. Segments of myelinated axons (my) are visible in the corners. 1900 X. Bundles of non-myelinated axons including those manifesting a neuroma phenotype are identified by * (axon that appose each other; axon sprouts) are shown in scLRP1$^{-/-}$ mice. Although the Schwann cell has produced some mesaxonal cytoplasm, many axons are in close opposition (asterisks). 4800 X. Quantification of (**B**) the axon numbers in Remak bundles (n=3–4 mice per genotype; *p<0.05, **p<0.01 one-way ANOVA with Tukeys post hoc); (**C**) total number of unmyelinated axons; (**D**) total number of myelinated axons; and (**E**) total number of DRG neurons. Data represent mean±SEM (n=3–4 mice per genotype; *p<0.05, **p<0.01, two tailed T-test).

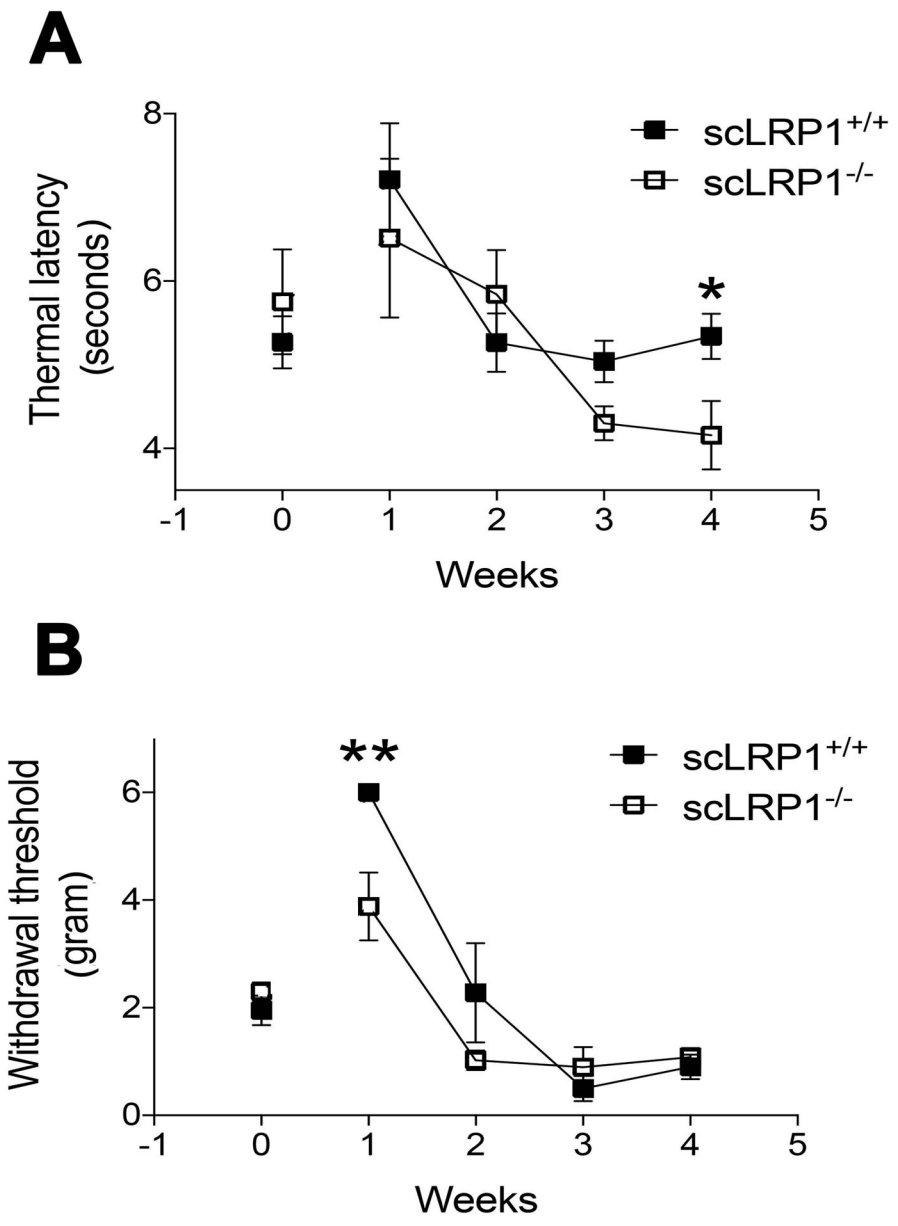

Figure 7.

Thermal and tactile allodynia develop in scLRP1$^{-/-}$ mice. (**A**) Thermal withdrawal latencies and (**B**) tactile withdrawal thresholds were evaluated in female scLRP1$^{+/+}$ and scLRP1$^{-/-}$ mice over 4 weeks. Data represent mean±SEM (n=4–7 mice group *p<0.05, **p<0.01 compared to sham control and naïve by two way repeated ANOVA followed by a Scheffe's test).