

Neuron-specific RNA-sequencing reveals different responses in peripheral neurons after nerve injury

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Abstract

Peripheral neurons are heterogeneous and functionally diverse, but all share the capability to switch to a pro-regenerative state after nerve injury. Despite the assumption that the injury response is similar among neuronal subtypes, functional recovery may differ. Understanding the distinct intrinsic regenerative properties between neurons may help to improve the quality of regeneration, prioritizing the growth of axon subpopulations to their targets. Here, we present a comparative analysis of regeneration across four key peripheral neuron populations: motoneurons, proprioceptors, cutaneous mechanoreceptors, and nociceptors. Using Cre/Ai9 mice that allow fluorescent labelling of neuronal subtypes, we found that nociceptors showed the greater regeneration after a sciatic crush, followed by motoneurons, mechanoreceptors and, finally, proprioceptors. By breeding these Cre mice with Ribotag mice, we isolated specific translates and defined the regenerative response of these neuronal subtypes after axotomy. Only 20% of the regulated genes were common, revealing a diverse response to injury among neurons, which was also supported by the differential influence of neurotrophins among neuron subtypes. Among differentially regulated genes, we proposed MED12 as a specific regulator of the regeneration of proprioceptors. Altogether, we demonstrate that the intrinsic regenerative capacity differs between peripheral neuron subtypes, opening the door to selectively modulate these responses.

eLife assessment

The **valuable** findings in this study show that subpopulations of peripheral sensory neurons display different capacities for regeneration after a similar injury. Nociceptor neurons have greater regeneration over mechanoreceptor, proprioceptors and motor neurons. This differential responsiveness of neuronal subtypes was traced to activation of different transcriptional programs, which were carefully analyzed and quantitated, resulting in **solid** evidence for the conclusions.

Introduction

Peripheral nerve injuries result in the loss of motor, sensory and autonomic function of denervated targets. Although peripheral neurons can regenerate after injury, most nerve lesions result in an incomplete functional recovery. Strategies to promote regeneration often do not take into account the heterogeneity of peripheral neurons. Motor and sensory neurons are very different in terms of function, molecular identity, and their response to injury. Moreover, peripheral sensory neurons have a wide range of functions and target organs, and single-cell RNA sequencing analyses have distinguished up to 11 subtypes in dorsal root ganglia (DRG) (Usoskin et al., 2015 [DOI](#); Renthall et al., 2020 [DOI](#)). Whereas the majority of DRG sensory neurons are cutaneous afferents, some innervate muscle (mainly proprioceptors) or other organs. Since neuron subtypes differentially respond to environmental cues and might activate distinct intrinsic regenerative programs, there is a need to investigate the regenerative response of specific neuron subtypes after a nerve injury.

Attempts to study variations in axon regeneration among subtypes of peripheral neurons have yielded some contradictory results. The finding that sensory neurons regenerate faster than motoneurons has robust evidence (Dolenc and Janko, 1976 [DOI](#); Madorsky et al., 1998 [DOI](#); Suzuki et al., 1998 [DOI](#); Kawasaki et al., 2000 [DOI](#); Negredo et al., 2004 [DOI](#); Brushart et al., 2020 [DOI](#)). In addition, unmyelinated fibers have been described to recover their function earlier than myelinated fibers (Navarro et al., 1994 [DOI](#)). In contrast, alternative work suggests that myelinated fibers regenerate at the same speed as unmyelinated fibers (Lozeron et al., 2004 [DOI](#); Moldovan et al., 2006 [DOI](#)) or even that motoneurons regenerate better than sensory neurons (da Silva et al., 1985 [DOI](#)). Given that sensory neurons comprise both myelinated and unmyelinated fibers, it is important to clarify the distinctions involving regeneration of different neuron subtypes.

After axotomy, peripheral neurons activate several signaling mechanisms such as the Ras/Raf/MAPK pathway (Sheu et al., 2000 [DOI](#); Obata et al., 2003 [DOI](#); Agthong et al., 2006 [DOI](#)), the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) pathway (Kimpinski and Mearow, 2001 [DOI](#); Murashov et al., 2001 [DOI](#); Edström and Ekström, 2003 [DOI](#)), the c-Jun N-terminal kinase (JNK)/c-Jun pathway (Kenney and Kocsis, 1998 [DOI](#)), or the cAMP/protein kinase A (PKA)/cAMP responsive element binding protein (CREB) pathway (Gao et al., 2004 [DOI](#); Chierzi et al., 2005 [DOI](#)). All these signaling pathways activate regeneration associated genes, including *Gap43* (Van der Zee et al., 1989 [DOI](#); Kawasaki et al., 2018 [DOI](#); Mason et al., 2022 [DOI](#)). This process enables neurons to switch to a pro-regenerative state in which axon regrowth is permitted. However, as neuron subtypes are thought to regenerate at different rates, the transcriptional regeneration mechanism activated by these neurons might be expected to differ. In fact, some regeneration-associated pathways have been shown to have specific relevance in neuron subtypes. For instance, medium-to-large DRG neurons show an increased activation of extracellular signal-regulated protein kinase (ERK) compared to small DRG neurons (Obata et al., 2003 [DOI](#), 2004 [DOI](#)). Importantly, the RhoA/Rho-kinase (ROCK) pathway may have a different role in motor and sensory regeneration given evidence that its inhibition enhances motor but not sensory axon regeneration (Joshi et al., 2015 [DOI](#)). Understanding the intrinsic regenerative differences between neuron subtypes may allow fine tuning of the specific regeneration of neuron subtypes, prioritizing the growth or guidance of specific subpopulations towards their target organs. Overall, neuron regeneration involves several levels of complexity driven by a large ensemble of molecules that may be challenging to differentiate among subtypes.

To address these challenges, we used two lines of genetically engineered mice that allowed interrogation of regenerative properties among peripheral neuron subtypes. Firstly, we described the regeneration of specific neurons after injury using TdTomato Cre reporter mice (Ai9). Breeding these to mice expressing Cre recombinase under the control of specific promoters allowed us to

study axonal regeneration in motoneurons (Choline acetyltransferase; *Chat*), proprioceptors (Parvalbumin; *Pvalb*), cutaneous mechanoreceptors (Neuropeptide Y receptor Y2; *Npy2r*) and nociceptors (Transient receptor potential vanilloid 1, *Trpv1*). We found significant differences in axonal regeneration of these neurons that suggested distinct intrinsic regenerative mechanisms. Secondly, we explored the neuron-specific gene expression of these neurons after a sciatic nerve injury *in vivo*. Cre-driver animals were crossed to Ribotag mice (Sanz et al., 2009 [↗](#)), which express a modified ribosomal protein L22 (Rpl22) in a Cre-dependent manner. Through immunoprecipitation assays, ribosomes and the associated mRNA from specific cell populations can be isolated and sequenced. Using this approach, we were able to describe the gene expression patterns of motoneurons, proprioceptors, mechanoreceptors, and nociceptors after a nerve injury. Amidst this approach and data, we explored the role of one of the differentially expressed transcripts, Mediator complex subunit 12 (*Med12*), a unique and unexplored protein, as a specific regulator of the regeneration of proprioceptors.

Methods

Animals

All experimental procedures were approved by the Universitat Autònoma de Barcelona Animal Experimentation Ethical Committee and followed the European Communities Council Directive 2010/63/EU and the Spanish National law (RD 53/2013). Mice were generated by breeding homozygous Ai9(RCL-tdT) mice (JAX stock #007909) (Madisen et al., 2010 [↗](#)) with different homozygous Cre-driver lines from The Jackson Laboratory (Bar Harbor, Maine, USA): ChAT-IRES-Cre (Choline acetyltransferase, JAX stock #006410) (Rossi et al., 2011 [↗](#)), B6 PVcre (Parvalbumin, JAX stock #017320), Npy2r-IRES-Cre (Neuropeptide Y receptor Y2, JAX stock #029285) (Barrozo et al., 2016 [↗](#)), and TRPV1-Cre (Transient receptor potential vanilloid 1, JAX stock #017769) (Cavanaugh et al., 2011 [↗](#)). We obtained four mice lines that expressed the red fluorescent protein TdTomato under the control of a specific neuronal promoter: ChAT-Cre/Ai9, PV-Cre/Ai9, Npy2r-Cre/Ai9, and TRPV1-Cre/Ai9, respectively. The same Cre-driver lines were bred to homozygous Ribotag mice (Sanz et al., 2009 [↗](#)). We obtained mice that expressed HA-tagged ribosomes in specific cell populations: ChAT-Cre/Ribotag, PV-Cre/Ribotag, Npy2r-Cre/Ribotag and TRPV1-Cre/Ribotag, respectively. Mice were housed in a controlled environment (12-hour light-dark cycle, 22±2°C), in open cages with water and food *ad libitum*.

Histological characterization of TRPV1-Cre/Ai9 mice

Three adult mice (8-12 weeks old) were euthanized with intraperitoneal sodium pentobarbital (30 mg/kg) and perfused with 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS). Lumbar DRGs and footpads were harvested and stored in PBS containing 30% sucrose at 4°C for later processing. DRGs were serially cut in a cryostat (15 µm thick) and picked up on glass slides, whereas footpads were cut (50 µm thick) and stored free-floating. Samples were hydrated with PBS for 10 minutes and permeabilized with PBS with 0.3% triton (PBST) for 10 minutes twice. Sections were blocked with 10% normal donkey serum in PBST for 1 hour at room temperature and then incubated with the primary antibody in PBST overnight at 4°C (Table 1 [↗](#)). Sections were washed with PBST three times and further incubated with a specific secondary antibody bound to Alexa 488 (1:200, Invitrogen) or Cy5 (1:200, Jackson ImmunoResearch) for 2 hours at room temperature (DRGs) or overnight at 4°C (skin). For IB4 immunostaining, an overnight incubation at 4°C with anti-lectin I (1:500; Vector, #AS2104) was done prior to the secondary antibody incubation. Finally, after three more washes in PBS, slides were mounted with Fluoromount-G mounting medium (Southern Biotech, 0100-01) and imaged with a confocal microscope (Leica SP5). In the DRG slices, we counted the number of sensory cells TdTomato-positive (TdTomato⁺) and how many of them co-labelled with the different markers using ImageJ software. We counted at least 929 cells per animal. For size distribution, we contoured 100 neurons of each type using ImageJ software. Skin was analyzed qualitatively, looking for the presence and distribution of fluorescence.

Sample	Thickness	Immunofluorescence
DRG	15 μ m	Parvalbumin (1:1000; Swant #PV-28)
		Neurofilament H (1:1000; Biolegend #801701)
		CGRP (1:200; Sigma-Aldrich #PC205L)
		Calbindin D-28K (1:200, Millipore #AB1778)
		Isolectin B4 (10 μ g/mL; Vector #L-1104)
		Anti-lectin I (1:500; Vector #AS2104)
Skin	50 μ m	PGP 9.5 (1:500; Springbio #E3340)

Table 1.

Antibodies used for histological validation of TRPV1-Cre/Ai9

Regeneration rate

Fifty-six adult mice (30 males and 26 females, 7- to 12-weeks old) were used for establishing the regeneration rate of the four different neuron subpopulations. Mice were anesthetized with intraperitoneal ketamine (90 mg/kg) and xylazine (10 mg/kg) and the right sciatic nerve was exposed through a gluteal muscle-splitting incision. Nerves were crushed 3 mm distal to the sciatic notch with fine forceps (Dumont no. 5) applied for 30 seconds. The lesion site was labelled with an epineural suture stitch (10-0 nylon suture, Alcon) and the muscle and the skin were closed (6-0 nylon suture, Aragó). Animals were monitored periodically until the end of the experiments. After 7 or 9 days, mice were euthanized with intraperitoneal pentobarbital (30 mg/kg) and perfused with 4% PFA in PBS (n=7 for each day and Cre/Ai9 mice). The right sciatic nerve and its extension as tibial nerve were harvested and stored in PBS with 30% sucrose. We also collected some contralateral nerves as controls (n=6 ChAT-Cre/Ai9, n=3 PV-Cre/Ai9, n=3 Npy2r-Cre/Ai9, n=3 Trpv1-Cre/Ai9). A segment from 12 mm to 17 mm from the lesion site was serially cut in 10 μ m longitudinal sections in a cryostat (Leica) and picked up in glass slides. The slides were mounted with Fluoromount-G mounting medium (SouthernBiotech, #0100-01) and visualized in an epifluorescence microscope (Nikon Eclipse Ni-E, Nikon, Tokyo, Japan) equipped with a digital camera (Nikon DS-RiE, Nikon, Tokyo, Japan) and Nikon NIS-Element BR software (version 5.11.03, Nikon, Tokyo, Japan). Using the software, we drew a line perpendicular to the nerve and counted the number of axons that crossed the line at 12 and 17 mm from the injury in one out of three sections.

In vitro neurite extension

Explants and organotypic cultures were obtained from postnatal mice (p7-p8) as previously described (Allodi et al., 2011 [DOI](#); Bolívar et al., 2021 [DOI](#)). Round coverslips were placed in 24-well plates and coated with poly-D-lysine (10 μ g/mL) overnight at 37°C. Then, coverslips were washed three times with sterile distilled water and dried at room temperature. A 25 μ L drop of collagen matrix composed by rat tail type I collagen solution (3.4 mg/mL; Corning, #354236) with 10% of minimum essential medium 10x (Gibco, #11430030) and 0.4% sodium bicarbonate (7.5%; Gibco, #25080-094) was placed on top of each coverslip. Plates were kept in the incubator at 37°C and 5% CO₂ for at least 2 h to induce collagen gel formation.

PV-Cre/Ai9, Npy2r-Cre/Ai9, and TRPV1-Cre/Ai9 postnatal mice were sacrificed with intraperitoneal pentobarbital (30 mg/kg) and their lumbar DRGs were dissected and placed in cold Gey's solution (Sigma, #G9779) supplemented with 6 mg/mL glucose. Connective tissue was eliminated and DRGs were placed in the previously prepared collagen matrices. An additional 25 μ L drop of collagen matrix was applied to cover the samples and the plates were left in the incubator for 45 minutes. Then, Neurobasal medium (Gibco, #21103049) supplemented with 1x B27 (Gibco, #17504044), 1x Glutamax (Gibco, #35050-038), 6 mg/mL glucose and 1x penicillin/streptomycin (Sigma, #P0781) was added and plates were incubated at 37°C and 5% CO₂. After 2 days in culture, explants were fixed with warm 4% PFA in PBS for 30 minutes at room temperature. After washing with tris-buffered saline (TBS), coverslips were detached from the plate and mounted onto glass slides using Fluoromount-G medium (Southern Biotech, #0100-01) and imaged with a confocal microscope (Leica SP5). All neurites in each DRG were semi-automatically measured in ImageJ, using the plugin SNT (Simple neurite tracer). Each individual DRG explant was treated as an experimental unit.

Cre/Ribotag immunofluorescence

Control mice ChAT-Cre/Ribotag, PV-Cre/Ribotag, Npy2r-Cre/Ribotag and TRPV1-Cre/Ribotag were perfused with 4% PFA in PBS. Lumbar DRGs were removed and stored in PBS containing 30% sucrose at 4° for later processing. A lumbar segment of the spinal cord from ChAT-Cre/Ribotag mice was harvested and post-fixed in 4% PFA in PBS before storage. Samples were cut in a cryostat

(15 μm thick) and processed for immunofluorescence as described above. Tissues were first incubated with rabbit anti-HA antibody (1:1000; ThermoFisher, #71-5500) overnight at 4°C and then with anti-rabbit 488 (1:200, AlexaFluor, A21206) for 2 hours at room temperature. Slides were imaged using an epifluorescence microscope (Olympus BX51, Olympus, Hamburg, Germany) equipped with a digital camera (Olympus DP50, Olympus, Hamburg, Germany).

Ribotag assay

Fifty-six adult ChAT-Cre/Ribotag, PV-Cre/Ribotag, Npy2r-Cre/Ribotag, and TRPV1-Cre/Ribotag mice (total of 30 females and 26 males, 8 to 11 weeks old) were anesthetized with intraperitoneal ketamine (90 mg/kg) and xylazine (10 mg/kg), and the right sciatic nerve was crushed as previously described. Then, the femoral nerve was exposed and crushed for 30 s above the bifurcation. The skin was closed with a 6-0 nylon suture (Aragó) and animals were monitored periodically until the end of the experiments. After 7 days, mice were euthanized with intraperitoneal pentobarbital (30 mg/kg), and L3, L4, and L5 DRGs (for PV-Cre/Ribotag, Npy2r-Cre/Ribotag, and TRPV1-Cre/Ribotag) or spinal cord (for ChAT-Cre/Ribotag) were dissected. Samples were placed on cold Gey's solution enriched with glucose (6 mg/mL) until they were used for the Ribotag assay. For isolation of sensory neurons RNA, L3-L5 DRGs from groups of 3 (Npy2r-Cre/Ribotag and TRPV1-Cre/Ribotag) or 4 mice (PV-Cre/Ribotag) were pooled and homogenized in 1 mL of homogenization buffer as described previously (Sanz et al., 2009). For motoneurons, the ipsilateral ventral horn of spinal cords from L3 to L5 from groups of 3 animals were pooled and homogenized in 1 mL of buffer. Female and male mice were used for the study, pooled in groups of the same sex. At least 4 pools of each condition and neuron type were used for the study (ChAT-Cre/Ribotag: 27 mice; PV-Cre/Ribotag: 36 mice; Npyr2-Cre/Ribotag: 24 mice; TRPV1-Cre/Ribotag: 24 mice) (Table 2). After centrifugation of the homogenate, 40 μL of the supernatant was stored as an input sample, whereas 4 μL of anti-HA antibody (Covance, #MMS-101R) was added to the remaining lysate and incubated for 4 hours at 4°C with rotation. Then, 200 μL of protein A/G magnetic beads (ThermoFisher, #88803) were washed and added to the lysate for 5 hours (DRG samples) or overnight (spinal cords) at 4°C with rotation. Samples were washed in a high-salt buffer to remove non-specific binding from the immunoprecipitate and beads were pulled out using a magnet. RNA was isolated from the samples using the RNeasy Micro Kit (Qiagen, #74004) and quantified with Quant-it RiboGreen RNA Assay Kit (ThermoFisher, #R11490). The integrity of the RNA was assessed using the 2100 Bioanalyzer system with the RNA 6000 Nano or Pico chips (Agilent Technologies).

qRT-PCR

1 μL of RNA was assayed using the TaqMan RNA-to-Ct 1-Step Kit (ThermoFisher, #4392938). Specific transcripts were detected using Taqman assays: *Actb* (Mm02619580_g1), *Fabp7* (Mm00445225_m1), *Chat* (Mm01221882_m1), *Pvalb* (Mm00443100_m1), *Npy2r* (Mm01956783_s1), *Trpv1* (Mm01246302_m1). Relative expression was obtained by normalizing to *Actb* RNA levels with the standard curve method.

Library preparation and RNA-sequencing

The Genomics Core Facility (Universitat Pompeu Fabra) performed the RNA quality control and RNA-seq. Validity of the samples was assessed with 4200 TapeStation System (Agilent). 100 ng of total RNA from each sample was used for preparing the libraries with the NEBNext Ultra II Directional RNA Library Prep kit (New England Biolabs, #E7760), using the rRNA depletion module. Libraries were validated with TapeStation and all samples were pooled and sequenced using the NextSeq 500 System (Illumina) in runs of 2x75 cycles, yielding at least 30 million reads per sample.

FASTQ files were tested for quality using FastQC (v0.11.9). The reads were aligned to the coding DNA reference database (Ensembl Mouse database, Genome assembly: GRCm39, release 102) using Salmon (v1.8.0). The nature of the pair-end library was checked using Salmon, detecting an ISR

	Control			Crush		
	<i>Female</i>	<i>Male</i>	<i>Total</i>	<i>Female</i>	<i>Male</i>	<i>Total</i>
ChAT-Cre/Ribotag	3	2	5	2	2	4
PV-Cre/Ribotag	2	2	4	3	2	5
Npy2r-Cre/Ribotag	2	2	4	2	2	4
TRPV1-Cre/Ribotag	2	2	4	2	2	4

Table 2.

Number of pools of each type used for the RNA-seq analysis

type: inward, stranded, and read 1 coming from the reverse strand. The Salmon option “validateMappings” was used. The quantified transcript reads were mapped to genes and imported to the R environment (R version 4.2.0) using the library “tximport” (v1.24.0). Then, the “DESeq2” library (v1.36.0) was used to perform the differential expression analysis. Volcano plots were plotted using the R library “EnhancedVolcano” (v1.14.0). Hierarchical clusters were performed using the R library “pheatmap” (v 1.0.12) using the different groups analyzed in the study. In each case, the 80 more variable genes among samples were used, performing the clustering both for the rows (genes) and columns (samples). The enrichment analysis was performed using the R library “STRINGdb” (v 2.8.4), using the genes quantified with an adjusted p-value lower than 0.05. Several ontologies and databases were used in this enrichment analysis: Biological Process (Gene Ontology), Molecular Function (Gene Ontology), Cellular Component (Gene Ontology), and KEGG Pathways.

Immunohistochemistry against some factors specifically upregulated in subsets of neurons

Animals ChAT-Cre/Tomato, and Npy2r-Cre/Tomato, either control or one week after suffering a nerve crush, were perfused and lumbar DRGs and a lumbar segment of the SC were extracted. Samples were post-fixed in 4% PFA in PBS before storage. Samples were cut in a cryostat (15 μ m thick) and processed for immunofluorescence as described above. Tissues were first incubated with rabbit anti-p75 antibody (1:100; Sigma Aldrich, #Ab1554) or rabbit ATF 2 antibody (1:100, ThermoFisher, #MA5-32022) overnight at 4°C and then with anti-rabbit 488 (1:200, AlexaFluor, A21206) for 2 hours at room temperature. Slides were imaged using an epifluorescence microscope (Olympus BX51, Olympus, Hamburg, Germany) equipped with a digital camera (Olympus DP50, Olympus, Hamburg, Germany).

DRG explants and trophic factors

DRG explants were done as described above (*In vitro neurite extension*). For testing the effect of trophic factors in neurite extension, collagen matrices were enriched with either 10 ng/mL NGF (Peprotech, #450-01), or 10 ng/mL BDNF (Peprotech, #450-02), 10 ng/mL NT-3 (Peprotech, #450-03). Plates were kept in the incubator at 37°C and 5% CO₂ for at least 2 h to induce collagen gel formation.

After 2 days in culture, explants were fixed with warm 4% PFA in PBS for 30 minutes at room temperature. After washing with TBS and TBS with 0.3% triton (TBST), matrices were incubated with hot citrate buffer for 1 hour. Then, samples were incubated with 50%, 70% and 100% methanol for 20 minutes each. Matrices were washed again and then incubated with rabbit anti-PGP9.5 (1:500, Springbio, #E3340) in TBST and 1.5% normal donkey serum for 48 hours at 4°C. After three more washes, samples were incubated with anti-rabbit 488 (1:200, AlexaFluor, #A21206) in TBST and 1.5% normal donkey serum overnight at 4°C. Finally, matrices were washed, and the coverslips were detached from the plate. Coverslips were mounted onto glass slides using Fluoromount-G medium (Southern Biotech, #0100-01) and imaged with an epifluorescence microscope (Olympus BX51, Olympus, Hamburg, Germany) equipped with a digital camera (Olympus DP50, Olympus, Hamburg, Germany) and a confocal microscope (Leica SP5). The longest TdTomato⁺ and PGP9.5⁺ neurites of each DRG were measured using ImageJ software. Each individual DRG explant was treated as an experimental unit.

DRG dissociated culture and siRNA

Adult male and female PV-Cre/Ai9 mice were sacrificed with intraperitoneal pentobarbital (30 mg/kg) and all their DRGs were dissected and placed in cold Gey’s solution (Sigma, #G9779) supplemented with 6 mg/mL glucose. DRGs were enzymatically dissociated in Ca and Mg free Hank’s medium with 10% collagenase A (Sigma, #C2674), 10% trypsin (Sigma, #T-4674) and 10% DNase (Roche, #11284932001) for 45 minutes at 37°C. Then, DRGs were mechanically digested by

pipetting. Enzymes were inhibited with 10% hiFBS in DMEM medium (Sigma, #41966052) and centrifuged at 900 rpm for 7 minutes. Pellet was resuspended with 1 mL of Neurobasal-A (Gibco, #10088022) and filtered through a 70 μ m cell mesh. The cell suspension was then carefully pipetted on top of 2 mL of 15% bovine serum albumin (BSA; Sigma, #A6003) in Neurobasal-A and centrifuged again. The pellet was resuspended in Neurobasal-A supplemented with 1x B27 (Gibco, #17504044), 6mg/mL of glucose, 1x Glutamax (Gibco, #35050-038) and 1x penicillin/streptomycin (Sigma, #P0781). Then, either 8×10^3 cells (for immunostaining) or 30×10^3 cells (for qPCR) were plated in 24-well plates with medium containing siRNAs (Sigma) and HiPerFect transfection reagent (Qiagen, #301704). The knockdown was achieved using four different siRNAs each at 50 nM ([Table 3](#)) whereas control wells contained scrambled siRNA at 200 nM. The transfection reagent and siRNAs were mixed at least 20 minutes before plating. Cells were cultured at 37°C and 5% CO₂ for 24 hours. Plates were previously coated with PDL (10 μ g/mL; Sigma, #P6407) overnight and with laminin (1 μ g/mL; Sigma, #L-2020) 2 hours at 37°C.

Immunofluorescence of dissociated cultures

Cells were fixed with 4% PFA in PBS at room temperature for 20 minutes. After washing with PBS and permeabilizing with PBST, wells were incubated in 10% normal donkey serum diluted in PBST for 30 minutes. Then, wells were incubated overnight at 4°C with mouse anti- β -III-tubulin (1:500, Biolegend, #MMS435P). After washing with PBST, cells were incubated with Alexa Fluor 488-conjugated anti-mouse antibody (1:200; Invitrogen, #A21202) for 1 hour at room temperature and washed again with PBS. Coverslips were detached from the plates and mounted in glass slides with DAPI Fluoromount G mounting medium (SouthernBiotech, #0100-20). We imaged neurons with an epifluorescence microscope (Olympus BX51, Olympus, Hamburg, Germany) equipped with a digital camera (Olympus DP50, Olympus, Hamburg, Germany). We imaged all TdTomato⁺ neurons and at least 12 random fields in each coverslip for β -III-tubulin neurons. Neurite length was automatically analysed using NeuroMath software. A total of four independent cultures were conducted and each one was treated as an experimental unit.

qPCR of dissociated cultures

Total RNA from cultures was isolated using the RNeasy Micro Kit (Qiagen, #74004) and quantified using a NanoDrop. 200-300 ng of RNA were reverse transcribed to cDNA using the High-capacity cDNA Reverse Transcription kit (ThermoFisher, #4368814). For determining the expression of Med12, iTaq Universal SYBR Green supermix (Bio-Rad, #1725124) was used. The geometric mean of the expression levels of *Actb* was used to normalize the expression of Cp values. The primers used are listed in [Table 4](#). Results from three independent cultures are reported and each culture was treated as an experimental unit.

Data analysis

GraphPad Prism 8 (version 8.0.2) was used for statistical analysis. The normal distribution of the samples was tested with either Shapiro-Wilk test. The statistical test used in each analysis is specified in the results section. All data are expressed as group mean $\pm$ standard error of the mean (SEM). Differences were considered statistically significant if $p < 0.05$.

siRNA	Sequence
Scrambled	CCUAAGGUUAAGUCGCCUCG
	CGAGGGCGACUUAACCUUAGG
Med12_1	AAGAACACCAUCUACUGUAAC
	GUUACAGUAGAUGGUGUUCUU
Med12_2	AAGAACGUCAACUCAAUCCU
	AGGAUUGAAGUUGACGUUCUU
Med12_3	AAGCAGCUAAUGCAUGAGGCA
	UGCCUCAGCAUUAGCUGCUU
Med12_4	AAGUGAAAGUGAGCGAGUAGA
	UCUACUCGCUCACUUUCACUU

Table 3.

Sequences of the siRNAs used in the cultures

Gene	Direction	Sequence
Actin	Forward	ctaaggccaaccgtgaaaag
	Reverse	accagaggcatacagggaca
Med12	Forward	agaaggttcaccaactgt
	Reverse	ctccttcttgaagatggaat
Smad7	Forward	cacagaggatcttgtcccg
	Reverse	ctggtctttcctcctgcgtt
Crmp2	Forward	cacaccagctaggagactt
	Reverse	gtttacccgtggtccttca
Dab2	Forward	tcctggagagtcctcagagc
	Reverse	acctttgaacctggccaaca
Serpine	Forward	atcgctgcacccttgagaa
	Reverse	atcgggctgagatgacaaa
Tgfr2	Forward	aaatggaagcccagaaagatgc
	Reverse	tgcaggacttctggtgtcg

Table 4.

Primers used for qPCR analysis

Results

Axon regeneration in motor and sensory neurons has different rates and patterns

A comparative study of regeneration in peripheral neuron subtypes is complex and requires specific markers to label neuron populations. We took advantage of genetic labeling to comparatively study four key peripheral populations. In our previous report, we characterized and validated ChAT-Cre/Ai9, PV-Cre/Ai9, and Npy2r-Cre/Ai9 mice for the study of motoneurons, proprioceptors, and cutaneous mechanoreceptors, respectively (Bolívar and Udina, 2022 [↗](#)). Here, we added TRPV1-Cre/Ai9 mice for the study of nociceptors, as described previously (Patil et al., 2018 [↗](#)). We corroborated that the labeled neurons in the DRG were mostly peptidergic ($42.8 \pm 1.2\%$ colabeling with CGRP) and non-peptidergic ($30.1 \pm 1.4\%$ IB4-reactive) small-diameter neurons. Additionally, and fluorescent axons were found in the skin as free endings (Supp Fig 1 [↗](#)).

The regeneration in these populations was established by crushing the sciatic nerve and counting the number of axons that had regenerated 7 and 9 days after the injury (Fig 1A [↗](#)). In control nerves, we found that nociceptors and mechanoreceptors were the populations with the most abundant axons, followed by proprioceptors and motoneurons. As the number of axons differ between populations in control conditions, we evaluated both the total number of regenerated axons (Fig 1C [↗](#)) and the relative regeneration compared to each control (Fig 1D [↗](#)). Seven days after the injury, the number of regenerated axons of the different subpopulation did not reach control values when assessed at a distance of 17 mm from injury site (Fig 1C [↗](#)). However, motoneurons and nociceptors achieved axon regrowth similar to controls at 12 mm from the injury site (Fig 1C [↗](#), $p > 0.05$ vs their control). In contrast, by 9 after crush, we found that all axon populations had regenerated to numbers comparable to uninjured controls at 12 mm, and only motoneuron and nociceptor axons reached control values at 17 mm (Fig 1C [↗](#), $p > 0.05$ vs their control). Therefore, motoneurons and nociceptors were the populations that recovered their number of axons earlier. Despite this similarity, the pattern of regeneration differed between these populations. Motoneurons regrew significantly more axons at 12 mm 9 days after the injury than in control conditions ($p = 0.038$), probably due to the collateral branching at the regenerative front, whereas nociceptors displayed an increase in axon number reaching a plateau at control levels. Since none of the populations reached control values at 17 mm and 7 days postinjury (dpi), we considered it the most discriminating evaluation point. At this time and distance, the relative number of regenerated motor axons was significantly lower than that of nociceptors ($p = 0.012$), indicating that nociceptors were the population with a greater axonal regeneration (Fig 1D [↗](#)). Although non-significant, mechanoreceptors showed a tendency to regenerate proportionately more than proprioceptors, at both times and distances (Fig 1D [↗](#)). This pattern was maintained in DRG explants *in vitro*, where nociceptors extended significantly longer neurites than other sensory neurons and proprioceptors were the population with shorter neurites (Supp Fig 2 [↗](#)). Altogether, these results indicated that nociceptors had the greater regeneration, followed by motoneurons and, finally, cutaneous mechanoreceptors and proprioceptors.

RNA isolation in Cre/Ribotag mice is neuron population-specific

We isolated the pool of actively translated mRNAs in specific neuron populations using the Ribotag assay (Fig 2A [↗](#)). We used the Cre-driver animals ChAT-Cre, PV-Cre, Npy2r-Cre, and TRPV1-Cre bred to Ribotag mice to specifically target the ribosomes of motoneurons, proprioceptors, cutaneous mechanoreceptors, and nociceptors, respectively. These animals showed the expression of the HA ribosomal tag in neurons either in the DRG (co-labeling with β -tubulin) or the spinal

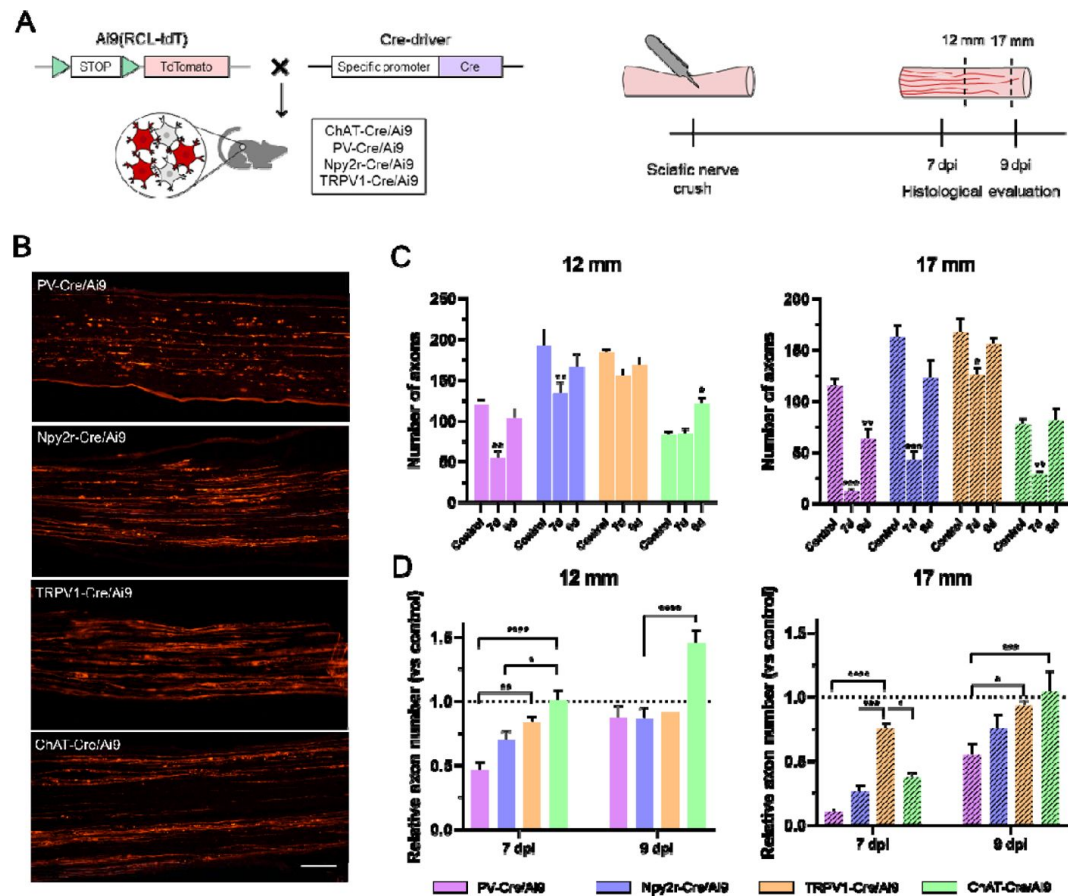


Figure 1.

Regeneration rate of peripheral neurons. A) Schematic representation of the transgenic mice and experimental design used in this study. B) Microscope images of longitudinal sections of regenerating nerves in the different Cre/Ai9 animals 7 days after injury. Images show a representative example of distal regeneration at 9 dpi. C) Quantification of the number of axons that regenerated at 12 mm (smooth bars) and 17 mm (stripped bars) at 7 or 9 dpi. Each color represents a different neuron subtype (purple: proprioceptors; blue: cutaneous mechanoreceptors; orange: nociceptors; green: motoneurons). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs each control group as calculated by 2-way ANOVA followed by Bonferroni's correction for multiple comparisons. D) Relative number of regenerated axons normalized by the control number of axons in each neuron type. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ as calculated by 2-way ANOVA followed by Bonferroni's correction for multiple comparisons. Scale bar: 100 μm

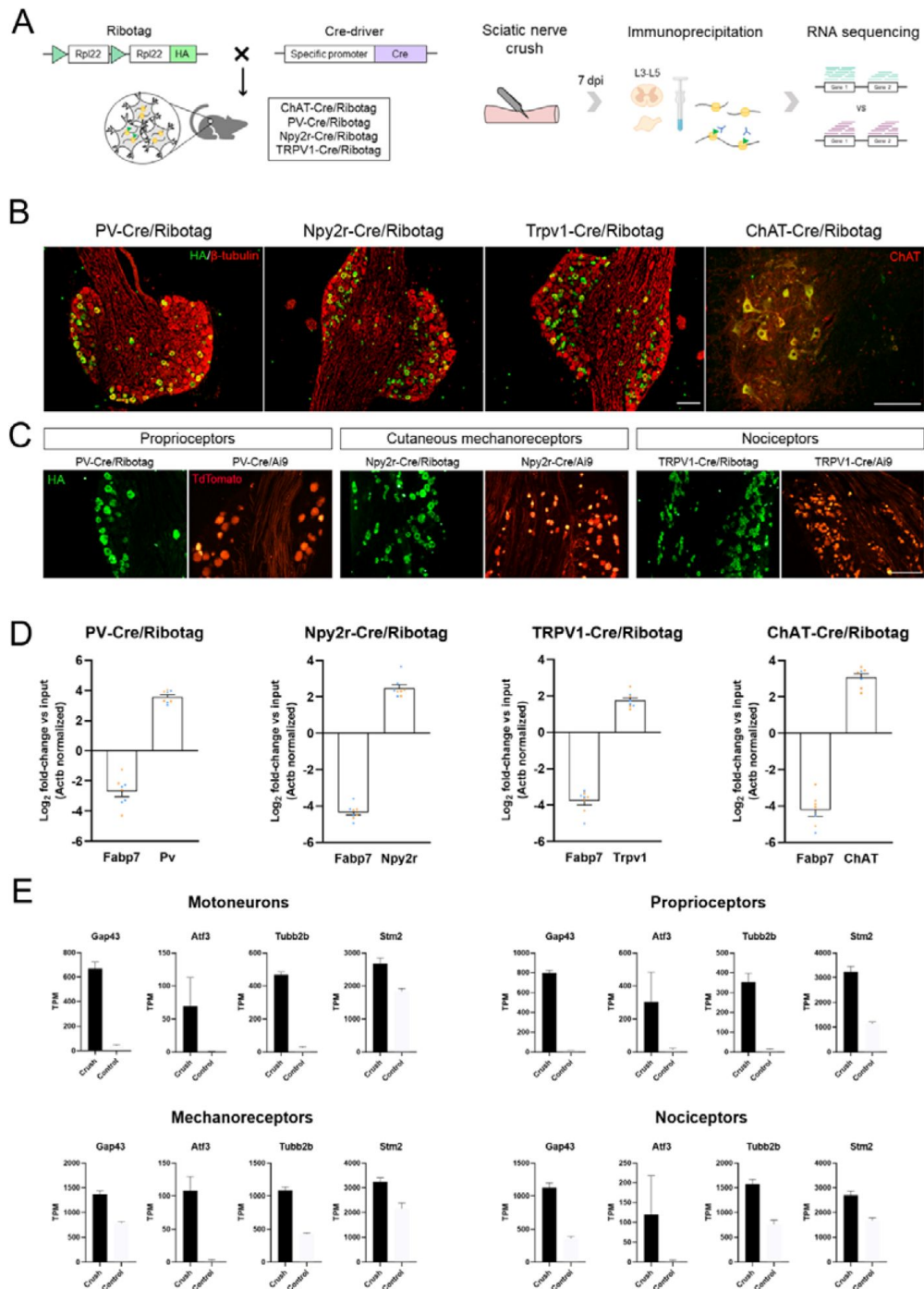


Figure 2.

Validation of the specificity of PV-Cre/Ribotag, Npy2r-Cre/Ribotag, TRPV1-Cre/Ribotag, and ChAT-Cre/Ribotag mice. A) Schematic representation of the mice and experimental design used in this experiment. B) Immunostaining against hemagglutinin (HA, green) shows the expression of tagged ribosomes in neuronal cells in the DRG (PV-Cre/Ribotag, Npy2r-Cre/Ribotag, and TRPV1-Cre/Ribotag) and in the motoneurons in the spinal cord (ChAT-Cre/Ribotag). In red, β -tubulin labels all cells in the DRG, and ChAT labels motoneurons in the spinal cord. C) Cre/Ribotag mice express HA (in green) in a similar pattern to the expression of TdTomato in Cre/Ai9 mice (in red). D) RT-qPCR reveals the enrichment of cell-type specific transcripts in each immunoprecipitate (*Pv*, *Npy2r*, *Trpv1*, *ChAT*) and the depletion of the glial transcript *Fabp7* in immunoprecipitates from all neuron populations. E) Transcripts per million (TPM) of regeneration markers. The expression of the transcripts *Gap43*, *Atf3*, *Tubb2b*, and *Stm2* is enriched in the immunoprecipitates from injured mice compared to control mice in all populations. Scale bar: 150 μ m.

cord (co-labeling with ChAT), with a similar pattern to their Cre/Ai9 counterparts (**Fig 2B,C**). After RNA isolation, RT-qPCR from immunoprecipitates (IPs) showed a several-fold enrichment of the cell-type specific transcripts *Chat*, *Pvalb*, *Npy2r*, and *Trpv1* in their respective IPs (**Fig 2D**). In contrast, the glial transcript *Fabp7* (Fatty acid binding protein 7) was depleted in all IPs, indicating that the ribosome isolation was neuron-specific.

The RNA-seq analysis showed that more than 3000 genes were differentially expressed in each subpopulation of neurons. Before further analysis of these data, we assessed its validity by checking the transcripts per million (TPM) of several markers. We confirmed that IPs from motoneurons had enriched transcripts of the well-established cell-type specific markers, including *Chat*, *Mnx1*, *Isl1*, *Nefh*, and *Tns1* (Supp **Fig 3**). *Tubb3* and *Uchl1*, which are pan-neuronal markers, were enriched in all sensory neurons, whereas *Pvalb*, *Npy2r*, and *Trpv1* transcripts were enriched in proprioceptors, mechanoreceptors, and nociceptors IPs, respectively. As expected, *Nefh* was enriched in proprioceptors but depleted in nociceptors. The transcripts encoded by the glial genes *Gfap*, *Fabp7*, *Tmem119*, *Mobp*, *Olig1*, and *Vim* were depleted in IPs (Supp **Fig 4**). Among IPs, we saw an enrichment of the transcripts for the regeneration markers *Gap43*, *Atf3*, *Tubb2b*, and *Stm2* in lesioned neurons compared to controls in all neuron types (**Fig 2E**). Finally, principal component analysis (PCA) revealed that samples clustered according to their experimental group (Supp **Fig 5**).

Peripheral neurons differentially activate regenerative programs

Compared to intact mice, a total of 3546, 4053, 3461, and 3281 were significantly differentially expressed in datasets from motoneurons, proprioceptors, mechanoreceptors, and nociceptors, respectively, 7 days after injury (Supp data 1, Supp Fig6,7). Many of these differentially expressed genes (DEGs) were enriched or depleted in more than one neuron type, whereas some of them were up or downregulated in a specific neuron type (**Fig 3A,B**). Since there was an elevated number of DEGs in each condition, we focused on the ones with the highest fold-change in each neuronal population. **Figure 3C-F** shows the DEGs that were significantly up or downregulated in each population with a $\log_2$ fold-change above 4 or below -4 but were regulated in the opposite direction in the other populations or the fold-change was lower than |1|. Some of these genes are particularly important for their potential implication in specific regeneration. For instance, we found that *Ngfr* (p75NTR), a neurotrophin receptor, was specifically enriched in lesioned motoneurons. This upregulation was confirmed by immunohistochemistry (Supp Fig 8). On the other hand, proprioceptors were the only population significantly upregulating *Nrp1* and *Nrp2* after injury, which are cell surface receptors for class 3 semaphorins. In nociceptors and cutaneous mechanoreceptors, some of the upregulated genes, such as *Il6ra* and *Atf2*, respectively, may be related with hyperalgesia. Increased number of ATF2 positive nuclei were also observed by immunohistochemistry in cutaneous mechanoreceptor neurons (Supp Figure 9).

In terms of specific regeneration, we subdivided peripheral neurons into “muscle neurons” or “cutaneous neurons”. This allowed us to study potential candidates that could prioritize the regeneration of groups of neurons towards the appropriate nerve branch required to reach their original target organs. We considered motoneurons and proprioceptors as muscle neurons, since these innervate the muscle, and cutaneous mechanoreceptors and nociceptors as cutaneous neurons because their most common target is the skin. In cutaneous neurons, we found 20 genes with a $\log_2$ fold-change above |2| that were not changed in muscle neurons or were regulated in the opposite direction in both groups (**Fig 3G**). Interestingly, only 2 of these genes were downregulated in cutaneous neurons: *Med12* and *Cacna1b*. In muscle neurons, we found 15 DEGs with a fold-change above |2| that were not changed in cutaneous neurons (**Fig 3H**). Finally, we evaluated the most differentially regulated genes in the three types of sensory neurons compared to motoneurons (**Fig 3I**). All these genes can be potential candidates to modulate the specific regeneration of these groups of neurons.

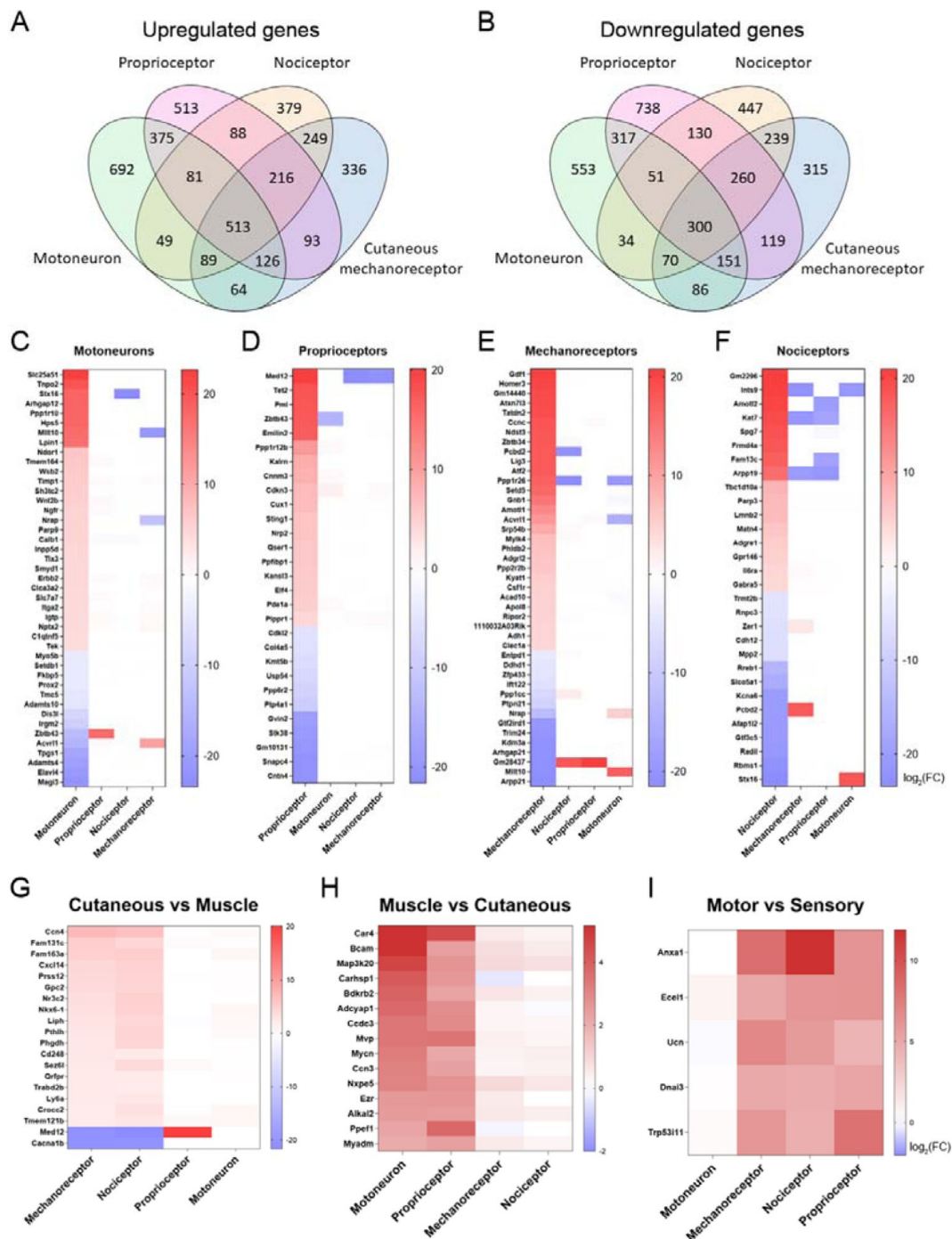


Figure 3.

Differentially expressed genes (DEGs) vary between neuronal subtypes. A–B) Number of genes that were commonly upregulated or downregulated between populations or uniquely expressed in one of the neuron subtypes. C–F) Significantly up or downregulated genes in each population with a $\log_2(\text{fold change})$ ($\log_2(\text{FC})$) above 4 or below –4. The genes shown in these graphs exclude those that are significantly regulated above |1| in the same direction in two or more neuron types. G–I) DEGs in groups of neuronal populations. DEGs with a $\log_2(\text{FC})$ above 2 or below –2 in cutaneous neurons (G), muscle neurons (H), or sensory neurons (I) are plotted from more upregulated to more downregulated.

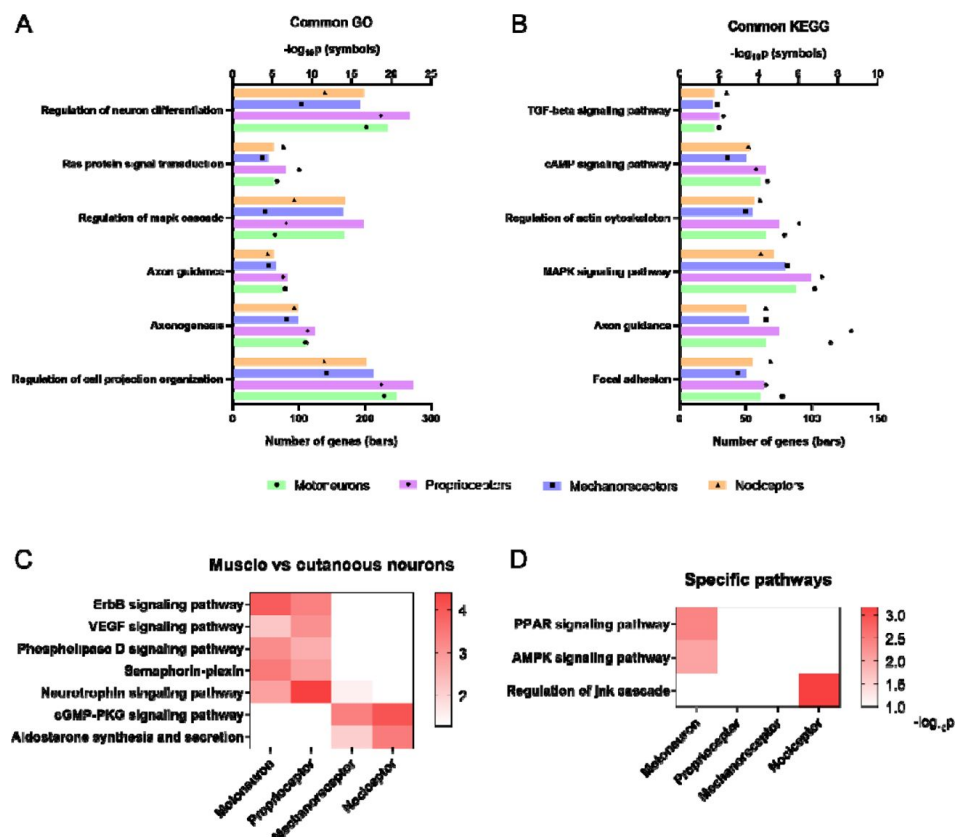


Figure 4.

Activation of relevant pathways in axon regeneration. A) Six of the most relevant Gene Ontology (GO) processes that are significantly activated by all the studied neurons after injury. B) Selection of relevant pathways enriched in neurons after injury according to the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. C) Some GO processes (semaphoring-plexin) and KEGG pathways (all the others) enriched in specific neuron groups. D) Examples of relevant pathways enriched in a specific neuron subtype. TGF: transforming growth factor, cAMP: cyclic adenosine monophosphate, MAPK: mitogen-activated protein kinases, VEGF: vascular endothelial growth factor, cGMP-PKC: cyclic guanosine monophosphate – protein kinase C, PPAR: peroxisome proliferator-activated receptor, AMPK: AMP-activated protein kinase, jnk: c-Jun N-terminal kinase.

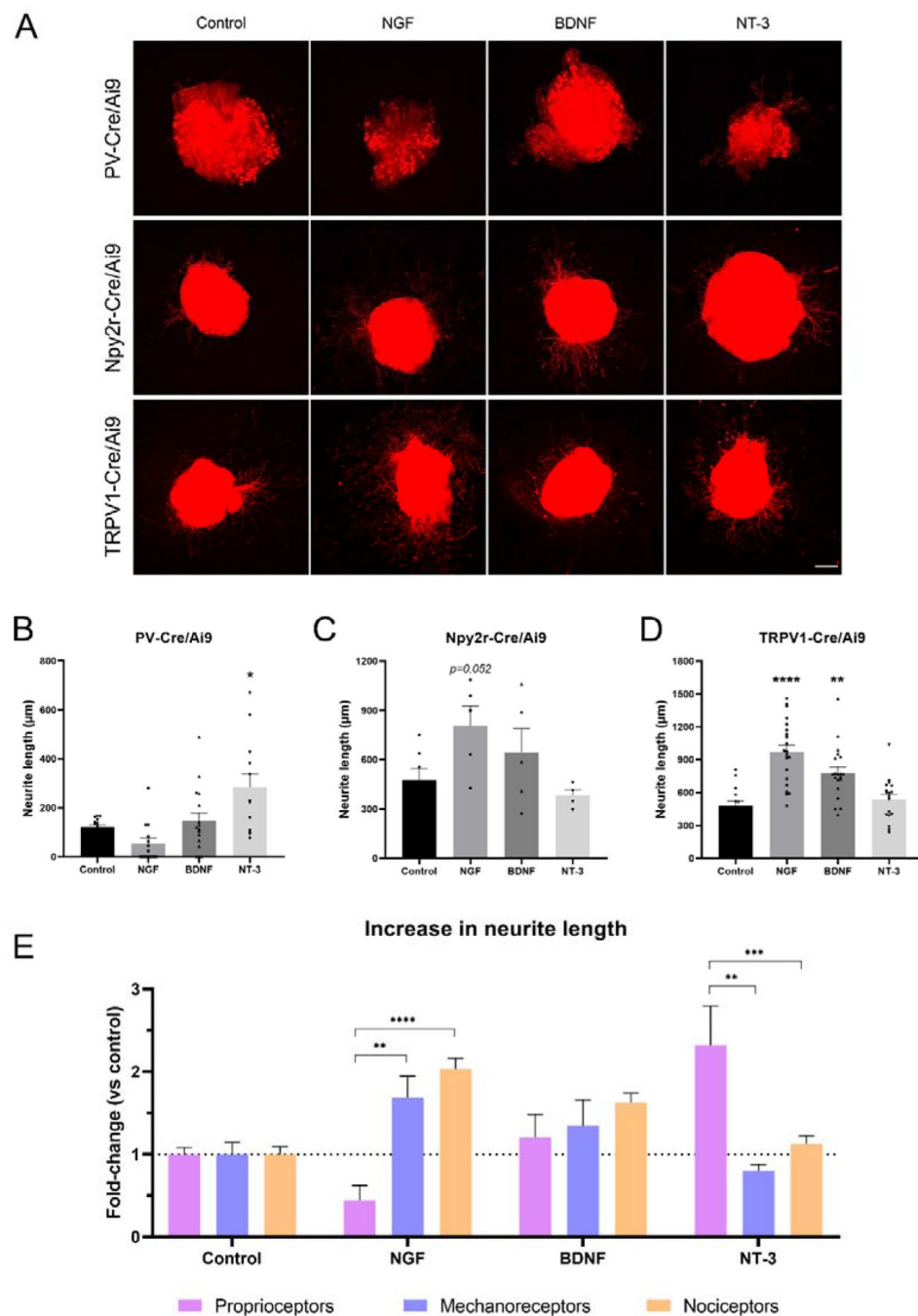


Figure 5.

Neurite extension in PV-Cre/Ai9, Npy2r-Cre/Ai9, and TRPV1-Cre/Ai9 DRG explants. A) Microscope images of the neurite outgrowth of each neuron population in explants with nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin-3 (NT-3). B-D) Quantification of the longest neurite in each condition. * $p < 0.05$ vs control as calculated by one-way ANOVA followed by Bonferroni's multiple comparisons test (PV-Cre/Ai9 and Npy2r-Cre/Ai9) or by Kruskal-Wallis test followed by Dunn's multiple comparisons test (TRPV1-Cre/Ai9). D) Comparison of the increase in neurite length in proprioceptors, mechanoreceptors, and nociceptors. The increase is plotted as a fold-change vs each control to compare the effect of the neurotrophic factors. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ vs proprioceptors as calculated by a 2-way ANOVA and followed by a Tukey's post hoc test. Scale bar: 200 µm.

Peripheral neuron subtypes differentially activate regenerative pathways

A combination of ontologies and databases were used in the enrichment analysis and the data are available in Supplementary material (Supp data 2). The caveat in these analyses is that many genes can be involved in multiple biological pathways and these databases might not entirely reflect the complete functional diversity of proteins. However, they provide a first approach for the interpretation of complex biological data. Gene Ontology (GO) analysis revealed significantly enriched terms associated with regeneration in all neuronal populations following injury, including cell projection organization, axon guidance and Ras protein signal transduction (**Fig 4A** [↗](#)). Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis showed that all neurons activate common pathways such as focal adhesion, MAPK, and cAMP signaling pathways (**Fig 4B** [↗](#)). There were some interesting GO terms and KEGG pathways that were differentially regulated between neuron types. Muscle neurons (motoneurons and proprioceptors) significantly regulated the ErbB and VEGF signaling pathways, which are potentially relevant for regeneration (**Fig 4C** [↗](#)). Importantly, GO analysis revealed a specific enrichment of the semaphorin-plexin pathway in these two neuron subtypes, a crucial pathway in neuron guidance during development and regeneration (**Fig 4C** [↗](#)). Cutaneous neurons showed a specific enrichment in cyclic guanosine monophosphate (cGMP)-dependent protein kinase (PKG) and aldosterone signaling pathways (**Fig 4C** [↗](#)). Other pathways were significantly regulated in specific neuron populations, such as the peroxisome proliferator-activated receptor (PPAR) or the AMP-activated protein kinase (AMPK) signaling in motoneurons. These two pathways are involved many cell processes including metabolism, cell survival, and neuroprotective functions and were not activated in sensory neurons. JNK pathway, which has been associated to neuropathic pain, was found enriched only in nociceptors (**Fig 4D** [↗](#)). This data highlights the heterogeneity in the regenerative response of the distinct peripheral neurons after a nerve injury.

Neurotrophic factors differentially influence sensory neurite outgrowth

Neurotrophins bind to Trk receptors, which are known to be differentially expressed in sensory neuron subpopulations. In our RNA-seq analysis, we found a differential expression of these receptors in our populations (Sup **table 1** [↗](#)). Therefore, we aimed to corroborate the differential effect of NGF, BDNF, and NT-3 (which bind TrkA, TrkB, and TrkC, respectively) in our three sensory neuron populations. First, we examined neurite extension in the DRG by labeling neurites with a pan-neuronal marker, PGP9.5 (Supp Fig 10). We found that 10 ng/mL NGF produced the larger increase in neurite length, being the mean longest neurite of $927.5 \pm 85.0 \mu\text{m}$ ($p < 0.0001$ vs control $441.7 \pm 32.0 \mu\text{m}$). We also observed a significant increase in neurite length with 10 ng/mL BDNF ($771.1 \pm 54.8 \mu\text{m}$, $p < 0.0001$) and 10 ng/mL NT-3 ($64.0 \pm 48.6 \mu\text{m}$, $p = 0.044$).

Next, we evaluated if these neurotrophic factors had a differential effect in distinct neuronal subtypes. The neurite length of proprioceptors was only significantly increased when adding NT-3 ($282.8 \pm 57.5 \mu\text{m}$ vs control $121.8 \pm 10.1 \mu\text{m}$, $p = 0.011$), but not NGF or BDNF (**Fig 5B** [↗](#)). For cutaneous mechanoreceptors, we did not find a significant increase in neurite outgrowth with any of the neurotrophic factors used (**Fig 5C** [↗](#)). However, NGF showed a tendency to increase the longest neurite in this population ($p = 0.052$). Contrarily, both NGF and BDNF significantly increased the neurite length of nociceptors ($973.5 \pm 63.8 \mu\text{m}$, $p < 0.0001$ for NGF; $780.1 \pm 54.4 \mu\text{m}$, $p = 0.002$ for BDNF, both vs control $479.2 \pm 45.2 \mu\text{m}$) (**Fig 5D** [↗](#)). The values of neurite length were normalized to their control groups for each neuron population and expressed as a fold-change to allow the direct comparison between groups (**Fig 5E** [↗](#)). Interestingly, we found that cutaneous mechanoreceptors and nociceptors had a similar pattern, which was clearly different from proprioceptors. The most specific growth factor for cutaneous neurons was NGF, which increased neurite length 1.69-fold in mechanoreceptors and 2.03-fold in nociceptors. In both cases, this

increase was significantly higher than in proprioceptors, which showed a 0.45-fold change ($p=0.007$ vs mechanoreceptors; $p<0.0001$ vs nociceptors). In contrast, the most specific factor for muscle sensory neurons was NT-3. Proprioceptors showed a 2.32-fold increase in neurite length in opposition to mechanoreceptors (0.8-fold change, $p=0.002$) and nociceptors (1.13-fold change, $p<0.0001$). Taken together, the findings supported established links between specific neurotrophin molecules and sensory neuron subtypes (Ernsberger, 2009 [↗](#)) and validated the specificity of our isolation approach.

MED12 specifically inhibits neurite extension of proprioceptors

Results of the RNA-seq showed that expression of *Med12* was upregulated in proprioceptors (20.1 $\log_2$ FC) and downregulated in mechanoreceptors and nociceptors (-21.0 and -21.6 $\log_2$ FC, respectively) after injury. In cancer cell lines, *Med12* has been described to inhibit TGF- β signaling (Huang et al., 2012 [↗](#)), an important pathway implicated in regeneration. Thus, we thought to determine its role in the regeneration of specific neuronal subtypes *in vitro* in naïve neurons. A mix of 4 different siRNAs was used to target *Med12* and its expression was evaluated by qPCR. We found that *Med12* was significantly downregulated using this approach ($p=0.001$), with a 57% decrease compared to the scrambled siRNA condition (Fig 6B [↗](#)).

Next, we used the fluorescence in PV-Cre/Ai9 mice to specifically evaluate neurite extension of proprioceptors (Fig 6C,D [↗](#)). We found that the longest neurite and the total neurite extension per neuron were significantly increased in the knockdown group ($p=0.039$ and $p=0.014$, respectively). To ensure that this effect was neuron-type specific, we analyzed neurite extension in non-proprioceptive neurons (PV) labelled with β III-tubulin. We did not find a significant change in the length of the longest neurite nor in total neuron extension, thus suggesting that MED12 specifically regulated proprioceptive neurite extension. We evaluated the expression of some genes implicated in the TGF- β pathway by qPCR (SMAD7, CRMP2, DAB2, SERPINE, TGFBR2), but we did not find a significant difference between the control and knockdown group (Sup Fig 11).

Discussion

Peripheral neurons are heterogeneous and have distinct functions and target organs. Here we show that, after axotomy, there is a specific response to injury that strongly varies between neuron types. We found differences in the regeneration ability of neuron subtypes after nerve injury, which were associated with an activation of distinct gene expression patterns. Previous studies have analyzed the transcriptome of peripheral neurons or the whole DRG after injury (Rozenbaum et al., 2018 [↗](#); Lemaitre et al., 2020 [↗](#)), but our results highlight the importance of the study of regeneration in specific neuron subtypes to improve specific regeneration after nerve injury.

In our crush study, we found that nociceptors were the peripheral population with greater axon growth. Unmyelinated fibers have been described to recover their function earlier than myelinated fibers (Navarro et al., 1994 [↗](#)). As our population of nociceptors includes mostly unmyelinated fibers, a faster regeneration rate of these fibers could explain the advantage of these neurons over other populations. Similarly, the regeneration rate of sensory neurons has been reported to be faster than that of motoneurons (Dolenc and Janko, 1976 [↗](#); Madorsky et al., 1998 [↗](#); Suzuki et al., 1998 [↗](#); Kawasaki et al., 2000 [↗](#); Negredo et al., 2004 [↗](#); Brushart et al., 2020 [↗](#)). Most of these studies, however, use functional assessments to determine the speed of regeneration, which can be influenced by the reinnervation capacity of the different fibers. Motoneurons also showed heightened growth since their axons reached control values at the same time as nociceptors. This is in accordance with previous reports stating that myelinated sensory fibers can regenerate at the same speed as unmyelinated fibers (Lozeron et al., 2004 [↗](#)). However, this population had a regeneration pattern distinct from the other neurons. At 12 mm, we found a

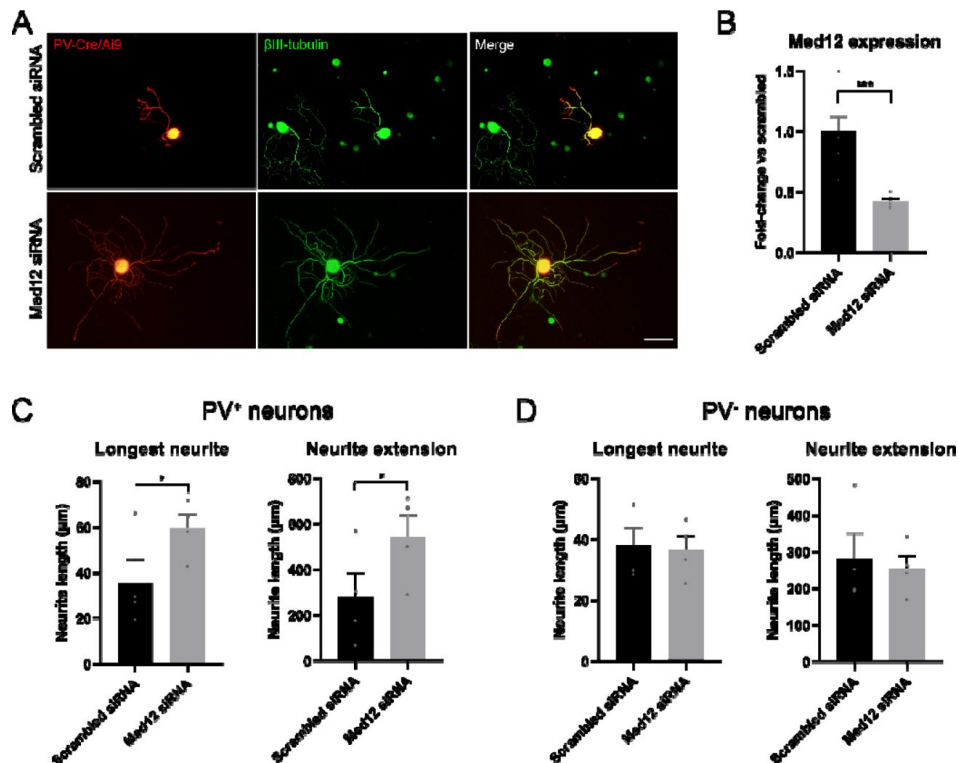


Figure 6.

Knockdown of Med12 in dissociated dorsal root ganglia (DRG) cultures. A) Microscope images from proprioceptors (in red) in cultures with scrambled or Med12 siRNA. β III-tubulin was used as a pan-neuronal marker (in green). B) Med12 expression measured by qPCR and expressed as fold-change vs scrambled. C) Quantification of the neurite length in PV neurons (fluorescent neurons in PV-Cre/Ai9). The mean of the longest neurite and the total length per neuron in each culture are plotted. D) Quantification of neurite length in PV neurons, labeled by β III-tubulin. * $p < 0.05$, *** $p < 0.001$ as calculated by t-test. Scale bar: 100 μ m.

significantly higher number of axons than in the control group which could be explained by the presence of regenerative collaterals. In fact, we have previously described that motoneurons extend more regenerative collaterals than proprioceptors, confirmed by the current work (Bolívar and Udina, 2022 [↗](#)). Cutaneous mechanoreceptors reached values of axon regrowth comparable to controls later than nociceptors and motoneurons. This finding differs from a previous work identifying that this population regenerates better than motoneurons after a femoral transection (Bolívar and Udina, 2022 [↗](#)). However, the approach and the territory used to assess regeneration differed, previously using retrotracers to evaluate numbers of regenerating femoral neurons, compared to direct counts of sciatic regenerating axons here. Since our cutaneous mechanoreceptors include myelinated and unmyelinated fibers, a more variable regeneration rate can be expected in these animals. We speculate that unmyelinated Npy2r⁺ fibers may regenerate faster than motoneurons, but this might be masked by the motor regenerative collaterals and the heterogeneity in Npy2r⁺ neurons. Finally, proprioceptors showed more limited regeneration. After the crush injury, this population exhibited a relatively lower proportion of axons compared to the other populations, both in terms of time and distance, which is in agreement with our previous experiments (Bolívar and Udina, 2022 [↗](#)).

Altogether these results indicate that sensory regeneration is inversely proportional to the fiber size: large, myelinated neurons regenerate less robustly than smaller and less myelinated neurons. Previous authors described this phenomenon by using cuff electrodes after a crush in the tibial nerve of cats (Krarup et al., 1989 [↗](#)) or with retrograde tracers after transection (Negredo et al., 2004 [↗](#)). As for motoneurons, our results suggest that, despite their large fiber size, their regeneration exceeds large-size DRG neurons but not small-size sensory neurons.

Axotomy initiates a massive response in neurons which involves several signaling pathways and the upregulation of RAGs. In our RNA-seq analysis, we saw a common upregulation of 515 transcripts and a downregulation of 300 transcripts across all studied populations. Among these, we found *Gap43*, *Tubb2a*, *Sprr1a*, *Jun*, *Stat3*, *Sox11*, *Atf3*, *Hspb1*, *Gfra1*, and *Gal*. All these genes are associated with regeneration after injury and growth cone dynamics (Tedeschi, 2012 [↗](#)). Surprisingly, most induced transcripts differed between neuronal populations: 75-80% of DEGs were specifically regulated in a single neuron type or in groups of neurons. These are potential factors explaining the differences in regenerative capacity between neurons subtypes. Based on these results, we studied two strategies to selectively modulate neurite growth *in vitro*: addition of neurotrophic factors and analysis of the impact of knockdown of a specific DEG, namely *Med12*.

Peripheral neurons express distinct Trk receptors according to their subtype. Broadly, peptidergic neurons express the NGF receptor TrkA, myelinated Aβ fibers have the BDNF receptor TrkB, and proprioceptors express NT-3 receptor TrkC. These neurotrophins have been described to be involved in the survival and/or neurite extension of peripheral neuron subpopulations (DiStefano et al., 1992 [↗](#); Terenghi, 1999 [↗](#)). For this reason, we aimed to corroborate that different neurotrophic factors would elicit a distinct response in our sensory neuron subpopulations. When neurotrophic factors were analyzed overall, evaluating the growth of all neurites, we found that NGF, BDNF, and NT-3 promoted sensory outgrowth. This is in agreement with previous reports showing an increased axon elongation in explants of rat DRG (Allodi et al., 2013 [↗](#); Santos et al., 2016b [↗](#), 2016a [↗](#)). In contrast, when we evaluated the neurite growth in each population separately, we saw that these factors had unique impacts. The neurite extension of proprioceptors was only promoted by NT-3. Contrarily, neither mechanoreceptors nor nociceptors showed an improved neurite extension when cultured in presence of this factor. NT-3 has been described as a “muscle factor” because of its trophic effect on proprioceptors and motoneurons (Ernfors et al., 1995 [↗](#); Braun et al., 1996 [↗](#); Genç et al., 2004 [↗](#); Taylor et al., 2005 [↗](#)). Here, we confirmed that an overall trophic effect of NT-3 in sensory neurons is largely accounted for by neurite growth from proprioceptors. Nociceptors demonstrated a great increase in neurite length with the presence of NGF and, to a lesser extent, of BDNF. Mechanoreceptors showed a similar response pattern, although it was non-significant. Therefore, NGF could be considered a “cutaneous factor”, as it

modulates specifically neurite extension of cutaneous neurons but not muscle neurons. In fact, NGF has been shown to act specifically on a subpopulation of small primary sensory neurons and on sympathetic neurons (Levi-Montalcini, 1987 [\[1\]](#)), and later studies confirmed that this factor increased sensory neurites, but not motor neurites (Allodi et al., 2013 [\[2\]](#); Santos et al., 2016b [\[3\]](#)). Thus, neurotrophic factors can be used to modulate differentially muscle and cutaneous sensory neurons.

Among the most differentially expressed genes, we found that *Med12* was strongly upregulated in proprioceptors but downregulated in nociceptors and mechanoreceptors. Since the regeneration of proprioceptors is limited, we thought that axotomy might trigger the expression of regeneration inhibitory factors in this population that slows this process. MED12 is a subunit of Mediator, a multiprotein complex that regulates transcriptional activity (Ding et al., 2008 [\[4\]](#)). Besides its known involvement in genomic signaling, cytoplasmic MED12 was described to inhibit TGF- β receptor 2 through a physical interaction (Huang et al., 2012 [\[5\]](#)). The TGF- β pathway is a positive regulator of regeneration since it contributes to the activation of the intrinsic growth capacity of neurons (Walshe et al., 2011 [\[6\]](#); Ye et al., 2022 [\[7\]](#)). Knowing MED12's involvement in the TGF- β pathway, we hypothesized that the upregulation of this protein could hinder axon regeneration in proprioceptors through inhibition of TGF- β receptor 2. When silencing *Med12*, we found a significant increase in the length of neurites that was specific to proprioceptors, with no discernible impact on other sensory populations. Since nociceptors and mechanoreceptors showed a strong downregulation of *Med12*, we believe that silencing this gene has little effect on these cells. Altogether, our data demonstrates that MED12 is a novel regulator of neuron-specific regeneration and can be a promising factor to improve proprioceptive regeneration after nerve injury. The mechanism by which MED12 regulates this process is, however, unknown. We did not observe a change in expression of some typical TGF- β mediators. This does not exclude this pathway as the mechanism of action of MED12 since the low percentage of proprioceptive cells could be masking the effects. Future investigations should focus on elucidating the specific mechanisms by which MED12 modulates regeneration and neurite outgrowth. As a unique strategy to enhance proprioceptive neuron plasticity, *in vivo* analysis of the functional impact of its knockdown will be of significant interest.

Besides *Med12*, we found a large sample of genes significantly regulated after an injury, and many of them could explain the differential response seen in the regeneration of peripheral neuron subtypes. From these, we focused on the differences between muscle and cutaneous neurons since these could help us improve specific regeneration towards their target organs.

Muscle neurons strongly upregulated *Bcam* and *Myadm*, among other genes. *Bcam* encodes a member of the immunoglobulin superfamily that binds to laminin (Udani et al., 1998 [\[8\]](#)). Although this protein has been mainly studied in erythrocytes (Wautier et al., 2007 [\[9\]](#); Bartolucci et al., 2010 [\[10\]](#)), laminin is the main substrate in the peripheral nerve and its differential expression might influence regeneration. MYADM has been described to be associated with lipid rafts (Capkovic et al., 2008 [\[11\]](#); Aranda et al., 2013 [\[12\]](#)), which are membrane domains involved in growth-factor signal transduction, axon guidance, and cellular adhesion (Tsui-Pierchala et al., 2002 [\[13\]](#)). In HeLa and PC3 cell cultures, MYADM is crucial for targeting RAC1 to these membrane rafts, facilitating cell migration (Aranda et al., 2013 [\[12\]](#)). Since RAC1 is one of the most important Rho GTPases favoring axon elongation, MYADM could be an interesting target for future regeneration studies. In contrast, one of the genes more upregulated in cutaneous neurons compared to muscle neurons was *Gpc2*, which encodes for a cell surface proteoglycan. In N2a cells, the interaction of GPC2 with midkine (Sorrelle et al., 2017 [\[14\]](#)), was shown to promote cell adhesion and neurite outgrowth (Kurosawa et al., 2001 [\[15\]](#)). This suggests that GPC2 could be a target to enhance cutaneous specificity in mechanoreceptors and nociceptors.

Given the plethora of DEGs identified in a single neuron subtype, several could account for their distinct regeneration patterns. Among the top motoneuron-specific upregulated genes we found *Ngfr* (p75^{NTR}), which was also found upregulated in previous motoneuron-specific RNA-seq (Shadrach et al., 2021 [DOI](#)). The functions of p75^{NTR} are complex and diverse since its activation by neurotrophins can have opposite effects, including both survival and apoptosis (Roux, 2002 [DOI](#); Chao, 2003; Gutierrez and Davies, 2011 [DOI](#)). NRP2 is a cell surface receptor for class 3 semaphorins, with high affinity to SEMA3C and SEMA3F (Chen et al., 1997 [DOI](#)). This receptor participates in axon guidance during development (Giger et al., 2000 [DOI](#); Gil and del Río, 2019 [DOI](#)), but its role in peripheral nerve injuries is largely unknown. In our study, we found a significant upregulation of *Nrp2* (and *Nrp1*) only in proprioceptors, the neuron population linked to less robust regenerative growth. Semaphorins are expressed in the nerve after an injury (Ara et al., 2004 [DOI](#)), and their repulsive effect through NRP2 could partially account for impaired growth in proprioceptive axons. In contrast, *Ndel1* was upregulated in all the studied neuron populations, except for proprioceptors. NDEL1 is a cytoskeleton integrator that participates in the formation of the vimentin-dynein complex during neurite extension (Shim et al., 2008 [DOI](#)). *In vivo* silencing of *Ndel1* after nerve injury resulted in reduced regeneration (Toth et al., 2008 [DOI](#)). Thus, the lack of upregulation of *Ndel1* in proprioceptors is in agreement with the limited regeneration observed in this population after a nerve injury.

Finally, the regenerative transcriptome from mechanoreceptors and nociceptors can also be useful to study candidates regulating allodynia and hyperalgesia after a nerve injury. We found a significant upregulation of *Atf2* in mechanoreceptors. Upregulation of this factor in small and medium DRG neurons was previously reported after injury, and its knock-down reduced tactile allodynia and thermal hyperalgesia after spinal nerve ligation (Salinas-Abarca et al., 2018 [DOI](#)). Moreover, we found a strong upregulation of *Il6ra* (Interleukin-6 receptor alpha or gp80) in nociceptors. In neuropathic pain models, the proinflammatory cytokine interleukin-6 (IL-6) is involved in hyperalgesia (Cunha et al., 1992 [DOI](#); Murphy et al., 1999b [DOI](#); Arruda et al., 2000 [DOI](#); Melemedjian et al., 2010 [DOI](#); Quarta et al., 2011 [DOI](#); Liu et al., 2019 [DOI](#)). Furthermore, some studies support that IL-6 is involved in the pro-regenerative response of sensory neurons (Hirota et al., 1996 [DOI](#); Murphy et al., 1999a [DOI](#), 2000 [DOI](#); Cafferty et al., 2004 [DOI](#); Cao et al., 2006 [DOI](#); Zorina et al., 2010 [DOI](#)). Thus, the high expression of *Il6ra* in nociceptors could be one of the contributing mechanisms to the greater regeneration of these neurons after nerve injury.

Altogether, we identified several DEGs not previously associated with the regenerative response. We also reported differences between neuron populations in genes that are known to be associated with a regenerative phenotype. Yet, it is worth noting that regenerative responses are dynamic, and we only analyzed the transcriptome 7 days after injury. Since we observed that regeneration rate varies between neurons, differences in gene expression could be influenced by the stage of regeneration. Analyzing different times after injury could help understanding some of the differences in the regenerative response of neuron populations. For instance, discriminating those set of genes that are specifically activated by a population after injury from the differential activation of the same set of genes at different regeneration stages. Furthermore, we studied four neuron populations, some of which encompass more than one neuron subtype and not all of them are found in the same microenvironment. Motoneuron's somas are found in the spinal cord, further away from the lesion, and are influenced by a completely different environment than sensory neurons. Therefore, the direct comparison of motor and sensory neurons is limited and challenging. However, here we used a well-established model of injury that resembles the physiological conditions. Despite these caveats, we think that our findings are a key step to decipher the intrinsic growth capacity of different types of peripheral neurons.

Conclusion

We characterized the regeneration of four key peripheral neuron subtypes from a histological and a genetic perspective. Our study aimed to identify specific mechanisms that could potentially be used in the future for modulating preferential regeneration of these neuron subtypes. Through our analysis, we were able to observe significant differences in the regeneration rate and gene expression after nerve injury that could be attributed to their distinct regenerative profiles. These findings provide valuable insights into the mechanisms driving the regeneration of different neuron subtypes and could serve as a basis for future studies focused on developing targeted approaches to promote specific types of regeneration.

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Joint Public Review:

Bolívar et al. set out to explore whether four distinct neuronal subtypes within the peripheral nervous system exhibit varying potentials for axon regeneration following nerve injury. To investigate this question, they harnessed the power of four distinct reporter mouse models featuring fluorescent labeling of these neuronal subtypes. Their findings reveal that axons of nociceptor neurons exhibit faster regeneration than those of motor neurons, with mechanoreceptors, and proprioceptors displaying the slowest regeneration rate.

To delve into the molecular mechanisms underlying this divergence in regeneration potential, the authors employed the Ribotag technique in mice. This approach enabled them to dissect the differential transcriptomes of these four neuronal populations after nerve injury, comparing them to uninjured neurons. Their comprehensive expression profiling data uncovers a remarkably heterogeneous response among these neuron subtypes to axon injury.

To focus on one identified target with a mechanistic experiment as a proof of concept, their analysis highlights a striking upregulation of MED12 in proprioceptors, leading to the hypothesis that this molecule may play an inhibitory role, contributing to the comparatively slower regeneration of proprioceptor axons when compared to other neuronal subtypes. This hypothesis gains support from their *in vitro* model, where siRNA-mediated downregulation of MED12 results in a significant increase in neurite outgrowth in proprioceptive neurons after plating in cell culture dishes.

Overall, this is an interesting study, and in conjunction with similar work from others will be highly valuable for neurobiologists studying how to modulate the regeneration of axons from distinct neuronal subtypes. The quality of data presentation appears to be very good in general, and the manuscript is appropriately written.

Comments on revised version:

Because there are multiple explanations for the differential regeneration responses, the authors have provided further discussion about how regeneration may be regulated *in vitro* and *in vivo*. The detection of a gene, *Med12*, which is unregulated in proprioceptive neurons, but not nociceptive and mechanoreceptors, gives support to the existence of specific programs of responses in the peripheral nervous system after injury. Further investigation is needed to define this responsiveness in detail.

Another response is the role of neurotrophins and their receptors. The authors have considered outcomes as a result of different Trk receptor signaling and also the effect of TGFβ and IL6 as cytokine modulators. Add to this list is the possibility that axon guidance molecules and downstream substrates may also play a role.

The original title was considered to be too broad and did not explain all the mechanistic aspects of this study. Therefore a revised title "Neuron-specific RNA-sequencing reveals different response in peripheral neurons after nerve injury" was used. It is appropriately suitable for the results reported in this manuscript.

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Author Response

The following is the authors' response to the original reviews.

We thank the reviewers and editors for their comments, as well as for the time dedicated to make useful suggestions that have contributed to improve the manuscript. We have responded to the concerns raised by the reviewers, and after that, we have also responded to the different points highlighted in the Recommendations for the authors:

Reviewer #1

While in vivo injury was used to assess regeneration from subsets of PNS neurons, different in vitro neurite growth or explant assays were used for further assessments. However, the authors did not assess whether the differential "regenerative" responses in vivo could be recapitulated in vitro. Such results will be important in interpreting the results.

We included a supplementary figure evaluating the neurite extension in vitro and updated the text accordingly.

Intriguingly, even in individual groups of PNS neurons, not all neurons regenerate to the same extent. It is known that the distance between the cell body and the lesion site affects neuronal injury responses. It would be interesting to test this in the observed regeneration.

Although it is true that the distance can affect the outcome, here we used a physiological model where all neurons are lesioned at the same point in the nerve. Not only distance is different for motoneurons, but also the microenvironment surrounding their somas and therefore the direct comparison of these neurons with sensory neurons is limited. We extended the discussion on this matter in the new manuscript.

Fig 1: The authors quantified the number of regenerating axons at two different time points. However, the total numbers of neurons/axons in each subset are different. The authors should use these numbers to normalize their regenerative axons.

Figure 1D shows the normalization of data from figure 1C (normalized against the number of control axons in each neuron type). This has been clarified in the text.

Fig 2-5: In explaining differential regeneration of individual groups of neurons, there are at least two possibilities: (1). Each group of neurons has different injury/regenerative responses; (2). The same set of injury/regenerative responses are differentially activated. Some data in this manuscript suggested the latter possibility. But some other data point in the opposite direction. It would be informative for the authors to analyze/discuss this further.

From our point of view, these two options can be considered differential response to injury and could be potentially used for the modulation of regeneration. However, if the second

possibility is correct, the regenerative program could be more influenced by the time chosen to study the response. Given the importance of this, we added some discussion about this topic.

Fig 6: Is it possible to assess the regenerative effects of knockdown Med12 after in vivo injury?

It is possible, but it is out of the scope of this work. Here, we aimed to describe the regenerative response and validate our data by testing a potential target for specific regeneration. Future studies will focus on the modulation of this specific regeneration both in vitro and in vivo.

Reviewer #2

It seems that the most intriguing outcome of this paper revolves around the role of Med12 in nerve regeneration. The authors should prioritize this finding. Drawing a conclusion regarding Med12's role in proprioceptor regeneration based solely on this in vitro model may be insufficient. This noteworthy result requires further investigation using more animal models of nerve regeneration.

The main goal of this work was to compare the regenerative responses of different neuron subpopulations. We modulated Med12 to validate our data and the potential of our findings. Unfortunately, investigating in depth the role of Med12 in regeneration is out of the scope of this paper. For this reason, we did not prioritise this finding here. As this finding was striking, we strongly agree that the next step should be studying how it modulates regeneration.

One critique revolves around the authors' examination of only a single time point within the dynamic and continuously evolving process of regeneration/reinnervation. Given that this process is characterized by dynamic changes, some of which may not be directly associated with active axon growth during regeneration, and encompasses a wide range of molecular alterations throughout reinnervation, concentrating solely on a single time point could result in the omission of critical molecular events.

We agree that this is probably the main limitation of this study, as we discussed in the text. We chose 7 days postinjury as a standard time point widely described in literature and to have a correlate with our histological data. Although the main aim was to compare populations, analyzing an additional time point after injury could add valuable information.

No concerns were expressed by that reviewer.

Recommendations for the authors:

The authors should assess whether the differential "regenerative" responses in vivo could be recapitulated in vitro.

We included a supplementary figure evaluating the neurite extension in vitro and updated the text accordingly.

Optional:

It will be interesting to test if the distances between the cell body and the lesion site contribute to the observed differences in individual subsets of PNS neurons.

Reviewer #3

No concerns were expressed by that reviewer.

Recommendations for the authors:

The authors should assess whether the differential "regenerative" responses in vivo could be recapitulated in vitro.

We included a supplementary figure evaluating the neurite extension in vitro and updated the text accordingly.

Optional:

It will be interesting to test if the distances between the cell body and the lesion site contribute to the observed differences in individual subsets of PNS neurons.

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Fig 2-5: In explaining differential regeneration of individual groups of neurons, there are at least two possibilities: (1). Each group of neurons has different injury/regenerative responses; (2). The same set of injury/regenerative responses are differentially activated. Some data in this manuscript suggested the latter possibility. But some other data point in the opposite direction. At least the authors should discuss these.

From our point of view, these two options can be considered differential response to injury and could be potentially used for the modulation of regeneration. However, if the second possibility is correct, the regenerative program could be more influenced by the time chosen to study the response. Given the importance of this, we added some discussion about this topic.

While the paper is technically well-executed, the conclusions and some of the findings appear to be incomplete and challenging to draw meaningful conclusions from. This manuscript presents some interesting findings, but the title is quite broad and may suggest that the authors have unveiled fundamental mechanisms explaining the varying regenerative abilities of peripheral axons. However, the results do not substantiate such a conclusion. Further comments and suggestions follow.

We eliminated the word "regenerative (response)" from the title, as it could lead to think that all changes seen in these neurons are related only to regeneration. We think that "Neuron-specific RNA-sequencing reveals different responses in peripheral neurons after nerve injury" highlights the differences between neurons that we found without misleading towards thinking that we described regenerative mechanisms in all neurons.

What's notably absent here is the validation of certain genes found with the ribosomes, especially those highlighted in the subsequent figures. The question arises as to whether the changes depicted in the figures align with changes in the DRGs in vivo. Is there concordance between the presence of these genes and their transcriptional changes? It would greatly enhance the study's value if the authors could show evidence of upregulation or downregulation of certain genes over time in tissue sections, utilizing techniques such as in situ hybridization or immunocytochemistry.

We selected some factors that were specifically upregulated in subsets of neurons to corroborated by immunohistochemistry these findings. Changes in the immunofluorescence of P75 in motoneurons and ATF2 in cutaneous mechanoreceptors, were evaluated in controls and animals that received a nerve crush one week before. Supplementary figures with the images have been added.

The authors discovered intriguing distinctions, such as the presence of specific signaling pathways unique to neurons projecting to muscle as opposed to those projecting to the skin. Among these pathways were those associated with receptor tyrosine kinases like VEGF, erbB, and neurotrophin signaling among others. The question now arises: do these pathways play a role in natural peripheral regeneration processes? To answer this, it is imperative to conduct in vivo studies. However, the authors employed an in vitro DRG neurite outgrowth assay to demonstrate that various types of neurons exhibit different responses to the presence of different neurotrophins. This does not reflect what actually happens in vivo. While neurotrophins indeed play a role in neuron survival and axon extension during development, their role in postnatal periods changes over time, and it remains unclear whether they play any role in the natural regenerative processes of the peripheral nerve. Therefore, this experiment may not be directly relevant in this case, especially during the early axon extension period of the regenerating axons. If the authors aim to establish a causal link with neurotrophin signaling, it becomes crucial to conduct in vivo experiments by manipulating the expression of key molecules like the receptors.

It has been widely described that different types of peripheral neurons have a differential expression of Trk receptors, even in the adult, and that these respond differentially to neurotrophins. In our study, we do not establish a causal relationship between the expression of Trk and neurite extension, but instead we show (as many others) that distinct neurons respond differentially to these neurotrophins. The fact that in vivo studies fail to show a clear effect does not necessarily mean that neurotrophins are not specific. It might mean that their effect is not strong enough to be a useful guide in the complex microenvironment found after an injury. For instance, NGF acts on TrkA (present in some neurons), but in vivo it has been shown to accelerate the clearance of myelin debris in Schwann cells (Li et al., 2020), which could facilitate regeneration of all type of axons, masking any potential specific effect on the subtypes of neurons expressing TrkA. In contrast, in an in vitro setting on neuronal cultures, the specific neuronal effect can be more evident.

Additionally, it's worth noting that another paper utilizing the same methodology and experimental setup (PMID: 29756027, "Translatome Regulation in Neuronal Injury and Axon Regrowth" by Rozenbaum et al.) exists. Are there any significant differences or shared findings with that study?

This study shows the transcriptomic response after an injury 4, 12 and 24 hours after an injury in a very similar experimental setup. They focus on comparing the neuronal vs the glial response to the injury, using a Ribotag line that tags ribosomes from all neurons in the DRG rather than specific neuron subtypes. As the time postinjury (24h vs 7 days) and the cell types studied are different, we could not directly compare our results. We did see an upregulation in both datasets of previously described growth-associated genes (Jun, Atf3, Sox11, Sprr1a, Gal...). We included the article in the references for its relevance in the topic.

It would be helpful for readers to illustrate the finding of the fastest axon regeneration of nociceptors by showing fluorescence micrographs of the nerve samples in addition to the graphs shown in Fig. 1 C/D.

In figure 1B, we show fluorescence micrographs of the nerves 7 days postinjury. As explained in the results, we counted the number of axons at 2 distances from the injury, we did not analyse the fastest axon. This is due to technical reasons: 7 days after the injury the fastest axon has surpassed our evaluation point, which was the further distance that we could assess in our experimental setting in a consistent manner. If the reviewer thinks that we need to include more images from our evaluations (from 9 dpi for example), we could prepare a new figure.

The labeling in Fig. 2B is confusing. Is the CHAT immunoreactivity shown in the last panel illustrated by green or red signals? Is the red signal counterstaining with beta-tubulin?

The labelling was changed in the figure to increase clarity.

The references to the supplementary data throughout the manuscript are confusing. For example, where can the "Supp data 2" be found? (mention on p. 14 in the merged pdf file). Are they referring to the Excel spreadsheets?

We divided the supplementary material in supplementary figures/table (found in the pdf) and supplementary data. Supplementary data refers to excel spreadsheets found outside the pdf file. We hope this will be clearer after the final formatting of the article.

What does the following statement on p. 14 mean?: "The caveat in these analyses was that molecular classification by these approaches may be arbitrary, and not reflective of protein repurposing." This reviewer notes that these databases consider the fact that components participate in different pathways.

Indeed, we aimed to explain that many proteins participate in different pathways, and this is a limitation of the enrichment analysis. We modified the sentence in the text.

First paragraph on p. 15: The PPAR and AMPK pathways have much broader roles, and are not only "related to fatty acid metabolism". This factual inaccuracy should be corrected in the manuscript.

The sentence has been corrected.

The authors should consider showing increased TGF-beta signaling in their neurons after downregulation of Med12 given the previous implication of TGF-beta signaling in axon regeneration.

We tried to demonstrate the effect of our knockdown in TGF-beta pathway by analyzing the expression of typical targets from this pathway by qPCR in our cultures. However, we could not detect any difference. We think that this can have two explanations: (1) as only a few cells upregulate Med12 whereas many cells downregulate it, the effect is masked (presumably only proprioceptors will have a significant difference in this pathway and, thus, it would be very difficult to see the effect), or (2) Med12 is not exerting its effect through this pathway. We added a supplementary figure with these data and discussed it in the manuscript.

It would be helpful to eliminate typos and improve syntax/grammar/style.

We revised the text to improve style.