

Peripheral regeneration of $A\beta$ low-threshold mechanoreceptors is limited despite activation of regenerative transcriptional pathways

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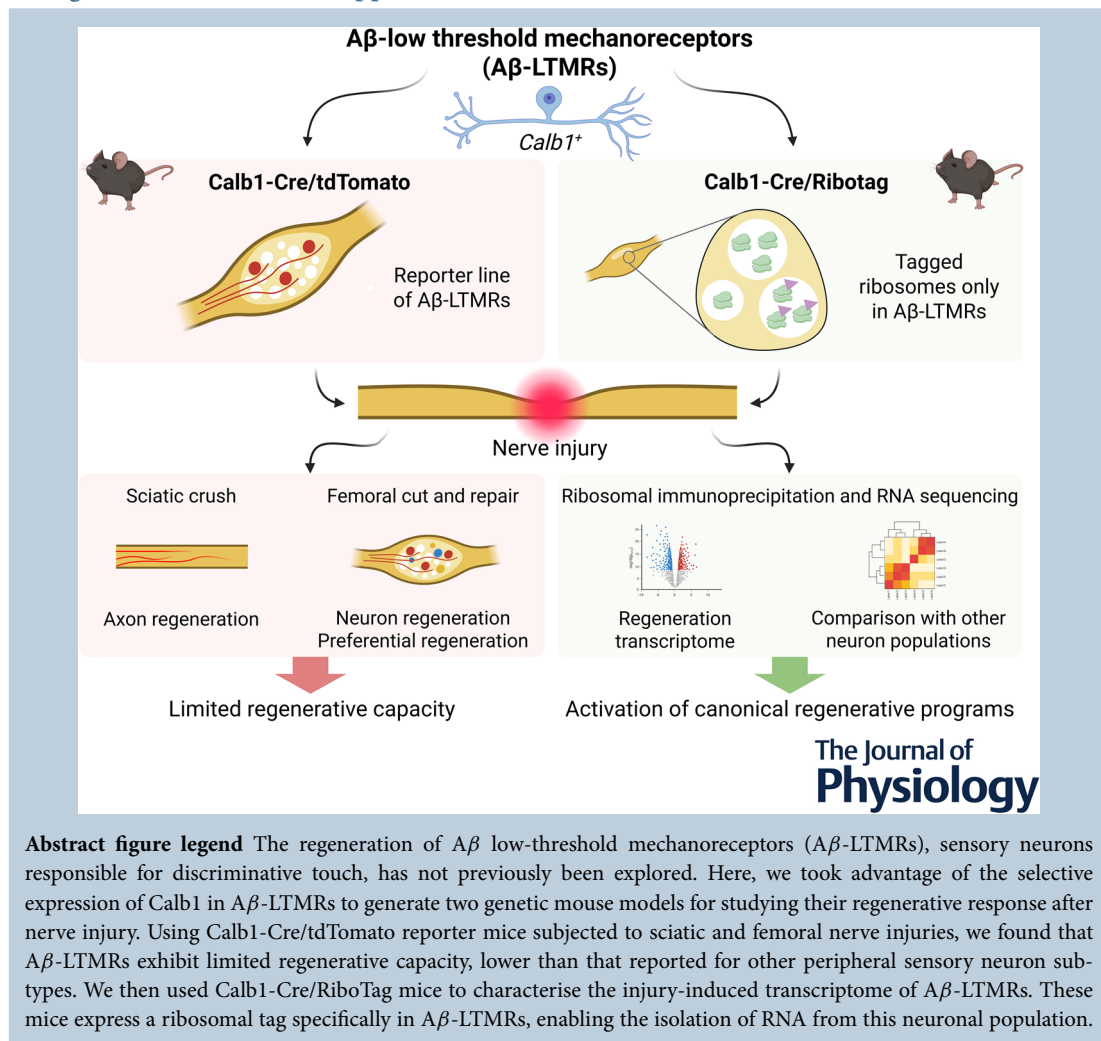
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Differential gene expression and pathway analyses revealed activation of canonical regeneration-associated programmes, similar to those observed in other sensory neuron subtypes. Together, these findings define the regenerative profile of $A\beta$ -LTMRs at both anatomical and transcriptomic levels, revealing limited regenerative capacity despite robust activation of classical regeneration-associated genes.

Abstract Peripheral neurons can regenerate after nerve injury, yet functional recovery is often incomplete due to non-specific or incomplete target reinnervation. Among sensory neuron subtypes, $A\beta$ low-threshold mechanoreceptors ($A\beta$ -LTMRs) mediate discriminative touch, a modality often incompletely restored after nerve injury. However, the mechanisms governing $A\beta$ -LTMR regeneration remain largely unexplored. Here, we characterised the anatomical and transcriptional features of $A\beta$ -LTMR regeneration after nerve injury. We assessed the extent of axonal regeneration after sciatic nerve crush and the preferential regeneration following femoral nerve transection using Calb1-Cre/tdTomato reporter mice. In both models, regeneration was incomplete, failing to reach control values. Moreover, $A\beta$ -LTMRs showed a modest but significant preferential regeneration to cutaneous rather than muscle pathways. To define the molecular programme underlying this response, we performed ribosome-bound RNA sequencing from Calb1-Cre/Ribotag mice 7 days after nerve crush. $A\beta$ -LTMRs upregulated canonical regeneration-associated genes, including *Atf3*, *Sprr1a*, *Gal*, and *Gap43*. Comparison with published RNA-seq datasets from other sensory neuron populations revealed only modest overlap, indicating that the core injury response shared between neurons is accompanied by a neuron subtype-specific programme. Together, these findings define the regenerative profile of $A\beta$ -LTMRs across anatomical and transcriptomic levels, revealing the limited regenerative capacity of this neuron subpopulation despite robust activation of classical regeneration-associated genes.

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Key points

- Peripheral neurons can regenerate after nerve injury, but functional recovery is often incomplete due to limited axonal regrowth and inaccurate target reinnervation.
- $A\beta$ low-threshold mechanoreceptors ($A\beta$ -LTMRs) mediate discriminative touch, a sensory modality that is frequently not fully restored following nerve injury.
- In this study, we investigated the regenerative capacity of $A\beta$ -LTMRs using complementary anatomical and transcriptomic approaches.
- We show that $A\beta$ -LTMRs exhibit limited regeneration after nerve injury, despite activating canonical regeneration-associated transcriptional programs.
- The results help us better understand how regenerative capacity differs between neuronal subtypes and highlight the importance of defining subtype-specific regenerative responses to improve axonal growth and promote accurate target reinnervation.

Sara Bolívar is a postdoctoral researcher at the University of Oxford, where her work focuses on peripheral neuron biology and neuropathic pain. Previously, she graduated in Biomedical Sciences from the Universitat Autònoma de Barcelona, where she also completed her MSc and PhD in Neuroscience within the Neuroplasticity and Regeneration Group. Her research has focused on neuronal responses to injury, with particular emphasis on the regenerative capacity of distinct peripheral neuron subtypes and the molecular mechanisms underlying regeneration. Looking ahead, she aims to advance our understanding of peripheral neuron function across injury states and contribute to new strategies for nerve repair and neuropathic pain management.



Introduction

Nerve injuries result in the loss of motor and sensory functions in the region that was innervated by the damaged nerve. Despite the capacity of peripheral axons to regenerate, functional recovery usually remains incomplete. The slow regeneration rate can delay the recovery and, even if axons reach distal targets, the lack of specificity in reinnervation can impair the function chronically. To achieve a successful regeneration, motor axons should reinnervate muscles, while sensory neurons need to reach sensory targets. Furthermore, sensory neurons in the dorsal root ganglia (DRG) are heterogeneous, having different targets, functions and transcriptional profiles (Qi et al., 2024; Usoskin et al., 2015). Given these considerations, understanding the mechanisms governing regeneration and axon guidance in specific cell-types is necessary to improve the functional recovery after a nerve injury.

Low-threshold mechanoreceptors (LTMRs) are a subtype of primary afferents that are activated by innocuous mechanical forces, encoding for light touch, pressure and vibration sensation. Not much is known about the regeneration of this subpopulation, in part because reliable labelling strategies to study these neurons have only recently become available. Classical studies have primarily distinguished between myelinated and unmyelinated fibres, suggesting that small neurons exhibit superior regenerative capacity (Krarup et al., 1989; Lozeron et al., 2004; Navarro et al., 1994). Similarly, studies comparing nociceptive and non-nociceptive populations indicate that regenerative responses are not uniform, particularly in the context of a conditioning lesion, with distinct behaviours observed among CGRP⁺, IB4⁺, and CGRP⁻/IB4⁻ neurons (Kalous & Keast, 2010; Seo et al., 2025). Together, these findings support the idea that intrinsic growth capacity varies across neuronal subtypes.

Understanding the behaviour of A β -LTMRs after injury can be crucial to recovery of fine sensory functions, such as spatial discrimination and fine motor interactions. Furthermore, A-fibres can induce mechanical allodynia after nerve injury (Dhandapani et al., 2018; Gautam et al., 2024; Xu et al., 2015), and their lack of regeneration can lead to aberrant C-fibre reinnervation that causes neuropathic pain (Gangadharan et al., 2022; Jeon et al., 2024). Thus, promoting accurate regeneration of A β -LTMRs could be crucial to both improve functional recovery and reduce neuropathic pain after nerve injury.

Over the last decade, efforts have been made to identify specific markers and genetic tools for studying peripheral neuron sub-populations. Single-cell (sc) and single-nucleus RNA sequencing (snRNA-seq) studies have identified at least 15 transcriptionally defined neuronal clusters in the DRG (Bhuiyan et al., 2024; Jung

et al., 2023; Qi et al., 2024; Renthal et al., 2020; Usoskin et al., 2015; Zeisel et al., 2018; Zhang et al., 2022; Zheng et al., 2019), highlighting the heterogeneity of primary afferents. From these markers, numerous genetic tools have emerged to allow the specific study of peripheral neuron subtypes (Qi et al., 2024), including the genetic manipulation of Calb1 to label A β rapid adapting (RA)-LTMRs (Zheng et al., 2019). This opens an opportunity for investigating the physiopathology of regeneration in these neurons.

In our previous work, we made a comparative characterisation of the regeneration of different neurons subtypes after nerve injury, including motoneurons, proprioceptors and nociceptors (Bolívar et al., 2024). We found a significant difference in the regeneration rate of these populations: proprioceptors showed the slowest regeneration rate, whereas nociceptors regenerated quicker. We found these functional differences were accompanied by distinct transcriptional profiles after nerve injury that could potentially explain the variable regeneration between neuron subtypes. Here, we aimed to extend our prior analysis by characterising the regeneration profile of an A β RA-LTMR sub-population. We used the genetically labelled mouse line Calb1-Cre/tdTomato (tdT) to define the regeneration rate and preferential reinnervation of these neurons after nerve injury. We then took advantage of the RiboTag mouse to isolate ribosome-bound transcripts from this neuronal subpopulation (Sanz et al., 2009, 2019) and examine their transcriptional response to injury. Our results demonstrate that A β RA-LTMRs exhibit limited regeneration *in vivo* following sciatic crush and femoral transection injuries. We further show that regenerating axons preferentially regenerate towards cutaneous pathways after transection. Notably, despite their modest regenerative capacity, this subpopulation activates a transcriptional programme associated with regeneration after injury, including significant upregulation of regeneration-associated genes (RAGs).

Methods

Animals

Double transgenic mice were generated by breeding homozygous Calb1-2A-dgCre-D (cat. no. 023531, The Jackson Laboratory, RRID:IMSR_JAX:023531) with homozygous Ai9(RCL-tdT) (cat. no. 007909, The Jackson Laboratory, RRID:IMSR_JAX:007909) or heterozygous RiboTag mice, kindly donated by Dr Elisenda Sanz (Universitat Autònoma de Barcelona, Spain). Cre expression was induced by intraperitoneal injection of trimethoprim (50 mg/ml; Sigma cat. no. T78883) dissolved in DMSO. Mice were treated for 3 consecutive days with a dose of 100 μ g per gram of body weight per

Table 1. Sample processing details and antibody specification for immunofluorescence analysis.

Sample	Thickness	Immunofluorescence
DRG	15 µm	Parvalbumin (1:1000; Swant cat. no. PV-28, RRID:AB_2315235) Neurofilament H (1:1000; Biolegend cat. no. 801701, RRID:AB_2564642) CGRP (1:200; Sigma-Aldrich cat. no. PC205L, RRID:AB_2068524) Calbindin D-28K (1:200; Millipore cat. no. AB1778, RRID:AB_2068336) Tyrosine hydroxylase (1:250; Millipore cat. no. AB1542, RRID:AB_90755) Isolectin B4 (10 µg/ml; Vector cat. no. L-1104, RRID:AB_2336498) Anti-lectin I (1:500; Vector cat. no. AS2104, RRID:AB_2314660) ATF3 (1:500; BioTechne cat. no. NBP1-85816, RRID:AB_11014863)
Skin	50 µm	PGP 9.5 (1:500; Springbio cat. no. E3340, RRID:AB_1661545)

day and used at least 48 h (Calb1-Cre/tdT) or 2 weeks (Calb1-Cre/Ribotag) after the injections.

Mice were housed in a controlled environment (12-h light-dark cycle, $22 \pm 2^\circ\text{C}$), in open cages with water and food *ad libitum*. 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 Spanish National law (RD 53/2013).

Immunofluorescence

Adult mice (8–12 weeks old) were killed with intraperitoneal sodium pentobarbital (30 mg/kg) and perfused with 4% PFA in phosphate buffered saline (PBS). Lumbar DRGs, footpads, and hairy skin were removed and stored in PBS containing 30% sucrose at 4°C for later processing. DRGs were serially cut in a cryostat (15 µm thick) and transferred to glass slides, whereas footpads were cut (50 µm thick) and stored free-floating. Samples were hydrated with PBS and permeabilized with PBS with 0.3% Triton X-100 solution. Sections were blocked with 10% normal donkey serum in PBST for 1 h at room temperature and then incubated with the primary antibody in PBST overnight at 4°C (Table 1). Sections were washed with PBST and further incubated with a specific secondary antibody bound to Alexa 488 (1:200, Invitrogen) for 2 h at room temperature (DRGs) or overnight at 4°C (skin). For IB4 immunostaining, an overnight incubation at 4°C with anti-lectin I was done prior to the secondary antibody incubation. Finally, after washing in PBS, slides were mounted with Fluoromount-G mounting medium (Southern Biotech, 0100–01) and imaged with a confocal microscope (Leica SP5) or an epifluorescence microscope (Olympus BX51, Olympus, Hamburg, Germany) equipped with a digital camera (Olympus DP50, Olympus, Hamburg, Germany). We counted the number of neurons tdTomato⁺ and how many of them co-labelled with the different markers using ImageJ software. Small (diameter <25 µm) and medium-to-large (diameter ≥ 25 µm)

neurons were quantified separately. For size distribution, we contoured 100 neurons using ImageJ software.

Femoral nerve transection and retrograde tracing

Adult Calb1-Cre/tdT mice (6 males and 11 females, 8–12 weeks old) were anaesthetized with intraperitoneal ketamine (90 mg/kg) and xylazine (10 mg/kg) and the left femoral nerve was exposed using an inguinal approach. The subcutaneous adipose tissue was carefully removed until the nerve and its ramifications were accessible. Then, the femoral nerve was cut above the bifurcation, 6 mm proximal to the entry of the quadriceps branch into the quadriceps muscle. Immediately afterwards, the injured nerve was repaired with 5–10 µl of fibrin glue. The glue was prepared by mixing thrombin (25 U/ml, ICN Biomedicals, cat. no. 0215416305) in calcium chloride (45 mmol/L, Sigma, cat. no. C1016), human fibrinogen (100 mg/ml, Sigma, cat. no. F3879), and bovine fibronectin (8 mg/ml, Sigma, cat. no. F4759) in a 2:1:1 ratio (Akhter et al., 2019; Guest et al., 1997). When the fibrin glue polymerized, the skin was closed with a 6-0 nylon suture (Aragó). Subcutaneous buprenorphine (0.1 mg/kg) was given as postsurgical analgesia twice a day for 3 days and animals were monitored periodically until the end of the experiments.

After 1 or 8 weeks, mice were anaesthetized to re-expose the left femoral nerve (1 week: $n = 6$, 8 weeks: $n = 8$). Additionally, some uninjured mice were included in the process as controls ($n = 3$). The quadriceps and the saphenous branches were cut at the same distance from the injury (approximately at 6 mm), just before the entry of the quadriceps branch into the muscle. The end of the muscle and cutaneous branch was soaked randomly in either Fluorogold (4%, Fluorochrome, cat. no. 52–9400) or True Blue Chloride (2.5%, Setareh Biotech, cat. no. 7120), and protected from the light for 45 min. To avoid spilling, we set up a small well using a piece of parafilm overlaid with a Vaseline ring. We introduced the nerve stump into the well, and we filled it with the retrograde tracers.

One week later, mice were killed with intraperitoneal pentobarbital (30 mg/kg) and perfused with 4% PFA in PBS. The ipsilateral L3 and L4 DRGs were extracted and stored in PBS with 30% sucrose at 4°C until they were serially cut in a cryostat (Leica, 15 μ m thick) and transferred to glass slides. We imaged DRGs with an epifluorescence microscope (Olympus BX51, Olympus, Hamburg, Germany) equipped with a digital camera (Olympus DP50, Olympus, Hamburg, Germany) and CellSens Digital Imaging software (version 1.9, Olympus, Hamburg, Germany). We counted the number of Calb1⁺ neurons that regenerated using the muscle or the cutaneous branch by counting the tdTomato⁺/TrueBlue⁺ or tdTomato⁺/Fluorogold⁺ neurons in the DRG. The experimenter counting the neurons was blinded to the tracer-branch sequence for each animal.

Sciatic nerve crush

Adult Calb1-Cre/tdT mice (3 males and 11 females, 8–12 weeks old) were anaesthetized 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 s. 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). Subcutaneous buprenorphine (0.1 mg/kg) was given as postsurgical analgesia twice a day for 3 days and animals were monitored periodically until the end of the experiments. After 7 or 9 days, mice were killed with intraperitoneal pentobarbital (30 mg/kg) and perfused with 4% PFA in PBS ($n = 7$ for each day). The right sciatic nerve and its tibial nerve extension were harvested and stored in PBS with 30% sucrose. We also collected some contralateral nerves as control. A segment was serially cut in 10 μ m longitudinal sections 12 mm to 17 mm from the lesion site in a cryostat (Leica) and transferred to glass slides. The slides were mounted with Fluoromount-G mounting medium (SouthernBiotech, cat. no. 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.

Translating ribosome affinity purification

Adult Calb1-Cre/Ribotag mice (total of 12 females and 9 males, 8–11 weeks old) were anaesthetized with intra-

peritoneal ketamine (90 mg/kg) and xylazine (10 mg/kg), and the right sciatic nerve was crushed as detailed above. 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. Subcutaneous buprenorphine (0.1 mg/kg) was given as postsurgical analgesia twice a day for 3 days. After 7 days, mice were killed with intraperitoneal pentobarbital (30 mg/kg), and L3–L5 DRGs were rapidly dissected. Samples were placed in cold Gey's solution enriched with glucose (6 mg/ml) until they were used for the Ribotag assay. L3–L5 DRGs from groups of 3 mice were pooled and homogenized in 1 ml of homogenization buffer as described previously (Sanz et al., 2009). Female and male mice were used for the study, pooled in groups of the same sex. After centrifugation of the homogenate, 40 μ l of the supernatant was stored as an input sample, whereas 4 μ l of anti-HA antibody (Covance, cat. no. MMS-101R) was added to the remaining lysate and incubated for 4 h at 4°C with rotation. Then, 200 μ l of protein A/G magnetic beads (ThermoFisher, cat. no. 88803) were washed and added to the lysate for 5 h at 4°C with rotation. Samples were washed in a high-salt buffer to weaken the antibody-magnetic bead binding and beads were removed using a magnet. RNA was isolated from the samples using the RNeasy Micro Kit (Qiagen, cat. no. 74004) and quantified with Quant-it RiboGreen RNA Assay Kit (ThermoFisher, cat. no. R11490). The integrity of the RNA was assessed using the 2100 Bioanalyzer system with the RNA 6000 Nano or Pico chips (Agilent Technologies).

Library preparation and RNA-sequencing

Library preparation and sequencing were performed by Lexogen NGS Services (Vienna, Austria). Libraries were prepared via the CORALL mRNA-Seq V2 preparation kit. All the samples were sequenced via the Illumina NextSeq 500 platform in runs of 2 $\times$ 150 bp, yielding at least 15 million reads per sample. The fastq files were tested for quality using FastQC (v0.11.9). Reads were aligned to the coding DNA reference database (Ensembl Mouse database, Genome assembly: GRCm39, release 103) using Salmon (v1.4.0). A total of 64 single-end samples were quantified. The quantified transcript reads were mapped to genes and imported to the R environment (version 4.4.0) using the library 'tximport' (v 1.32.0). Then, the 'DESeq2' library (v1.44.0) was used to perform the differential expression analysis. Volcano plots were plotted using the R library 'EnhancedVolcano' (v 1.22.0). Only genes with more than 100 counts among all samples were used in the differential analysis. The raw data are available on the NCBI Sequence Read Archive (SRA) (BioProject ID PRJNA1424702).

Gene set enrichment was performed using the R library 'STRINGdb'5, using the databases offered by the String platform6. Only proteins with a *P* value lower than 0.05 are used in the enrichment. Pathway analysis was made using Bioconductor libraries 'KEGGREST' and 'pathview'. The KEGG7,8 database was accessed in this process using RESTful web services with the R package. All genes in the comparison were evaluated according to their *P* value using a Wilcoxon test for each pathway, to produce a list of KEGG pathways with a given *P* value related to the original *P* value of the gene and the ratio of the genes matched in the pathway with respect with the total number of genes in that pathway.

For gene set enrichment analysis (GSEA) we customized a list of RAGs that have been described extensively in the literature (Allodi et al., 2012; Ma & Willis, 2015; van Kesteren et al., 2011) to evaluate if these genes were differentially and concordantly expressed 7 days after injury. The list of RAGs was: *Adcyap1*, *Arg1*, *Atf3*, *B2m*, *Basp1*, *Bdnf*, *Bid*, *Ccl2*, *Cdc42*, *Cdkn1a*, *Cebpd*, *Calca*, *Creb1*, *Dpysl2*, *Drd4*, *Fmr1*, *Gal*, *Gap43*, *Gdnf*, *Hspb1*, *Il6*, *Itgb5*, *Itgb7*, *Jun*, *Klf7*, *Lgals1*, *Mtl5*, *Ngfr*, *Rac1*, *Smad1*, *Snip1*, *Sox11*, *Stat3*, *Syn1*, *Timp1*, *Trp53*. GSEA was performed using the R Package Cluster profiler from BioCManager.

Correlations with our previous RNA-seq dataset were assessed using Pearson's correlation. Genes with $\log_2$ fold-change values greater than 10 or less than -10 were excluded in this analysis to minimize potential confounding effects.

Statistical analysis

GraphPad Prism 8 (version 8.0.2) was used for the statistical analysis of the histology data. The normal distribution of the samples was tested with Shapiro-Wilk test. The statistical test used in each analysis is specified in the results section, and the experimental unit (*n*) corresponds to animal number. All data are expressed as group mean $\pm$ standard deviation (SD). Differences were considered statistically significant if *P* < 0.05.

Results

Calb1-Cre/tdT mice preferentially label low-threshold mechanoreceptors

The Calb1-Cre transgenic mouse has been described as a tool for studying A β RA-LTMRs (Zheng et al., 2019). To corroborate this, we performed immunostaining in the DRG of Calb1-Cre/tdT reporter mice against markers of specific neuron subtypes (Fig. 1). While this line was originally described as labelling a single neuronal population, our analysis revealed two

clearly distinct fluorescent populations: medium-to-large neurons with a diameter above 25 μ m (hereafter 'large') and small neurons below 25 μ m (Fig. 1B). Large neurons co-labelled predominantly with the A β -LTMR marker Calb1 ($85.84 \pm 2.85\%$) and with NFH ($85.58 \pm 0.96\%$). A substantial proportion of these neurons were also positive for the marker Pvalb ($41.03 \pm 4.51\%$), which is classically associated with proprioceptors but is also expressed in a subset of RA-LTMR (de Nooij et al., 2013; Wu et al., 2019). We cannot exclude the possibility that a fraction of these tdTomato⁺/Pvalb⁺ neurons correspond to proprioceptors, as a subset of Ia proprioceptors has been reported to express Calb1 (Wu et al., 2021).

In contrast, small neurons co-labelled mostly with Calb1 ($89.59 \pm 2.17\%$), but only $6.54 \pm 0.62\%$ were NFH⁺. These also did not show expression of Pvalb ($1.57 \pm 0.85\%$). Neither large nor small neurons co-labelled with the nociceptive markers IB4 ($0.29 \pm 0.29\%$) and CGRP ($3.78 \pm 2.05\%$) (Fig. 1C–E). These data are consistent with published scRNA-seq showing Calb1 expression in two distinct DRG neuron subtypes Ntrk3^{high}+Ntrk2 and Rxfp1 clusters, Fig. A1) (Bhuiyan et al., 2024).

Next, we evaluated axonal distribution in the femoral nerve and skin. We found that most fluorescent axons in the femoral nerve were in the saphenous branch. In hairy skin, axons were found in hair follicle receptors (Fig. 1F). Altogether, these observations indicate that fluorescent neurons in Calb1-Cre/tdT mice fit the profile of cutaneous afferents, labelling predominantly (although not exclusively) RA-LTMRs.

A β -LTMRs preferentially regenerate toward cutaneous targets

We assessed whether Calb1⁺ neurons exhibit preferential regeneration into a specific nerve branch after injury. We transected and repaired the femoral nerve above its bifurcation and, at 1 or 8 weeks post-injury, applied distinct retrograde tracers distal to the lesion in both the cutaneous and the muscle branch. Tissue was collected 1 week after tracer application to allow transport to the soma, and we then quantified neurons that had regenerated into the muscle branch, the cutaneous branch, or both (Fig. 2A and B). Labelling therefore reflects the state of regeneration at the time of tracer application and is presented in the figure accordingly (1 and 8 weeks).

In intact nerves, we corroborated that most retrotraced neurons projected to the cutaneous branch (27 ± 4.93 neurons in muscle branch versus 141 ± 19.52 neurons in cutaneous branch), as expected for a population enriched in A β -LTMRs. One week after injury, we did not observe significant differences between the number of neurons found in the muscle and the cutaneous

branch (14.17 ± 3.37 neurons and 22.33 ± 5.32 neurons, respectively). Conversely, by 8 weeks neurons showed a significant preferential regeneration towards the cutaneous branch (25.13 ± 2.42 neurons in muscle branch versus 39.5 ± 5.43 neurons in the cutaneous branch, $P = 0.039$) (Fig. 2C). Although modest ($59.96 \pm 4.32\%$ of cutaneous preference; Fig. 2D), this preference was consistent across most animals (Table 2). Thus, A β -LTMRs show a preferential regeneration towards

their original branch comparable to that described for motoneurons and other sensory afferents.

Calb1 + neurons have limited regeneration after nerve injury

To establish the regenerative capacity of Calb1⁺ neurons, we performed a sciatic nerve crush and quantified the number of tdTomato⁺ axons at two distances distal from

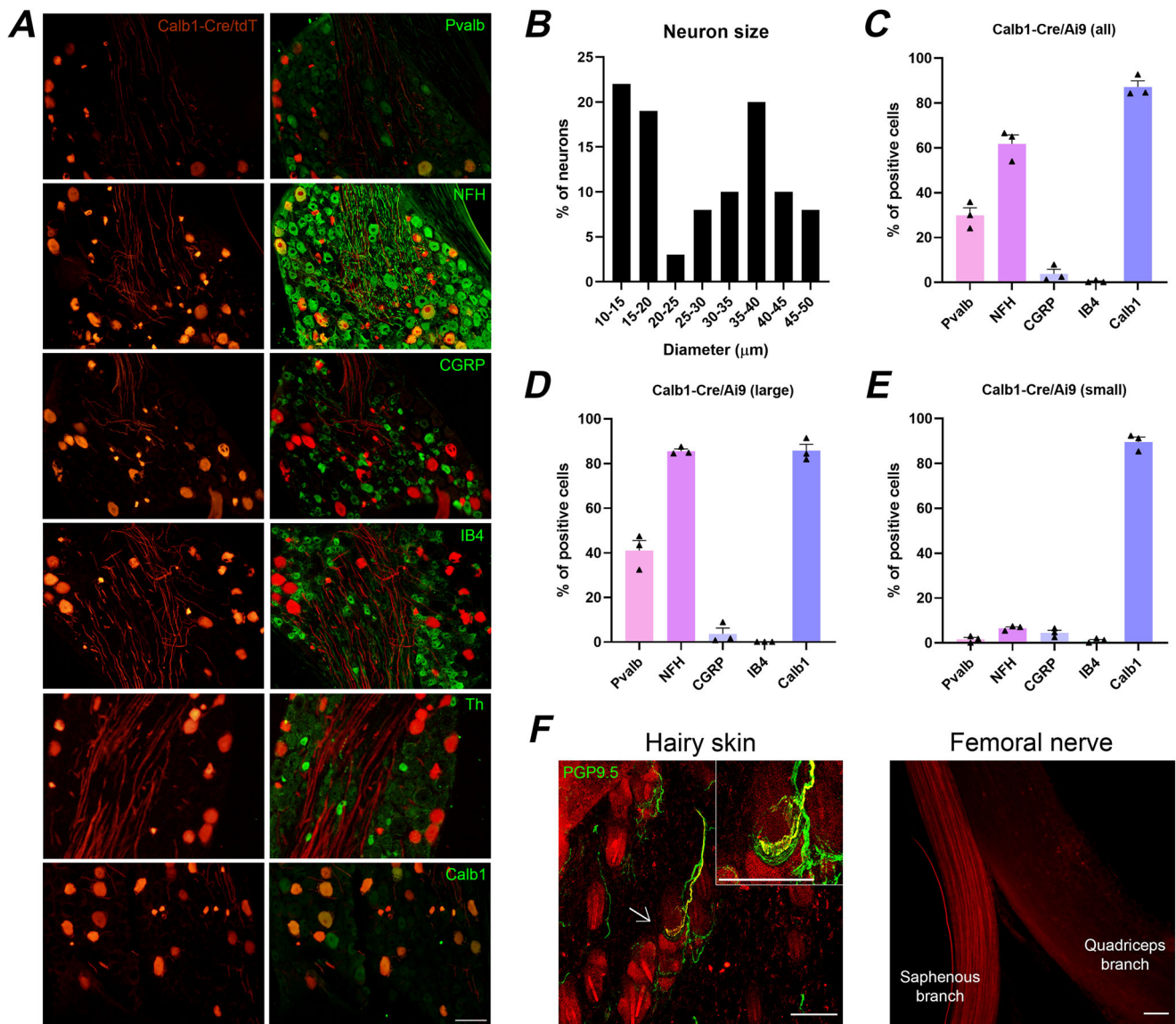


Figure 1. Characterization of Calb1-Cre/tdT mice

A, Immunostainings against specific markers in the DRG (parvalbumin, Pvalb; neurofilament heavy chain, NFH; calcitonin gene-related peptide, CGRP; isolectin B4, IB4; tyrosine hydroxylase, Th; calbindin-1, Calb1). The first column shows tdTomato fluorescence, whereas the second column shows the merge of the reporter and the staining. **B**, Size distribution of tdTomato⁺ neurons in the DRG. **C–E**, Quantification of tdTomato co-localization with the indicated markers in all neurons **C**, large neurons (>25 μ m diameter; **D**), and small neurons (<25 μ m, **E**) ($n = 3$). **F** Representative images of hairy skin and femoral nerve. The arrow indicates a tdTomato⁺ axon (yellow) associated with a hair follicle (PGP9.5 immunostaining is shown in green). Scale bar: 100 μ m.

the lesion site (Fig. 3A). At 7 days post-injury (dpi), only $29.61 \pm 5.24\%$ and $5.98 \pm 1.65\%$ of the total axons had reached 12 and 17 mm, respectively. By 9 dpi, regeneration increased to $59.47 \pm 9.13\%$ at 12 mm and $51.60 \pm 6.96\%$ at 17 mm (Fig. 3C and D). At both distances, there was a significant increase in the number of axons between 7 dpi and 9 dpi ($P = 0.003$ at 12 mm and $p < 0.001$ at 17 mm), but none of the conditions reached control values. Notably, at 9 dpi there was no significant difference between 12 mm and 17 mm ($P = 0.706$), consistent with a

regeneration plateau. We further compared these results with our previously published datasets on nociceptor, proprioceptor, and motoneuron regeneration (Bolívar et al., 2024), and found that Calb1⁺ neurons regenerated significantly less than nociceptors and motoneurons, but at levels comparable to those of proprioceptors (Fig. A2).

Finally, we analysed the total number of neurons that regenerated into both the muscle and the cutaneous branch following femoral nerve transection and repair using the retrograde tracer labelling (Fig. 2A). Incomplete

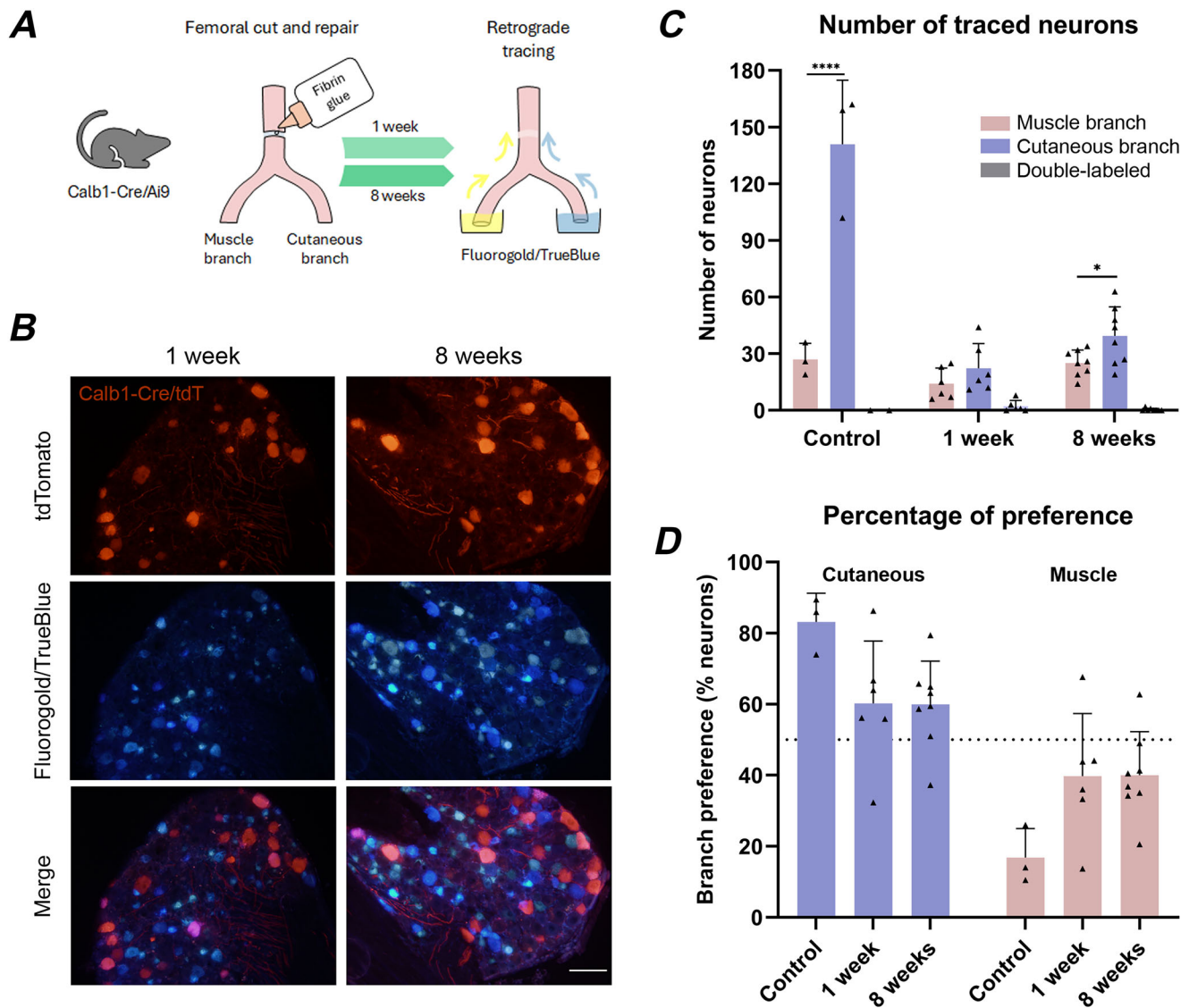


Figure 2. Preferential regeneration of Calb1⁺ neurons

A, Schematic representation of the experimental design. B, Representative DRG images at 1 week ($n = 6$) and 8 weeks ($n = 8$) after nerve transection. Neurons were counted when tdTomato (red) co-localized with TrueBlue (blue) or Fluorogold (yellow). Those tdTomato⁺ neurons showing both TrueBlue and Fluorogold were counted as double-labelled. C, Quantification of neurons whose axons projected to the muscle branch, the cutaneous branch, or both. D, Percentage of retrotraced neurons in the muscle or cutaneous branch calculated over the total number of regenerated neurons. Muscle preference is plotted on the right and cutaneous preference on the left. Differences between branches were assessed by two-way ANOVA followed by a Sidak *post hoc* test. * $P < 0.05$, **** $P < 0.0001$ (muscle vs. cutaneous branch). Scale bar: 100 μ m.

Table 2. Ratio of animals with preference to regenerate through the cutaneous or muscle branch 1 and 8 weeks after section and repair of the femoral nerve.

Preferred branch	Touch mechanoreceptors	
	1 week	8 weeks
Cutaneous	5/6	6/8
Muscle	1/6	1/8
Equal	0/6	1/8

regeneration was also evident in this model, with only $23.02 \pm 4.14\%$ and $38.69 \pm 3.52\%$ of neurons regenerating into the distal branches at 1 and 8 weeks, respectively (Fig. 3E). Together, these data indicate that A β -LTMRs display limited regenerative capacity and fail to fully recover after peripheral nerve injury.

Regenerative transcriptional programme activated by A β -LTMRs

We isolated the pool of actively translated mRNAs in Calb1⁺ neurons using the Ribotag assay (Fig. 4A). Calb1-Cre mice were crossed with RiboTag mice to generate double-transgenic animals expressing a hemagglutinin (HA) tag specifically in ribosomes of this neuronal population. By immunoprecipitating the tagged ribosomes from DRG homogenates, we isolated neuron-specific mRNAs and performed RNA-seq to compare naive *versus* nerve crush samples.

To assess the specificity of the strategy, we confirmed that the RNA from control immunoprecipitates (IPs) had enriched transcripts of the well-established A β -LTMR transcripts *Calb1*, *Nefh*, *Nefm* and *Nefl* compared to the input (whole DRG) (Fig. 4D). Conversely, IPs were depleted for markers of other neuronal subtypes (*Scn10a*, *Mrgprd*, *Calca*, *Pvalb*, *Th*), as for glial markers (*Fabp7*,

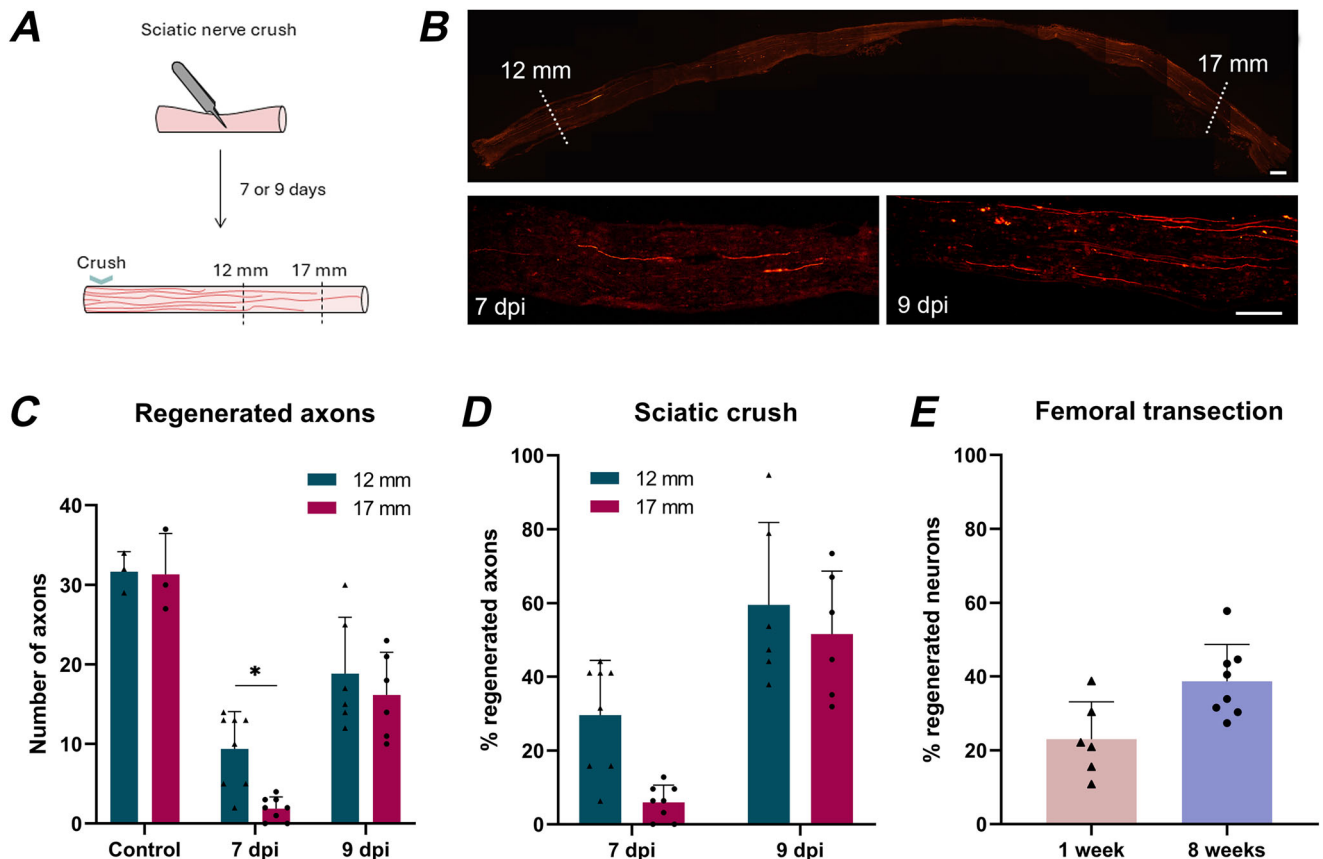


Figure 3. Regenerative capacity of A β -LTMRs

A, Schematic representation of the experimental design. B, Longitudinal sections of tibial nerves from Calb1-Cre/tdT mice at 7 days (top image and bottom left; $n = 8$) and 9 days (bottom right; $n = 6$) post-injury (dpi). C and D, number C, and percentage D, of tdTomato⁺ axons in the tibial nerve after injury. E, Percentage of neurons that regenerated 1 or 8 weeks after femoral transection, assessed by retrograde tracers. Percentages were normalized to the total number of traced neurons in uninjured animals (i.e. without femoral nerve injury). Differences in axon regeneration were assessed by two-way ANOVA followed by a Sidak *post hoc* test. * $P < 0.05$ (proximal vs. distal). Scale bar: 100 μ m.

Dcn, *Plp1*) (Fig. 4E). Principal component analysis (PCA) revealed that samples clustered according to their experimental group (Fig. 4B).

Seven days after nerve crush, 1941 genes were differentially expressed in Calb1⁺ neurons (Fig. 5A,

Supplementary data 1). Despite the limited regeneration of these neurons, we found an enrichment of regeneration associated genes (RAGs) in the crush condition (*Sprr1a*, *Atf3*, *Gap43*, *Tubb2b*) (Fig. 5B). Pathway analysis revealed significant activation of regeneration-related processes,

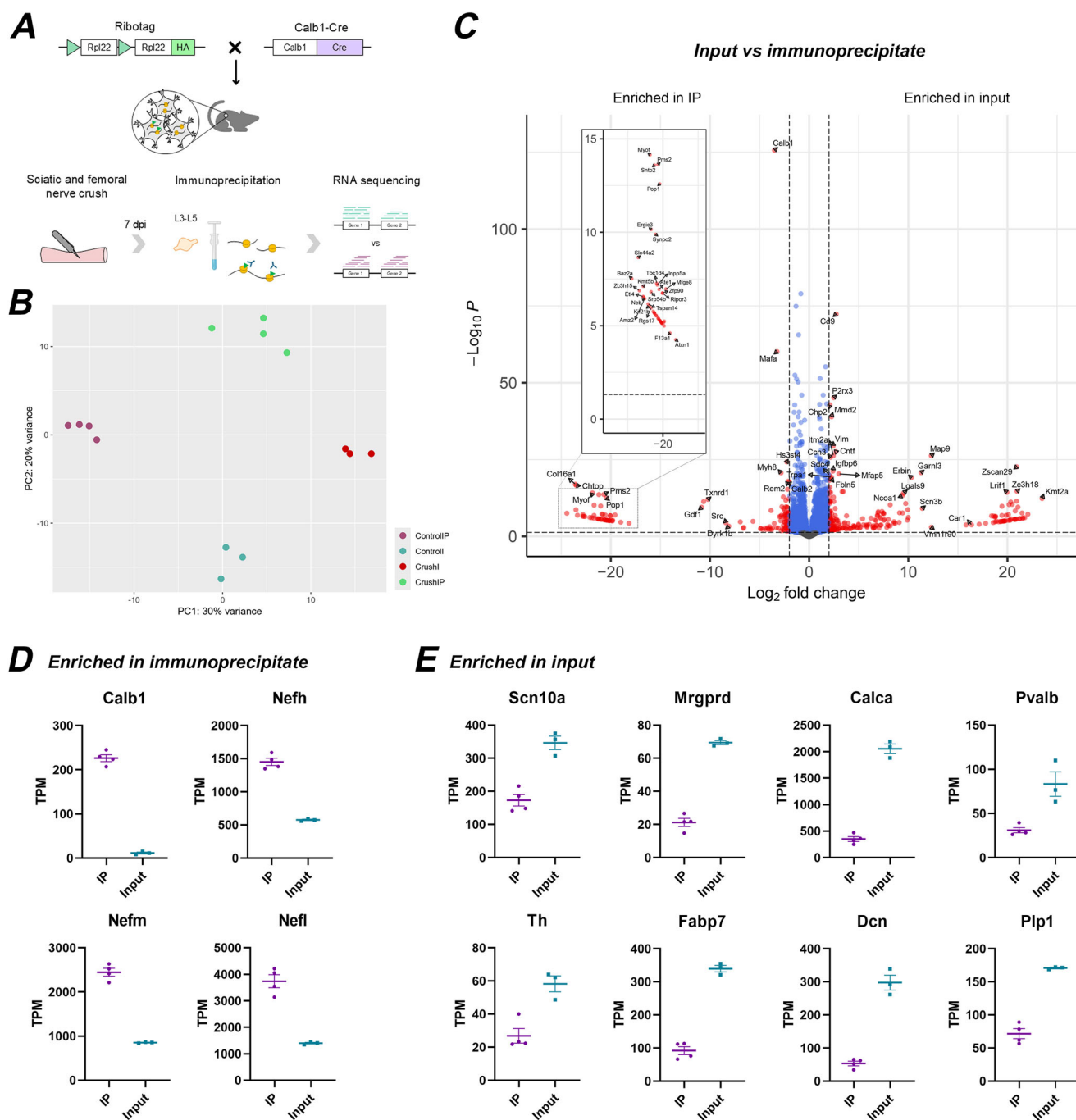


Figure 4. Validation of the immunoprecipitate specificity in naive Calb1-Cre/Ribotag mice

A, Schematic representation of the experimental design. **B**, Principal component analysis (PCA) of the samples showing segregation of samples by experimental group. **C**, Volcano plot comparing input (whole DRG) versus immunoprecipitate (IP; Calb1⁺ neurons) showing significant differentially expressed genes in blue (log₂ fold-change < 2) and red (log₂ fold-change > 2). **D** and **E**, transcripts per million (TPM) for representative genes enriched in the IP **D**, or enriched in the input **E**.

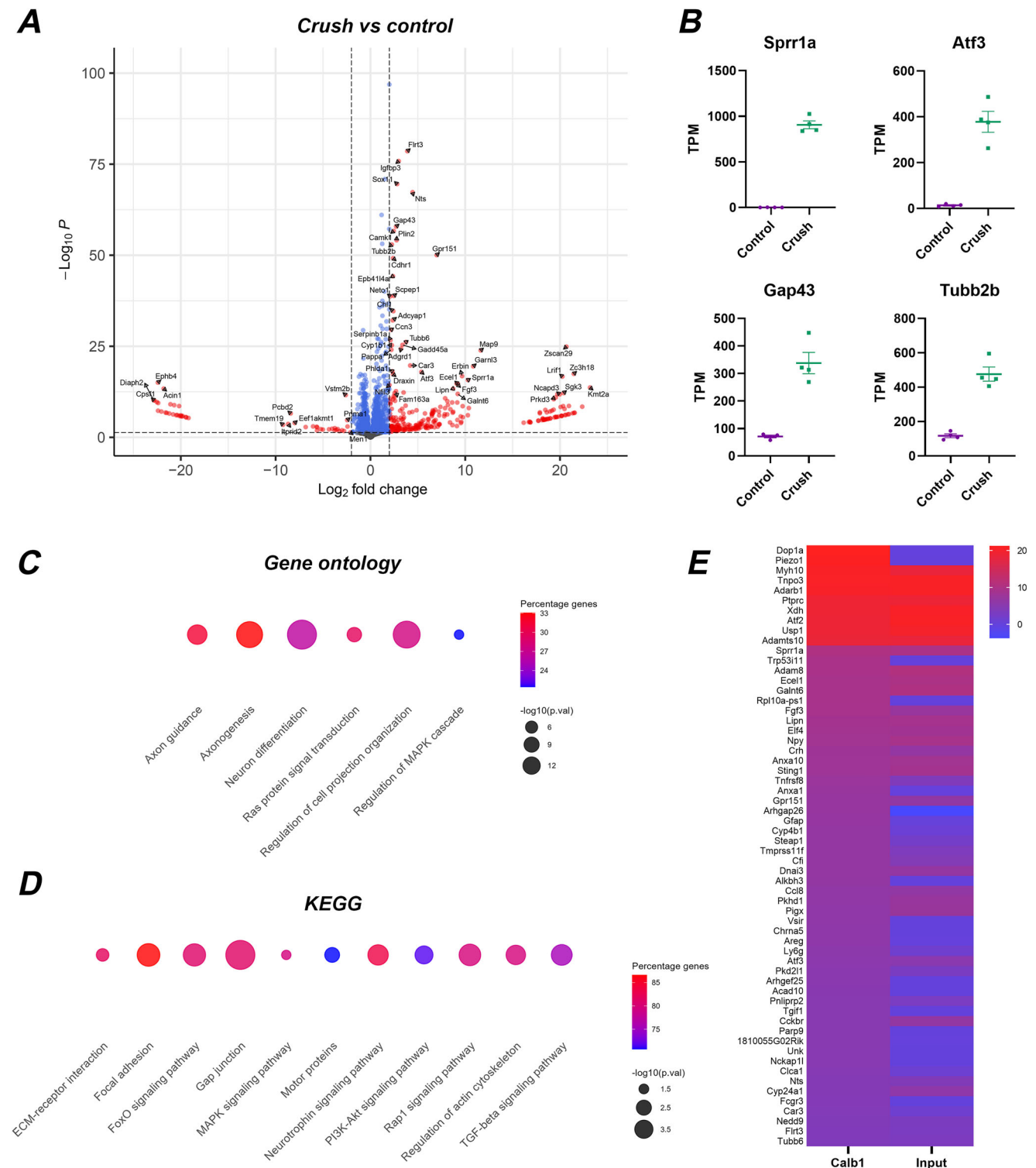


Figure 5. Injury-induced transcriptional changes in A β -LTMRs

A, Volcano plot of the control versus nerve crush DRG immunoprecipitates showing significantly differentially expressed genes in blue ($\log_2$ fold-change < 2) and red ($\log_2$ fold-change > 2). B, Transcripts per million (TPM) for representative genes enriched after crush. C and D, gene ontology C, and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment D, analysis showing pathways activated after nerve injury. E, Top upregulated genes in Calb1 $^{+}$ neurons after injury and their corresponding changes in input (whole DRG) samples (expressed in $\log_2$ fold-change).

including Axon guidance, Axonogenesis and Regulation of cell projection organization (Fig. 5C and D).

Because peripheral neuron populations exhibit distinct regeneration behaviours despite sharing the same micro-environment, we hypothesized that population-specific transcriptional programmes could underlie these physiological differences. Indeed, although some of the top upregulated genes after injury overlapped with those observed in the input, we found that many genes such as *Dop1a* and *Piezo1* displayed distinct activation patterns relative to input (Fig. 5E). *Dop1a* encodes a protein that helps direct the transport of vesicles and organelles within the cell (Mahajan et al., 2019), and it has also been suggested to play a role in myelinogenesis (Tanaka et al., 2014), whereas *Piezo1* is a mechanosensitive cation channel required for mechanotransduction (Coste et al., 2010; Murthy et al., 2017). Together, these findings indicate that $A\beta$ -LTMRs engage a partially specific regenerative transcriptional programme after nerve crush.

Distinct transcriptional responses in peripheral neuronal subtypes

We have previously investigated the regeneration profile and transcriptional response to injury of different peripheral neuron populations using the same experimental approaches employed here for $A\beta$ -LTMRs (Bolívar & Udina, 2022; Bolívar et al., 2024). Specifically, we analysed the transcriptional response of proprioceptors (*Pvalb*⁺ neurons), nociceptors (*Trpv1*⁺-lineage neurons), a population enriched for A high-threshold mechanoreceptors (*Npy2r*⁺-lineage) and motoneurons (MNs). We correlated the log₂ fold-change (log₂FC) of differentially expressed genes after injury across populations and found a significant but modest correlation between gene expression in *Calb1*⁺ neurons and the other populations (Fig. 6A–E), suggesting a partially distinct injury response. As expected, the *Calb1* transcriptome correlated more strongly with the other sensory afferents than with motoneurons. Notably, the correlation was highest between *Calb1*⁺ and *Pvalb*⁺ neurons despite their distinct target organs, suggesting that similarities in function (low-threshold mechanosensation), size (medium to large neurons) and/or myelination may be more relevant to this response. Among the remaining populations, the strongest correlation was observed between *Trpv1*⁺ and *Npy2r*⁺, followed by *Pvalb*⁺ and motoneurons (Fig. 6F).

Finally, we wondered whether the limited regeneration of LTMRs might reflect insufficient activation of RAGs. A targeted gene set enrichment analysis (GSEA) based on a literature-curated RAG list revealed significant positive enrichment of RAGs in *Calb1*⁺ neurons after injury (Fig. 6G), comparable to that observed in the

other populations examined (Fig. A3). To validate RAG activation, we assessed expression of the canonical RAG ATF3 in *Calb1*⁺ neurons by immunofluorescence (Fig. A4). Using retrogradely traced DRG samples 7 days after femoral nerve transection and repair, we found that ATF3 was expressed in retrograde tracer-positive neurons, which had regenerated to the tracer application site, but also in retrograde tracer-negative neurons, which had either failed to regenerate or regenerated significantly more slowly.

Therefore, despite having a subtype-specific injury transcriptome, $A\beta$ -LTMRs activate canonical RAGs to a similar extent across populations, indicating that RAG activation alone is insufficient to explain their limited regenerative capacity.

Discussion

The *Calb1*-Cre mouse line was originally described as a tool to label $A\beta$ RA-LTMRs (Zheng et al., 2019). In our hands, however, this line labelled two distinct neuronal subpopulations: one composed of large neurons and another of small neurons. We confirmed that large neurons exhibited expected features of $A\beta$ RA-LTMRs, including cutaneous targets and expression of histological markers associated with this neuron type. Interestingly, we found an additional labelled population of small-diameter, cutaneous neurons. Previous scRNA-seq studies have identified a *Rxfp1*⁺ cluster expressing *Calb1*. These *Rxfp1*⁺ neurons represent a poorly characterized, low-abundance sensory neuron subtype (Wang et al., 2021, 2023; Zeisel et al., 2018). The discrepancy with previous reports may result from differences in the timing of trimethoprim-induced transgene activation, as previous studies induced expression at P18–P19 (Zheng et al., 2019), whereas our induction was performed in adult mice (7–9 weeks old). As our main goal was to improve target selectivity, we analysed both populations together as cutaneous neurons. Future studies could leverage recently developed genetic tools (Qi et al., 2024) to investigate more precisely defined subtypes.

After a nerve transection, endoneurial tubes are disrupted and regenerating axons rarely re-enter their original pathways to the appropriate targets (Brushart & Mesulam, 1980). However, it has been described that neurons can exhibit a preferential regeneration towards their original target organ (Bolívar & Udina, 2022; Brushart, 1988, 1993; Brushart et al., 1998; Li et al., 2025; Madison et al., 1996, 2007; Maki et al., 2005; Martini et al., 1994; Mears et al., 2003; Robinson & Madison, 2003, 2005, 2006, 2013a, 2013b; Uschold et al., 2007). Classically, this has been demonstrated in motoneurons, where axons preferentially grow through muscle pathways after transection. In our previous work, we confirmed

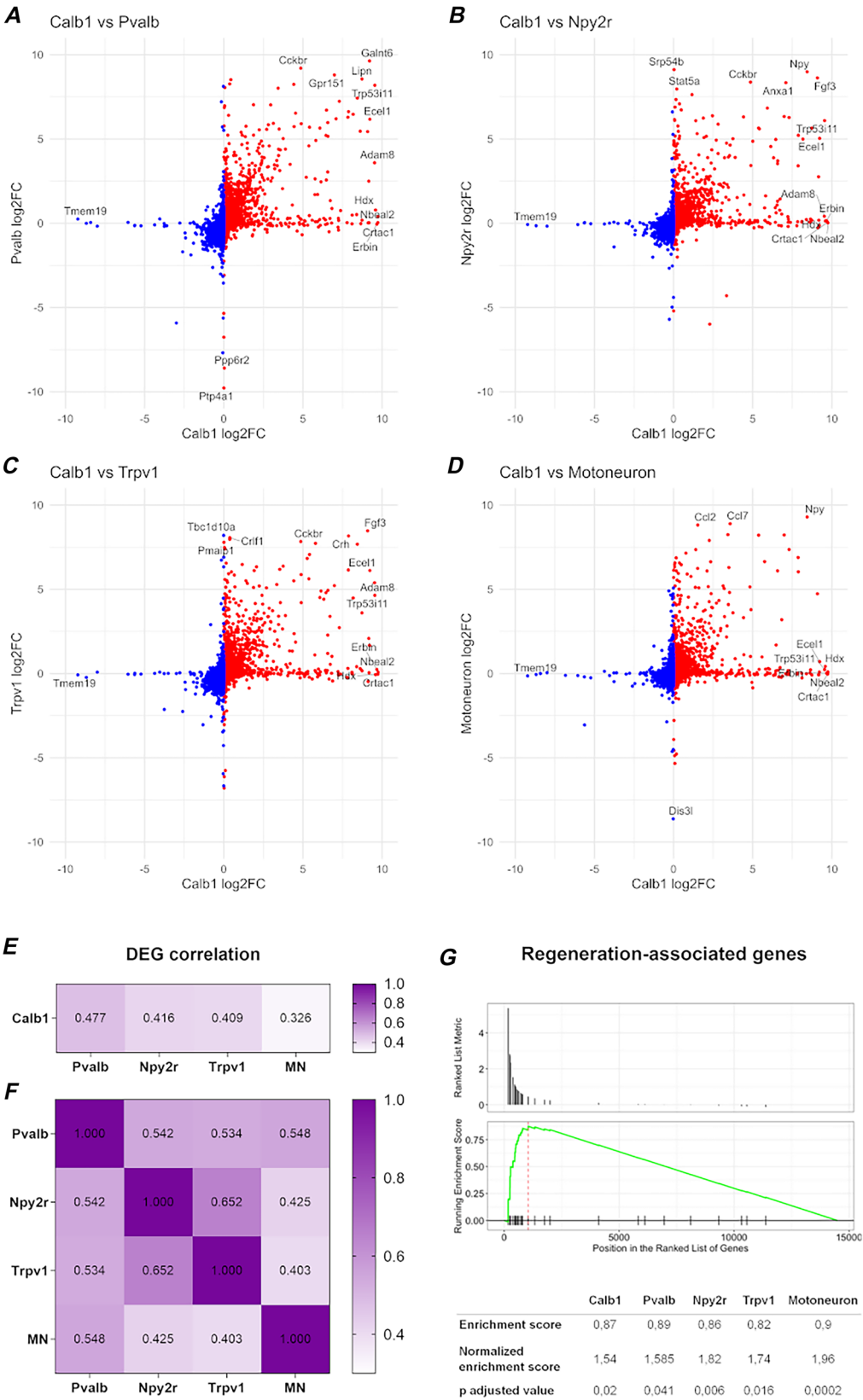


Figure 6. Cross-population comparison of injury-induced gene expression changes
A–D, Scatter plots comparing the log₂ fold-change (log₂FC) of the differentially expressed genes (DEGs) after injury in Calb1+ neurons versus proprioceptors (Pvalb, A), an A high-threshold mechanoreceptor-enriched

population (Npy2r, B), nociceptors (Trpv1, C) and motoneurons (MN, D). (E) Heatmap of Pearson correlation coefficients (r) between DEGs in Calb1+ neurons and each neuron population. All correlations were significant ($P < 0.0001$). (F) Heatmap of pairwise Pearson correlations among neuronal subtypes from Bolívar et al. (2024). All correlations were significant ($P < 0.0001$). (G) Gene set enrichment analysis (GSEA) of a literature-curated set of regeneration-associated genes (RAGs) in Calb1+ neurons, with a summary of enrichment metrics across all populations.

that this phenomenon also occurs in some primary afferent subtypes, which show selectivity to regenerate towards their original target organ (Bolívar & Udina, 2022). Here we extended this work by showing that A β RA-LTMRs preferentially regenerate into cutaneous pathways after injury. This preference varies across neuron subtypes: proprioceptors and motoneurons are attracted to muscle pathways, whereas cutaneous afferents prefer cutaneous branches (Bolívar et al., 2024; Madison et al., 1996). Several studies have described differences in the trophic factors secreted by denervated Schwann cells from muscle and cutaneous branches (Brushart et al., 2013; Höke et al., 2006), as well as molecular differences between Schwann cell subtypes (Jesuraj et al., 2012; Martini et al., 1992, 1994; Saito et al., 2005, 2010). These factors, together with trophic cues from target organs (Madison et al., 2007; Uschold et al., 2007), are likely key factors driving this regeneration selectivity. Nonetheless, all regenerating axons, regardless of subtype, are found in the same post-injury microenvironment and thus are exposed to comparable trophic support (aside from potential local differences in satellite glial cells or macrophages). Therefore, we believe that intrinsic differences in the regenerative programmes activated by these subtypes should influence this process.

Similarly, we have consistently found that neuronal subtypes regenerate at different rates. Calb1+ neurons showed a strikingly low regeneration rate and even incomplete regeneration after a sciatic crush and femoral transection. This regeneration is comparable to that seen in proprioceptors and substantially poorer than that of nociceptors. Classical studies using cuff electrodes suggested that small afferents regenerate more effectively than large sensory neurons (Krarup et al., 1989; Negredo et al., 2004). Functional studies likewise indicated a faster, more complete functional recovery in unmyelinated compared to myelinated fibres (Navarro et al., 1994). More recently, incomplete regeneration of LTMRs after injury has been reported (Gangadharan et al., 2022; Jeon et al., 2024). This deficit is unlikely to be explained by neuronal loss, as large-diameter neurons are largely preserved after nerve injury (Cooper et al., 2024). This lack of regeneration can have detrimental consequences: beyond limiting functional recovery, insufficient regeneration may enable aberrant reinnervation of denervated territories by nociceptive fibres, contributing to neuropathic pain (Jeon et al., 2024).

Does a differential transcriptional response to injury explain these differences? Previous snRNA-seq studies identified a conserved response across DRG neurons after nerve injury, with cells clustering into an 'injured' transcriptional state (Renthal et al., 2020). Our data are consistent with the existence of a conserved core response, but they also reveal a subtype-specific regulation. Comparing the injury-induced transcriptome of Calb1+ neurons with our previous RNA-seq data from other populations, we found only a modest correlation among DEGs. It is important to note that part of this divergence likely reflects baseline transcriptional differences between neuron subtypes. While Renthal et al. (2020) defined a common transcriptional response across neurons, our population-specific comparisons (injured vs. uninjured within each subtype) inherently capture subtype-restricted changes. For example, the downregulation of subtype-specific identity markers will only be detected within the corresponding population, contributing to the divergence observed between neuron subtypes. Nevertheless, beyond these expected differences, many DEGs may represent subtype-specific components of the injury response, potentially underlying the distinct regenerative capacities observed across neuronal populations.

Given the limited regeneration capacity of A β -LTMRs, we asked whether this population shows reduced expression of classical RAGs. Instead, we observed a significant increase in RAGs, including *Gap43*, *Sprr1a* and *Atf3*. GSEA using a literature-based RAG list showed similar activation across all populations, including Trpv1-Cre/tdT which displays a much faster regeneration rate. Thus, transcriptional changes in canonical RAGs cannot explain differences in regeneration speed. We hypothesize that these differences may arise from differential regulation of additional, not-yet-characterized RAGs, or from divergence at the protein level (expression, localization, or post-translational modification) not captured by our approach. For example, *Sting1* was selectively upregulated in A β -LTMRs and proprioceptors after injury, the two subtypes with the poorest regeneration. Although the neuronal role of *Sting1* is not completely understood, there is some evidence suggesting it can shape regeneration-degeneration dynamics. In experimental autoimmune encephalomyelitis, neuronal *Sting1* activation has been linked to neurodegeneration through the activation of ferroptosis (Woo et al., 2024),

and ferroptosis was among the pathways enriched in our dataset after injury (Supplementary data 1). Furthermore, Sting1 global knockout has been shown to enhance functional recovery after nerve injury (Morozzi et al., 2021). These observations suggest Sting1 as a candidate modulator of regenerative capacity. In addition, we observed a subtype-specific upregulation of Atf2. Given prior evidence linking *ATF2* to injury-evoked mechanical allodynia and thermal hyperalgesia (Salinas-Abarca et al., 2018), this gene may be relevant to subtype-specific consequences of injury. In addition, it is important to consider that some of the classical RAGs (e.g., *ATF3*) are also activated in other neuronal injury models such as diabetic and chemotherapy-induced neuropathy, which are not necessarily associated with axon regeneration (Chang et al., 2019; Schinke et al., 2026; Wright et al., 2004). This might reflect the fact that these genes are injury-induced, and although many are needed to activate the intrinsic growth state of the neurons, this alone is not sufficient to explain their regenerative response.

Alternatively, size and the degree of myelination may explain some of the injury-specific responses. During Wallerian degeneration, myelin debris is cleared by macrophages, neutrophils and Schwann cells (Lindborg et al., 2017). Inhibitory myelin-associated molecules and extracellular matrix components, such as

myelin-associated glycoprotein or chondroitin sulphate proteoglycans, can hinder axon regeneration (Mears et al., 2003; Mukhopadhyay et al., 1994; Niederöst et al., 2002; Sarhane et al., 2019; Torigoe & Lundborg, 1998; Zuo et al., 2002). In this context, unmyelinated axons might have a physical advantage, as their endoneurial tubes require less debris clearance. Together with the greater energetic demands of larger, myelinated axons, this could contribute to slower regeneration of myelinated sensory afferents.

This work has some limitations. First, we used Calb1-Cre mice to study A β -LTMRs. Although this line preferentially labels A β -LTMRs, it also labels a second cutaneous population. While these data are relevant for target selectivity, more refined genetic tools would help isolate A β -LTMRs with greater specificity. Second, we analysed the transcriptome at a single time point (7 days after sciatic crush). A time course and additional injury paradigms (e.g., nerve transection) would better define the dynamics and regeneration profile of the A β -LTMR response. Finally, examining the transcriptome at the axonal compartment would be valuable, as local translation at growth cones may be particularly relevant for the regenerative response and could help explain subtype-specific differences in regeneration after nerve injury.

Appendix

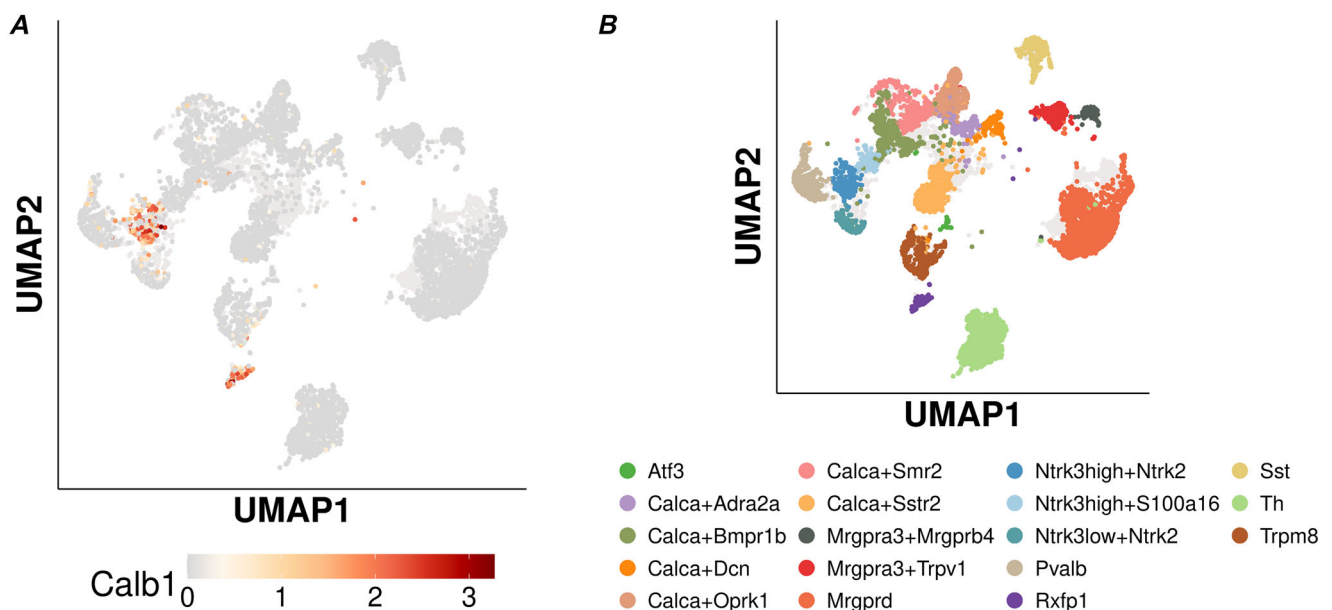


Figure A1. Calb1 gene expression in DRG neurons from Bhuiyan et al. (2024) scRNA-seq data. A, Calb1 expression is restricted to two distinct neuronal clusters. B, Cluster annotations identify these Calb1⁺ clusters as Ntrk3high+Ntrk2 and Rxfp1.

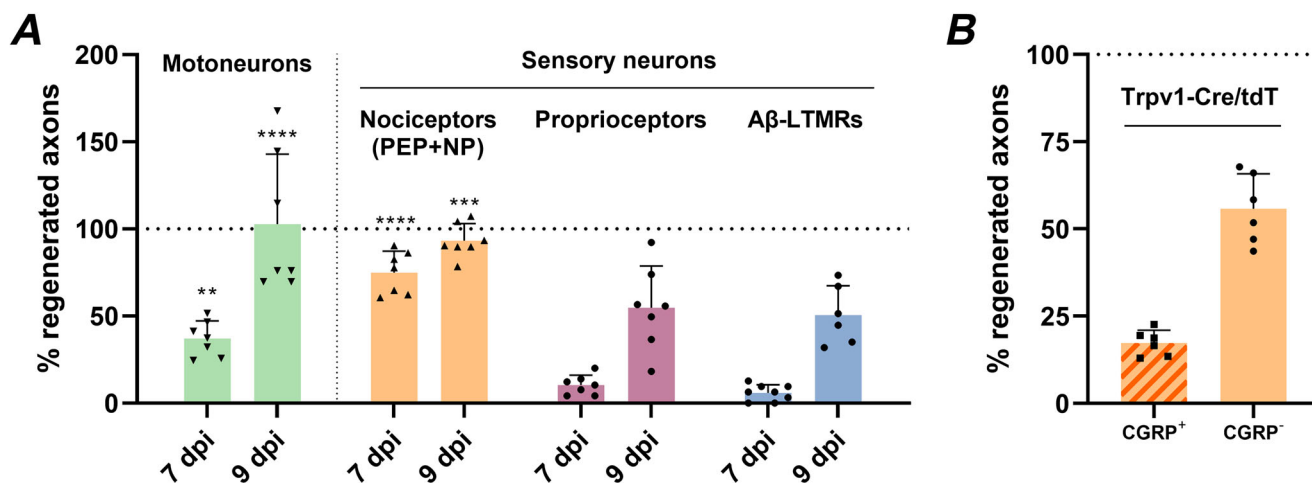


Figure A2. Comparison of the regenerative capacity of different neuron subtypes following sciatic nerve crush

(A) Percentage of regenerated axons reaching 17 mm distal to the lesion site at 7 and 9 days post-injury (dpi). Data from Bolívar et al. (2024) were reanalysed to compare the Aβ-LTMRs (Calb1-Cre/tdT) with other populations: motoneurons (Chat-Cre/tdT), nociceptors (Trpv1-Cre/tdT) and proprioceptors (Pvalb-Cre/tdT). The nociceptor population comprises peptidergic (PEP) and non-peptidergic (NP) neurons. (B) Percentage of tdT⁺/CGRP⁺ and tdT⁺/CGRP⁻ fibres from Trpv1-Cre/tdT mice regenerated at 7 dpi. Differences in axon regeneration were assessed using two-way ANOVA followed by a Sidak's *post hoc* test. ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001 (other populations vs. Aβ-LTMRs).

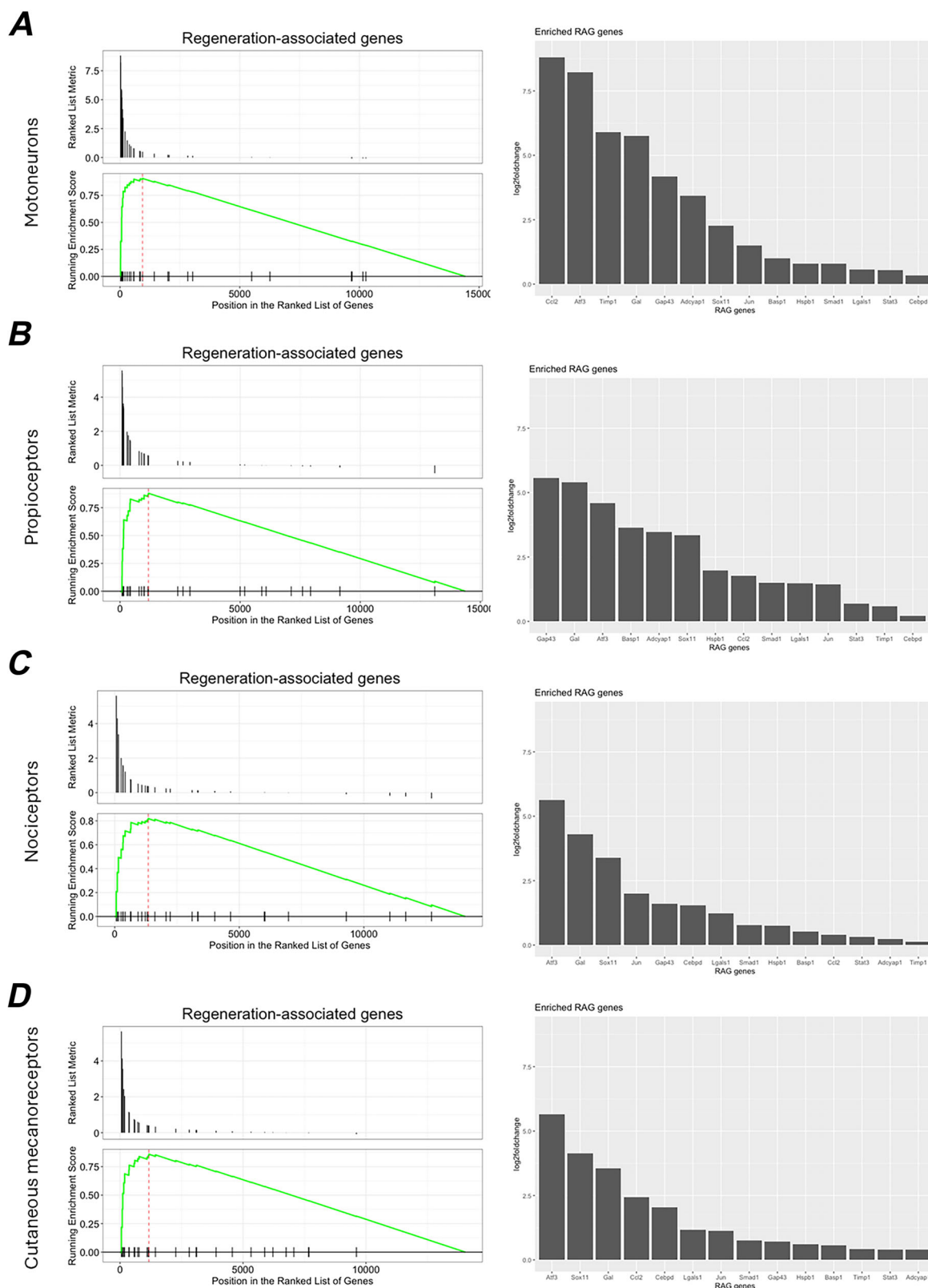


Figure A3. Gene set enrichment analysis of regeneration-associated genes in different neuron populations after injury

Gene set enrichment analysis of a literature-curated set of regeneration-associated genes in motoneurons (A), proprioceptors (Pvalb⁺ neurons) (B), nociceptors (Trpv1⁺ neurons) (C), and cutaneous mechanoreceptors (Npy2r⁺ neurons) (D).

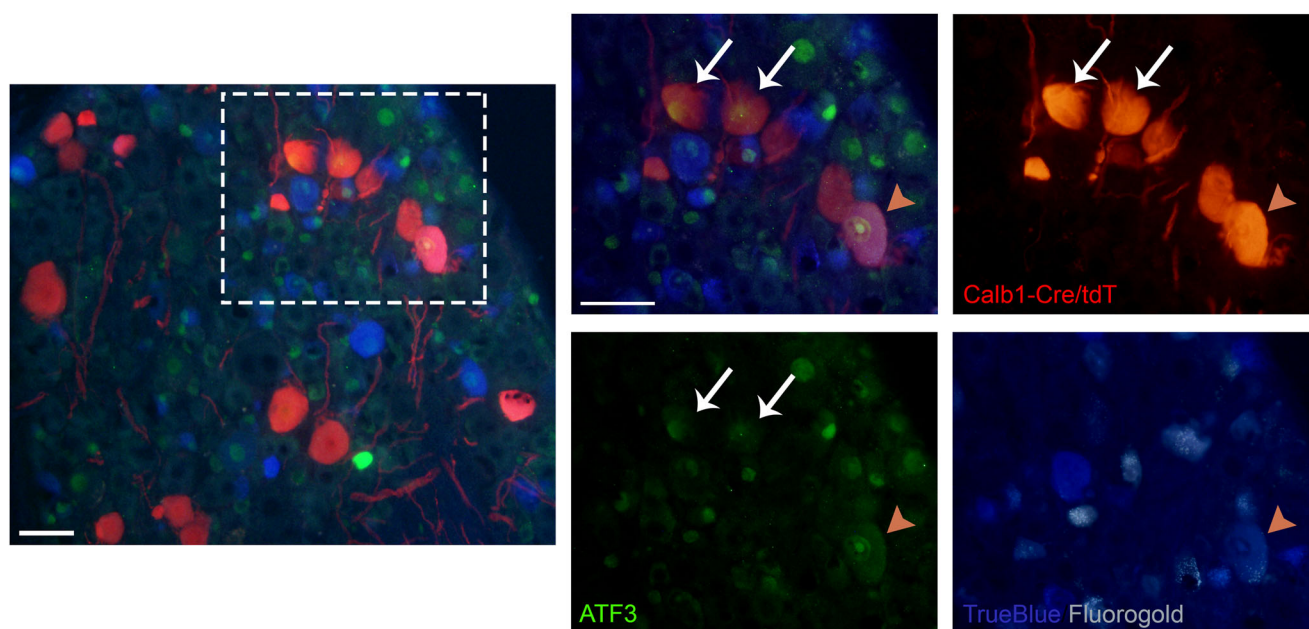


Figure A4. ATF3 expression in Calb1+ neurons after femoral nerve injury

Representative images of DRG sections 7 days after femoral nerve transection and repair, showing ATF3 expression (green) in both retrograde tracer-positive (magenta: red and blue; orange arrowhead) and tracer-negative Calb1⁺ neurons (red, white arrows).

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Additional information

Data availability statement

Sequencing data have been deposited in SRA under accession code PRJNA1424702.

Competing interests

The authors declare no competing financial interests.

Author contributions

S.B.: conceptualization, data curation, formal analysis, validation, investigation, visualization, methodology, software,

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Additional supporting information can be found online in the Supporting Information section at the end of the HTML view of the article. Supporting information files available:

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