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Research paper

Preferential regeneration and collateral dynamics of motor and sensory neurons after nerve injury in mice

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ABSTRACT

Specificity in regeneration after peripheral nerve injuries is a major determinant of functional recovery. Unfortunately, regenerating motor and sensory axons rarely find their original pathways to reinnervate appropriate target organs. Although a preference of motor axons to regenerate towards the muscle has been described, little is known about the specificity of the heterogeneous sensory populations. Here, we propose the comparative study of regeneration in different neuron subtypes. Using female and male reporter mice, we assessed the regenerative preference of motoneurons (ChAT-Cre/Ai9), proprioceptors (PV-Cre/Ai9), and cutaneous mechanoreceptors (Npy2r-Cre/Ai9). The femoral nerve of these animals was transected above the bifurcation and repaired with fibrin glue. Regeneration was assessed by applying retrograde tracers in the distal branches of the nerve 1 or 8 weeks after the lesion and counting the retrotraced somas and the axons in the branches. We found that cutaneous mechanoreceptors regenerated faster than other populations, followed by motoneurons and, lastly, proprioceptors. All neuron types had an early preference to regenerate into the cutaneous branch whereas, at long term, all neurons regenerated more through their original branch. Finally, we found that myelinated neurons extend more regenerative sprouts in the cutaneous than in the muscle branch of the femoral nerve and, particularly, that motoneurons have more collaterals than proprioceptors. Our findings reveal novel differences in regeneration dynamics and specificity, which indicate distinct regenerative mechanisms between neuron subtypes that can be potentially modulated to improve functional recovery after nerve injury.

Significance statement

The lack of regeneration specificity after nerve transections is one of the largest challenges in nerve regeneration, which directly affects functional target regeneration. Most studies have focused on the regenerative environment, but only a few have examined the potential differences in the intrinsic growth response of different peripheral neurons. Using reporter mice, we demonstrate that three paradigmatic populations of peripheral neurons (motoneurons, proprioceptors, and cutaneous mechanoreceptors) share a common mechanism guiding their axons to the cutaneous branch shortly after injury, but at long term this mechanism differs, and each neuron type prefers to regenerate through the "correct" branch. Our findings evidence distinct regenerative mechanisms that could be potentially modulated to guide axons, thus improving functional recovery after nerve transection.

1. Introduction

Peripheral neurons can regenerate after nerve injury, but functional recovery might fail if reinnervation is not specific. After a transection, axons elongate following environmental cues from Schwann cells and target organs (Bolívar et al., 2020). Classical studies in mammals have described a "preferential motor reinnervation" (PMR), a preference of the motoneurons to regenerate towards the muscle branch rather than to the cutaneous branch of the nerve (Brushart, 1988, 1993; Madison et al., 2007; Madison et al., 1996). However, this phenomenon is controversial and depends on many factors such as the species (Martini et al., 1994; Robinson and Madison, 2006), the age of the animal (Brushart, 1988; Robinson and Madison, 2006), or the repair method (Robinson and Madison, 2005; Robinson and Madison, 2003).

Most of these studies have focused on PMR, but only a few investigated preferential regeneration in sensory neurons. Peripheral sensory neurons are a highly heterogeneous population that includes

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proprioceptors, several touch receptors, and nociceptors and, therefore, these can be found in both the cutaneous and the muscle branch of the nerve in uninjured nerves. Thus, there is not a general "correct path" for all the dorsal root ganglia (DRG) neurons and addressing the question of specific regeneration is more complex. There are, however, few studies suggesting that sensory axons might preferentially regenerate through the cutaneous branch (Maki et al., 2005; Maki et al., 1996; Mears et al., 2003), although the diversity of DRG neurons was not taken into account. Furthermore, muscle afferents may have a preference for the muscle branch (Brushart et al., 2005; Madison et al., 1996). However, the evidence is scarce and further studies are needed to confirm that preferential sensory regeneration exists, especially in mice.

Reporter mice that express a fluorescent protein in specific neuron subpopulations can facilitate the study of regeneration in the different types of neurons. Genetic labeling of the neuron soma can help us differentiate the preference in regeneration in different types of neurons in the DRG. Moreover, fluorescence in the axons of these neurons allows us to study collateral branching during regeneration, a process that might be relevant in specificity (Brushart et al., 1998; Redett et al., 2005; Robinson and Madison, 2013).

In the present study, we aimed to take advantage of reporter transgenic mice to investigate PMR and preferential sensory regeneration. We have examined specificity in motoneurons and in two sensory populations that have different target organs: proprioceptors, which innervate muscle spindles, and cutaneous mechanoreceptors, whose endings are found mainly in the skin. Our results show that there is a common mechanism in regeneration that guides neurons towards the cutaneous branch shortly after injury. At long term, we find that motoneurons and proprioceptors have preference for the muscle branch, whereas cutaneous mechanoreceptors prefer the cutaneous branch. These results extend the classical concept of PMR to sensory neurons and show that there might be an intrinsic neuronal mechanism guiding them towards specific target organs after an injury.

2. Methods

2.1. 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.

Double transgenic mice were generated by breeding homozygous Ai9 (RCL-tdT) mice (#007909) with different homozygous Cre-driver lines from The Jackson Laboratory (Bar Harbor, Maine, USA): ChAT-IRES-Cre (#006410), B6 PV-Cre (#017320) and Npy2r-ires-Cre (#029285). We obtained three mice lines that expressed the red fluorescent protein TdTomato under the control of a specific neuronal promoter: ChAT-Cre/Ai9, PV-Cre/Ai9, and Npy2r-Cre/Ai9, respectively. Mice were housed in a controlled environment (12-h light-dark cycle, 22 ± 2 °C), in open cages with water and food ad libitum.

2.2. Surgical procedures

Fifty-three adult female (20) and male (37) mice (7- to 12-weeks old) were used to evaluating the preferential regeneration of some neuron subtypes (Fig. 1). Mice were anesthetized 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 afterward, the injured nerve was repaired with 5–10 μl of fibrin glue. The glue was prepared by mixing thrombin (25 U/ml, ICN Biomedicals) in calcium chloride (45 mmol/l, Sigma), human fibrinogen (100 mg/ml, Sigma), and bovine fibronectin (8 mg/ml, Sigma) 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). Animals were monitored periodically until the end of the experiments.

After 1 or 8 weeks, mice were anesthetized to re-expose the left femoral nerve (1 week: $n = 7$ Pv-Cre/Ai9, $n = 5$ Npy2r-Cre/Ai9; 8 weeks: $n = 15$ Pv-Cre/Ai9, $n = 14$ Npy2r-Cre/Ai9, $N = 7$ ChAT-Cre/Ai9). Additionally, some uninjured mice were included in the process as controls ($n = 3$ Pv-Cre/Ai9 and 3 Npy2r-Cre/Ai9). 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 in Fluorogold (4%, Fluorochrome) and TrueBlue (2.5%, Setareh Biotech), respectively, and protected from the light for 45 min. To avoid spilling, we set up a small well using a piece of parafilm covered by a Vaseline circumference, we introduced the nerve stump into the well and we filled it with the retrotracers. Control experiments were performed to confirm both retrotracers labeled neurons to the same extent. We applied TrueBlue in the muscle branch and Fluorogold in the cutaneous branch and counted the traced motoneurons and sensory neurons, finding no significant differences (Supplementary Fig. 1).

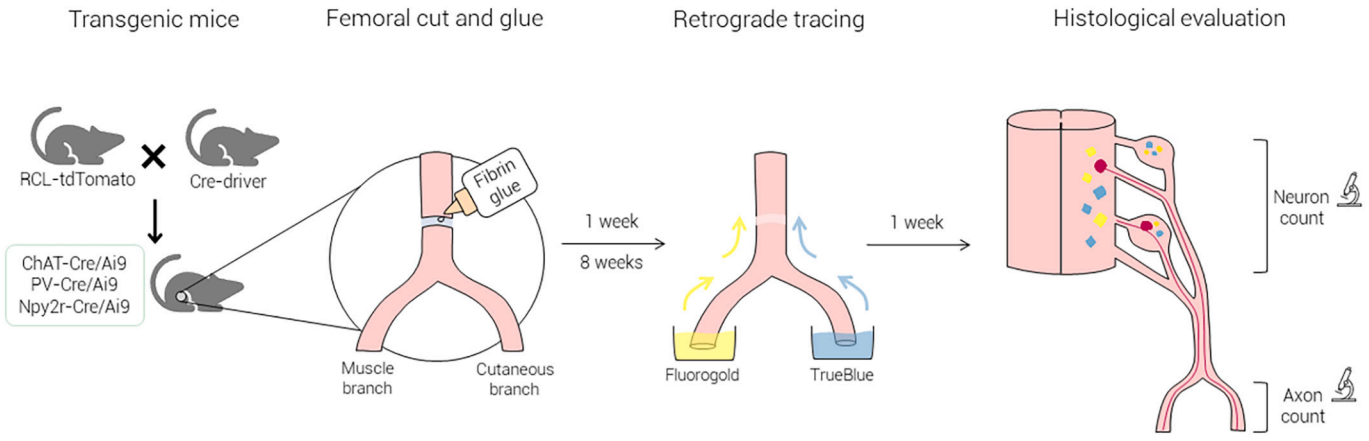

Fig. 1. Experimental design of the study. Transgenic mice were obtained by breeding RCL-TdTomato mice and different Cre-driver lines. The femoral nerve was transected and glued proximally to the bifurcation. After 1 or 8 weeks, the nerve was re-exposed and the retrograde tracers Fluorogold and TrueBlue were applied to the muscle and cutaneous branch, respectively. Histological evaluation was performed 1 week later. We counted the number of retrotraced TdTomato$^+$ neurons in each animal and the axons found in the terminal branches of the nerve.

2.3. Histological evaluation

Characterization of transgenic mice. Three naïve mice of each type were euthanized with intraperitoneal pentobarbital (30 mg/kg) and perfused with 4% paraformaldehyde in PBS. Lumbar DRGs, spinal cords, tibial anterior and soleus muscle, and skin from the hind paw were harvested and stored in PBS with 30% sucrose at 4 °C. DRGs, spinal cords (15 μm), and muscles (20 μm) were serially cut in a cryostat (Leica) and collected in glass slides. The skin was also cut in a cryostat (60 μm thick) and stored free-floating in PBS. Samples were permeabilized with PBS and 0.3% Triton X-100 (PBST) and blocked with 1.5% normal donkey serum (Vector Laboratories). DRGs were incubated overnight at 4 °C with mouse anti-neurofilament H antibody (1/000, Biolegend), rabbit anti-calbindin D-28 K (1/200, Millipore), rabbit anti-Parv (1/800, Swant), rabbit anti-CGRP (1/200, Millipore) or Griffonia Simplicifolia Lectin I (IB4, 10 μg/m, Vector). Muscles were incubated with either mouse anti-sdMyHC (1/100, DSHB) or α-bungarotoxin AlexaFluor 488 conjugate (1:200, Invitrogen). The skin was incubated with rabbit anti-PGP9.5 (1:500, Cedarlane). Slices were washed and incubated with appropriate secondary anti-mouse or anti-rabbit antibodies (AlexaFluor 488, Invitrogen) for 2 h at room temperature. For IB4, a previous incubation with rabbit anti-IB4 (1:500, Vector) was needed. After washing, slides were mounted with Fluoromount-G medium (Southern Biotech) and imaged with a confocal microscope (Leica SP5, 20×, z-step size 1 μm).

Preferential regeneration. One week after applying the retrotracers, PV-Cre/Ai9 and Npy2r-Cre/Ai9 mice were euthanized with intraperitoneal pentobarbital (30 mg/kg) and perfused with 4% paraformaldehyde in PBS. The ipsilateral L3 DRGs were extracted and stored in PBS with 30% sucrose at 4 °C, whereas lumbar spinal cords were harvested and post-fixed for 3 h before storage. Spinal cords and DRGs were serially cut in a cryostat (Leica, 15 μm thick) in a longitudinal orientation and picked up in glass slides. We photographed 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). All neurons were counted in one out of three slices (every 45 μm). We calculated the percentage of sensory neurons that regenerated using the muscle or the cutaneous branch by counting the TdTomato+/TrueBlue+ or TdTomato+/Fluorogold+ neurons in the DRG and dividing them by the total counted number of retrotraced neurons. The spinal cords of the same animals were used to count the motoneurons that regenerated through each branch.

Axon collaterals. A subset of the long-term animals (8 weeks post-injury) was used to calculate the number of axon collaterals per neuron. After perfusion, the ipsilateral L2 and L3 DRGs and the femoral nerves of PV-Cre/Ai9 animals were harvested and stored in PBS with 30% sucrose at 4 °C. ChAT-Cre/Ai9 femoral nerves and lumbar spinal cords were extracted, the latter being post-fixed before storage. DRGs and spinal cords were processed as described above. The distal branches of the femoral nerve were put on glass slides and mounted with Fluoromount. We applied pressure on the coverslips to flatten the nerves and image the distal part in the confocal microscope (Leica SP5, 20×, z-step size 0.38 μm). The number of collaterals in the cutaneous branch was calculated by dividing the number of TdTomato$^+$ axons in the saphenous nerve by the number of TdTomato$^+$/TrueBlue$^+$ neurons. Likewise, the collaterals in the muscle branch were obtained by dividing the number of axons in the quadriceps branch by the number of TdTomato$^+$/Fluorogold$^+$ neurons. All neuron counts were corrected using the Abercrombie correction (Abercrombie, 1946), being the correction factor $t/((t + H)ssf)$, where t is the section thickness, H is the mean high of the object and ssf is the sampling factor. H was estimated by measuring the area of the soma of 100 neurons of each type and determining the mean diameter of each neuronal population (Table 1).

Table 1
Mean diameter of motoneurons and proprioceptors.

Animal	Neuronal type	Mean diameter	Correction factor
ChAT-Cre/Ai9	Motoneurons	27,540 ± 0,54	1,057
PV-Cre/Ai9	Proprioceptors	33,454 ± 0,72	0,928

2.4. Data analysis

GraphPad Prism 8 (version 8.0.2) was used for the statistical analysis. The normal distribution of the samples was confirmed with Shapiro-Wilk test ($p > 0.05$). Sex differences among regenerated motoneurons were assessed by a t-test. Percentage of regeneration, preferential regeneration, and axon collaterals were analyzed using two-way ANOVA, followed by a post hoc Tukey or Sidak test (indicated in the figures). Differences were considered statistically significant if $p < 0.05$. All data are expressed as group mean ± standard error of the mean (SEM).

3. Results

3.1. Reporter mice label different neuronal subtypes

Choline acetyltransferase (ChAT) and parvalbumin (PV) are well-known markers of motoneurons and proprioceptors, respectively, and reporter transgenic mice driven by their promoters have been shown to label these neuron subpopulations (Blum et al., 2021; Woo et al., 2015). These neurons innervate the muscles and, thus, in the femoral nerve, their axons are found in the muscle branch. We observed that ChAT-Cre/Ai9 animals expressed fluorescence in the motoneurons of the ventral horn of the spinal cord (Fig. 2A), but not in the DRGs. The area distribution of these neurons revealed two peaks, indicating that both γ- and α-motoneurons were labeled (Fig. 2B). Contrarily, PV-Cre/Ai9 showed fluorescence in large-diameter neurons in the DRG (Fig. 2B). These neurons co-labeled mostly with the proprioceptive marker parvalbumin (84.62 ± 2.44%) and the marker for myelinated neurons NFH (82.37 ± 0.62%) (Fig. 2C). Furthermore, 9.82 ± 1.05% of these neurons were positive for the Aβ-LTMR marker, Calb1. In the muscle, we saw that ChAT-Cre/Ai9 had fluorescence in fibers innervating neuromuscular junctions, whereas PV-Cre/Ai9 fluorescence co-labeled with slow developmental myosin heavy chain, a muscle spindle marker (Fig. 2A). Applying retrotracers to uninjured femoral nerves, we corroborated that all motoneuron and essentially all proprioceptive axons (97.69 ± 0,82%) were found in the muscle branch of the nerve in our animal models (Fig. 2D).

Axons of touch mechanoreceptors that innervate skin receptors are found in the cutaneous branch of the femoral nerve. Npy2r reporter mice have been described to label low-threshold mechanoreceptors (LTMR) and nociceptors (Arcourt et al., 2017; Li et al., 2011). We characterized our Npy2r-Cre/Ai9 reporter mice and found that these animals label large- and small-diameter DRG neurons (Fig. 2B). Terminal axons could be found in the skin either as free endings or associated with hair follicles (Fig. 2A). In the DRG, 40.7 ± 1.39% of Npy2r$^+$ neurons were myelinated, whereas 61 ± 1.66% of the neurons co-labeled with the peptidergic marker CGRP (Fig. 2C). Only 7.23 ± 0.69% of the Npy2r neurons were positive for the non-peptidergic marker IB4 and 1.54 ± 0.4% for Calb1. We observed that most axons of Npy2r$^+$ neurons were in the cutaneous branch (82.68 ± 2,17%), but 17.32% of these neurons did not have their axons in this branch (Fig. 2D). Altogether, we used this transgenic animal as a reporter of cutaneous mechanoreceptors (including touch and nociceptive neurons).

3.2. Regeneration does not differ between females and males but varies between neuron subtypes

Regeneration was assessed in both females and males in all the

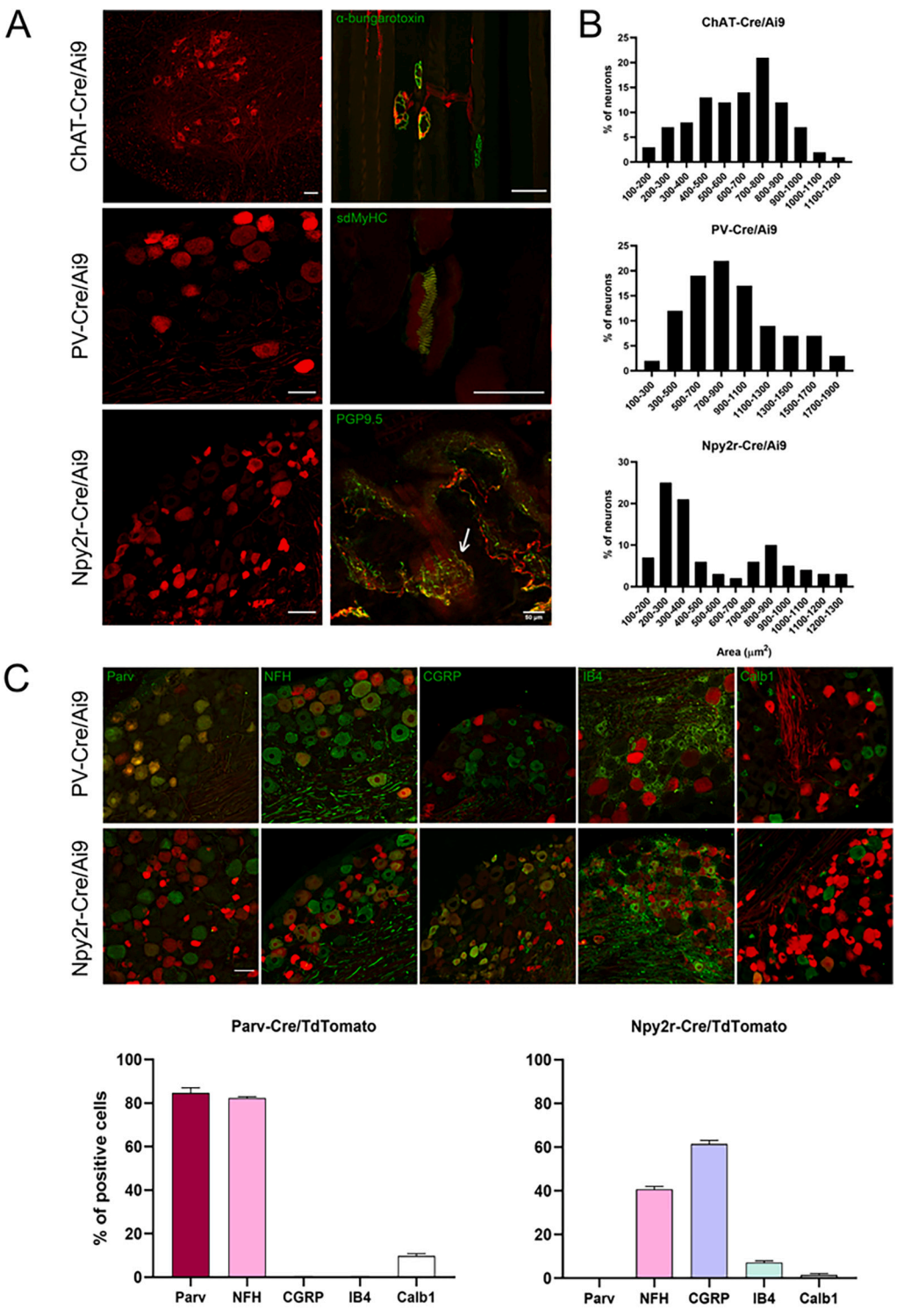

Fig. 2. Characterization of ChAT-Cre/Ai9, PV-Cre/Ai9, and Npy2r-Cre/Ai9 transgenic mice. A) Microscope images of the neuron somas in the spinal cord or the DRG and the terminal axons in the target organs. ChAT-Cre/Ai9 mice express fluorescence in the motoneurons of the ventral horn of the spinal cord and distally in the muscles, where their axons co-label with neuromuscular junctions (green). PV-Cre/Ai9 fluorescence is found in large neurons of the DRG and their axons co-label with sdMyHC, a marker of the muscle spindle (green). Sensory neurons of Npy2r-Cre/Ai9 mice are found in the DRG and their axons in the skin, either as free endings or associated with hair follicles (indicated by the arrow, PGP9.5 in green). B) Size distribution of neurons according to their somatic area. C) Microscope images and percentage of co-labeling of TdTomato^{+} neurons in the DRG with the markers parvalbumin (Parv), NFH, CGRP, IB4, and Calb1 (n = 3/group). D) Percentage of neurons that have their axons in the muscle (orange) or cutaneous branch (purple) in uninjured nerves (n = 3/group). Scale bar: 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

studies (Fig. 3A). To ensure that the counted number of regenerated neurons does not differ between sexes, we analyzed the total number of regenerated motoneurons 8 weeks after the injury (Fig. 3B). We chose motoneurons because the three transgenic mice (ChAT-Cre/Ai9, PV-Cre/Ai9, and Npy2r-Cre/Ai9) were used for the retrotracer counts in the spinal cord. We found that regeneration did not differ between females and males ($p = 0.7884$) and included both sexes for the statistical analysis of the following experiments.

The percentage of regenerated motoneurons, proprioceptors, and cutaneous mechanoreceptors was assessed by counting the total number of retrotraced neurons that regenerated after the injury, including those in the cutaneous branch, the muscle branch, and those double-labeled. After 1 week, 51.43 ± 11.7% of the cutaneous mechanoreceptors regenerated up to the evaluation point, significantly more than motoneurons (24.96 ± 6.27%, $p = 0.017$) and proprioceptors (13.64 ± 4.45%, $p < 0.001$). Eight weeks after the injury, the population that had regenerated more was the Npy2r^{+} (89.29 ± 6.83%), followed by motoneurons (72.92 ± 1.64%, $p = 0.012$ vs Npy2r) and, lastly, proprioceptors (51.03 ± 3.57%, $p < 0.001$ vs motoneurons and Npy2r).

3.3. Motor and sensory neurons have preferential regeneration

The proportion of neurons that regenerated through the muscle branch, the cutaneous branch, or both were assessed 1 and 8 weeks after the injury (Fig. 4). One week after the injury, all the studied neuron populations regenerated up to the evaluation point more through the cutaneous branch than the muscle branch (motoneurons: 21.8 ± 7.73 vs 9.13 ± 2.03, $p = 0.006$; proprioceptors: 7.06 ± 2.64 vs 2.14 ± 0.77, p = 0,046; cutaneous mechanoreceptors: 146.8 ± 44.68 vs 39.6 ± 11.03, $p = 0.002$, cutaneous vs muscle branch). At long term, neurons showed a preferential regeneration towards the branch they originally innervated. Motoneurons and proprioceptors regenerated significantly more in the muscle branch (motoneurons: 40.50 ± 1.68 vs 46.76 ± 1.26, $p = 0.023$; proprioceptors: 12.99 ± 1.20 vs 20.04 ± 1.58, $p < 0.0001$, cutaneous vs muscle branch). Contrarily, more cutaneous mechanoreceptors regenerated in the cutaneous than in the muscle branch of the nerve (193 ± 18.96 vs 118.36 ± 8.88, $p = 0.0003$, cutaneous vs muscle branch). There was some variability between animals, but most of them showed the same tendency to project to the "preferred" branch (Table 2).

3.4. Motoneurons extend more collaterals than proprioceptors

We counted the number of fluorescent axons in the cutaneous and muscle branch of the ChAT-Cre/Ai9 and PV-Cre/Ai9 animals 8 weeks after the femoral nerve injury to compare two myelinated populations (Fig. 5). For the estimation of the number of collaterals, we divided the number of axons by the number of traced neurons in each animal (Fig. 4). Since Npy2r-Cre/Ai9 animals have an extensive number of axons, most of them unmyelinated, these animals were not included in this analysis. We found that motoneurons had a mean of 2.25 (± 0.12) sprouts in the muscle branch and 2.46 (± 0.22) in the cutaneous branch. Proprioceptors extended significantly fewer collaterals in the muscle (1.57 ± 0.14, $p = 0.023$) compared to motoneurons and, although non-significant, these showed the same tendency in the cutaneous branch (1.90 ± 0.18, $p = 0.064$). The two-way ANOVA revealed a significant effect of the branch ($p = 0.0498$), but multiple comparisons failed to find differences between the collaterals in the different branches.

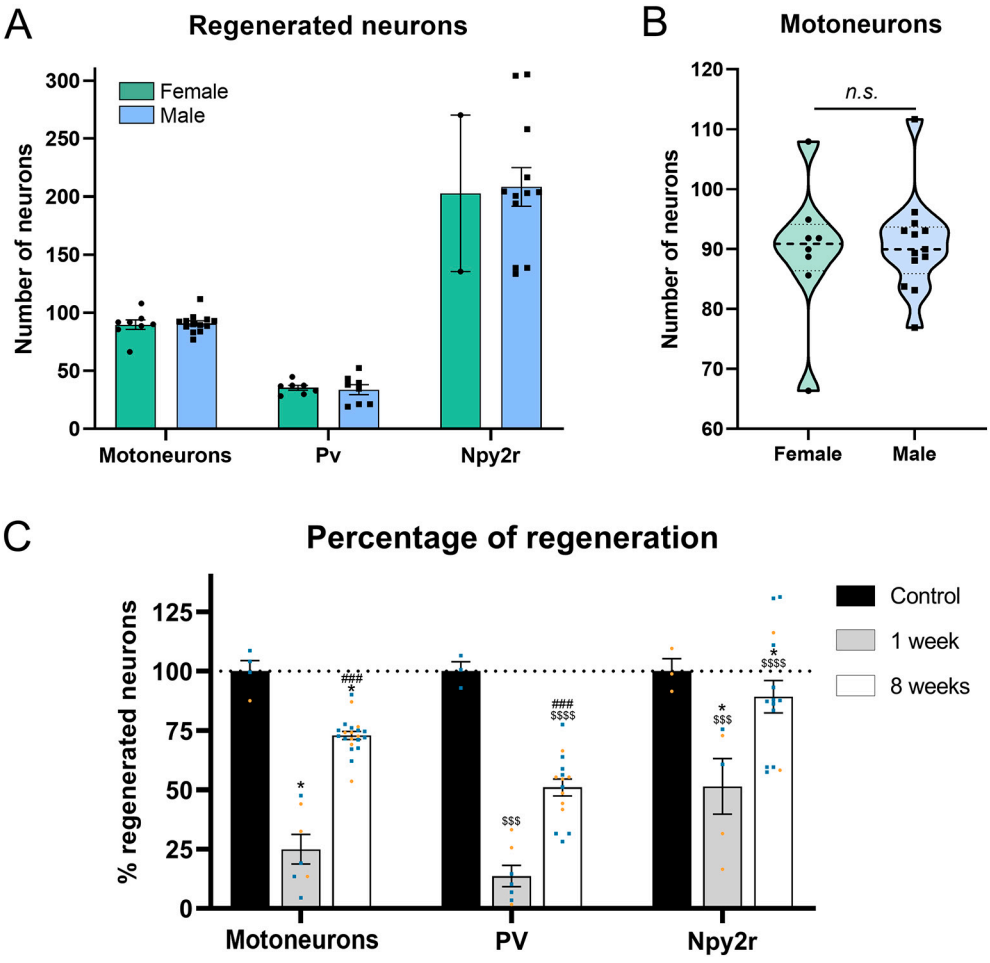

Fig. 3. Total regeneration in both femoral branches. A) Regeneration in females and males 8 weeks after the injury. The total counted number of neurons that regenerated in both branches of the femoral nerve 8 weeks after the injury of each neuronal type is similar between females (green bars) and males (blue bars). B) No differences were found in the number of motoneurons that had regenerated 8 weeks after the injury between males and females (*t*-test, $p = 0.788$). C) Percentage of neurons that regenerated 1 or 8 weeks after injury. Complete regeneration (100%) is considered when reaching control values. Individual values are represented as orange circles (females) and blue squares (males). Two-way ANOVA and Tukey post hoc test indicated differences between neuron types. *$p < 0.05$ motoneurons vs Npy2r; $^{\$\$\$}p < 0.001$, $^{\$\$\$\$}p < 0.0001$ PV vs Npy2r; $^{\#\#\#}p < 0.001$ motoneurons vs PV. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

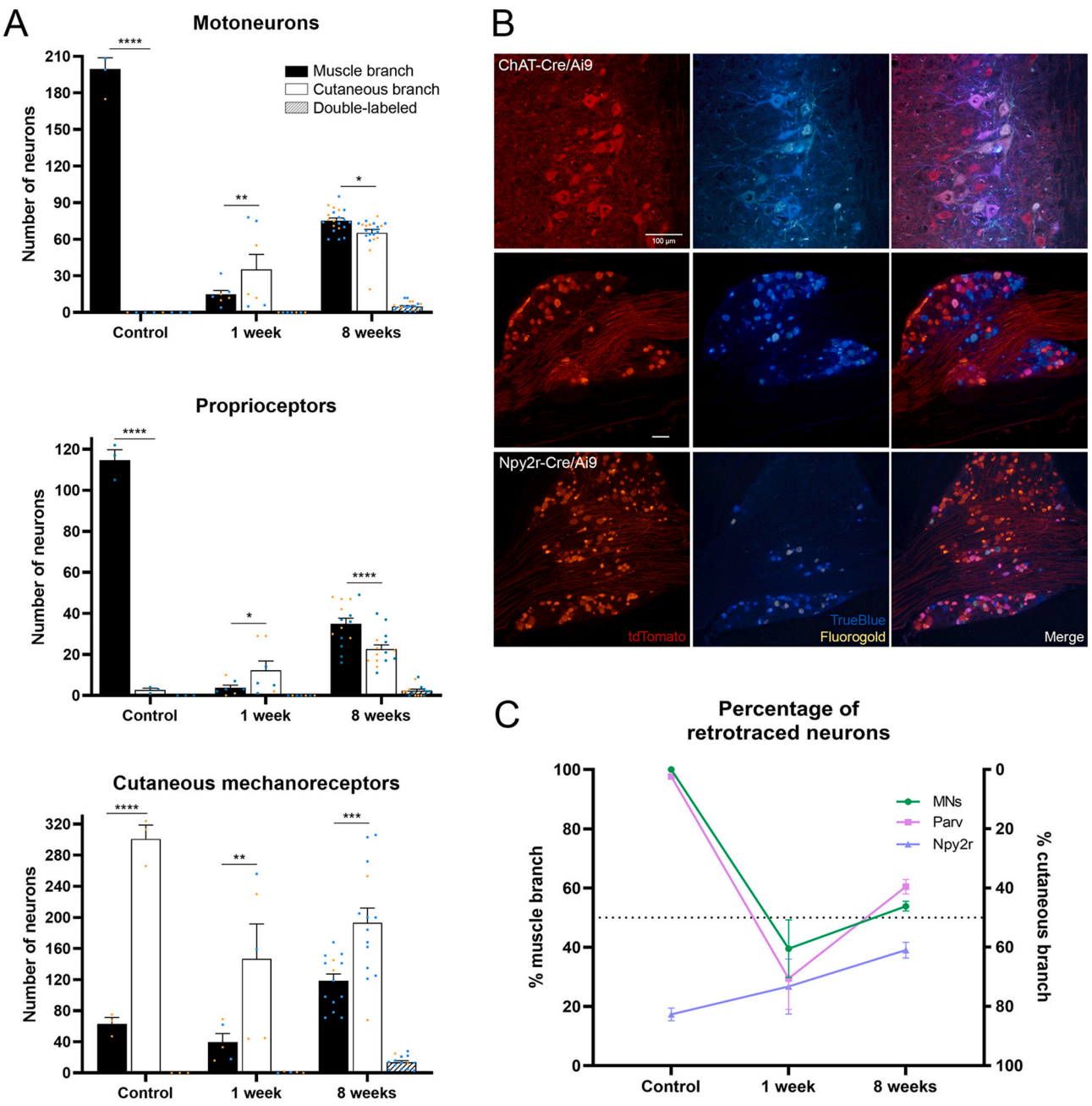

Fig. 4. Evaluation of the preferential regeneration of motoneurons, proprioceptors, and cutaneous mechanoreceptors. A) Counted number of neurons of each type in the muscle branch (black bars), the cutaneous branch (white bars), or both (striped bars). Individual values are represented as orange circles (females) and blue squares (males). B) Microscope images of the somas of motoneurons, proprioceptors, and cutaneous mechanoreceptors 8 weeks after the injury. Neurons were counted when the TdTomato (red) co-localized with TrueBlue (blue) or Fluorogold (yellow). C) Percentage of retrotraced neurons in the muscle or the cutaneous branch calculated over the total number of regenerated neurons. The preference for the muscle branch is represented in the left axis, whereas the cutaneous preference is shown in the right axis. Differences between branches were assessed by a two-way ANOVA followed by a Tukey post hoc test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ muscle vs cutaneous branch. Scale bar: 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Number of animals showing preference towards each branch.

Preferred branch	Motoneurons		Proprioceptors		Cutaneous mechanoreceptors	
	1 week	8 weeks	1 week	8 weeks	1 weeks	8 weeks
Cutaneous	5/7	4/21	5/7	2/15	4/5	13/14
Muscle	2/7	16/21	2/7	12/15	1/5	1/14
Equal	0/7	1/21	0/7	1/15	0/5	0/14

4. Discussion

In this study, we used reporter mice to examine peripheral regeneration of motor and different sensory neurons. Our findings indicate that each type of neuron has an intrinsic mechanism that leads to a regenerative preference towards its original target.

4.1. Reporter mice for the study of regeneration

Preference in regeneration has been controversial over the decades.

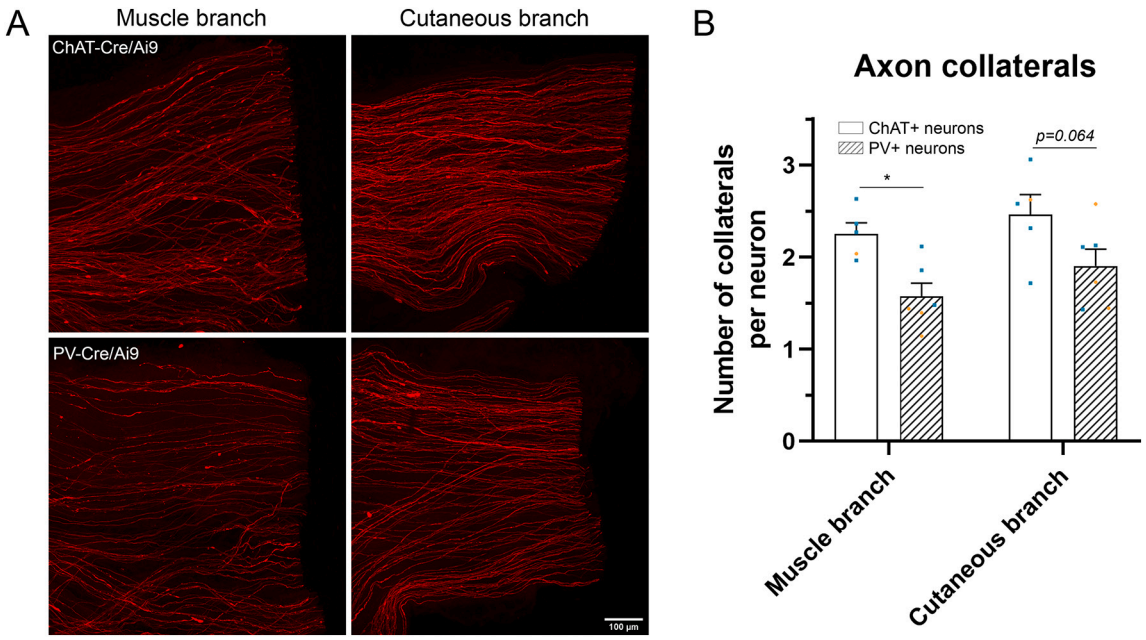

Fig. 5. Axon collaterals of motoneurons and proprioceptors in the lesioned femoral nerve. A) Microscope images of the distal branches of the femoral nerve 8 weeks after the axotomy. Motor (ChAT-Cre/Ai9) and proprioceptive (PV-Cre/Ai9) axons are labeled in red. B) Regenerative sprouts in each type of neuron were calculated by dividing the number of fluorescent axons by the number of traced somas in each branch. Individual values are represented as orange circles (females) and blue squares (males). Two-way ANOVA was followed by Sidak's post hoc test. $*p < 0.05$, $**p < 0.01$ $ChAT^+$ neurons vs PV^+ neurons. Scale bar: 100 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Brushart first reported the existence of PMR in rats 35 years ago (Brushart, 1988), but results in mice were not as clear (Mears et al., 2003; Robinson and Madison, 2006; Robinson and Madison, 2003). Additionally, little attention has been paid to sensory specificity. In the DRG, neurons are remarkably heterogeneous and have diverse target organs. Whereas muscle spindles are innervated by proprioceptors, LTMR innervate specialized structures in the skin (Li et al., 2011). Therefore, we need to find strategies to study each neuron subtype particularities.

Three lines of reporter mice were used in our study, each labeling a different neuron. Among sensory neurons, we chose two populations that should regenerate through opposite branches. Our results confirmed that PV-Cre/Ai9 mice labeled proprioceptors as previously described (Woo et al., 2015). In contrast, Npy2r-Cre/Ai9 mice were used to study cutaneous mechanoreceptors.

We found that the population labeled in these mice was heterogeneous, including small and large neurons. Previous reports described that the marker Npy2r was associated with large diameter Aβ-LTMR (Li et al., 2011), but we found that only 1.54% of the labeled neurons were $Calb1^+$. Our results are in line with recent reports that suggest that Npy2r is a marker for A-fiber nociceptors and A-high threshold mechanoreceptors (HTMR) (Arcourt et al., 2017; Chiu et al., 2014; Patil et al., 2018). These studies showed similar co-labeling with the markers NFH, CGRP, and IB4 and found terminal free endings and some lanceolate endings in the skin. The discrepancies between studies might be related to the different techniques used to design the transgenic mice (Li et al., 2011). Although our Npy2r-Cre/Ai9 mice do not specifically label this Aβ-LTMR population, the fluorescent neurons are mainly projecting to the cutaneous branch and do not overlap with those from PV-Cre/Ai9 mice. Thus, $Npy2r^+$ neurons represent a heterogeneous population of mechanoreceptors, myelinated and unmyelinated, that generally innervate the skin. Therefore, we used these mice to comparatively study preferential regeneration in another sensory neuron subpopulation.

Importantly, some LTMR and HTMR innervate muscles and articulations so a proportion of these neurons projecting to the muscle branch is expected. Specifically, we found 17.32% of these neurons are in the muscle branch. Being the natural condition, this increases the complexity of the evaluation of sensory preferential regeneration.

4.2. *Regeneration speed of neurons*

We found that the regeneration speed can widely vary depending on the type of fiber. One week after the transection, more cutaneous mechanoreceptors arrived at the point where we applied the retrograde tracers compared to motoneurons and proprioceptors. Unmyelinated fibers have been described to recover their function earlier than myelinated fibers (Navarro et al., 1994). As our cutaneous mechanoreceptors include myelinated and unmyelinated neurons, a faster speed in the regeneration of unmyelinated fibers could explain their advantage over motoneurons and proprioceptors. Similarly, sensory neurons have been reported to regenerate faster than motoneurons (Brushart et al., 2020; Dolenc and Janko, 1976; Kawasaki et al., 2000). In contrast, using retrograde tracers, it has been described that motoneurons regenerate better than sensory neurons (da Silva et al., 1985) and that myelinated sensory fibers can regenerate at the same speed as unmyelinated fibers (Lozeron et al., 2004). These contradictions can be explained by the heterogeneity of the sensory neurons. We found a greater advantage in the regeneration of cutaneous mechanoreceptors compared to proprioceptors than to motoneurons. Therefore, regeneration should be analyzed in the different types of neurons considering sensory neurons diversity.

We also observed that the number of neurons that regenerated 8 weeks after the injury differed between populations. Regenerating axons had sufficient time to reach the evaluation point after 8 weeks. However, we found that whereas cutaneous mechanoreceptors and motoneurons had substantially regenerated (89.29 and 72.92%, respectively), only 51.03% of proprioceptors were able to arrive at the distal point. An explanation for this phenomenon can be related to the worse regenerative capability of these neurons. If motoneurons regenerated and reinnervated target muscles faster than proprioceptors, the regenerative environment would change. Schwann cells might re-establish contact

with axons and return to a mature non-reactive state. In this case, the still regenerating proprioceptive axons would not have the proper pro-regenerative conditions to continue extending towards the muscle. Thus, those neurons with slower regeneration rates might fail to continue extending their axons in a less permissive environment and their regeneration would remain incomplete.

4.3. Preferential regeneration in motor and sensory neurons

It has been hypothesized that PMR in the femoral nerve occurs in two phases. During the first weeks, motor axons grow into both distal branches, forming collaterals (Brushart et al., 1998; Brushart, 1993; Uschold et al., 2007). This phase results from pathway-axon interaction (Redett et al., 2005) and collaterals might prefer the branch with the most relative amounts of basal lamina (Robinson and Madison, 2009). Then, PMR might be achieved by pruning the collaterals from the cutaneous branch while maintaining those in their correct path.

Here, we analyzed PMR and sensory preference at earlier times than previous studies and found that more neurons arrived at the cutaneous branch evaluation point after 1 week than to the muscle branch, independently of the neuron population. This favors the hypothesis that during the initial phases of regeneration, collaterals prefer the branch with more Schwann cells. Even though the femoral nerve has a similar cross-sectional area between branches, there are proportionally more myelinated axons in the cutaneous branch than in the muscle branch (Takahashi et al., 1999). More myelinated axons in a branch mean that more Schwann cells are available for motoneurons to establish contacts and probably also more trophic support coming from these denervated cells (Robinson and Madison, 2009; Takahashi et al., 1999). Furthermore, several studies describe that Schwann cells from muscle and cutaneous branches secrete distinct trophic factors (Brushart et al., 1998; Höke et al., 2006). Whereas denervated muscle nerves express higher levels of pleiotrophin (PTN), cutaneous nerves predominantly upregulate hepatocyte growth factor (HGF), brain-derived neurotrophic factor (BDNF), and nerve growth factor (NGF) (Brushart et al., 2013; Höke et al., 2006). BDNF is required for axonal growth and myelination (Zhang et al., 2000) and has been shown to improve motor axon regeneration (Boyd and Gordon, 2003), so it could be driving motor and sensory sprouts towards the cutaneous branch.

In contrast, at long term we found a preference of axons towards their correct branch: motor and proprioceptive neurons regenerated more through the muscle branch, whereas cutaneous mechanoreceptors were more prone to regenerate into the saphenous nerve. This preference could depend on the type of trophic factors secreted by the distal target organs (Madison et al., 2007; Uschold et al., 2007) or the differences between Schwann cells of each branch (reviewed in Bolívar et al., 2020). In contrast to classical studies, we did not find a high number of double-labeled neurons neither 1 week nor 8 weeks after the injury. This might contradict the collateral pruning hypothesis since our results do not corroborate that neurons extend many collaterals to both branches that could account for the later specificity. Contrarily, our data suggest that signals from target organs predominate over those generated by Schwann cells. These signals can arrive via axonal transport and diffusion-driven movement, being the latter more significant (Madison and Robinson, 2014). If this is the case, different types of neurons would differentially respond to factors released by target organs. Denervated muscle has been shown to increase the expression of glial cell line-derived neurotrophic factor (GDNF), fibroblast growth factor (FGF), and HGF (Lie and Weis, 1998; Yamaguchi et al., 2004; Zhao et al., 2004), whereas denervated skin upregulates NGF (Mearow et al., 1993). However, evidence that these factors can specifically attract one type of axon is not consistent in the literature (reviewed in Allodi et al., 2012).

Finally, most studies addressing specific regeneration have focused on the differences between the motor and sensory branches, but not on the intrinsic differences between neuronal populations. If axons have a selective preference for a substrate or trophic factor, their regenerative capacity must be different. Our data reveal that not only motor axons, but also proprioceptors and cutaneous mechanoreceptors preferentially regenerate towards their correct branch. However, although significant, this preference is remarkably low (53.88% for motoneurons, 60,44% for proprioceptors, 60,97% for cutaneous mechanoreceptors) and, hence, hardly relevant in terms of functional outcome. A better knowledge of the intrinsic growth programs of different neurons would help us design strategies to enhance this preferential regeneration and, thus, the functional recovery after nerve lesions.

4.4. Collateral branching in myelinated neurons

After axotomy, regenerating axons have been described to sprout, sending collaterals to up to 10 endoneurial tubes (Witzel et al., 2005). Sprouting is a pathfinding mechanism for the regenerating neurons since increasing collaterals provide more opportunities for the axons to find their correct targets. After reinnervation, sprouts are reduced in a process that can take several months (MacKinnon et al., 1991). In our model, motoneurons and proprioceptors showed some collaterals in both branches of the nerve after axotomy that varied from 1.14 to 3.06 per neuron. Classical studies described an average of five daughter axonal sprouts (Fu and Gordon, 1997). However, a significant degree of reinnervation is expected 8 weeks after the injury, and that could explain the smaller number of collaterals in our samples.

We found that the cutaneous branch contained more collaterals than the muscle branch. This phenomenon was previously described for motoneurons by Redett et al. (2005) in a rat femoral nerve injury model with DRG excision. Here, the use of reporter mice allowed us not only to study collaterals without the need to manipulate sensory axons but also to study sprouting in a specific sensory population.

Trophic factors such as NGF or BDNF have been described to increase axonal sprouting (Boyd and Gordon, 2003; Diamond et al., 1992; Gallo and Letourneau, 1998; Streppel et al., 2002). Interestingly, these factors are significantly upregulated in the cutaneous but not in the muscle branch (Höke et al., 2006). Thus, the differential expression of factors in cutaneous and muscle Schwann cells might influence the presence of regenerating collaterals. Additionally, 8 weeks after the lesion many axons can arrive to target organs. Both motoneurons and proprioceptors can reinnervate muscles, but they are not able to find a target in the skin. If these sprouts are a pathfinding mechanism, neurons reinnervating the muscle would start reducing the sprouts when they reach their target organ, which would explain the lower number of motor and proprioceptive collaterals in the muscle branch.

Our analysis indicated a significant difference in collaterals not only between branches but also between neuronal populations. We found that motoneurons extended more sprouts than proprioceptors in the muscle branch. Interestingly, motoneurons increase the expression of BDNF receptor trkB after axotomy (Fernandes et al., 1998; Kobayashi et al., 1996), as well as the neurotrophin low-affinity receptor $p75^{NTR}$ (Johnson et al., 1999). If NGF and BDNF are partly responsible for increasing axonal sprouting, the increased expression of their receptors in motoneurons may explain the increased number of collaterals found in this population.

5. Conclusion

In this study we showed that, shortly after injury, there is a common mechanism in regeneration that guides axons towards the cutaneous branch. At long term, axons prefer to regenerate through their “correct” branch. Moreover, regenerative capability and sprout dynamics differ between neuron subtypes. Our results evidence different regenerative mechanisms of peripheral neurons, that can be potentially modulated to improve functional recovery after nerve injury.

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Declaration of Competing Interest

The authors declare no competing financial interests.

Data availability

Data will be made available on request.

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