Segregation of motor and sensory axons regenerating through bicompartamental tubes by combining extracellular matrix components with neurotrophic factors

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Abstract
Segregation of regenerating motor and sensory axons may be a good strategy to improve selective functionality of regenerative interfaces to provide closed-loop commands. Provided that extracellular matrix components and neurotrophic factors exert guidance effects on different neuronal populations, we assessed in vivo the potential of separating sensory and motor axons regenerating in a bicompartamental Y-type tube, with each branch prefilled with an adequate combination of extracellular matrix and neurotrophic factors.

The severed rat sciatic nerve was repaired using a bicompartamental tube filled with a collagen matrix enriched with fibronectin (FN) and brain-derived neurotrophic factor (BDNF) encapsulated in poly-lactic co-glycolic acid microspheres (FN + MP.BDNF) in one compartment to preferentially attract motor axons and collagen enriched with laminin (LM) and nerve growth factor (NGF) and neurotrophin-3 (NT-3) in microspheres (LM + MP.NGF/NT-3) in the other compartment for promoting sensory axons regeneration. Control animals were implanted with the same Y-tube with a collagen matrix with microspheres (MP) containing PBS (Col + MP.PBS).

By using retrotracer labelling, we found that LM + MP.NGF/NT-3 did not attract higher number of regenerated sensory axons compared with controls, and no differences were observed in sensory functional recovery. However, FN + MP.BDNF guided a higher number of regenerating motor axons compared with controls, improving also motor recovery. A small proportion of sensory axons with large soma size, likely proprioceptive neurons, was also attracted to the FN + MP.BDNF compartment. These results demonstrate that muscular axonal guidance can be modulated in vivo by the addition of fibronectin and BDNF.

KEYWORDS
axon regeneration, axonal guidance, extracellular matrix, motor axons, neurotrophic factors, sensory axons, Y-tube

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Neuroprosthetic devices have been evolving in the last years in order to replace and restore limb functionality to amputee patients. Advanced neuroprostheses offer a high number of degrees of freedom and sensors (Carrozza et al., 2006); however, these robotic innovations are not matched by a suitable neural interface technology. Thus, there is still need for closed-loop commands for precise execution of movements and incorporating input to the sensory neurons (SNs), that is, the integration of both SN and motor neuron (MN) functions on the interface device. Therefore, the topographical separation of regenerating axons from SN and MN in mixed nerves could be a good approach to selectively stimulate or record different neural signals by means of regenerative interfaces to obtain improved neuroprosthetic control (Delgado-Martínez et al., 2017).

Previous studies described the existence of a preferential motor reinnervation, in which regenerating motor axons tend to enter into distal muscle branches in higher proportion than into cutaneous branches (Brushart, 1988). Similarly, the muscle branch was also more prone to be reinnervated by muscular than by cutaneous sensory afferents (Madison, Archibald, & Brushart, 1996). This phenomenon of preferential reinnervation has been attributed to the specific interactions between regenerating axons and the environment generated in the denervated distal nerve branches (Brushart, Gerber, Kessens, Chen, & Royall, 1998). Several studies have focused in elucidating different contributors for the preferential motor reinnervation effect. Schwann cells have been suggested to have a specific identity that can be recognized by regenerating axons due to differential motor or sensory phenotypes and expression of particular surface receptors. Thus, the human natural killer (HNK-1) carbohydrate epitope is expressed strongly in Schwann cells of motor axons in the adult rodent, but rarely in those associated to sensory axons, whereas the neural cell adhesion molecule (NCAM) is present exclusively in sensory nerve fascicles and not in motor fascicles (Martini, Schachner, & Brushart, 1994; Martini, Xin, Schmitz, & Schachner, 1992; Saito, Nakao, Takayama, Toyama, & Asou, 2005). However, Schwann cells de-differentiate during Wallerian degeneration and tend to lose that preferential marker expression within days after nerve transection in motor and sensory fascicles (Lago, Rodríguez, Guzmán-Lenís, Jaramillo, & Navarro, 2007; Saito et al., 2005). Another point of view suggests that it is the relative level and type of trophic support provided by each nerve branch and the target organ that determine whether motor or sensory axons regrow in that particular branch (Madison, Robinson, & Chadaram, 2007). Interestingly, Hoke and colleagues (2006) demonstrated that Schwann cells of sensory and motor nerves produce different growth factors at baseline and respond differently during denervation and when reinnervated by cutaneous or motor axons. Thus, we hypothesized that the local sustained application of specific guidance and trophic factors might result in higher probability of directed regeneration of motor or sensory axons than grafting different populations of Schwann cells, that lost their distinct phenotype after denervation and thus have a limited effect (Höke et al., 2006; Lago et al., 2007).

Important, nerve growth factor (NGF) and neurotrophin-3 (NT-3) have predominant attractive properties, mediated by their TrkA and TrkC receptors, for SN both in vitro and in vivo (Bloch, Fine, Bouche, Zum, & Aeberscher, 2001; Gallo, Lefcort, & Loterneau, 1997; Lotfi, Garde, Chouhan, Bengali, & Romero-Ortega, 2011; Moore, MacSween, & Shoichet, 2006), whereas brain-derived neurotrophic factor (BDNF) promotes MN outgrowth in vitro (Alldini, Guzmán-Lenís, Hernández, Navarro, & Udina, 2011) and has attractive properties mediated by TrkB receptors for regenerating MNs (Henle et al., 2011; Li et al., 2005; Song et al., 1997; Yuan et al., 2003). In addition, different extracellular matrix (ECM) components constitutes of the endoneurial tubes and the basal lamina play a role in the promotion of preferential axon outgrowth. For instance, laminin substrate mainly promotes sensory axon outgrowth in vitro (Gardiner, 2011; Plantman et al., 2008), whereas fibronectin enhances motor and proprioceptive axon outgrowth (González-Pérez et al., 2016). Therefore, the add of different combination of neurotrophic factors and ECM components in a Y-tube model could be an interesting approach for segregating motor and sensory axons. In fact, a previous study used a Y-tube model to separate regenerating TrkC-expressing proprioceptive from TrkA-expressing nociceptive neurons by the addition of NT-3 and NGF, respectively (Lotfi et al., 2011).

In the present study, a sciatic nerve transection was repaired with a bicompartmental tube containing a different combination of ECM and neurotrophic factors in each distal branch. Based on our previous results (Santos, González-Pérez, et al., 2017; Santos, Giudetti, et al., 2016; Santos, González-Pérez, et al., 2016), each compartment was filled prior to implantation with a collagen matrix containing either fibronectin with poly-lactic co-glycolic acid (PLGA) microspheres releasing BDNF (FN + MP.BDNF) in one side in contact with the distal peroneal nerve (P), or laminin with PLGA microspheres releasing NGF and NT-3 (LM + MP.NGF/NT-3) at the other side in contact with the tibial and sural nerves (T + S). We assessed whether regenerated motor axons preferentially grew in the first branch and sensory axons in the second branch, compared with control condition in which both branches contained the same collagen matrix and PLGA microspheres filled with PBS (Col + MP.PBS).

2 MATERIALS AND METHODS

2.1 Ethics statement

The experimental procedures were approved by the ethical committee of the Universitat Autònoma de Barcelona in accordance with the European Communities Council Directive 2010/63/EU. Female Sprague–Dawley rats (250–300 g) were used for the in vivo studies. They were kept on standard laboratory conditions with a light-dark cycle of 12:12 hr and ad libitum access to food and tap water. All efforts were made to minimize pain and animal discomfort during surgery and treatments.

2.2 Fabrication of the bicompartmental tube and implantation

The Y-tube consisted of a silicone tube divided in two sections by a flat polyimide film. Silicone tubes (8 mm long, 2 mm i.d.) were cut longitudinally along 4 mm, and a polyimide film (8 mm long, 2 mm wide, 30 μm thick) was placed along the midline to divide the distal half tube into
two separated chambers. To prevent distal axon reorganization, 4 mm of the polyimide film extended out of the tube (see Figure 1a,b). The slit cut of the tube was closed with silicone adhesive to hold the film in place.

NGF, NT-3, and BDNF were encapsulated in PLGA microspheres (MPs) as previously described (Giudetti, del Valle, Navarro, & Micera, 2014). MPs containing NGF and NT-3 were added to a collagen type I solution (3 mg/ml; #354236, Corning) supplemented with laminin 20% (Sigma) to reach a final concentration of 1 μg/ml for each trophic factor. Similarly, BDNF containing MPs were added to a collagen solution supplemented with fibronectin 20% (BD Biosciences) to reach a final concentration of 2 μg/ml for BDNF. In the experimental group, each branch of the Y-tube was filled with one of the above solutions (Figure 1b). In the control group, both branches were filled with collagen gel mixed with MPs containing PBS (Col + MP.PBS; Figure 1a).

Tubes were maintained vertically for 12 hr before surgery in order to promote fibril alignment during gel formation (Verdú et al., 2002).

Animals were anaesthetized with ketamine/xylacine (90/10 mg/kg i.p.), the sciatic nerve was exposed at the midthigh, transected and a portion of 3 mm resected. The proximal sciatic nerve stump was sutured to the proximal end of the bicompartamental tube. The distal stump was carefully dissected to separate the P, T, and S nerve branches; then the distal P nerve was inserted in the compartment containing FN + MP.BDNF, whereas the T + S nerves were inserted in the compartment containing LM + MP.NGF/NT-3 (Figure 1c). The nerve stumps were fixed with one 10-0 suture stitch, leaving an interstump distance of 6 mm. The wound was closed by planes with silk sutures. Animals were kept for 90 days to allow axonal regeneration.

2.3 | Assessment of skin sensory reinnervation

The progression of nociceptive reinnervation of the hindpaw was assessed by means of the pinprick test and thermal algometry at 7, 30, 45, 60, 75, and 90 days postinjury (dpi). For the pinprick test, animals were gently kept in a cloth with the sole of the injured paw facing upward, and the skin was stimulated with a needle from proximal to distal at specific sites of the lateral side of the plantar surface (Cobianchi, de Cruz, & Navarro, 2014). Positive withdrawal responses were taken as sign of skin functional reinnervation and recorded only when clear pain reaction was triggered by the stimulation. A composite score was calculated as the mean number of responses per group at each day of testing.

Thermal sensitivity was assessed using a Plantar test algesimeter (Ugo Basile, Comerio, Italy). Rats were placed into a plastic box with an elevated plexiglass floor. The beam of a lamp was pointed to the lateral part in the hindpaw plantar surface. Intensity was set to low power (40 mW/cm²) with a heating rate of 1 °C/s to elicit activation of unmyelinated fibres as described before (Cobianchi et al., 2014). A cut-off time for the stimuli was set at 20 s to prevent tissue damage. Heat pain threshold was calculated as the mean of three trials per test site, with a 5-min resting period between each trial, and expressed as the latency (in seconds) of paw withdrawal response.

2.4 | Assessment of motor reinnervation

Functional reinnervation of target muscles was assessed at 90 dpi before the retrotracer application. Briefly, animals were anaesthetized with ketamine/xylacine, and subdermal steel needle electrodes were placed transcutaneously at the sciatic notch for electrical stimulation using single monophasic pulses of 100 μs duration (Synergy Medelec, Viasys HealthCare). The compound muscle action potentials (CMAPs) of tibialis anterior (TA), gastrocnemius medialis (GM), and plantar interossei (PL) muscles were recorded using steel needles in monopolar configuration (Santos, Wieringa, Moroni, Navarro, & Del, 2017). The amplitude and latency of the M-wave were measured, and the contra-lateral intact limb was used as control. The rat body temperature was maintained by means of a thermostated warming flat coil throughout the test.

2.5 | Retrograde labelling and neuronal counting

To quantify motor- and sensory-regenerated neurons, rats were anaesthetized with ketamine/xylacine, the sciatic nerve was carefully dissected, and the silicone tube removed. The regenerated nerve branches were transected at the distal part, separated from the polyimide wall, and dipped in 5 μl of Fluorogold (FG; 5%; Fluorochrome Inc.) or True Blue (TB; 5%; Setareh Biotech) for 1 hr inside a vaseline well.

![Figure 1](image_url)  
**FIGURE 1**  Schematic drawing showing the design of the bicompartamental Y-tube and the contents of each branch (a) in the control group and (b) in the experimental group. (c) Micrograph of the implanted Y-tube filled with a collagen gel at both distal branches of the tube. (d) Micrograph of a regenerated nerve after the extraction of the tube prior to retrotracer application.
Retrotracer application was counterbalanced in order to minimize possible differences between retrotracers efficacy (Zele, Sketelj, & Bajrović, 2010). After retrieval of the well, the area was rinsed with saline to clean any remnants of the tracer and the wound sutured. Animals were allowed to survive for 7 days for accumulation of the tracer in the soma of spinal motoneurons and dorsal root ganglion (DRG) SNs. Then, rats were deeply anaesthetized and transcardially perfused with 4% paraformaldehyde in phosphate buffered saline (PBS). The lumbar segment (L3–L6) of the spinal cord and the L4 and L5 DRG were removed, postfixed at 4 °C in the same fixative solution for 1 hr, and transferred to 30% sucrose in PBS. The cord and DRG were cut in a cryostat longitudinally in 40 and 20 μm thick sections respectively, mounted on slides, heated at 35 °C for 1 hr and stored at −20 °C in the dark. Finally, sections were observed with an Olympus BX51 fluorescence microscope under UV light, and the number of FG, TB, and double-labelled (DL) neurons were counted in every third section following the fractionator principle (Gundersen, 1986).

2.6 | Morphometric analysis

The identification of labelled SNs was done using Fiji software (Schindelin et al., 2013). RGB pictures were converted to L*a*b* colour space to extract the “a*” channel, containing the information of the tracer fluorescence. The resulting image was smoothed using a median filter. A binary image was generated using a mid-grey adaptive local thresholding procedure. Soma clusters were extrapoalted using a watershed algorithm. Neuron-like objects were then identified by the “Analyze particles” tool of the software and manually verified. The data about number of objects and their area and “a*” value were further analysed in SPSS 22.0 (IBM Corp., USA). Objects were clustered using a k-means procedure according to two independent features, area and mean value of the “a*” channel. From the area, three groups were selected: small (<600 μm²), medium (600–1200 μm²), and large size (>1200 μm²), according to previously defined size criteria (Fukuoka, Kondo, Dai, Hashimoto, & Noguchi, 2001). From the value of the “a*” channel, objects were separated into TB⁺, FG⁺, and DL⁺. TB⁺ and FG⁺ objects were assigned to the corresponding reinnervating branch based on the experimental settings.

2.7 | Data analysis

Data are presented as mean ± SEM. Results were statistically analysed by using GraphPad Prism (GraphPad Software, USA). Student t test and one- and two-way analysis of variance followed by Bonferroni’s post hoc tests for comparison between groups were used. Statistical significance was considered when p value was <.05.

3 | RESULTS

3.1 | Sensory and motor functional recovery

Sensory functional recovery, evaluated by mechanical and thermal tests, did not show differences between control and experimental groups. For pinprick test, the first responses were observed at 30 dpi, without significant differences between groups at any time points (Figure 2a). Similarly, withdrawal responses in the plantar test reappeared at 30 dpi, and no differences were observed during the follow-up between groups (Figure 2b). These results indicate that the branch containing LM + MP.NGF/NT-3 did not promote higher regeneration of SNs to the T + S nerves in comparison with the control condition.

Electrophysiological tests were performed to assess reinnervation of the TA, GM, and PL muscles (Figure 3). The amplitude of the TA CMAP (Figure 3a) was higher in FN + MP.BDNF than in the control group (27.5 ± 2.9 mV and 22.3 ± 2.7 mV, respectively, p < .05) suggesting that a higher number of MNs had regenerated towards the chamber which contained FN + MP.BDNF and was sutured to the P nerve that innervates the TA muscle. In contrast, the GM and PL CMAPs (Figure 3b,c) were higher in the control than in the LM + MP.NGF/NT-3 condition (p < .05 for the GM CMAP) indicating that a lower number of MNs had regenerated towards the T + S nerves with LM + MP.NGF/NT-3 in comparison with the control condition.

3.2 | MN- and SN-directed regeneration

At the final time point, we observed that all the animals had a regenerated nerve within the Y-tube that bifurcated in each compartment created by the polyimide film to distally reinnervate the P nerve at one side and T + S nerves at the other side (Figure 1d).

Retrotracer application confirmed that in all the rats, MNs and SNs had regenerated through the Y-tube, as judged by the presence of TB⁺, FG⁺, and DL⁺ neurons (Figure 4a–c) in SC and DRG sections (Figure 4d–g). Data on the total number of retrolabelled neurons for each condition are shown in Table 1. The number of regenerated neurons was about 8,500 in both groups, but there were differences

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**FIGURE 2** (a) Plot of the pinprick score in the control and experimental groups during follow-up. (b) Latency of withdrawal response to thermal algometry test in the lateral part of the paw during follow-up. Data expressed as mean ± SEM.

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between branches and distribution. Thus, in the experimental group, about 1,200 more neurons regenerated their axons in the branch towards the P nerve and correspondingly less towards the T + S nerves than in the control group.

When counting the number of retrolabelled MNs, in the control group, 74.1 ± 1.2% of the MNs regenerated towards the T + S distal stump, whereas only 10.0 ± 4.1% regenerated towards the P nerve. In addition, 15.8 ± 6.4% MNs were DL suggesting the formation of axonal sprouts that grew through both branches of the tube. On the other hand, in the experimental group, 53.5 ± 6.5% of regenerated MNs grew towards the FN + MP.BDNF branch connected to the P nerve (p < .001 vs the control condition, Figure 4h). Consequently, less MNs regenerated through the LM + MP.NGF/NT-3 compartment (36.6 ± 6.5%; p < .001 vs the control condition). There were no differences in the number of DL MNs between the two groups.

Regarding retrolabelled SNs, in the control group, 67.0 ± 4.4% grew towards the T + S distal stump, and only 23.9 ± 9.0% towards the P distal nerve, whereas 9.0 ± 1.5% were DL neurons. The addition of different combinations of ECM and NTFs into separate branches of the Y-tube had a slight but not significant influence on regeneration of sensory axons, since 56.8 ± 14% were labelled from the LM + MP.NGF/NT-3 branch, 34.7 ± 9.3% from the FN + MP.BDNF branch, and 8.43 ± 2.2 from both chambers (Figure 4i).

These results demonstrate that the addition of LM + MP.NGF/NT-3 did not exert a significant effect attracting regenerating SNs, whereas the addition of FN + MP.BDNF significantly attracted more regenerating MNs compared with the control condition.

### 3.3 FN + BDNF preferentially attract large SNs

Since SNs are constituted by a heterogeneous population (Usoskin et al., 2015), we performed a morphometric analysis to characterize differences in size of SNs that regenerated at different branches of the bicompartmental tube.

The frequency distribution of regenerative SNs showed no differences in soma size of SNs that regenerated in the LM + MP.NGF/NT-3 branch compared with the control gel (Figure 5a). However, SNs that regenerated in the FN + MP.BDNF gel towards the P nerve showed a larger soma size profile than in the control gel. Then, we grouped the regenerated SNs in small, medium, and large size to further characterize the composition of each regenerating branch (Figure 5d–f). The only significant difference in size was observed between FN + MP.BDNF and Col + MP.PBS conditions for large SNs (p < .05, Figure 5f). These results demonstrate that the combination of FN and BDNF promoted the directed growth of large sensory axons, which are related with muscular afferents (Taylor, Holdeman, Weltmer, Ryals, & Wright, 2005).

### 4 DISCUSSION

In this study, we have used a bicompartamental tube model that forces the regenerating axons to grow along a different branch. When the axons regenerate within the tube, they may choose between two branches with a different milieu to grow. By manipulating the composition of the inside matrix of each branch, we attempted to preferentially attract the regenerating motor and sensory axons and then selectively guide them towards different distal nerves and their corresponding targets. In accordance with similar studies with gap defects (Clements et al., 2009; Meyer et al., 2016; Santos, González-Pérez, Navarro, & del Valle, 2016), the division of the tube in two separated compartments did not suppose an obstacle for nerve regeneration as all the operated animals showed regeneration distal to the tube and evidence of functional reinnervation of skin and muscles. Tube repair leaving a short gap between proximal and distal nerve stumps was first presumed to allow axons to be guided towards their original distal fascicle by means of neurotropic diffusible factors (Evans, Bain, Mackinnon, Makino, & Hunter, 1991; Rende, Granato, Lo Monaco, Zelano, & Tecesca, 1991). However, later studies proved that tube repair by itself did not improve the selectivity of regeneration with respect to direct suture when correct alignment between proximal and distal stumps was achieved (Bodine-Fowler, Meyer, Moskovitz, Abrams, & Botte, 1997; Valero-Cabrè et al., 2001, 2004).

Therefore, we have evaluated here if the addition of a different combinations of ECM molecules and NTFs into a collagen matrix was able to selectively promote the regeneration of MN and SN towards different distal fascicles. We chose the two matrix combinations based on previous in vitro findings using organotypic cultures that demonstrated a preferential effect of fibronectin and BDNF to promote neurite growth from MNs and of laminin and NGF/NT-3 to promote neurite growth of SNs (González-Pérez et al., 2016; Santos, González-Pérez, Giudetti, Micera, Udina, Del Valle, & Navarro, 2017). The distal insert was selected to accommodate the size of the distal
Representative micrographs of neurons retrolabelled with (a) True Blue, (b) Fluorogold, and (c) double labelled (DL). Representative micrographs of motor neurons retrolabelled in the spinal cord in (d) control and (f) experimental conditions and of sensory neurons retrolabelled in DRG in (e) control and (g) experimental conditions. In this case, Fluorogold was applied to the T + S distal nerve and True Blue to the P distal nerve. Histogram of the number of regenerated (h) motor neurons in the spinal cord and (i) sensory neurons in the DRG in both control and experimental groups. Data expressed as mean ± SEM. ***p < .001

| Table 1 | Average number of motor and sensory neurons that regenerated axons within each branch of the Y-tube towards the distal inserts of T + S or P nerves |
|---------|--------------------------------------------------------------------------------------------------|
|         | Control                                                                                          | Experimental                                                               |
|         | T + S | P | DL | T + S | P | DL |
| MN      | 654.6 ± 11.4 | 88.6 ± 36.6 | 139.6 ± 56.9 | 339.6 ± 60.8 | 497.2 ± 60.1 | 91.4 ± 31.47 |
| SN      | 5173.6 ± 343 | 1846.6 ± 864.1 | 698.3 ± 117.3 | 4263.2 ± 1069.7 | 2609.2 ± 700.5 | 633 ± 166.64 |

Note. DL, double labelled; MN = motor neuron; SN, sensory neuron.
nerve with the number of axons of each type. Since there are many more sensory than motor axons in the sciatic nerve, the largest distal nerve (tibial + sural) was placed in the compartment for preferential growth of sensory axons, whereas the smaller peroneal nerve was placed in the compartment for motor axons.

Retrotracer labelling quantification of regenerated MN and SN when placing the control collagen matrix in the tube branches showed no significant differences between branches connected distally with the T + S nerves and the P nerve. Moreover, about 15% of MN and 9% of SN generated regenerative sprouts that grew towards both branches. Due to the fact that T, S, and P are mixed nerves, no preferential influence on MN or SN regeneration was expected (Brushart, 1993; Brushart et al., 1998). The higher percentage of MN and SN regenerating to the T + S branch than to the P branch can be attributed to the larger calibre of the former that contains larger number of Schwann cells that generate higher concentration of trophic and tropic factors in the distal nerve (Abernethy, Rud, & Thomas, 1992; Robinson & Madison, 2004; Takahashi, Maki, Yoshizu, & Tajima, 1999; Uschold, Robinson, & Madison, 2007).

On the other hand, in the experimental group, we found that adding FN + MP.BDNF at one branch of the tube and LM + MP.NGF/NT-3 to the other branch resulted in higher number of MNs that regenerated their axons towards the first condition compared with the control group. In accordance, higher CMAP amplitudes were recorded in TA muscles of animals treated with FN + MP.BDNF and lower CMAPS for the LM + MP.NGF/NT-3 indicating that more motor fibres had reinnervated the muscles provided by the P nerve than those by the T nerve. The addition of FN + MP.BDNF to the branch in contact with the P nerve was able to partially overcome the size attracting effect of the T + S nerves and attracted more than 50% of motor axons. In fact, BDNF is a potent neurotrophic factor for MNs regeneration (Allodi et al., 2011; Boyd & Gordon, 2002; Vögelin et al., 2006), and it has been reported that the same intracellular mechanisms that promote axonal growth and cell survival are related with axonal guidance.
substrates (González et al., 2011). Furthermore, fibronectin also preferentially promotes motor axon outgrowth in vitro (González-Pérez et al., 2016). In addition, it is possible that FN or BDNF act not only directly on the regenerating fibres but also they may promote the recruitment of motor-related Schwann cells (Jesuraj et al., 2012). The enhancing effect shown is of relevance given the reduced regeneration of motor fibres compared with sensory fibres in tube repair of mid to long gaps (Madorsky, Swett, & Crumley, 1998; Navarro, Verdú, & Buti, 1994).

Contrary to our hypothesis, addition of laminin and NGF/NT-3 was not able to promote attraction of sensory axons, and in fact, a non-significantly lower proportion of regenerating SNs was labelled from the TS branch than with the control collagen gel. This result was corroborated by lack of differences in sensory functional recovery between both control and experimental groups. The lack of effect of LM + MP.NGF/NT-3 on SNs was not expected as previous studies described a trophic and tropic guidance effect for laminin and NGF and NT-3 individually assessed (Gallo et al., 1997; Santos, Giudetti, Micera, Navarro, & del Valle, 2016, Santos, González-Pérez, Navarro, & del Valle, 2016; Turney & Bridgman, 2005; Webber et al., 2008). One possible reason could be related with the heterogeneous populations of SNs (Usoskin et al., 2015) that reside in the DRG, in which approximately 70% of neurons express Trk receptors but of different subtypes whereas 30% are nonpeptidergic neurons and respond to Glial cell-derived neurotrophic factor (GDNF) (Tucker & Mearow, 2008). Laminin substrate did not show effects on nonpeptidergic neurons as they express α7 integrin receptor at low levels (Gardiner et al., 2005); but there are no studies assessing fibronectin for guidance of nonpeptidergic axons. In addition, among the Trk + SNs, a low percentage of neurons express only one type of Trk receptor (23%), whereas coexpression of different Trk receptors is more abundant (47%) (Karchewski, Kim, Johnston, McKnight, & Verge, 1999). Then, the attracting effect mediated by the addition of LM + NGF/NT-3 could be masked by the heterogeneity of the DRG neuronal populations, which may be nonresponding to NGF and NT-3 or responding to BDNF, so that sorting sensory axons is a complex task. Moreover, it has been reported that mRNA for NGF and also for BDNF, among others, was expressed vigorously by denervated cutaneous nerves (Höker et al., 2006). Therefore, it may be well considered that the increased supply of both factors one at each branch of the Y-tube did not allow for an effective preferential guidance for sensory axons.

A first analysis of the differential growth of sensory axons was attempted by subdividing the regenerated SNs by soma size. Indeed, we observed a slight increase of medium and large SNs towards the FN + BDNF branch. It should be taken into account that proprioceptive neurons, characterized by their large size diameter (Taylor et al., 2005; Tucker & Mearow, 2008), respond to NT-3 (Lotfi et al., 2011), but also preferentially extend neurites on fibronectin containing substrates (González-Pérez et al., 2016). Taking into account that the FN + MP.BDNF branch attracted a high number of motor axons, it is plausible to think that such combination promotes also regeneration of large SNs that are directed towards muscular targets. Indeed, during early development, muscle sensory axons grow slightly later and become adjacent to motor axons elongating along the muscular nerve to innervate the same muscle, reflecting specific attractions between them mediated by changes in expression of cell adhesion molecules.

In contrast, developing cutaneous sensory axons bundle together and project along individual cutaneous nerves (Honig, Frase, & Camilli, 1998).

In conclusion, we demonstrate here that nerve regeneration is successful in a bicompartamental tube, in which the two branches contain a matrix with different components. We found that motor axons regeneration was promoted preferentially in the branch containing fibronectin and BDNF. The same branch also attracted axons from large SNs that might correspond to proprioceptive neurons. On the other hand, the addition of laminin and NGF/NT-3 in the other branch did not promote the growth of sensory axons. Further studies are justified to enhance the effects obtained to segregate different functional populations of axons that may be of benefit for an advanced regenerative interface.

**ACKNOWLEDGEMENTS**

This research was supported by European Union FP7-NMP project MERIDIAN under contract 280778 and FP7-ICT project NEBIAS under contract number 611687, TERCEL (RD12/0019/0011) and CIBERNED (CB06/05/1105) funds from the Instituto de Salud Carlos III of Spain, and FEDER funds. ICN2 is supported by the Severo Ochoa programme from Spanish MINECO (Grant SEV-2013-0295) and is funded by the CERCA Programme/Generalitat de Catalunya. The authors thank the technical help of Monica Espejo, Marta Morell, and Jessica Jaramillo.

**CONFLICT OF INTEREST**

The authors declare no financial or personal relationships between themselves and others that might bias their work. Hence, the authors declare no conflict of interest regarding the publication of this article.

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