



HAL
open science

BDNF-Gene Transfected Schwann Cell-Assisted Axonal Extension and Sprouting on New PLA-PPy Microfiber Substrates

Fernando Gisbert Roca, Franck M. André, Jorge Más Estellés, Manuel Monleón Pradas, Lluís M. Mir, Cristina Martínez-Ramos

► To cite this version:

Fernando Gisbert Roca, Franck M. André, Jorge Más Estellés, Manuel Monleón Pradas, Lluís M. Mir, et al. BDNF-Gene Transfected Schwann Cell-Assisted Axonal Extension and Sprouting on New PLA-PPy Microfiber Substrates. *Macromolecular Bioscience*, 2021, 10.1002/mabi.202000391 . hal-03028419v1

HAL Id: hal-03028419

<https://hal.science/hal-03028419v1>

Submitted on 27 Nov 2020 (v1), last revised 29 Nov 2021 (v2)

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

BDNF-gene transfected Schwann cells-assisted axonal extension and sprouting on new PLA-PPy microfibre substrates

*Fernando Gisbert Roca, Franck M. André, Jorge Más Estellés, Manuel Monleón Pradas, Lluís M. Mir and Cristina Martínez-Ramos**

F. Gisbert Roca, Prof. Jorge Más Estellés, Prof. M. Monleón Pradas, Dr. C. Martínez Ramos
Universitat Politècnica de València
Centro de Biomateriales e Ingeniería Tisular
Camino de Vera s/n, 46022, Valencia, Spain
E-mail: cris_mr_1980@hotmail.com

Prof. Jorge Más Estellés, Prof. M. Monleón Pradas
CIBER-BBN, Centro de Investigación Biomédica en Red-Bioingeniería, Biomateriales y
Nanomedicina
Spain

Dr. Franck M. André, Prof. Lluís M. Mir
Université Paris-Saclay
CNRS, Institut Gustave Roussy, Metabolic and systemic aspects of oncogenesis (METSYS)
94805, Villejuif, France

Keywords: bioengineering, fibers, polypyrroles, conducting polymers, tissue engineering

The work here reported analyses the effect of increased efficiency of brain-derived neurotrophic factor (BDNF) production by electroporated Schwann cells (SCs) on the axonal extension in a co-culture system on a biomaterial platform that can be of interest for the treatment of injuries of the nervous system, both central and peripheral. Rat SCs are electrotransfected with a plasmid coding for the BDNF protein in order to achieve an increased expression and release of this protein into the culture medium of the cells, performing the best balance between the level of transfection and the number of living cells. Gene-transfected SCs show an about 100-fold increase in the release of BDNF into the culture medium, compared to non-electroporated SCs. Co-cultivation of electroporated SCs with rat dorsal root ganglions (DRGs) is performed on highly aligned substrates of polylactic acid (PLA) microfibres coated with the electroconductive polymer polypyrrol (PPy). The co-culture of DRGs with electrotransfected SCs increase both the axonal extension and the axonal sprouting from DRGs neurons compared to the co-culture of DRGs with non-electroporated SCs. Therefore, the use of PLA-PPy highly

aligned microfibre substrates pre-seeded with electrotransfected SCs with an increased BDNF secretion is capable of both guiding and accelerating axonal growth.

1. Introduction

Injuries produced at the central nervous system (CNS) or at the peripheral nervous system (PNS) are very difficult to treat because they involve cell loss, development of neurotoxic environments and a multiplicity of factors only partially known, which makes spontaneous regenerative processes extremely unlikely[1,2]. For this reason, hope for progress towards a greater effectiveness in the regeneration of the nervous system lies in multi-combinatorial solutions that use cell transplantation and biomaterials to guide axonal growth and protect transplanted cells[3,4], as well as the use of different types of stimuli (mechanical, electrical, biochemical) that trigger and accelerate the necessary processes[5–8].

Biomaterials interact with cells essentially topographically, through adhesion and mechanotransduction signals[9,10], and they can direct axonal growth towards a target through the mechanical constriction of cells[11,12]. However, biomaterials cannot supply the multitude of growth factors and biochemical signals that are necessary for axonal growth and regeneration[13–16]. This mission can be achieved with the support of auxiliary cells that behave as factories for the biological factors, and whose decisive role in axonal growth is well known[17,18]. Thus, Schwann cells (SCs) are essential in both development and regeneration in the PNS, and oligodendrocytes are essential in the CNS[19–22]. SCs secrete, among others, angiogenic factors and neurotrophic factors, such as BDNF and NGF, necessary for axonal regeneration[23–25].

In this work, the process of axonal extension in the presence of SCs seeded on a biomaterial substrate is studied. This biomaterial has electroconductive characteristics, so it is susceptible to electrical excitation and to transmit this type of stimuli. Moreover, it is essential to understand the behaviour of the SCs that adhere to the biomaterial, and to check if their trophic factors

1 secretary capacity remains unchanged. The planned strategy was to develop a methodology to
2 enhance this secretory role of SCs, and to check its effect on the extension of axons from dorsal
3 root ganglion (DRG) neurons over growth-guiding biomaterials. Specifically, a bioactive
4 device based on the combination of a biocompatible substrate formed by highly aligned
5 electroconductive microfibrils and genetically modified support cells had to be developed with
6 the aim of accelerating the repair of lesions both in the CNS and in the PNS. On the one hand,
7 the topography can guide axonal growth to maximize the linear distance that regenerating axons
8 cover. On the other hand, the use of genetically modified support cells to increase their secretion
9 of growth factors allows to increase and accelerate the axonal growth of the neurons seeded on
10 the device.
11

12 Electrically conductive substrates based on intrinsically conductive polymers have been
13 extensively studied in order to improve the axonal extension of neurons and induce a greater
14 release of neurotrophins by glial cells when subjected to an exogenous electric field[26–28].
15 Among all electroconductive polymers, there are well-studied ones like polypyrrole (PPy),
16 polyaniline (PANI) and poly(3,4-ethylenedioxythiophene) (PEDOT). Herein, we sought to use
17 poly-L-lactic acid (PLA) - polypyrrole (PPy) electroconductive highly aligned substrates based
18 on microfibrils since we have previously observed that they offer an enhanced axonal guidance
19 and growth versus flat, non-aligned, or smaller fibre sized substrates[29]. In addition, the use
20 of electroconductive substrates offers the possibility of its electrically stimulation in future
21 experiments.
22

23 As host polymer of the substrates, PLA is a low cost, renewable, environmentally friendly and
24 biodegradable biomaterial[30,31]. Furthermore, its biocompatibility is critical in order to use
25 PLA-PPy substrates for tissue engineering applications[32,33]. To obtain the substrates the
26 coating of the PLA microfibrils with PPy via in situ polymerization seemed a good approach,
27 since PPy is an intractable and brittle solid with poor mechanical processability[34]. With this
28 strategy we can exploit both the intrinsic electrical conductivity of PPy and the better
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1 mechanical properties of the insulating host polymer[34–36]. It is important to note that PPy is
2 an interesting electroconductive material due to its high electrical conductivity, long-term
3 ambient stability, good biocompatibility, low cost and facile synthesis by chemical
4 polymerization[34,37–40]. Furthermore, PPy has been commonly used in biomedical
5 applications, especially in nerve tissue engineering scaffolds, due to its good biocompatibility
6 and high electrical conductivity[35,41–43].

7
8
9
10
11
12
13
14 Regarding the topography of the substrates, we focus on the use of aligned substrates because
15 they offer a high axonal orientation, whereas in a flat substrate such as a glass cover or a
16 substrate with randomly oriented fibres, axons grow unoriented[11,12,35,44–46]. In addition,
17 the diameter of the fibres that form the substrate is an important parameter since it influences
18 the neurite outgrowth and the Schwann cell migration from DRGs[47–49]. We have previously
19 studied different fibre diameters, concluding that micrometer sized fibres (10 μm diameter)
20 provides the best axonal growth[29]. Here, we have grouped in parallel 10 μm diameter
21 microfibres with polycaprolactone (PCL) bands that act as fasteners in the extremes to obtain
22 rail shaped microfibre bundles. PCL was used as fastening material for the microfibres bundles
23 due to its low melting point (around 60°C), making possible the fabrication of the microfibre
24 bundles. PCL is a biodegradable and biocompatible polymer that has been commonly used for
25 biomedical applications as an implantable biomaterial[50,51].

26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43 Axonal extension is accompanied and influenced by interaction with glial cells such as the
44 SCs[17]. These cells are naturally present in the peripheral nervous system, forming the myelin
45 sheath around the axons and playing a key role in neuronal survival and axonal
46 regeneration[18,23–25]. In case of injury, SCs are necessary to achieve the axonal regeneration
47 since they form regeneration columns (called Büngner’s bands) that guide the regenerating
48 axons[19,20]. SCs also release cytokines as LIF and IL-6 that promote the survival of
49 neurons[23]. In addition, SCs secrete different neurotrophic factors like nerve growth factor
50 (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3), among
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1 others[23–25]. The BDNF protein is necessary for the continued survival and phenotypic
2 maintenance of neurons[52], promotes survival of dorsal root ganglion neurons[53], enhances
3 neurogenesis[53] and promotes nerve healing and axon growth, improving axonal
4 regeneration[54–58].
5
6
7

8
9 Different studies have successfully achieved the genetic modification of different types of cells
10 to increase the expression and release of growth factors by the modified cells[56,59–61].
11

12 Biological, chemical and physical techniques can be employed to achieve the genetical
13 modification of cells[62]. Biological virus-mediated transfection is the most used technique in
14 clinical research, since it is highly efficient and it easily achieves a stable transfection[62,63].
15
16
17

18 With this technique, the foreign DNA is integrated into the genome of the cell and it replicates
19 as the host genome does, enabling a sustainable transgene expression[64,65]. However, it has
20 been reported that viral vectors integrate into the host genome randomly, which may disrupt
21 tumour suppressor genes, activate oncogenes, or interrupt essential genes, leading to a
22 malignant transformation[62,66,67]. For these reasons, non-viral transfection techniques as
23 electroporation have been widely applied[62,68].
24
25
26
27
28
29
30
31
32
33
34
35

36 The transfection of SCs to achieve a greater expression and release of BDNF has been studied
37 by various authors using retroviral vectors, observing an increase in the secretion of BDNF by
38 infected cells compared to control cells[59,60]. SCs transfection using electroporation
39 techniques to achieve an increased BDNF secretion in vitro has not been previously studied.
40
41
42
43
44
45

46 Electroporation is a physical technique that offers a safe and fast alternative to achieve the
47 introduction of the plasmid of interest into the target cells and to transiently express the protein
48 that it encodes[62,68,69]. In our case, a transient transfection of SCs in order to obtain an
49 increased secretion of BDNF by these cells should be sufficient since the DRG culture duration
50 is shorter than two weeks and there is no need of the protein expression for a long time.
51
52
53
54
55
56
57

58 Finally, in order to study the axonal growth in the context described here above, DRGs are
59 interesting to use as neuron source because the DRG body edge can be taken as the starting
60
61
62
63
64
65

point for the axon's growth, facilitating the measurement of axonal extension. Furthermore, the migration of cells from the DRG body (mostly SCs) generates a cellular environment that favours axonal growth and survival[47,49].

In the frame of optimized biological conditions using genetically modified rat Schwann cells co-cultured (or not) with rat DRGs, we evaluated thus the new highly aligned microfibre based substrates, hoping to reach a large axonal sprouting and a sustained and directional axonal growth along these substrates.

Table 1. Detail of the variable parameters of the 2 control conditions and the 7 protocols explored.

	C1	C2	P1	P2	P3	P4	P5	P6	P7
Number of cells	$5 \cdot 10^5$	$1 \cdot 10^6$	$5 \cdot 10^5$	$1 \cdot 10^6$					
SMEM (% v/v) / Water (% v/v)	100/0	100/0	96/4	44/56	44/56	44/56	44/56	25/75	44/56
Plasmid mass (μg)	0	0	4.5	4.5	4.5	50	50	50	50
Electric field amplitude (V/cm)	0	0	1,000	1,000	1,500	1,500	2,000	2,000	2,000

2. Experimental section

2.1. Electroporation of rat Schwann cells

The plasmid pCMV-GFP (3,487 bp) containing the GFP reporter gene under the cytomegalovirus (CMV) promoter was produced by PlasmidFactory (Bielefeld, Germany) and stored at -20°C at a concentration of $1 \mu\text{g plasmid}/\mu\text{l}$ of Mili-Q® ultrapure water. The gene transfection of rat Schwann cells (SCs; P10301, Innoprot) in passage 5 using the pCMV-GFP plasmid was carried out with the BIORAD gene pulser (Gene Pulser Xcell Electroporation System, BIORAD) applying 8 square pulses with a duration of $100 \mu\text{s}/\text{pulse}$ and a frequency of 1 Hz in a $100 \mu\text{l}$ total volume cuvette with 1 mm between electrodes. The time between the electroporation and the dilution of the cells in culture medium and their introduction into the incubator was 15 min. Apart from these parameters which were kept constant, we varied the

1 other parameters: number of cells; amplitude of the applied voltage (thus of the electric field);
2 volume of Spinner Minimum Essential Medium (SMEM, Thermo Fisher Scientific); volume of
3 Milli-Q® ultrapure water and mass (thus amount) of plasmid. As indicated in Table 1, two
4 controls with a different number of cells (C1 - C2) and seven different electroporation protocols
5 (P1 – P7) were studied in order to determine the best conditions for the electrotransfection of
6
7 the Schwann cells, based on our previous experience on other cell types[70,71]. The goal of
8 these experiments was to produce the maximum amount of transfected living cells which is a
9 trade between the level of electroporation/transfection and the number of cells remaining alive.
10
11 A detailed explanation of the results obtained for each protocol is described in section 3.1.
12
13

14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

Once the best electroporation parameters were obtained using the pCMV-GFP plasmid (protocol P7), it was applied again to rat SCs in passage 5 using the BDNF-SEP plasmid [72] which was a gift from Ryohei Yasuda (Addgene plasmid # 83955 ; <http://n2t.net/addgene:83955> ; RRID:Addgene_83955). We purified this plasmid from Escherichia coli-transformed cells using the NucleoBond PC2000 EF kit (Macherey-Nagel, Hoerd, France) and then we diluted it in Milli-Q® ultrapure water. Light absorption at 260 nm was used to determine DNA concentration and the quality of the plasmid was assessed by calculating the ratios of light absorption at 260/280 nm and 260/230 nm. It was stored at a concentration of 4.3 µg plasmid/µl Milli-Q® ultrapure water.

After the electroporation of SCs, control and gene-transfected SCs were diluted in normal Schwann cell culture medium and introduced in an incubator at 37 °C with a humid atmosphere containing 5% CO₂. All the cells from a single electroporation cuvette were introduced in a single T25 culture flask.

2.2. Quantification of pCMV-GFP plasmid transfection results

SCs transfected with the pCMV-GFP plasmid were analyzed at different times: after 1, 4, 6 and 11 days of cell culture. Firstly, cells were observed using an inverse microscope (Axio Observer

1 Z1, Carl Zeiss Microscopy) to appreciate the green fluorescence of transfected cells. Then, after
2 removing the culture medium and thus the dead cells, culture flasks were washed with PBS and
3
4 a trypsin/EDTA solution (T/E; 25200-072, Life Technologies) was then added to break the cell-
5
6 matrix and cell-cell interactions in order to remove the cells from the bottom of the culture flask.
7
8 After centrifugation at 1,080 rpm for 5 min, the pellet was resuspended in Schwann cell culture
9
10 medium (P60123, Innoprot) and the total number of cells was counted. Half of the cells in
11
12 suspension were re-seeded in a T25 flask and the rest were analysed by flow cytometry (10,000
13
14 events were recorded) using the green fluorescence channel (excitation 488 nm, emission 530
15
16 nm) in a C6 Accuri flow cytometer (BD, San José, California, USA). As a result, the number
17
18 of living cells (total number of cells), the level of transfection (percentage of cells showing
19
20 green fluorescence) and the number of transfected living cells (multiplication of the two
21
22 previous parameters) were collected.
23
24
25
26
27
28

29 **2.3. Preparation of PLA-PPy microfibre bundles**

30 Highly aligned PLA microfibre (MF) bundles were manufactured by grouping 1,200 PLA MFs
31
32 with a diameter of 10 μm (Aitex, Spain). PCL bands that acted as fasteners were used at the
33
34 extremes of the MFs to maintain the alignment and the lane-shaped disposition. PCL bands
35
36 were placed in a solid state on both extremes of the bundle and melted so, once cooled, the MFs
37
38 were attached by the PCL bands. MF substrates with dimensions of 17 mm x 3 mm (length x
39
40 width) were used. The auxiliary PCL bands that maintain the MF together are not considered
41
42 when defining the dimensions of the MF substrates.
43
44
45
46
47
48

49 MF substrates were coated with the electrically conductive polymer PPy via in situ
50
51 polymerization. Firstly, the substrates were immersed in deionized water and a fixed vacuum
52
53 was applied until the substrates stopped floating. With this procedure the introduction of water
54
55 inside the spaces between fibres was achieved to obtain a homogeneous coating of all MFs, not
56
57 only the superficial ones. Next, each substrate was introduced into a polypropylene tube with
58
59
60
61
62
63
64
65

1 an aqueous solution of pyrrole monomer (Py, Sigma-Aldrich 131709) and sodium para-toluene
2 sulfonate (pTS, Sigma-Aldrich, 152536) that acted as dopant. It was followed by ultrasonication
3
4 for 1 min to allow the membrane to be saturated with Py/pTS aqueous solution. The substrates
5
6 were incubated with shaking at 4°C for 1 h. The ratio between the substrates area (length x
7
8 width) and the final volume of the Py/pTS aqueous solution was 0.6 cm²/ml and a concentration
9
10 of 14 mM was used for both Py and pTS. Then, an aqueous solution of ferric chloride (FeCl₃,
11
12 Sigma-Aldrich 157740) that acted as oxidant was added and incubated with shaking at 4°C for
13
14 24 h for the polymerization and deposition of PPy on the PLA substrates. The ratio between the
15
16 substrates area (length x width) and the final volume of the FeCl₃ aqueous solution was 0.6
17
18 cm²/ml and a concentration of 38 mM of FeCl₃ was used. PPy-coated membranes were washed
19
20 with deionized water with agitation for 10 min for three times and ultrasonicated for 30 min in
21
22 deionized water for three times. Finally, the membranes were dried in a desiccator with a fixed
23
24 vacuum at 40°C for 2 days.
25
26
27
28
29
30

31 32 **2.4. Morphological characterization by field emission scanning electron microscopy** 33 **(FESEM)** 34

35
36 In order to characterize the surface morphology of PLA-PPy substrates a field emission
37
38 scanning electron microscope (FESEM; ULTRA 55, ZEISS Oxford Instruments) was employed.
39
40 During the 24 h prior to the test the samples were desiccated under vacuum conditions to avoid
41
42 interferences caused by evaporated water. After that, samples were placed on the top of a carbon
43
44 tape creating a carbon bridge between the samples and the carbon tape and they were coated
45
46 with a thin layer of platinum. Finally, the images were taken applying a voltage of 2 kV.
47
48
49
50

51 52 **2.5. Substrates sanitization and preconditioning** 53

54 To sanitize the PLA-PPy MF substrates prior to the cell culture, they were immersed in 70%
55
56 ethanol (ET00021000, Scharlab) for 3 washes of 10 min. Following, 4 washes of 10 min with
57
58 sterile Milli-Q® ultrapure water were applied to remove the ethanol residues.
59
60
61
62

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

Finally, the substrates were preconditioned by immersion in Dulbecco's Modified Eagle Medium with a high glucose level (4.5 g/L) (21331020, Life Technologies) supplemented with 10% of Fetal Bovine Serum (10270- 106/A3381E, Life Technologies) and 1% Penicillin/Streptomycin (15140122, Life Technologies) and incubation at 37°C for 24 h in a humidified atmosphere containing 5% CO₂.

2.6. Schwann cells culture on PLA-PPy substrates

The culture of rat Schwann cells (SCs) in passage 5 and BDNF-SEP transfected SCs (eSCs) 24 h after electroporation was performed on PLA-PPy MF substrates (n=12 per group) during 24 h in order to allow the cells to attach to the substrates before the DRG seeding.

Firstly, the culture medium was removed in order to discard dead cells. Next, the cell culture flasks containing the cells were washed with PBS and then a trypsin/EDTA solution (T/E; 25200-072, Life Technologies) was incorporated to break the cell-matrix and cell-cell interactions with the objective of detach cells from the bottom of the flasks. Finally, a centrifugation at 1080 rpm for 5 min was applied and the pellet was resuspended in Schwann cell culture medium (P60123, Innoprot). After counting the number of cells present, SCs and eSCs were seeded with a seeding density of 100,000 cells per substrate through the deposit of 2 drops of 5 µl (50,000 SCs per drop) at equidistant points from the substrate extremities to spread the cells along the substrate, specially at its extremes (**Figure 1**). Each substrate was placed in a different well of a P12 well plate (Nunc A/s, Roskilde, Denmark) containing 1 ml of Schwann cell culture medium. Finally, the samples were introduced in an incubator at 37°C with a humid atmosphere containing 5% CO₂ for 24 h, until the next day in which the DRGs were seeded.

2.7. Dorsal root ganglions culture on PLA-PPy substrates

Dorsal root ganglions (DRGs) from 5 days old Wistar rats were extracted and seeded on the centre of PLA-PPy substrates (Figure 1). Three different groups were studied (n=12 per group):

1 DRGs seeded on substrates without pre-seeded SCs (DRG), DRGs seeded on substrates pre-
2 seeded with SCs (SCs + DRG) and DRGs seeded on substrates pre-seeded with BDNF-SEP
3
4 transfected SCs (eSCs + DRG). Each substrate was placed in a different well of a P12 well plate
5
6 containing 3 ml of neurobasal culture medium (21103-049, Thermo Fisher Scientific) with 2%
7
8 D-(+)-Glucose 0.56 M (G8644, Sigma-Aldrich), 0.25% L-Glutamine 200 mM (25030024,
9
10 Thermo Fisher Scientific), 1% Penicillin/Streptomycin (15140122, Life Technologies) and 1%
11
12 Fetal Bovine Serum (10270- 106/A3381E, Life Technologies). Finally, the samples were
13
14 introduced in an incubator at 37 °C with a humid atmosphere containing 5% CO₂ for 5 days to
15
16 study the axonal growth and axonal extension.
17
18
19
20
21
22

23 **2.8. Quantification of BDNF secretion**

24 The quantity of BDNF present in the cell culture supernatant of the three studied groups (DRG,
25
26 SCs + DRG and eSCs + DRG) was quantified after 1, 2 and 5 days of cell culture. For it, 100
27
28 µl of culture medium were removed from every well at each time and the total BDNF
29
30 Quantikine ELISA Kit (DBNT00, R&D Systems) was employed (n=3) for the quantification.
31
32 A standard curve was performed to convert the obtained absorbance into BDNF concentration.
33
34
35
36
37

38 **2.9. Immunostaining of SCs and DRGs**

39 After culture, cells were fixed with 4% paraformaldehyde (PFA; 47608, Sigma-Aldrich) for 20
40
41 min at room temperature. Then, 3 washes of 10 min with PB 0.1M were carried out and the cell
42
43 membrane was permeabilized by the use of a blocking buffer composed of PB 0.1M with 3%
44
45 bovine serum albumin (BSA; A7906, Sigma-Aldrich) and 0.1% Tween-20 (P1379, Sigma-
46
47 Aldrich) for 1 h at room temperature.
48
49

50 SCs were stained with rabbit monoclonal anti-S100 beta antibody (ab52642, abcam, 1/200
51
52 dilution) and neurons were stained with mouse monoclonal anti-beta III tubulin antibody
53
54 (ab7751, abcam, 1/500 dilution), and then incubated at 4°C overnight. Secondary antibodies,
55
56 goat anti-rabbit IgG Alexa Fluor® 555 (A-21429, Thermo Fisher Scientific, 1/200 dilution) and
57
58
59
60
61
62
63
64
65

1 goat anti-mouse IgG Alexa Fluor® 488 (A-11029, Thermo Fisher Scientific, 1/200 dilution),
2 were used for another 2 hours at room temperature in the darkness. Afterwards, samples were
3
4 incubated with DAPI (D9564, Sigma-Aldrich, 1/1000 dilution) for 10 min to mark the cells'
5
6 nuclei. The imaging was performed employing an inverse microscope (Axio Observer Z1, Carl
7
8 Zeiss Microscopy).
9
10

11 **2.10. Quantification of axonal extension, axonal sprouting and SCs coverage**

12
13 The methodology followed for the quantification of axonal extension and axonal sprouting is
14
15 explained in detail in[29]. Below we proceed to explain it briefly.
16
17

18
19 Using the ImageJ/FIJI image processing software [73] both the maximum length and the area
20
21 of axons were measured. On the one hand, the maximum length of axons was considered as the
22
23 distance between the edge of the DRG body and the end of the longest axon (studying both the
24
25 maximum of both sides and the sum of both sides). On the other hand, the area of the axons
26
27 was calculated by subtracting the area of the DRG body from the total area of the DRG.
28
29

30
31 In addition, the axonal sprouting was quantified by analysing the DRG images using an in-
32
33 house software developed under MATLAB R2018a (The MathWorks, Inc.). The centre and the
34
35 left and right edges of the DRG body were obtained as the maximum point of the relative
36
37 intensity and as the maximum and minimum values of the relative intensity gradient,
38
39 respectively. Then, three different parameters were obtained: the distance covered by the
40
41 Sprouts between the point that corresponds to the centre of the DRG body (I_{centre}) and the point
42
43 where I_{centre} is reduced by 75% (S_{centre} , Equation 1), the distance covered by the Sprouts between
44
45 the point that corresponds to the edge of the DRG (I_{edge}) and the point where I_{edge} is reduced by
46
47 75% (S_{edge} , Equation 2) and the area under the curve (AUC) between the point that corresponds
48
49 to the edge of the DRG body (I_{edge}) and the point where I_{centre} is reduced by 80% (S_{AUC} , Equation
50
51 3). All the parameters were obtained for both sides of the DRG body and the sum of both values
52
53 was considered.
54
55
56
57
58
59
60
61
62
63
64
65

$$S_{centre} = Distance \left(I_{centre}, \frac{I_{centre}}{4} \right) \quad (1)$$

$$S_{edge} = Distance \left(I_{edge}, \frac{I_{edge}}{4} \right) \quad (2)$$

$$S_{AUC} = Area \left(I_{edge}, \frac{I_{centre}}{5} \right) \quad (3)$$

In addition, the length and the area of the SCs coverage formed on the substrate were quantified. These parameters were obtained for both sides of the DRG body and the mean of both values was considered.

2.11. Statistical analysis

Results are expressed as mean \pm standard error of the mean (SEM). The statistical analysis of the results was performed with GraphPad Prism® software using the one-way ANOVA test together with a multiple sample mean comparison (Tuckey's multiple comparisons test with a significance degree of 95%) in order to reveal significant differences between conditions. Statistically significant differences are indicated by *, **, *** or ****, indicating a p-value below 0.05, 0.01, 0.001 or 0.0001, respectively.

3. Results and discussion

3.1. Optimization of electroporation parameters of Schwann cells using the pCMV-GFP plasmid

In order to obtain the electroporation parameters that provide the maximum number of transfected living cells, a scan of the electroporation conditions that were considered more critical (applied electric field, amount of plasmid, amount of water and number of cells) was carried out based on our previous experience[70,71]. The rest of parameters (number, shape, duration and frequency of pulses and time between the electric pulses delivery and the dilution and introduction of the cells in the incubator) remained unmodified. Cuvettes with a volume of

100 μ l and a gap between electrodes of 1 mm were used to apply the highest electric field amplitudes with small currents.

P1, P2 and P3 protocols were performed to know the effect of the amount of water and the applied electric field in the case of low quantities of cells and plasmid. On the one hand, it was observed (**Figure 2**) that increasing from 4% to 56% the volume percentage of water (P1 vs P2) considerably improved both the level of transfection and the number of transfected living cells, thanks to increasing the difference in concentrations between the inside and the outside of cells. On the other hand, according to Figure 2, increasing the applied electric field from 1,000 V to 1,500 V (P2 vs P3), keeping the volume percentage of water at 56%, further improved the level of transfection and the number of transfected living cells.

With the P4 protocol the effect of increasing the amount of plasmid was studied, increasing the plasmid mass from 4.5 μ g to 50 μ g (P3 vs P4). As can be seen in Figure 2, it increased the level of transfection, but the higher cell death led to obtain a quantity of transfected living cells similar to that obtained with the P3 protocol.

To study the effect of increasing the applied electric field from 1,500 V to 2,000 V (P4 vs P5) protocol P5 was carried out. As it can be observed in Figure 2, an increase of the level of transfection was obtained with a low increase of cell death, resulting in the increase of the transfected living cells. Therefore, SCs are able to tolerate this applied electric field quite well.

With the P6 protocol, the volume percentage of water was increased from 56% to 75% (P5 vs P6). According to Figure 2, the higher water content slightly improved the level of transfection, but the higher level of cell death caused a considerable decrease in the number of transfected living cells. Consequently, SCs cannot tolerate this high quantity of water in the cell culture medium.

Since the objective was to achieve the largest number of transfected living cells, the P5 protocol was modified and the number of cells present in the electroporation cuvette was doubled from 500,000 to 1 million cells (protocol P7). The P7 protocol derives from the P5 protocol because

1 the latter provided already a high level of transfection and the highest number of transfected
2 living cells when 500,000 cells were used. As a result, a level of transfection slightly higher to
3
4 the one obtained with P5 protocol was obtained, with a quite low cell death, using the same
5
6 amount of DNA. This, together with the higher number of cells that were used, led to a very
7
8 high number of transfected living cells, doubling the results obtained with P5 protocol.
9
10 Therefore, the P7 protocol was chosen to carry out the electroporation of SCs with the BDNF-
11
12 SEP plasmid (section 3.2), since it provided the largest number of transfected living cells per
13
14 cuvette and per μg of DNA. As it can be observed in Figure 2, the survival of the SCs transfected
15
16 using the P7 protocol is very high, similar to the survival in the C2 control group, at least for
17
18 11 days. In addition, the images of fluorescence microscopy reveal not only the level of
19
20 transfection, but also that the transfected SCs survive well and display a healthy morphology.
21
22
23
24
25
26
27

28 **3.2. Quantification of secreted BDNF by Schwann cells transfected with the BDNF-SEP** 29 **plasmid**

30
31 Using the P7 electroporation protocol (Table 1), SCs in passage 5 were transfected with the
32
33 BDNF-SEP plasmid. Next, SCs and eSCs (24 hours after electroporation) were seeded on PLA-
34
35 PPy MF substrates (**Figure 3**) by 2 equidistant drops of 5 μl (50,000 SCs per drop) to spread
36
37 the cells along the substrate, specially at its extremes. As can be observed in Figure 3, PLA-
38
39 PPy microfibrils have a high alignment thanks to the PCL bands that act as fasteners at its
40
41 extremes. In addition, there is a homogeneous coating with PPy in the form of fine grain texture
42
43 that can be seen in the whole surface, with just some loose aggregates that mostly disappear
44
45 with repeated washing.
46
47
48
49
50

51 To allow cell adhesion, SCs and eSCs were cultured on PLA-PPy substrates for 24 hours. Then,
52
53 DRGs were seeded in the middle of three different substrates (one DRG per substrate):
54
55 substrates without precultured cells (DRG), substrates with a previous culture of SCs during 24
56
57 h (SCs + DRG) and substrates with a previous culture of eSCs during 24 h (eSCs + DRG). One,
58
59 two and five days after the DRG seeding, the quantity of BDNF present in the cell culture
60
61
62
63
64
65

1 supernatant was measured by ELISA. As it can be observed in **Figure 4A**, when DRGs are seed
2 alone without precultured SCs (DRG) there is a weak liberation of BDNF from SCs that migrate
3 from the DRG body. However, when DRGs are seeded in substrates with precultured SCs (SCs
4 + DRG) there is an increase of the secreted BDNF due to the higher amount of SCs that are
5 present (precultured SCs and SCs that come from the DRG). This corroborates that SCs secrete
6 naturally the BDNF protein, but at low quantities.

7
8
9
10
11
12
13
14 Regarding the group with a previous culture of eSCs (eSCs + DRG) the secretion of BDNF to
15 the culture medium greatly increases (Figure 4B). As it can be observed, when genetically
16 unmodified SCs are used, the quantity of BDNF present in the medium after 5 days of cell
17 culture is around 8 pg/ml, while when eSCs are used the quantity of BDNF increases to around
18 800 pg/ml (100 times more). With this result we can confirm that the BDNF-SEP plasmid has
19 been correctly taken up by almost of the cells and that the transcription machinery of SCs is
20 able to express the BDNF protein. In addition, the SCs are able to naturally secrete this excess
21 of BDNF protein into the culture medium, secreting much more BDNF than non-electroporated
22 SCs.
23
24
25
26
27
28
29
30
31
32
33
34
35

36 In order to check the presence of precultured SCs and eSCs, fluorescent images (β III Tubulin,
37 DAPI and S 100 β) of samples after 5 days of DRG culture were studied (**Figure 5**). Firstly, a
38 global image of each sample is presented for each group (A-C for the DRG group, D-F for the
39 SCs + DRG group and G-I for the eSCs + DRG group). Secondly, two different zones are
40 presented for each sample: one at the end of the axons (A1-C1 for the DRG group, D1-F1 for
41 the SCs + DRG group and G1-I1 for the eSCs + DRG group) and another far away from the
42 axons (A2-C2 for the DRG group, D2-F2 for the SCs + DRG group and G2-I2 for the eSCs +
43 DRG group).

44
45
46
47
48
49
50
51
52
53
54
55
56 For the DRG group the SCs are only present very near to the axons, with no SC in areas where
57 axons are not present (Figure 5A-C). This indicates that axons grow together with SCs that
58 migrate from the DRG body, and therefore, the presence of SCs is a necessary requirement for
59
60
61

1 axonal growth. However, for the SCs + DRG (Figure 5D-F) and eSCs + DRG (Figure 5G-I)
2 groups, SCs are present far away from the axons, indicating that these cells are the pre-seeded
3 ones, and not SCs that come from the DRG body. In order to confirm this presence of the pre-
4 seeded SCs (both unmodified and genetically transfected) we measured the length and the area
5 of the SCs coverage present on the substrate after 5 days of DRG culture (**Figure 6**). Both the
6 length and the area of the SCs coverage are clearly larger for the groups with pre-seeded SCs
7 (SCs + DRG and eSCs + DRG), confirming the presence of the pre-seeded SCs. Furthermore,
8 there is no statistically significant difference in the length or in the area covered by the SCs
9 between the SCs + DRG and eSCs + DRG groups, which is indicative of the good survival of
10 the eSCs.
11

12 In addition, there is a great increment of axonal growth when precultured eSCs are used (eSCs
13 + DRG group) when compared with the DRG cultured without precultured SCs (DRG group)
14 (Figure 5). For the DRG + SCs group there is an intermediate behaviour. This indicates that the
15 precultured SCs are able to support the axons, increasing the axonal growth rate. In addition,
16 the increased secretion of BDNF by eSCs to the culture medium increases even more the axonal
17 growth rate. A quantification of axonal extension and sprouting for the three groups is done in
18 section 3.3.
19

20 **3.3. Quantification of axonal extension and axonal sprouting on PLA-PPy substrates**

21 In order to quantify the axonal growth on the three different groups (DRG, SCs + DRG and
22 eSCs + DRG), both the axonal extension and the axonal sprouting were quantified. It is
23 important to study these two characteristics because the axonal extension refers to the maximum
24 length that axons can reach, and the axonal sprouting refers to the quantity of axons that are
25 emerging from the DRG body. To do so, six different parameters were measured from
26 fluorescent microscope images (β III Tubulin) of samples after 5 days of DRG culture. On the
27 one hand, the maximum length of axons (maximum and sum of both sides) and the area of
28

1 axons were obtained to quantify the axonal extension. On the other hand, the parameters S_{centre} ,
2 S_{edge} and S_{AUC} were obtained to study the axonal sprouting. As it can be observed in Figure 5,
3
4 the high alignment of PLA-PPy substrates results in a high directionality of axons in the
5
6 direction of the fibres, maximizing the lineal distance that axons travel.
7

8
9 As it can be observed in **Figure 7**, the presence of precultured SCs is critical in order to increase
10
11 the axonal extension, since the maximum length of axons (sum of both sides) displays a 91.8%
12
13 increase and the area of axons a 126.2% increase when the SCs are present. According to Figure
14
15 5, when DRGs are seeded alone without precultured SCs, the axons arrive as far as the SCs that
16
17 migrate from the DRG body. This proves that axons do not grow without the direct support of
18
19 SCs and so their growth rate depends directly on the migration rate of SCs from the DRG body.
20
21 However, when precultured SCs are present in areas far away from the DRG body, axons are
22
23 able to increase their growth rate thanks to the support of the precultured SC cells, since axons
24
25 do not have to wait for SCs from the DRG. The effect of precultured SCs is also noticeable for
26
27 axonal sprouting, since an increase in S_{centre} , S_{edge} and S_{AUC} of 57.1%, 25.8% and 68.3%,
28
29 respectively, is observed when precultured SCs are present. This indicates an effect of
30
31 precultured SCs that are near the DRG body, so the axons do not need to wait for the SCs
32
33 migration from the DRG body and can start to grow earlier.
34
35

36
37 Regarding the effects of eSCs, we observe that, in addition to the effect observed when non-
38
39 electroporated SCs are used, the higher concentration of BDNF that is present in the cell culture
40
41 medium when eSCs are used is able to further increase the axonal growth, observing an increase
42
43 of both axonal extension and axonal sprouting. For the eSCs + DRG group, an increase of
44
45 34.7% and 40.1% was observed in the maximum length of axons (sum of both sides) and in the
46
47 area of axons, respectively, comparing with the SCs + DRG group. Therefore, the BDNF-rich
48
49 culture medium produces an additional increase in the growth rate of axons, so they can growth
50
51 faster. In addition, an increase of 27.6%, 30.0% and 40.6% was observed for S_{centre} , S_{edge} and
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1 SAUC, respectively, for the eSCs + DRG group when compared with the SCs + DRG group. This
2 shows an increase of axonal sprouting, so more axons migrate from the DRG body.
3

4 Thus, with the eSCs + DRG group we are observing the combined effect of the high alignment
5 of the substrate, the preculture of SCs and the continuous release of BDNF by these cells that
6 increases neuronal regeneration and protection. Therefore, this group would be the one
7 considered to be used as a device for the regeneration of lesions in the nervous system.
8
9 Furthermore, it should be noted that thanks to the SCs electrotransfection we are achieving an
10 effective and regular release of large amounts of BDNF during the first 10-15 days after its
11 implantation in vivo. This is of great importance since the first days after the injury occurs are
12 the most critical ones in the process of remodelling and regenerating an injury of the nervous
13 system. In addition, thanks to the continuous release of BDNF by SCs, it is possible to have a
14 stable BDNF-rich cell environment, solving the problem of the short half-life and the rapid
15 diffusion of BDNF when it is supplied directly in the lesion and avoiding the use of a continuous
16 infusion pump which could lead to greater wound complications and an increased risk of
17 infection[74,75].
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37

38 **4. Conclusions**

39 We have obtained a SCs electroporation protocol that provides a high level of transfection with
40 low cell death, obtaining a large number of live transfected cells. Thanks to this protocol, SCs
41 have been successfully transfected with a plasmid encoding the BDNF protein, which is
42 necessary for the continued survival and maintained phenotype of neurons, promotes survival
43 of dorsal root ganglion neurons, enhances neurogenesis and promotes nerve healing and axonal
44 growth, improving axonal regeneration. Electroporated SCs have been able to both express
45 higher quantities of BDNF protein and secrete them naturally into the culture medium. This has
46 not been previously reported using electroporation techniques with this type of cells in vitro. In
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1 addition, the quantity of BDNF secreted by electroporated SCs was very high, multiplying by
2 100 the level of BDNF secretion of non-electroporated SCs.
3

4 The co-culture of DRGs with non-electroporated and electroporated SCs revealed that the use
5 of PLA-PPy MF substrates precultured with non-electroporated SCs increases both axonal
6 extension and axonal sprouting, but when electroporated SCs are used, there is an additional
7 increment of them. The preculture of SCs is critical so that the axons that grow from the DRG
8 neurons do not have to wait for the SCs that migrate from the DRG body and can be
9 accompanied by the precultured SCs that are present on the substrate, being able to considerably
10 increase its growth rate. Furthermore, when the precultured SCs that are present on the substrate
11 have been previously electroporated with the plasmid encoding the BDNF protein, they release
12 large additional quantities of BDNF to the culture medium, which helps to obtain an additional
13 increase of axonal growth.
14
15
16
17
18
19
20
21
22
23
24
25
26
27

28 Therefore, our system based on a PLA-PPy highly aligned microfibre substrate pre-seeded with
29 electroporated SCs with an increased BDNF secretion is capable of both guiding and
30 accelerating axonal growth and regeneration. For this, the device can be of application for the
31 treatment of injuries of the nervous system, both central and peripheral. The device can be used
32 either alone or in combination with other devices such as conduits for an inside nerve guidance.
33
34 In addition, the electrical conductivity of PLA-PPy microfibres allows the electrical stimulation
35 of the device in future studies.
36
37
38
39
40
41
42
43
44
45
46
47
48

49 **Author contributions**

50
51 **Fernando Gisbert Roca:** Methodology, Software, Formal analysis, Investigation, Writing –
52 Original Draft, Visualization. **Franck M. Andre:** Methodology, Writing – Review & Editing,
53 Supervision. **Jorge Más Estellés:** Writing – Review & Editing, Supervision, Funding
54 acquisition. **Manuel Monleón Pradas:** Conceptualization, Writing – Review & Editing,
55 Supervision, Funding acquisition. **Lluís M. Mir:** Methodology, Writing – Review & Editing,
56 Supervision, Funding acquisition. **Cristina Martínez-Ramos:** Conceptualization,
57 Methodology, Writing – Review & Editing, Supervision, Funding acquisition.
58
59
60
61
62
63
64
65

Competing interests

The authors declare no competing interests.

Acknowledgements

The authors acknowledge financing from the Spanish Government's State Research Agency (AEI) through projects DPI2015-72863-EXP and RTI2018-095872-B-C22/ERDF. FGR acknowledges the scholarship FPU16/01833 and the short stay mobility aid EST18/00524 of the Spanish Ministry of Universities. FGR also acknowledges the hosting at the Vectorology and Anti-cancer Therapies Centre (UMR 8203 CNRS). We thank the Electron Microscopy Service at the UPV, where the FESEM images were obtained.

Received: ((will be filled in by the editorial staff))

Revised: ((will be filled in by the editorial staff))

Published online: ((will be filled in by the editorial staff))

References

1. E. a Huebner, S. M. Strittmatter, *Results Probl. Cell Differ.* **2009**, *48*, 339.
2. K. S. Houschyar *et al.*, *Plast. Surg. Int.* **2016**, *2016*, 1.
3. W. Daly, L. Yao, D. Zeugolis, A. Windebank, A. Pandit, *J. R. Soc. Interface* **2012**, *9*, 202.
4. G. C. W. de Ruiter, M. J. A. Malessy, M. J. Yaszemski, A. J. Windebank, R. J. Spinner, *Neurosurg. Focus* **2009**, *26*, E5.
5. M. D. Tang-Schomer, *Brain Res.* **2018**, *1678*, 288.
6. M. D. Sarker, S. Naghieh, A. D. McInnes, D. J. Schreyer, X. Chen, *Prog. Neurobiol.* **2018**, *171*, 125.
7. I. A. Kim *et al.*, *J. Biosci. Bioeng.* **2006**, *101*, 120.
8. A. W. English, G. Schwartz, W. Meador, M. J. Sabatier, A. Mulligan, *Dev. Neurobiol.* **2007**, *67*, 158.
9. P. M. Tsimbouri, L. E. McNamara, E. V. Alakpa, M. J. Dalby, L. A. Turner, *Tissue Eng. Second Ed.* **2014**, doi:10.1016/B978-0-12-420145-3.00007-9.
10. H. Amani *et al.*, *Adv. Mater. Interfaces* **2019**, *6*, 1.
11. W. Zhu, F. Masood, J. O'Brien, L. G. Zhang, *Nanomedicine Nanotechnology, Biol. Med.*

2015, 11, 693.

12. Y. S. Lee, G. Collins, T. Livingston Arinzeh, *Acta Biomater.* **2011**, 7, 3877.
13. A. Markus, T. D. Patel, W. D. Snider, *Curr. Opin. Neurobiol.* **2002**, 12, 523.
14. P. Lu, M. H. Tuszynski, *Exp. Neurol.* **2008**, 209, 313.
15. M. Lykissas, A. Batistatou, K. Charalabopoulos, A. Beris, *Curr. Neurovasc. Res.* **2007**, 4, 143.
16. B. S. Bregman, M. McAtee, H. N. Dai, P. L. Kuhn, *Exp. Neurol.* **1997**, 148, 475.
17. M. R. Freeman, *Curr. Opin. Neurobiol.* **2006**, 16, 119.
18. E. Pompili *et al.*, *Eur. J. Histochem.* **2020**, 64.
19. K. R. Jessen, R. Mirsky, A. C. Lloyd, *Cold Spring Harb. Perspect. Biol.* **2015**, 7, 1.
20. J. A. Gomez-Sanchez *et al.*, *J. Neurosci.* **2017**, 37, 9086.
21. M. Bradl, H. Lassmann, *Acta Neuropathol.* **2010**, 119, 37.
22. D. M. McTigue, R. B. Tripathi, *J. Neurochem.* **2008**, 107, 1.
23. N. El Seblani, A. S. Welleford, J. E. Quintero, C. G. van Horne, G. A. Gerhardt, *J. Neurosci. Methods* **2020**, 335, 108623.
24. K. R. Jessen, P. Arthur-Farraj, *Glia* **2019**, 67, 421.
25. G. Nocera, C. Jacob, *Cell. Mol. Life Sci.* **2020**, 1, doi:10.1007/s00018-020-03516-9.
26. C. E. Schmidt, V. R. Shastri, J. P. Vacanti, R. Langer, *Proc. Natl. Acad. Sci. U. S. A.* **1997**, 94, 8948.
27. J. Huang, Z. Ye, X. Hu, L. Lu, Z. Luo, *Glia* **2010**, 58, 622.
28. H. T. Nguyen *et al.*, *J. Biomed. Mater. Res. - Part A* **2014**, 102, 2554.
29. F. Gisbert Roca, J. Más Estellés, M. Monleón Pradas, C. Martínez-Ramos, *Int. J. Biol. Macromol.* **2020**, 163, 1959.
30. D. E. Henton, P. Gruber, J. Lunt, J. Randall, *Nat. Fibers, Biopolym. Biocomposites* **2005**, 48674, 527.
31. J. Lunt, *Polym. Degrad. Stab.* **1998**, 3910, 145.

- 1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
32. Y. Ramot, M. Haim-Zada, A. J. Domb, A. Nyska, *Adv. Drug Deliv. Rev.* **2016**, *107*, 153.
 33. D. da Silva *et al.*, *Chem. Eng. J.* **2018**, *340*, 9.
 34. L. X. Wang, X. G. Li, Y. L. Yang, *React. Funct. Polym.* **2001**, *47*, 125.
 35. J. Y. Lee, C. A. Bashur, A. S. Goldstein, C. E. Schmidt, *Biomaterials* **2009**, *30*, 4325.
 36. A. Esfandiari, *World Appl. Sci. J.* **2008**, *3*, 470.
 37. T. H. Le, Y. Kim, H. Yoon, *Polymers (Basel)*. **2017**, *9*.
 38. M. Mattioli-Belmonte *et al.*, *Mater. Sci. Eng. C* **2005**, *25*, 43.
 39. G. Sabouraud, S. Sadki, N. Brodie, *Chem. Soc. Rev.* **2000**, *29*, 283.
 40. C. Li, H. Bai, G. Shi, *Chem. Soc. Rev.* **2009**, *38*, 2397.
 41. S. Aznar-Cervantes *et al.*, *Bioelectrochemistry* **2012**, *85*, 36.
 42. J. F. Zhou *et al.*, *Neural Regen. Res.* **2016**, *11*, 1644.
 43. P. M. George *et al.*, *Biomaterials* **2005**, *26*, 3511.
 44. Y. Wang *et al.*, *Chin. Med. J. (Engl)*. **2011**, *124*, 2361.
 45. Y. Zou *et al.*, *ACS Appl. Mater. Interfaces* **2016**, *8*, 12576.
 46. Y. Xu, Z. Huang, X. Pu, G. Yin, J. Zhang, *Cell Prolif.* **2019**, *52*, 1.
 47. H. B. Wang, M. E. Mullins, J. M. Cregg, C. W. McCarthy, R. J. Gilbert, *Acta Biomater.* **2010**, *6*, 2970.
 48. G. T. Christopherson, H. Song, H. Q. Mao, *Biomaterials* **2009**, *30*, 556.
 49. S. Gnani *et al.*, *Mater. Sci. Eng. C* **2015**, *48*, 620.
 50. M. A. Woodruff, D. W. Huttmacher, *Prog. Polym. Sci.* **2010**, *35*, 1217.
 51. C. X. F. Lam, D. W. Huttmacher, J. T. Schantz, M. A. Woodruff, S. H. Teoh, *J. Biomed. Mater. Res. A* **2009**, *90*, 906.
 52. C. Zuccato, E. Cattaneo, *Nat. Rev. Neurol.* **2009**, *5*, 311.
 53. D. K. Binder, H. E. Scharfman, *Growth Factors* **2004**, *22*, 123.
 54. K. Hanamura, A. Harada, R. Katoh-Semba, F. Murakami, N. Yamamoto, *Eur. J. Neurosci.* **2004**, *19*, 1485.

- 1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
55. T. Lopatina *et al.*, *PLoS One* **2011**, *6*.
56. P. Lu, L. L. Jones, M. H. Tuszynski, *Exp. Neurol.* **2005**, *191*, 344.
57. R. M. Lindsay, *J. Neurosci.* **1988**, *8*, 2394.
58. S. Liu *et al.*, *Acta Biomater.* **2017**, *60*, 167.
59. P. Menei, C. Montero-Menei, S. R. Whittmore, R. P. Bunge, M. B. Bunge, *Eur. J. Neurosci.* **1998**, *10*, 607.
60. S. T. Sayers, N. Khan, Y. Ahmed, R. Shahid, T. Khan, *J. Mol. Neurosci.* **1998**, *10*, 143.
61. L. A. Hohsfield, S. Geley, M. Reindl, C. Humpel, *J. Immunol. Methods* **2013**, *391*, 112.
62. T. K. Kim, J. H. Eberwine, *Anal. Bioanal. Chem.* **2010**, *397*, 3173.
63. A. Pfeifer, I. M. Verma, *Annu. Rev. Genomics Hum. Genet.* **2001**, *2*, 177.
64. S. Hacein-Bey-Abina *et al.*, *N. Engl. J. Med.* **2002**, *346*, 1185.
65. J. Roesler *et al.*, *Blood* **2002**, *100*, 4381.
66. N. B. Woods *et al.*, *Blood* **2003**, *101*, 1284.
67. S. Hacein-Bey-Abina *et al.*, *N. Engl. J. Med.* **2003**, *348*, 255.
68. C. Rosazza, S. H. Meglic, A. Zumbusch, M.-P. Rols, D. Miklavcic, *Curr. Gene Ther.* **2016**, *16*, 98.
69. M. Aspalter *et al.*, *J. Neurosci. Methods* **2009**, *176*, 96.
70. A. Liew *et al.*, *Hum. Gene Ther. Methods* **2013**, *24*, 289.
71. L. L. Lesueur, L. M. Mir, F. M. André, *Mol. Ther. - Nucleic Acids* **2016**, *5*, e291.
72. S. C. Harward *et al.*, *Nature* **2016**, *538*, 99.
73. J. Schindelin *et al.*, *Nat. Methods* **2012**, *9*, 676.
74. J. Guan *et al.*, *Biomaterials* **2012**, *33*, 1386.
75. E. Palasz *et al.*, *Int. J. Mol. Sci.* **2020**, *21*.

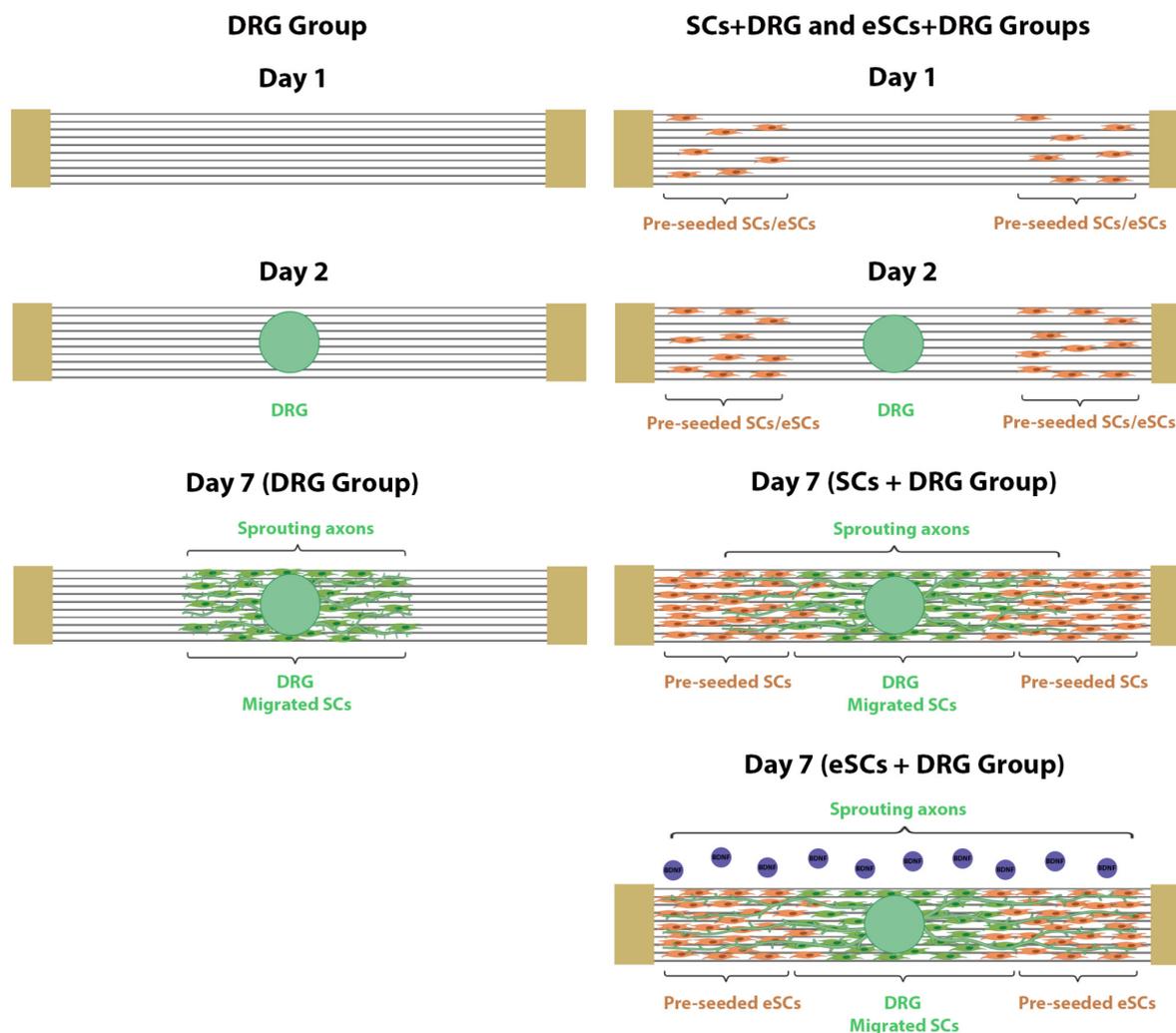


Figure 1. Scheme of the different stages of cell culture. First (day 1) the Schwann cells (SCs) and BDNF-SEP transfected Schwann cells (eSCs) are seeded on the substrate extremes for the SCs + DRG and eSCs + DRG groups, respectively. After 24 hours (day 2) one DRG is seeded in the centre of the substrate for all groups. Evaluation is performed after another 5 days of culture (day 7). The neurons present in the DRG body extend their axons over the surface than the SCs covered on the substrate. In the case of the DRG group, the SCs coverage is formed only by SCs that migrated from the DRG body, while in the case of the SC + DRG and eSCs + DRG groups the SCs coverage is made up of both cells that migrated from the DRG body and the pre-seeded Schwann cells (SCs and eSCs, respectively). The presence of pre-seeded SCs is capable of increasing the speed of axonal growth, since the sprouting axons can be accompanied in their growth by the pre-seeded SCs and are not limited by the speed of migration of the SCs originating from the DRG body. Furthermore, in the case of the eSCs + DRG group, the eSCs possess an increased secretion of BDNF that increases even more the axonal growth rate. Created with BioRender.com

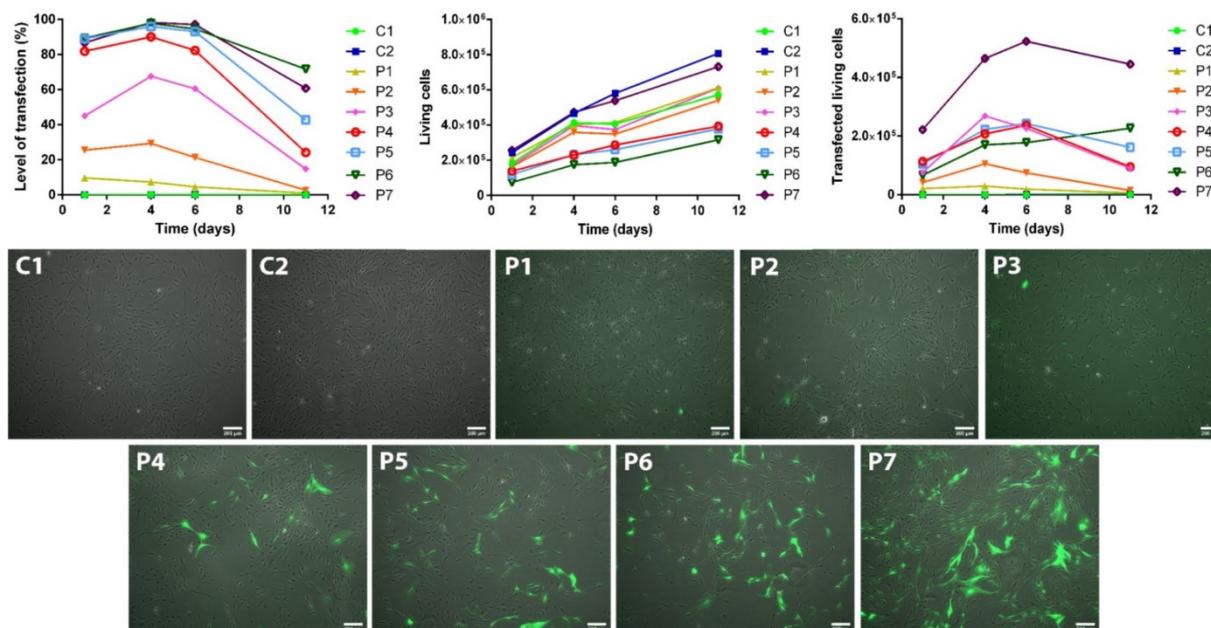


Figure 2. Level of transfection, living cells and transfected living cells obtained with the electroporation protocols explained in Table 1. Increasing the quantity of water, the quantity of plasmid and the applied electric field increased the level of transfection but with the cost of a higher cell death. The quantity of transfected living cells was the parameter used to obtain the best balance. To obtain the highest quantity of living cells, a protocol with a high level of transfection (P5) was used but doubling the number of cells (P7). Images of fluorescence microscope after 4 days of cell culture are also included, where can be visually appreciated that the electroporation protocol P7 provides the highest quantity of transfected living cells.

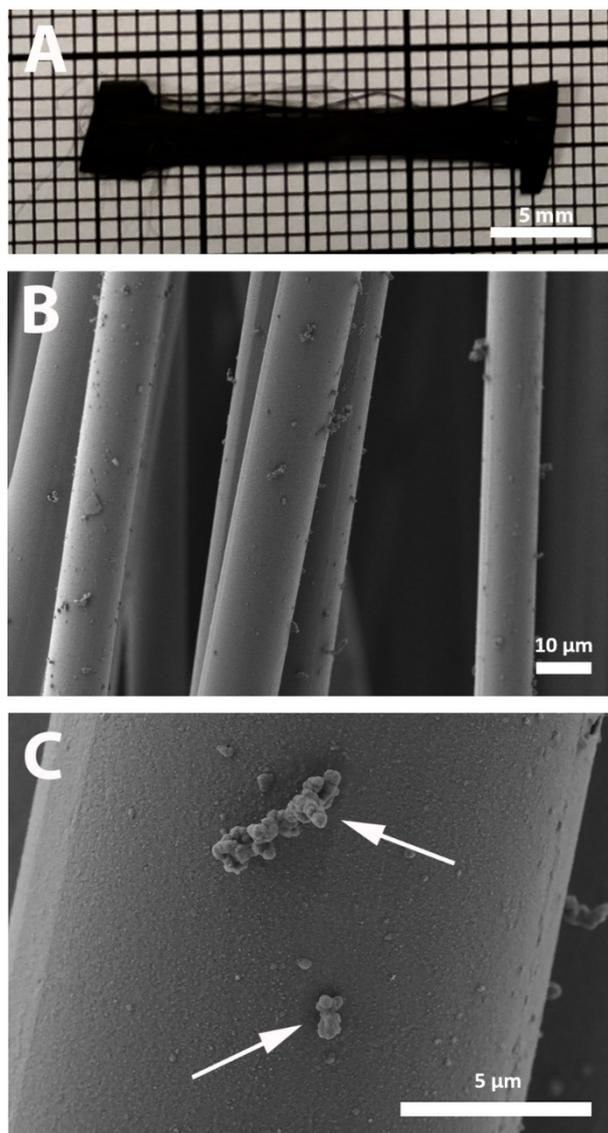


Figure 3. Macroscopic (A) and microscopic (B and C) images of PLA-PPy MF substrates used for the co-culture of SCs, eSCs and DRGs. PLA MFs (10 μm diameter) were arranged together with a rail shaped form using PCL bands at the extremes that acted as fasteners and coated with PPy via in situ polymerization. A homogeneous PPy coating can be observed, forming a fine grain texture. Just some loose aggregates (arrows in C) are present and most of them disappear with repeated washing. Scale bars = 5 mm (A), 10 μm (B) and 5 μm (C).

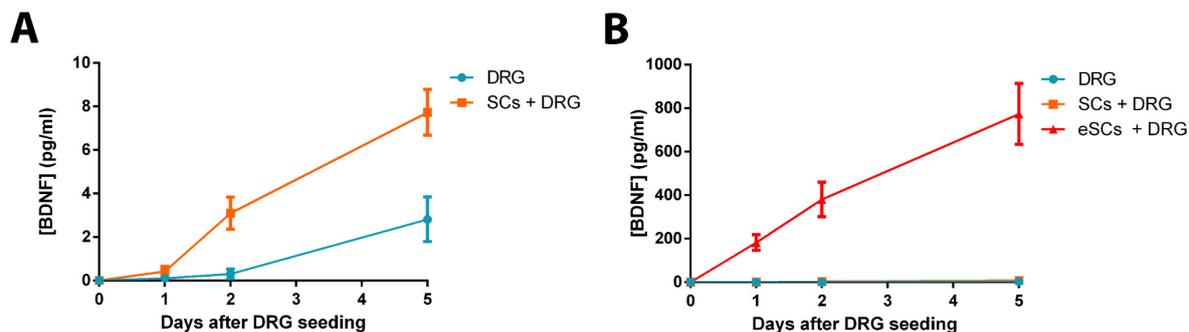


Figure 4. A: Magnified detail of B. B: Quantity of BDNF present in the culture medium after 1, 2 and 5 days of cell culture. As can be observed, the BDNF gene transfection of SCs increases a lot the quantity of BDNF protein that is secreted to the culture medium. Values are expressed as mean \pm SEM.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

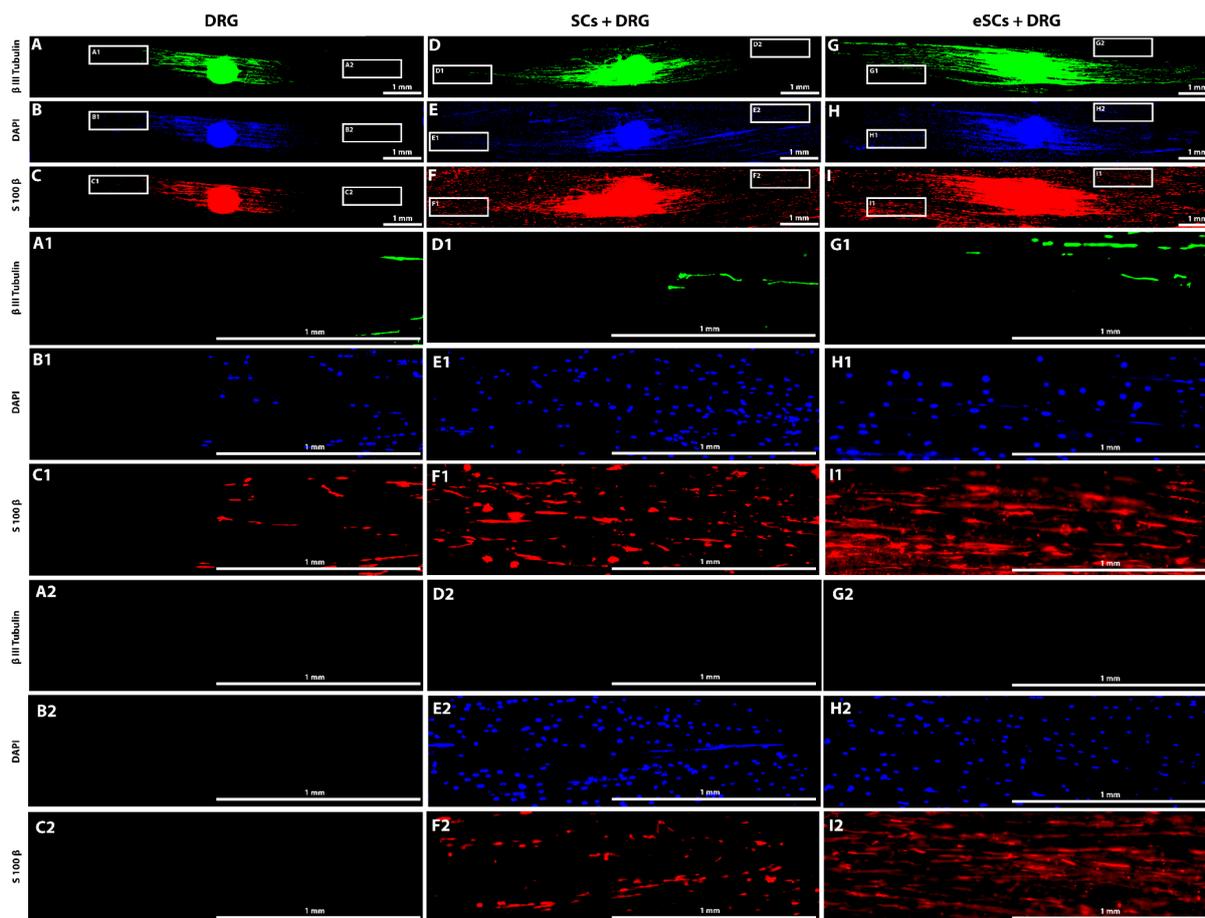


Figure 5. Fluorescent microscope images of samples after 5 days of DRG culture. Samples are marked with β III Tubulin to observe the neurons (green colour), DAPI to observe the cell nuclei (blue colour) and S 100 β to observe the Schwann cells (red colour). Firstly, a global image of one representative sample is presented for each studied group: DRG (A-C), SCs + DRG (D-F) and eSCs + DRG (G-I). As can be observed, there is a great increment of axonal extension and axonal sprouting when eSCs are used (G) when compared with the sample without SCs (A). For the sample with non-electroporated SCs (D) there is an intermediate behaviour. Therefore, the co-culture of the DRG with non-electroporated SCs increases axonal growth, but the use of eSCs increases this growth even more. The presence of pre-cultured SCs and eSCs is corroborated with image details at two different places of the sample: one at the end of the axons (A1-C1 for the DRG group, D1-F1 for the SCs + DRG group and G1-I1 for the eSCs + DRG group) and another far away from the axons (A2-C2 for the DRG group, D2-F2 for the SCs + DRG group and G2-I2 for the eSCs + DRG group). As can be observed, at the DRG group SCs are only present very near to the axons, indicating that the SCs are migrating from the DRG body along with the axons. However, for SCs + DRG and eSCs + DRG samples, the SCs are present far away from the axons, indicating that those cells are the pre-seeded ones, and not SCs that come from the DRG body. Scale bars = 1 mm.

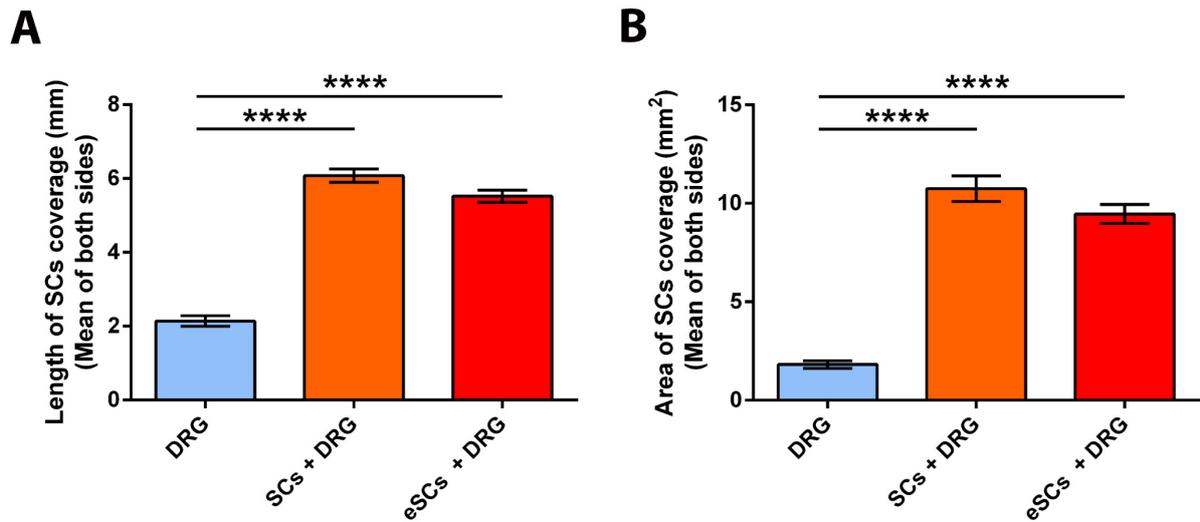


Figure 6. Length (A) and area (B) of the SCs coverage present on the substrate after 5 days of DRG culture. As can be observed, the presence of the pre-seeded SCs and eSCs is confirmed by the greater length and area covered by SCs on the substrate for the SCs + DRG and eSCs + DRG groups, respectively. Values are expressed as mean \pm SEM.

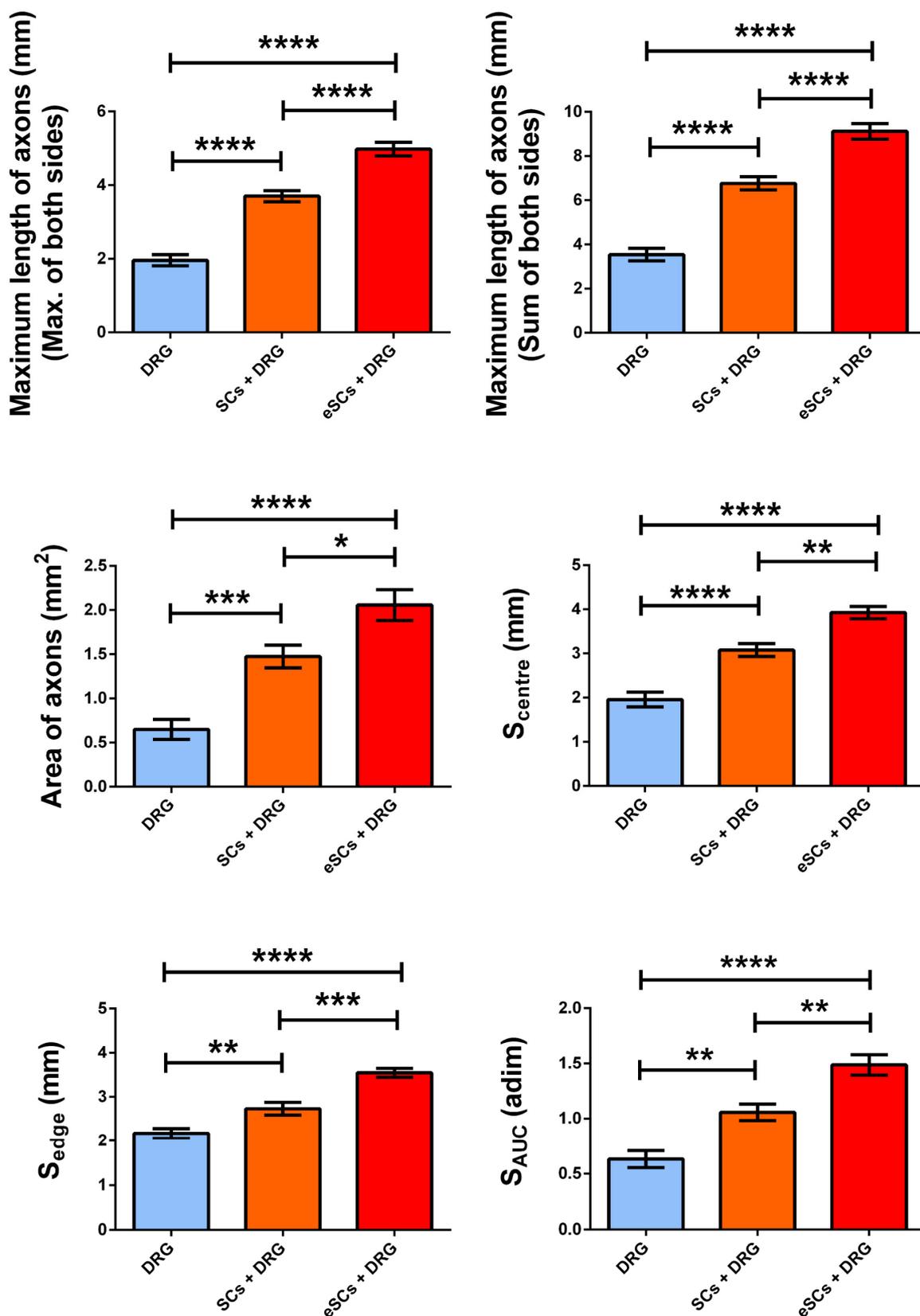


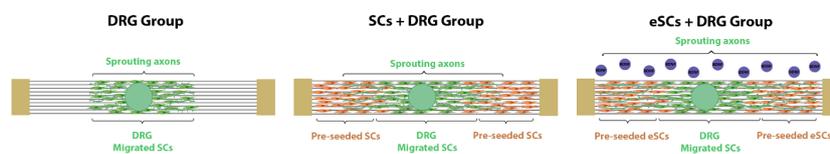
Figure 7. Quantification of axonal extension (maximum length of axons and area of axons) and axonal sprouting (S_{centre} , S_{edge} and S_{AUC}) after 5 days of DRG culture. The higher values of BDNF protein secreted by eSCs to the culture medium enhanced both axonal extension and axonal sprouting. Values are expressed as mean \pm SEM.

A new biomaterial platform that can be of interest for the treatment of injuries of the nervous system is developed. It combines the use of a substrate based on highly aligned microfibrils that guide the axonal growth with the use of gene-transfected Schwann cells with an increased secretion of the brain-derived neurotrophic factor (BDNF) protein that accelerates axonal growth.

Fernando Gisbert Roca, Franck M. André, Jorge Más Estellés, Manuel Monleón Pradas, Lluís M. Mir and Cristina Martínez-Ramos*

BDNF-gene transfected Schwann cells-assisted axonal extension and sprouting on new PLA-PPy microfibre substrates

ToC figure





Click here to access/download
Supporting Information
TOC.tif

