Combined Influence of Gelatin Fibre Topography and Growth Factors on Cultured Dorsal Root Ganglia Neurons

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Running title: effects of substrate and drugs on DRG neurons

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ABSTRACT:

Nerve guidance channels facilitate nerve regeneration and represent an attractive alternative to nerve graft. Actually, nano- and micro-structured biomaterials for nerve reconstruction have gained much attention, thanks to recent discoveries about topography effects on cell behaviour and morphology. Electrospun fibres have been proposed as filler or structural component for nerve guidance channels, principally due to their similarity with extracellular matrices which facilitate nerve regeneration. Among several tested biomaterials, gelatin has been used to prepare fibres able to support Schwann cell migration and neurite outgrowth. In this work, the effects of gelatin fibre size on axon elongation and Schwann cell migration have been tested using dorsal root ganglia cultures. Moreover, we analysed how fibres might affect the expression of specific neuronal subtype markers in sensory neuron cultures and how the combined effect of substrate and biological cues affects neurite growth and gene expression. Data show that fibre topography differentially affects both neurite outgrowth and gene expression and suggest that fibre size and topography associated to specific growth factor exposure might be used to select neuron subpopulations and favour the axonal growth of specific neurons.

KEYWORDS: gelatin fibres, electrospinning, nerve regeneration, neuronal subtypes, growth factors, NGF, BDNF, GDNF

INTRODUCTION:

Post-traumatic peripheral nerve injuries continue to be a major topic in reconstructive nerve microsurgery (Geuna et al., 2013). Over the last years many progresses have been made in surgical nerve reconstruction, but functional recovery following severe lesions is still unsatisfactory with very poor clinical results (Schlosshauer et al., 2006). In case of severe injury, when the nerve ends cannot be sutured together without tension, different approaches are used to restore nerve continuity and support nerve regeneration. The current gold standard for nerve reconstruction consists in autologous nerve graft, which provides mechanical support and a permissive and stimulating environment for nerve regeneration, since it contains Schwann Cells (SCs), basal lamina, neurotrophic factors and adhesion molecules (Lundborg, 2004, Gordon et al., 2011). Nevertheless, despite high functional recovery, this technique has some disadvantages such as donor site morbidity, autograft material availability and size mismatch problems (Battiston et al., 2009, Raimondo et al., 2011, Geuna et al., 2013).

Because of these multiple drawbacks, nerve microsurgery is oriented towards the introduction of biomimetic nerve guidance channels (NGCs), which provide mechanical support for SCs and regrowing axons. Hollow NGCs are successful in repairing short nerve gaps, but they do not support nerve regeneration over long distances. Thus, a second generation of NGCs were developed with a more complex structure, which may include wall modifications or the use of internal fillers that mimic the native nerve structure (Geuna et al., 2004, Tos et al., 2007, de Ruiter et al., 2009, Konofaos and Ver Halen, 2013, Dalamagkas et al., 2016). In particular, fillers include the insertion of nano/micro- fibres and hydrogels, or the supplement of cells, extracellular matrix components (i.e. laminin, fibronectin and collagen) or growth factors in

order to enhance nerve regeneration process inside NGCs (Bhardwaj and Kundu, 2010, Bacakova et al., 2011, Harvey et al., 2013, Rahmany and Van Dyke, 2013).

For NGC production, natural polymers are often preferred to synthetic ones because of their biocompatibility and the presence of bio-functional cues on their surface (Ciardelli and Chiono, 2006). Among them, gelatin (a natural polymer obtained by thermal denaturation of collagen), is biocompatible, biodegradable, does not induce immune-rejection problems and maintains on its surface molecular cues that play key role in regulating cell behaviour (Ciardelli and Chiono, 2006, Tonda-Turo et al., 2013). In a previous study, we prepared gelatin nano- and micro-metric fibres, cross-linked with γ-glicidoxypropyltrimethoxysilane (GPTMS), through the electrospinning technique (Tonda-Turo et al., 2013, Gnavi et al., 2015a, Gnavi et al., 2015b, 2016) and we demonstrated that fibres support the adhesion, proliferation and survival of several type of cells, such as primary rat Schwann cell, the Schwann cell line RT4-D6P2T, the sensory neuron-like cell line 50B11 and neonatal olfactory bulb ensheating cells.

Several studies demonstrated that micro-to-nano scale biomaterial topography may modulate SC migration and axon growth (Gupta et al., 2009, Xie et al., 2009, Gnavi et al., 2015a, Simitzi et al., 2017). Besides biomaterial topography, also chemotactic cues influence cell survival, motility, elongation and invasion. Therefore, chemotactic cues can be incorporated into NGCs to enhance the regeneration process (de Ruiter et al., 2009, Deumens et al., 2010). The main neurotrophic factors synthesized and secreted by SCs during the nerve regeneration process are nerve growth factor (NGF), glial-cell line derived neurotrophic factor (GDNF), brain derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) (Boyd and Gordon, 2003, Deumens et al., 2010, Richner et al., 2014),which promote different neural responses by the specific binding to cell surface receptors, such as low affinity receptor p75, Trk receptors (Trk-A, Trk-B and Trk-

C receptors) and cRET. The response to growth factors may vary among neurons depending on the receptors expressed by the different neuron subtypes. Indeed dorsal root ganglia (DRG) contain different neuron subtypes that can be classified on the basis of their function (nociceptive, mechanoreceptor, thermoreceptor, proprioceptive), cell-body diameter (small and large), neurotransmitter (peptidergic and non-peptidergic) and biomolecular markers expression. NGF promotes survival, differentiation and neurite outgrowth of sensory and sympathetic neurons and SC migration (Sofroniew et al., 2001, Sarkar et al., 2013, Richner et al., 2014). GDNF promotes both motor and sensory neuron survival and outgrowth (Oppenheim et al., 1995, Richner et al., 2014). BDNF supports motor neuron survival and promotes axonal growth of motor and sensory neurons (Oudega and Hagg, 1999, Richner et al., 2014, Harvey et al., 2015, Keefe et al., 2017). NT-3 supports survival, growth, and differentiation of neurons, and promotes the formation of neuronal synapses (Maisonpierre et al., 1990, Keefe et al., 2017).

In this study, we prepared random gelatin-GPTMS cross-linked electrospun fibres with a diameter of 300 nm or 1300 nm. Using neonatal DRG explants we studied *in vitro* the influence of micro- and nano- gelatin fibre topography on cell migration and axonal outgrowth. Combining fibre topography with chemotactic cues, we investigated the effects of gelatin fibre substrate and concomitant stimulation with growth factors on cell migration, axon elongation and neuron marker expression.

MATERIAL AND METHODS:

Fibre preparation

Gelatin type A (from porcine skin) and GPTMS were supplied by Sigma-Aldrich.

Random cross-linked gelatin fibres of 300 nm and 1300 nm diameter were prepared as previously described (Tonda-Turo et al., 2013, Gnavi et al., 2015a). Briefly, gelatin was dissolved in demineralised water at 50°C to obtain the desired concentration (15% or 20% w/v) and 137 µl or 183 µl of GPTMS were added to the solution, respectively. Then, solution was mixed for 1 hour before spinning (GL-GPTMS). The electrospinning system used for fibre preparation consists of an isothermal chamber equipped with: a high voltage generator (PS/EL30R0 1.5-22 Glassman High Voltage) providing a voltage of 0 to 30 kV and a current of 0 to 1.5 mA with reversible polarity; a volumetric pump (KDS210, KD Scientific); an electrode; a mobile syringe support; a syringe and a 1.5 mm thick flat aluminium collector. Electrospun scaffolds were prepared using a vertical electrospinning set-up and a 1.5 mm-thick flat fixed aluminium collector. 15% w/v GL-GPTMS solution was spun at flow rate 10 µl min^{-1} to yield fibres of 300 nm diameter (F300) while the 20% w/v GL-GPTMS solution was spun at flow rate 15 µl min^{-1} to obtain fibres of 1300 nm diameter (F1300). Both solutions were spun at 50°C, 30 kV, and nozzle-collector distance 15 cm. Before cells seeding, fibre samples were sterilized by overnight (O/N) exposure to UV irradiation (UV lamp Technoscientific Co., wavelength 254 nm) and then incubated O/N in the culture medium.

Animals

All procedures were approved by the Bioethics Committee of the University of Torino, by the Institutional Animal Care and Use Committee of the University of Torino, and by the Italian

Ministry of Health, in accordance with the European Communities Council Directive (2010/63/EU), the National Institutes of Health guidelines and the Italian Law for Care and Use of Experimental Animals (DL26/14).

Dissociated DRG preparation

DRG were harvested from P1-P3 Wistar rat pups. Briefly, after animal decapitation, the skin on the back was removed to reach the vertebral canal, the canal was exposed and then longitudinally cut, proceeding from the neck to the tail; the spinal cord was removed and DRG were easily collected from intervertebral foramina. During the harvesting phase DRG were stored in Hank's balanced salt solution (HBSS, w/o Mg and Ca, PAA Laboratories GmbH). Enzymatic dissociation was performed incubating DRG at 37°C for 20 min in HBSS with 0.125% trypsin (Sigma-Aldrich) and 0.05% DNase (Roche), followed by a further incubation at 37°C for 20 min with the addition of 0.06% of collagenase type IV (Sigma-Aldrich). The reaction was stopped adding N2 medium (Dulbecco's Modified Eagle's Medium [DMEM] F-12, 1% pen/strep, 0.25% bovine serum albumin, 2 mM L-glutamine, 1 mM sodium pyruvate, 1× N2 supplement; all provided by Sigma-Aldrich) with 3% fetal bovine serum (FBS, Gibco). Then, DRG were mechanically dissociated through fire-polished glass pipette. Obtained neurons were counted at light microscopy and seeded on the different biomaterials.

Dissociated DRG culture

Dissociated DRG were used for *in vitro* analysis. Glass coverslips were coated with poly-L-lysine (PLL, P1275, Sigma-Aldrich) or with gelatin fibres and placed in 24 multiwells plate. Each coverslip was then covered, before cell seeding, with a layer of NVR gel (Neural and Vascular Reconstruction) (Shahar et al., 2011), a hydrogel composed mainly by laminin and

ialuronic acid, compatible with neuronal growth, diluted 1:2 in N2 medium. For neurite outgrowth assay, a drop of 5000 neurons was seeded on NVR-gel layer, and left to grow for 24 hours, as previously described (Morano et al., 2014). NVR gel avoids collapsing of DRG drops and it acts as vehicle for growth factor delivery as it might be used for *in vivo* application.

Nevertheless, since NVR gel negatively affects RNA extraction efficiency, for gene expression analysis dissociated DRG were homogeneously seeded directly on the differently coated-coverslips (5000 cells/well) and left to grow for 24 hours in N2 medium alone or with different factors.

Neurotrophic factors added to cell medium or to NVR-gel (factors were mixed with N2 medium during gel preparation) are: NGF (Millipore); NT-3 (Abcam); BDNF (Peprotech); GDNF (Peprotech). Each factor was used at a final concentration of 50 ng/ml. A mix of BDNF, NGF, NT3 (25 ng/ml each, MIX) was also tested.

Immunofluorescence

After 24 hours incubation, dissociated DRG were fixed in 4% paraformaldehyde (PFA, Sigma-Aldrich) for 20 min at room temperature. Samples were incubated in blocking solution (normal goat serum, DAKO X0907, diluted 1:100 in PBS containing 0.1% Triton X-100) for 1 hour at room temperature. Cells were stained by O/N incubation with the following primary antibodies: anti-mouse β-tubulin monoclonal antibody (diluted 1:100, in PBS, Sigma-Aldrich) and anti-rabbit S-100β polyclonal antibody (diluted 1:600 in PBS, Sigma-Aldrich). Goat-anti-mouse IgG (H+L) AlexaFluor488 (diluted 1:200 in PBS, Invitrogen) and goat-anti-rabbit IgG (H+L) Cy3 (diluted 1:200 in PBS, Jackson) secondary antibody were incubated for 1 hour at room

temperature. Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) diluted 1:1000 in PBS.

DRG culture image processing

The image processing analysis of immunofluorescence data was performed using *ImageJ* software. For each DRG drop, the following parameters were measured: i) number of SCs; ii) neurite number; iii) total area occupied by neurites; iv) neurite length. The SC number was manually evaluated by counting the number of S100β positive cells around the DRG explant-like body. The analysis of all the other parameters was performed using *Neurite-J*, an *ImageJ* plug-in developed for semi-automatic axonal growth quantification in organotypic culture, based on the Sholl method (Torres-Espin et al., 2014), following the producer's instructions. The number of neurites corresponds to the Nmax value given by the program, which represents the number of intersection observed at a distance of 25 μm from the drop area. Total area occupied by neurites was given by the sum of values automatically calculated for different distances from the drop zone. The neurite length was calculated as mean of the 10 maximum neurite distance reached by the axons sprouting from the DRG culture drop body (intersections of the neurites with a series of circles automatically traced at the distance of 25 μm). All values are expressed as means ± SEM (standard error of the mean).

RNA isolation, cDNA preparation and quantitative real-time PCR (qRT-PCR) analysis

Total RNA was extracted from dissociated DRG cultures with TRizol (Invitrogen) following the manufacturer's instructions. For each sample 1 μg total RNA was reverse transcripted (RT) in 25 μl containing: 1× RT-Buffer, 7.5 μM random primers, 0.5 mM dNTPs, 40U RNase Out Inhibitor and 200U RevertAid Reverse Transcriptase (Fermentas). The reaction protocol is the following:

10 min at 25°C, 1 hour at 42°C, 10 min at 70°C. The cDNA was diluted 1:10 with water and stored at -20°C.

qRT-PCR analysis was performed using a StepONE detection system (Applied Biosystems). The reaction was carried out in 10 µl, containing 1 µl diluted cDNA (corresponding to 10 ng starting RNA), 1x Taq Universal SYBR Green Mix (Applied Biosystems) and 900 nM forward and reverse primers. The reaction was performed at 95°C for 10 min, 40 cycles of denaturation at 95°C for 15 sec followed by primer annealing and elongation at 60°C for one minute. Dissociation curve was analysed to check the accuracy of the amplification. Data were analysed using the ΔΔCt relative quantification method (Ronchi et al., 2016) and TATA-box Binding Protein (TBP) and ubiquitin (UBC) genes were used as housekeeping genes. Primers were designed using Annhyb software and produced by Invitrogen; primer sequences are reported in Table 1.

Statistical methods

A biological triplicates were performed for in vitro experiments of cultured DRG on NVR-gel and for expression analysis. All data are reported as means ± SEM.

The statistical analysis was carried out using *GraphPad Prism* Software. Student T-test or Two-way ANOVA analysis were used, as specified in figure legends. Two-ways ANOVA analysis consider both substrate and drug variables.

RESULTS:

The substrate influences both neurite growth and neuron marker expression

We investigated *in vitro* the influence of gelatin fibre size on neuron culture. Gelatin fibres of two different diameters were tested: nanometric (F300) and micrometric (F1300) fibres. Dissociated neonatal DRG cells were seeded for 24 hours on PLL-coated coverslips (control condition) or on gelatin fibres in a layer of hydrogel, the NVR-gel, suitable for neuron survival and neurite outgrowth. Neurite outgrowth was analysed by immunocytochemistry, to quantify the number and the length of the grown neurites (Fig. 1A, B). The neurite number is higher when cells are seeded on nanometric fibres compared to control condition, while the length of the grown neurites results shorter (Fig. 1A). However, the total area occupied by neurites does not vary among the groups. The number of positive cells for S100β, a specific marker for SCs, was also assessed, as an index of cells migrated from the DRG drop to the surrounding environment. Fibre substrates do not affect cell migration (Fig. 1A). No statistical relevant differences were detected between the two tested types of fibres for all investigated parameters.

Moreover, we investigated the expression of several neuronal markers in DRG cells seeded on nanometric and micrometric gelatin fibres or on PLL-coated coverslips. DRG were dissociated and cells were seeded on the selected biomaterial for 24 hours. For this experiment we did not use NVR gel, which interferes with RNA extraction, thus we observed the effect given only by gelatin fibres. Through qRT-PCR, we checked the expression level of the following receptors, markers for DRG neuron subpopulations: GLUR5 (non-peptidergic, nociceptor), TRKA

(peptidergic, nociceptor), TRKB (mechanoreceptors), TRKC (proprioceptors), cRET (mechanoreceptors, non-peptidergic nociceptor), and p75 (low-affinity nerve growth factor receptor) (Table 2). Among all investigated receptors, only TRKA and TRKB expression is significantly influenced by the type of substrate (Fig. 2). In particular, TRKA expression is reduced in dissociated DRG seeded on micrometric substrate, while TRKB expression is higher in cells seeded on nanometric fibres.

Double effects of drug and substrate in neuron culture

With the aim to promote nerve regeneration, we evaluated the combined effect of substrate and growth factor administration (Fig. 3). We investigated the effects of several growth factors, used either alone (NGF, BDNF, GDNF and NT-3) or in combination (a mixture of BDNF, NGF and NT-3, that has been named MIX). The effect of these growth factors has been tested in combination with fibre topography features (random nano- or micro-fibres). The expression of different genes, including growth factor receptors, has been evaluated. The NVR gel was easily mixed with growth factors before culture preparation. We analysed neurite number, neurite length, neurite area and migrated cells in DRG culture exposed to growth factors for 24 hours. The combination of substrate and growth factor does not influence the number of migrated cells in all the evaluated cases (see Supplementary, S1). Regarding neurite outgrowth, we did not find differences between the two types of fibres (Fig. 3). Curiously, depending on PLL or gelatin substrate the effect of growth factor administration is different (Fig. 3 and S1). In the control group, NGF treatment corresponds to an increase of neurite area, but this effect is not visible when cells are seeded on gelatin fibres. After BDNF treatment, the neurite length and the neurite area of DRG seeded on micrometric fibres are lower in comparison to control group. Moreover, in GDNF treatment condition the differences between control and fibre groups are extremely

evident: we found that GDNF combined with fibre substrate corresponds to lower neurite number, shorter neurites and lower value for neurite area as compared with GDNF combined with PLL substrate. After NT-3 treatment, the neurite area results lower in DRG seeded on nanometric fibres compared to control condition. The comparison among substrates showed that, after MIX administration, neurite length and neurite area values are lower in gelatin fibre groups in comparison to PLL group.

The effect of growth factor administration on neuronal marker expression was also investigated (Fig. 4, and S2) thus demonstrating that TRKA, TRKC, cRET and p75 are not influenced by the combination of substrate and growth factor administration. On the other hand, the expression of GLUR5 and TRKB is differently modulated depending on the kind of substrate and on which growth factor was administered. In particular, GLUR5 expression increases in DRG seeded on fibre substrates and treated with NGF, BDNF, NT-3 and MIX. TRKB expression is higher in F1300 in comparison to the control group, after BDNF treatment; also, NT-3 treatment determines an increase of TRKB mRNA levels in fibre groups in comparison to control condition.

DISCUSSION:

It is now extremely clear that surface topography, as well as other physical cues, influences the morphology, the behaviour and the growth of neurons and glial cells (Simitzi et al., 2017). Thus, exploring the effect of different substrates on neuronal cultures proved to be necessary to choose

the biomaterial arrangement that better supports the nerve regeneration inside a nerve guidance channel (de Ruiter et al., 2009). Fibre structures represent suitable fillers for nerve scaffolds, thank to their architecture that mimics the native extracellular matrix. Furthermore, during their production, it is possible to control several parameters, such as fibre diameter, porosity and mechanical properties which allow to regulate the cell-material interaction (Bhardwaj and Kundu, 2010, Rahmany and Van Dyke, 2013, Skoog et al., 2017).

We previously demonstrated that gelatin fibres of 300 nm or 1300 nm of diameter support SC survival and neuronal growth (Gnavi et al., 2015a), indicating that gelatin is a suitable biomaterial for nerve application as also observed by other authors (Liu et al., 2004, Jiang et al., 2010, Kohn-Polster et al., 2017, Tao et al., 2017). The aim of this study is to investigate the applicability of gelatin fibres combined with hydrogel for growth factor delivery, as filler for nerve conduits. NVR hydrogel has been shown to be suitable for nerve culture and optimal for growth factor inclusion and release (Morano et al., 2014). The neurite outgrowth is visible both on nanometric and micrometric fibres; however, on nanometric fibres neurites are perceptively shorter, in agreement with our previous data (Gnavi et al., 2015a). Moreover, a higher number of neurites has been observed in cells seeded on gelatin nanofibres suggesting that these fibres promote axon sprouting rather than axon elongation. The number of migrated SCs is similar for control group and fibrous substrates, in contrast with our previous results which indicate a superior migration rate and cell motility on micrometric fibres (F1300) in comparison to nanometric fibres (F300) (Gnavi et al., 2015a). However, the differences might depend on the used culture protocol or cell model. In the previous published articles, we used adult DRG explants, primary SC culture from median nerve or SC lines (i.e. RT4-D6P2T).

A gene expression analysis focused on specific neuronal population markers was performed. In literature, several data are available about topography influence on SC behaviour and morphology and neurite outgrowth (Wang et al., 2010, Ren et al., 2013, Simitzi et al., 2017), while the related gene expression is less investigated. However, for other cell types it was reported that the topography induces a gene expression shift, crucial for stimulating cell differentiation or cell specific behaviour (Mendonca et al., 2009, Gasiorowski et al., 2010). Here, we seeded dissociated DRG on gelatin fibres and we observed a change in TRKA and TRKB expression, with differences between the two types of investigated fibres. These two receptors were found to be down-regulated in DRG after nerve injury (Richner et al., 2014). In this regard, the up-regulation of TRKB observed in cells seeded on nanometric fibres may be encouraging, indicating a rescue of the down-regulation observed *in vivo* after axotomy. However, since these two receptors, as the other analysed receptors, are used to identify different neuron populations, we can postulate that the substrate might selectively promote the survival of specific neuron population: nanometric fibres might facilitate the attachment and the growth of TRKB positive neurons (mechanoreceptors neurons), like large type A neurons, while micrometric fibres might not support the survival of neurons expressing TRKA, such as nociceptive small dark type B neurons. Anyway, we have to consider that also satellite glial cells express TRKA and their survival could be also influenced by the type of material used for the culture.

Additionally, the introduction of biochemical cues on biomaterial surface is a successful way to efficiently induce specific cell behaviour such as cell survival, cell differentiation or cell growth (Li and Folch, 2005, Krick et al., 2011). The stimulation with growth factors combined with fibre substrate has demonstrated its efficacy for different biological processes, among which nerve regeneration (Bruggeman et al., 2017, Olvera et al., 2017), and specific growth factors might be

used to guide the axonal growth of specific neuron population. Here, we tested individually several growth factors, whose receptors are expressed in different sensory neuron populations. We also tested a mixture of three "classic" neurotrophins NGF, BDNF and NT-3 (MIX condition). Taking advantage of the NVR gel we administrated the growth factors to DRG cultures and we analysed the neurite outgrowth. Curiously, the combination of fibres with GDNF led to poor neurite growth, as demonstrated by the low number and shorter neurites and a small occupied area detected in comparison to the control group. It is well known that GDNF sustains motor neuron survival and growth (Boyd and Gordon, 2003), however, it has been recently demonstrated that both low and high doses of GDNF are able to induce neurite outgrowth in cultured rat DRG (Santos et al., 2016). In cultured neurons, the expression of cRET is not changed in cells seeded on fibres, thus we can exclude that the absence of a GDNF-based effect on fibres is due to a reduced number of GDNF-responsive neurons. A possibility might be the interaction between the biomaterial and the factor itself, which influences the GDNF availability for the DRG cultures. However, a gelatin-based hydrogel mixed with GDNF has been already used for sub ventricular zone injection, where it has induced neurite outgrowth at the interface with tissue and matrix (Fon et al., 2014).

Moreover, while NGF treatment corresponds to an increase of neurite area in neurons seeded on control substrate, on micrometric fibres this effect is not visible. This result might be due to a decrease of axonal sprouting on fibres, but we cannot exclude a reduction of NGF-responsive neurons, as suggested by the lower expression level of TRKA in neurons on micrometric fibres (not influenced by NGF treatment). However, also for nanometric fibres we observed a reduction of neurite area after NGF treatment, without a reduction of TRKA expression.

BDNF treatment combined with micrometric fibres results in shorter neurites and reduced neurite area. Interestingly, we observed that in the same condition the expression level of TRKB is higher in comparison to control (neurons seeded on PLL and treated with BDNF) suggesting that BDNF stimulates the expression of its receptor, but this does not correspond to an increased neurite growth. In DRG seeded on nanometric fibres we found high TRKB expression levels. However, the treatment with BDNF does not correspond to higher neurite growth compared to control condition; concomitantly, we observed a reduction of TRKB mRNA quantity. BDNF might have a distinct effect in stimulating neurite growth depending on the type of neuron population, in fact this was observed for BDNF ability to induce myelination, demonstrating that this ligand has a positive effect on NGF-dependent neurons and an inhibitory effect on TRKB positive neurons (Xiao et al., 2009).

The mixture of BDNF, NGF and NT-3 did not reveal a greater effect in comparison to the single factors and it seems inhibiting neurite elongation and sometimes also neurite sprouting in DRG seeded on gelatin fibres. Thus, other combinations need to be tested to find the mix of factors that effectively stimulates sensory neuron outgrowth.

Overall, data suggest that micrometric fibres are preferable compared to nanometric fibres for nerve application, since micrometric fibres do not limit neurite length and reduce the expression of TRKA, associated to nociceptive neurons. Additional experiments are necessary to understand how the fibre type influences gene expression or acts as a positive or negative selector of neuron subpopulations. Future experiments will be focused on the analysis of axon elongation induced by fibre substrate on the different subpopulations of sensory neurons. These data are particularly relevant and useful, especially to determine how gelatin fibres are a suitable materials for nerve repair, able to avoid an overstimulation of nociceptive neuron growth and to promote motor,

mechanical and proprioceptive neuron growth. In this regard, in the future approaches, motor neuron growth on gelatin fibres after growth factor stimulation will also be tested.

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FIGURE LEGENDS

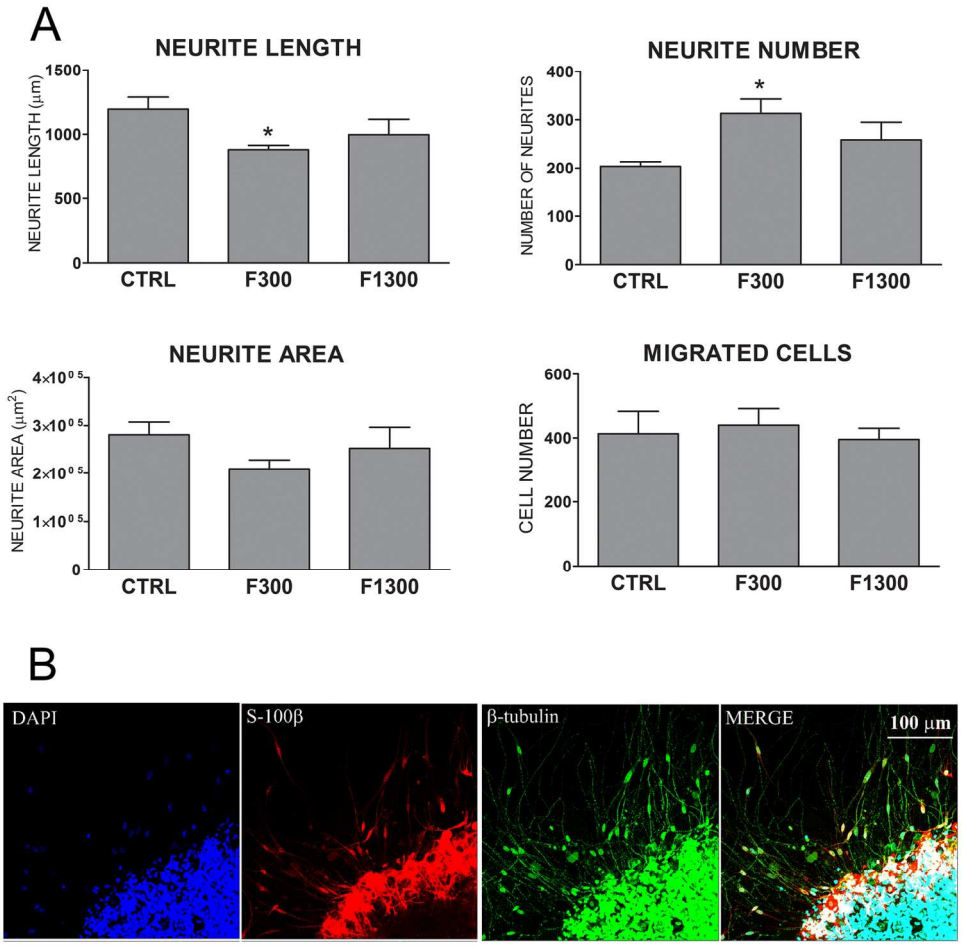
Fig 1. Effects of different substrates on neurite outgrowth. A. The diagrams report the data obtained in a biological triplicate experiment in which adult dissociated DRG were seeded on PLL-coated coverslips (control condition, CTRL) or nanometric (F300) or micrometric (F1300) fibre-coated coverslips in a layer of NVR hydrogel for 24 hours. Values are presented as means ± SEM. Statistical analysis: Student T-test versus control group: * $p \leq 0.05$ **B.** Representative pictures of cultured dissociated DRG.

Fig 2. The substrate affects neuron subpopulation marker expression in DRG cultures. The graphs show the expression levels of six known markers for neuron subpopulations present in rat DRG. Dissociated DRG were cultured on PLL-coated (control condition, CTRL) or nanometric

(F300) or micrometric (F1300) fibres-coated coverslips for 24 hours, then the expression of neuron markers was quantified through qRT-PCR. Value are presented as means ± SEM. Statistical analysis: Student T-test versus control group: $ $p \leq 0.05$; Student T-test between fibre groups: * $p \leq 0.05$.

Fig 3. Neurite outgrowth in DRG culture is influenced both by substrate and treatment. The graphs show the results of biological triplicate analysis of several parameters regarding neurite outgrowth. Dissociated rat DRG were seeded on PLL (CTRL) or nanometric (F300) or micrometric (F1300) gelatin fibres and in a layer of NVR gel mixed with several growth factors. Neurite length, neurite number and the area occupied by neurite were calculated for each condition. Value are presented as means ± SEM. UNT= untreated (without growth factor stimulation). Statistical analysis: Two Ways Anova plus Bonferroni Post Hoc Test: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

Fig 4. The combination of substrate and drug differently affects neuron marker expression. The expression of several neuron markers was investigated through qRT-PCR. The graphs report the results of a biological triplicate, in which dissociated DRG were cultured on PLL-coated (control condition, CTRL) or nanometric (F300) or micrometric (F1300) fibre-coated coverslips and stimulated for 24 hours with different growth factors. The black line at zero value corresponds to the control expression value observed in DRG cultured on PLL-coated coverslips without factor stimulation. TRKB and GLUR5 expression is influenced both by substrate and growth factor used. Values are presented as means ± SEM. Statistical analysis: Two Ways Anova plus Bonferroni Post Hoc Test: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.



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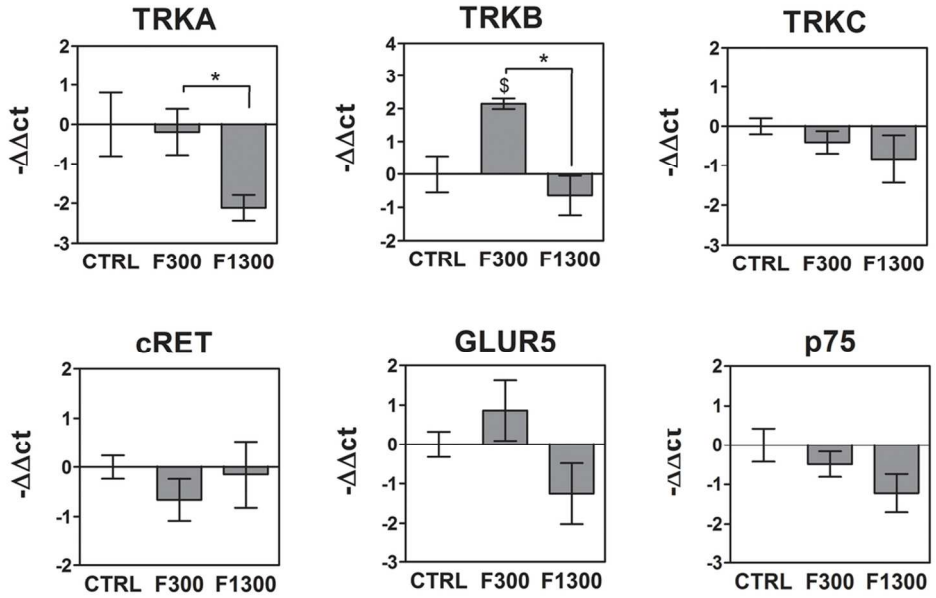

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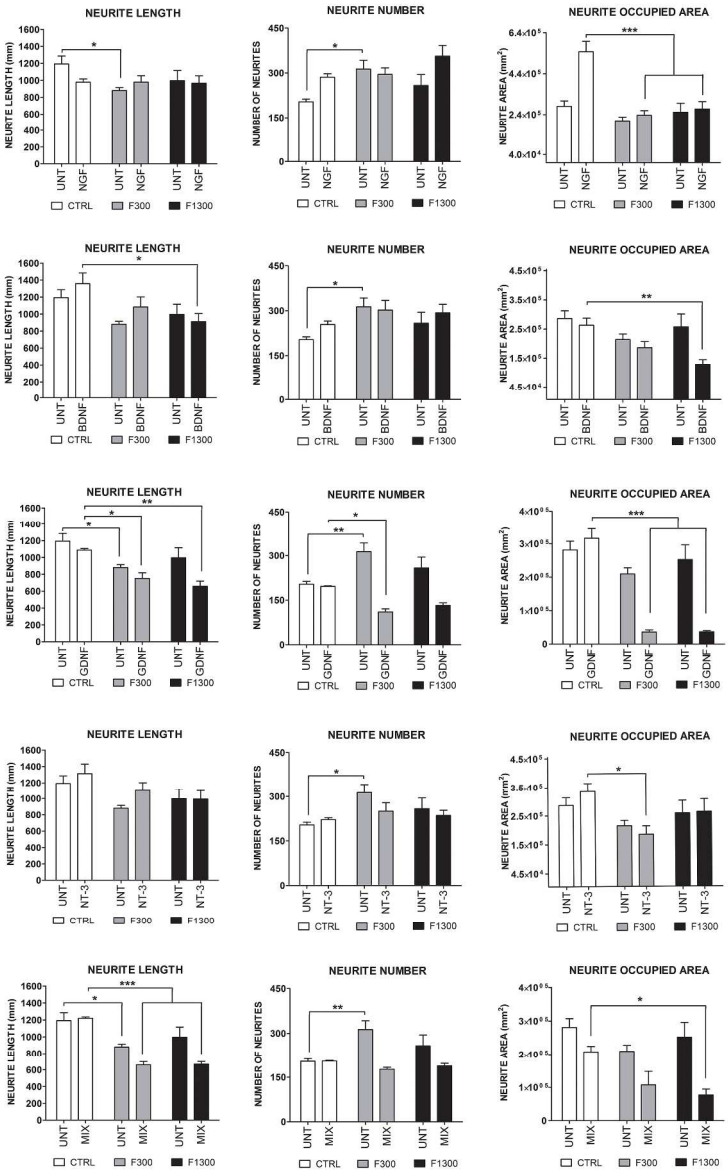
100x65mm (300 x 300 DPI)



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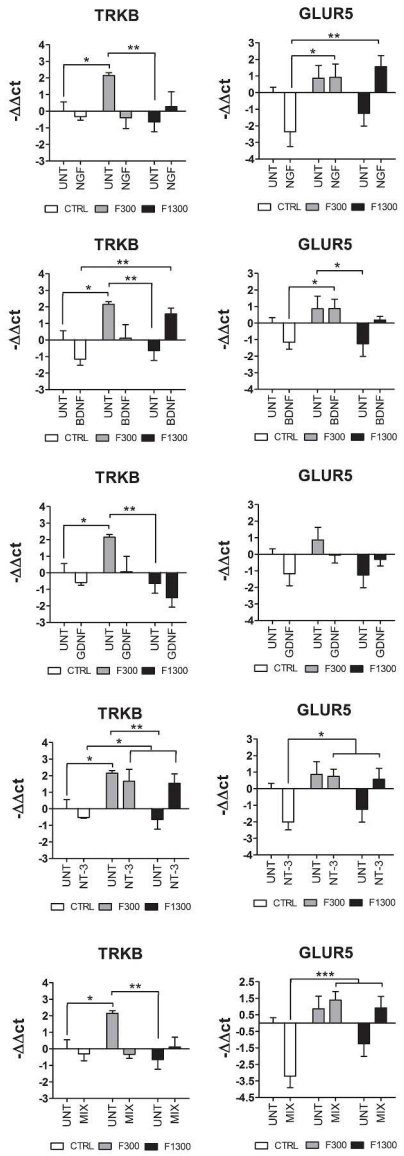
101x158mm (600 x 600 DPI)



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129x354mm (600 x 600 DPI)

GENE	FORWARD PRIMER	REVERSE PRIMER
TBP	5′-TAAGGCTGGAAGGCCTTGTG-3′	5′-TCCAGGAAATAATTCTGGCTCATAG-3′
UBC	5′-TCGTACCTTTCTCACCACAGTATCTAG-3′	5′-GAAAACTAAGACACCTCCCCATCA-3′
c-RET	5′-CCATGCACAATTACAAGCTG-3′	5′-CATTGACCAGGACTACTAGC-3′
GLUR5	5′-GAATGACAAAGGGGAATGGA-3′	5′-ATGCTTTCTCCCGTACGTA-3′
TRK-A	5′-CCCTCTGGCGAACCTGCAG-3′	5′-CCCTTCCTCCACCGTGAGG-3′
TRK-B	5′-CGTGGCATTTCCGAGGTTGG-3′	5′-AGCCCCACGTAAGCTTCGAC-3′
TRK-C	5′-AGCCCCACGTAAGCTTCGAC-3′	5′-GCATTGAGTGTGTGCAGGCC-3′
p75	5′-AGCAGACCCATACGCAGACTG-3′	5′-TCTCTACCTCCTCACGCTTGG-3′

Table 1. Primer sequences

42x12mm (600 x 600 DPI)

MOLECULAR MARKER	RECEPTOR	LIGAND	FIBRE TYPES
cRET	RA-mechanoceptor non-peptidergic nociceptor	GDNF	Aβ, C
GLUR5	Non-peptidergic nociceptor	Glutamate	C
TrkA	Peptidergic nociceptor	NGF	Aδ, C
TrkB	SA-mechanoceptor RA-mechanoceptor	BDNF	Aβ
TrkC	Proprioceptor	NT-3	Aβ
p-75	Low-affinity nerve growth factor receptor	All neutrophins	All types

RA: rapidly adapting
SA: slowly adapting

Table 2: markers for DRG neuron subpopulations.

128x130mm (600 x 600 DPI)