Full title:

Motor and Sensory Schwann Cell Phenotype Commitment is Diminished by Extracorporeal Shockwave Treatment *in vitro*

Running title:

ESWT Reduces Schwann Cell Phenotype Commitment

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Abstract

Background and aim: The gold standard for peripheral nerve regeneration uses a sensory autograft to bridge a motor/sensory defect site. For motor nerves to regenerate, Schwann cells (SC) myelinate the newly grown axon. Sensory SCs have a reduced ability to produce myelin, partially explaining low success rates of autografts. This issue is masked in pre-clinical research by the excessive use of the rat sciatic nerve defect model, utilizing a mixed nerve with motor and sensory SCs. Aim of this study was to utilize extracorporeal shockwave treatment as a novel tool to influence SC phenotype.

Methods: SCs were isolated from motor, sensory and mixed rat nerves and *in vitro* differences between them were assessed concerning initial cell number, proliferation rate, neurite outgrowth as well as ability to express myelin.

Results: We verified the inferior capacity of sensory SCs to promote neurite outgrowth and express myelin-associated proteins. Motor Schwann cells demonstrated low proliferation rates, but strongly reacted to pro-myelination stimuli. It is noteworthy for pre-clinical research that sciatic SCs are a strongly mixed culture, not representing one or the other. Extracorporeal shockwave treatment (ESWT), induced in motor SCs an increased proliferation profile, while sensory SCs gained the ability to promote neurite outgrowth and express myelin-associated markers.

Interpretation: We demonstrate a strong phenotype commitment of sciatic, motor and sensory SCs *in vitro*, proposing the experimental use of SCs from pure cultures to better mimic clinical situations. Furthermore we provide arguments for using ESWT on autografts to improve the regenerative capacity of sensory SCs.

Key words: Schwann cells; Schwann cell phenotype; Extracorporeal Shockwave Treatment; Peripheral Nerve Regeneration;

Introduction

Peripheral nerve lesions are a frequent cause of hospitalization, as they occur with an incidence of approximately 300.000 cases annually in Europe and thus represent a major burden to patients and health-care systems[1].

The peripheral nervous system has been shown to have a remarkable potential to regenerate. One of the key players involved, and at the same time a limiting factor for the regeneration of long distance gaps[2], are Schwann cells. In the uninjured nerve, Schwann cells are closely associated with axons, either myelinating axons, enabling saltatory conduction[3] or providing trophic support for axons as so called "Remak cells"[4,5]. However, after nerve injury, Schwann cells undergo a phenotype change, recently described as transformation into "Büngner cells"[6]. In the Büngner cell state, Schwann cells facilitate the removal of myelin axonal remnants by recruitment of macrophages and myelinophagy[7,8]. Also, they proliferate and align to build the bands of Büngner[9], which act as guiding structures for the regenerating axon. After the axon has elongated along the bands of Büngner, Schwann cells complete the regeneration process by remyelinating the newly formed axons[6]. However, Schwann cells display a limited proliferative capacity. A reason for unsatisfying axonal regeneration in long-distance gaps is the reduced capacity of Schwann cells to form full-length bands of Büngner, and therefore fail to guide the outgrowing axon[2]. Several studies have shown reduced capacity of supporting axonal regeneration by long-term denervated Schwann cells due to atrophy[10], down-regulation of key factors such as neuregulin-1[10,11] and other neurotrophic factors[12,13]. Furthermore, Gordon et al. report a significant decline in number of non-neuronal cells in long-term denervated nerve stumps in the rat[14]. Although regeneration has been reported after

time periods of up to 26 months, successful regeneration and meaningful recovery of function is strongly impaired with increasing delay of repair[15].

The gold standard in the clinics to treat peripheral nerve injuries involving segmental tissue loss is nerve autografting, where a sensory nerve (e.g. sural nerve) is harvested and transplanted to the defect site. However, especially in long distance gaps the functional outcome is often not satisfactory[16].

It has been described that the phenotype of the graft – motor or sensory- influences the functional outcome[17,18], meaning that motor axons favor a motor nerve environment. Since transplantation of motor nerves is rarely feasible, novel strategies are sought to improve sensory nerve grafts, e.g. by pushing phenotypical commitment of sensory Schwann cells into motor-like Schwann cells to improve their regenerative capacity.

One strategy that has become increasingly popular in regenerative medicine over the last years is extracorporeal shockwave treatment (ESWT). ESWT has its origin in the field of urology for the destruction of renal stones[19], however, in clinics and pre-clinics, beneficial effects have been reported in treatment of various medical conditions such as non-union fractures[20–22], ischemia-induced tissue necrosis[23], or chronic wounds[24,25]. Shockwaves are sonic pulses, characterized by an initial rise in pressure, reaching a positive peak of up to 100 MPa within 10 ns, followed by a negative amplitude of up to - 10 MPa and a total life cycle of less than 10 µs. Biological responses are thought to be triggered by the high initial pressure, followed by subsequent tensile force and the resulting mechanical stimulation[26].

ESWT has been successfully used to improve axonal regeneration after autologous nerve grafting in a pre-clinical sciatic nerve defect model[27]. Based on these findings we investigated

the behavior of sciatic Schwann cells after ESWT treatment in a previous *in vitro* study and found alterations in proliferation as well as in expression of Büngner-cell associated markers (e.g. p75 or c-Jun), among others[28]. However, both the sciatic nerve defect model as well as sciatic-nerve derived Schwann cells do not reflect the clinical situation in terms of motor and sensory graft mismatch. In order to improve the translatability of our previous research, the aim of this study was to assess differences between Schwann cells isolated from the sciatic nerve (motor and sensory mixed nerve)[28], the saphenous nerve (purely sensory) and the femoral motor nerve (afferent and efferent fibers from/to the quadriceps muscle) in the presence and absence of ESWT.

Materials and Methods

Unless indicated otherwise, all reagents were purchased from Sigma Aldrich and of analytical grade.

Shockwave treatment of nerve tissue and Schwann cell isolation

All animals were euthanized according to established protocols, which were approved by the City Government of Vienna, Austria in accordance with the Austrian Law and Guide for the Care and Use of Laboratory Animals as defined by the National Institute of Health.

For *ex vivo* shockwave treatment an unfocused electro-hydraulic device was used (Dermagold 100, applicator OP155, MTS Medical, Germany) in a water bath set-up (described by[29–31]), allowing reproducible physical propagation and application of shockwaves *in vitro* (figure 1). Adult male Sprague Dawley rats were used to harvest the sciatic nerves (mixed: motor and cutaneous; further referred to as “sciatic”) as well as the motor branch (afferent and efferent

fibers of the quadriceps muscle; further referred to as “motor”) and cutaneous branch (purely sensory; further referred to as “sensory”) of the femoral nerves. For ESWT application, 15 ml centrifuge tubes containing the whole nerves in phosphate buffered saline (PBS) were placed centered inside the water bath, 5 cm in front of the applicator. Subsequently, unfocused shockwaves were applied using the parameters chosen according to previous experiments[27]: 300 pulses at an energy level of 0.10 mJ/mm^2 with a frequency of 3 Hz. The corresponding contralateral nerves served as controls and were placed accordingly in centrifuge tubes in 37°C warm water for the time of treatment to ensure equal sample treatment.

After ESWT treatment, Schwann cells were isolated from treated and non-treated nerve tissues according as previously described[28]. Briefly, the epineurium was removed and nerve wet weight was assessed (Sartorius, Austria). Nerves were strained, minced and incubated with 0.025% collagenase 1 for 1 hour at 37°C. Subsequently cells were centrifuged at 400 x g for 5 minutes and washed with Dulbecco’s Modified Eagle Medium (DMEM) containing 10% fetal calf serum (FCS; PAA, Austria). For culturing, cells were resuspended in DMEM-D-valine (Cell Culture Technologies, Switzerland), supplemented with 10% FCS, 2 mM L-Glutamine, 1% Pen/Strep, 1% N_2 supplement (Invitrogen, Germany), 15 µg/mL bovine pituitary extract, 3 µM forskolin (further referred to as “Schwann cell medium”). Finally, the cell suspension was seeded onto plates coated with poly-L-lysin and laminin.

Culture conditions for Schwann cells

Schwann cells were left to adhere and establish a proliferative phenotype for 21 days. Schwann cell medium was added on day 5 after isolation, and was partially (50%) changed on day 9, 13 and 17. Subsequent splitting of cells was performed for 9 passages as follows: cells

were detached using a cell scraper, centrifuged at 1000 rpm for 3 minutes and seeded at a density of $4x10^4$ cells/cm^2 on plates previously coated with poly-L-lysin. Medium was partially (50%) changed every third day and cells were split every sixth day.

Evaluation of Schwann cell yield from sciatic and femoral nerves

To evaluate cell yield, cells were counted after 19 days in culture using a Bio-Rad TC20™ automated cell counter (Bio-Rad Laboratories Inc., US). Non-viable cells were identified and excluded by trypan blue staining. Cell count was normalized to 100 mg nerve wet weight assessed before isolation.

Proliferative capacity of Schwann cells derived from sciatic, motor and sensory nerves

Proliferation of Schwann cells was assessed by calculating the population doublings (PD) and by using a 5- bromo-2-deoxyuridine uptake assay (BrdU; Cell Proliferation ELISA assay Kit; Roche Diagnostics, Switzerland). For PD cells were detached, counted and reseeded in a concentration of $4x10^4$ cells/cm^2 poly-L-lysine-coated plates, and cultivated for six days. PD were calculated as described by Gillies et al.[32]. BrdU assay was performed according to manufacturer's instructions. Briefly, 96 well plates were coated with poly-L-lysine and cells were seeded in quintuplicates at a density of $3x10^4$ cells/cm^2 in 100 µl Schwann cell medium. 36 hours after cell seeding, medium was collected for the neurite outgrowth assay and stored at -20°C. Cells were incubated with Schwann cell medium containing 100 µM BrdU for 24 hours. Culture plates were fixated with FixDenat® solution for 30 min and subsequently incubated with anti-BrdU POD antibody solution for 60 minutes at room temperature. After washing with PBS twice, tetramethyl benzidine was added as substrate for 30 minutes. The

reaction was stopped with 1 M H_2SO_4 and absorption was measured at 450 nm with 690 nm as reference wavelength on a microplate reader (Tecan Sunrise; Tecan, Switzerland).

Morphological evaluation of cultured Schwann cells

Schwann cell cultures in passage 4 were assessed for morphological differences using phase contrast imaging (Leica DMI6000B) and ImageJ analysis. Similar fields from 5 independent cultures were analyzed for cell body size (size of cell body without processes) and total length of Schwann cells.

Flow cytometric analysis

Purity and phenotypical marker expression of the Schwann cell cultures were evaluated with flow cytometry for common Schwann cell markers: anti-S100b (rabbit polyclonal; Dako, Denmark), anti-p75 NGFR (goat polyclonal; Santa Cruz Biotechnology, US), anti-P0 (rabbit polyclonal; Santa Cruz Biotechnology) and anti-GFAP (rabbit monoclonal; Abcam, UK). Antibodies were labeled with Allophycocyanin (APC) (Lynx Rapid Conjugation Kit, ABD Serotec, UK). For analysis, cells were washed with PBS, detached using a cell scraper, centrifuged at 1000 rpm for 3 minutes and stained with the respective antibodies for 30 minutes (on ice and protected from light). Stained cells were washed twice and flow cytometric analysis (10.000 events) was performed with a BD FACS Canto II (Becton Dickinson, USA). Data was evaluated using Flowjo Version 8.8 (Tree Star Inc, USA).

Neurite outgrowth assay

To evaluate effects of Schwann cell secretome on neurite outgrowth, the

neuroblastoma/glioma hybrid cell line NG108-15 was stimulated using Schwann cell conditioned medium ($3x10^5$ cells/ml for 36 hours). NG108-15 cells were seeded in 12-well plates at a density of $1x10^4$ cells/cm^2 and left to adhere for 4 hours in NG108-15 expansion medium (DMEM high glucose, 10% FCS, 1% L-Glutamin, 1% Pen/Strep). Subsequently, cells were washed with PBS and conditioned medium was added to stimulate neurite outgrowth. Unconditioned Schwann cell medium, expansion medium as well as expansion medium containing 20 ng/mL nerve growth factor (NGF; Peprotech, Austria) served as controls. After 48 hour incubation neurite outgrowth was evaluated: five equivalent fields per group were analyzed using ImageJ software, assessing percentage of cells expressing neurite and average neurite length.

Activation switch

In passage 4, the activation status and the capacity of sciatic, motor and sensory Schwann cells to switch activation status (proliferating to pro-myelinating) were assessed. Cells were divided in two groups, seeded at a density of $2x10^4$ cells/cm^2 and left to adhere for 24 hours. Subsequently medium was changed to fresh Schwann cell medium and basic medium without supplements (DMEM-D-valine supplemented with 10% FCS, 2 mM L-Glutamine, 1% PenStrep), favoring the proliferating or the pro-myelinating status, respectively. Proliferation rate was assessed using BrdU assay as described above. Expression of Schwann cell markers S100b, p75, MAG (rabbit polyclonal, Abcam) and P0 was assessed 5 days after medium switch with flow cytometry.

Statistics

All data in this study are shown as mean ± standard deviation (SD) and were tested for normal distribution. Statistical analysis was performed –depending on groups analyzed- using one-way ANOVA (analysis of variance) or two-way ANOVA followed by Tukey's range test for significant differences between the means. Significance was considered at $p<0.05$. For statistical calculations GraphPad Prism 5 for Mac OS X, Version 5.0b (GraphPad Software, Inc., USA) was used.

Results

Cell Yields increase with ESWT treatment

To evaluate differences between Schwann cell phenotypes as well as effects of ESWT on the isolation efficacy, cells of all three phenotypes were counted after 19 days in culture. It can be seen in figure 2A that there was no significant difference between the yields of the sciatic and the sensory Schwann cells ($1.6x10^6$ and $1.3x10^6$ cells per 100 mg nerve respectively), while the cell yield of motor Schwann cells was significantly lower ($5.7x10^5$ cells per 100 mg nerve). ESWT treatment resulted in a significant increase in all groups compared to the respective control group (increase between 42.1 and 92%).

Proliferation was assessed by cell count (population doubling time; figure 2B) as well as BrdU assay (figure 2C) over 8 passages. Results of both revealed significant differences for all phenotype groups between untreated control and ESWT group. Untreated motor Schwann cells proliferated significantly less than untreated sciatic and sensory Schwann cells, reflected in both, population doubling time and BrdU. Within the ESWT treatment group no significant

difference between the phenotypes could be observed. Furthermore, it can be seen in figure 2B/C how proliferation rates of untreated Schwann cells decrease over time, while proliferation rates for ESWT treated Schwann cells remain at a comparable level.

Schwann cells phenotypical morphology changes after ESWT

Schwann cells from all groups, independent of phenotype and ESWT treatment displayed typical bipolar Schwann cell morphology with elongated processes (figure 3A). Analysis of light microscopy images concerning cell body size (figure 3B) showed a general trend towards smaller cell bodies in the ESWT treated groups, with significant differences between control and ESWT in the sciatic Schwann cell culture. Furthermore, significant differences were found between sciatic/motor and sensory Schwann cell cultures, with sensory Schwann cells displaying a smaller cell body (Mean sciatic: 156.6±5.8 μm^2; motor: 161.8±3.7 μm^2; sensory: 131.6±6.8 μm^2). Assessing Schwann cell total length (figure 3C), no differences between the control groups were found.

ESWT diminishes phenotypical marker expression patterns

To determine purity and activation status of phenotypically different Schwann cells, expression of Schwann cell specific marker S100b, as well as the markers low affinity neurotrophic factor receptor p75 and glial fibrillary acidic protein (GFAP) (proliferative/regenerative phenotype), and P0 (myelinating phenotype) were assessed *in vitro* over 8 passages (passages 2 to 8) with flow cytometry (figure 3A&B). Initial purity, determined by expression of S100b in passage 2, did not differ between the different phenotypes (sciatic: 58.68±12.55%; motor: 58.9±13.07%; sensory 56.48±6.9%, figure 4B),

but was increased in the respective ESWT treated groups (sciatic: 71.8±4.6%; motor: 73.8±4.3%; sensory 73.5±5.8%). Purity steadily decreased in all untreated groups over time, following a relatively similar pattern over the passages, to an expression level of S100b below 20% at passage 9 (sciatic: 15.2±6.4%; motor: 17.2±4.4%; sensory 19.4±6.2%). In contrast to the untreated groups, the ESWT treated groups remained at a comparable level over the passages, with expression of S100b above 70% at passage 9 (sciatic: 73.3±3.9%; motor: 72.7±7.3%; sensory 19.4±8.1%).

Concerning the activation status, the following observations were made: untreated cultures of sciatic and sensory phenotype followed a similar pattern of p75 expression, starting at around 35% at passage 2, slightly increasing in passage 3 to above 40% and then continuously decreasing to an expression level below 20% at passage 9. Motor Schwann cells showed a significantly lower expression level at passage 2 (11.6±3.8%) and passage 3 (25.1±8.3%), remaining at a comparable level around 15-20% until passage 9. A similar expression behavior could be observed for GFAP, where an increase was observed until passage 4 (sciatic: 37.3±7.4% to 51.9±8.1%; sensory: 33.2±4.1% to 46.6±6.7%), followed by a steady decrease until passage 9 in the untreated groups of sciatic and sensory phenotype Schwann cells. Expression of GFAP in motor phenotype cultures was significantly lower in P2 than in sensory cultures and increased over time to around 30% (29.5±8.3% in P4), followed by a decrease after passage 4. In contrast to p75 and GFAP, P0 was significantly increased in motor Schwann cells (37.5±11.3%) at early passages compared to sciatic and sensory cultures (sciatic: 19.7±8%; sensory: 9.76±7.1%). Differences between the phenotypes in P0 expression levels diminished after passage 5. Extracorporeal shockwave treatment lead to a higher

expression of p75 in all phenotypes compared to the respective control groups and expression levels did not decrease over time. Furthermore, as opposed to untreated groups, no significant differences between phenotypes of the ESWT treated groups could be observed. P0 levels of all phenotypes were below 10% in passage 2 (sciatic: 5.3% ±2.6; sensory: 9.7% ±5.2) and decreased further over time to expression levels under 3% at passage 9. The absence of differences between the ESWT treated groups has to be pointed out especially for motor Schwann cells, as ESWT treatment increased expression of p75 by 53.4% and decreased expression of P0 by 28.4%.

Sensory Schwann cells stimulate neurite outgrowth after ESWT

To assess the potential of phenotypically different Schwann cells to stimulate neurite outgrowth, NG108-15 cells were incubated with medium conditioned by sciatic, motor and sensory Schwann cells with and without ESWT. As it can be seen in the representative phase contrast micrographs in figure 5A, conditioned medium of all groups induced neurite outgrowth to a certain extent. Quantification revealed significant differences of all groups to the two negative controls (NG108-15 expansion medium and Schwann cell medium) concerning the percentage of cells expressing neurites as well as average neurite length (figure 5B&C). Differences between the Schwann cell phenotypes were observed as follows: medium conditioned by motor Schwann cells induced significantly more neuritogenesis in NG108-15 than conditioned medium from sensory Schwann cells (sciatic: 50.2±1.5%; sensory: 34.1%±7.7%), and the expressed neurites were significantly longer (motor: 123.6 ±10.5µm; sensory: 91±8.6µm). Treated with ESWT, significant increases could be found in both parameters, percentage of cells expressing neurites (increase in percent: sciatic: +14.6%;

motor: +11.2%; sensory: +26%;) as well as average neurite length (increase in µm: sciatic: +52.3 µm; motor: +34.5 µm; sensory: +54.7 µm), compared to the respective untreated group. Unlike the untreated groups, no differences between the phenotypes in the ESWT treated groups could be observed.

Activation switch

Schwann cell cultures of different phenotypes were evaluated concerning their ability to switch from a proliferating state into a pro-myelinating state, using a minimal stimulus. Therefore, in passage 4 cells were deprived of pro-proliferative stimulus and evaluated after a 5 days incubation period. Flow cytometric analyses of S100b revealed no significant changes within phenotypes between proliferating and pro-myelinating Schwann cells (figure 6), maintaining the differences between ESWT treated groups and controls observed before.

The proliferation associated marker p75 was significantly less expressed in sciatic cultures (32.2±4.6% to 22.4±5.3%) in pro-myelination medium, however, no significant decrease was found in motor and sensory cultures (motor: 18.3±5.1% to 12.6±2.1%; sensory 30.6±3.1% to 20.9±1.8%) in comparison to proliferation medium; Treated with ESWT, all Schwann cell phenotypes displayed a significant change in p75 expression (sciatic: 75.4%±6.1% to 16.1±2.5%; motor: 51.8±4.2% to 8.6±2.2%; sensory: 56.8±6.6% to 12±2.8%). We observed an increase in p75 expression in the proliferation medium and a decrease in pro-myelination medium, compared to untreated cultures.

MAG showed a significantly higher expression by untreated sciatic and motor Schwann cells in pro-myelination medium (sciatic: 14.5±3.4% to 28.7±7.1%; motor 23.9±3.2% to

67.5±5.4%), but no significant increase in the sensory Schwann cell culture (10.4±2.8% to 18.38±7.5%). ESWT resulted in a reduced expression of MAG of all Schwann cell cultures in the proliferation medium and an increased expression in pro-myelination medium (except for motor SCs). All ESWT treated Schwann cell phenotypes significantly increased expression of MAG in comparison to proliferation medium (sciatic: 4.4±2.1% to 62.6±4.3%; motor: 6.2±1.4% to 71.5±4.6%; sensory: 2.8±1.5% to 66.1±10.2%). P0 revealed significant changes in the untreated group only for motor Schwann cells (24.62±7.1% to 63.45±5.6%). Sciatic and sensory Schwann cells increased P0 expression in pro-myelination medium when treated with ESWT (sciatic: 6.7±3.1% to 63.9±5.3%; sensory: 5.1±2% to 68.5±4.5%). Interestingly, differences between the phenotypes within the untreated group could be observed: as both markers MAG and P0 were expressed significantly higher by motor Schwann cells than by the other phenotypes. Treated with ESWT, all Schwann cell cultures showed significantly increased expression of MAG and P0 in the pro-myelination condition, independent of their phenotype. Proliferation behavior after switch to pro-myelinating status was also assessed and lead to following observations: all groups -independent of phenotype and treatment- displayed significantly lower proliferation after 5 days in pro-myelination medium. However, untreated sensory Schwann cells were found to proliferate significantly more than motor Schwann cells.

Discussion

The regeneration of axons as well as motor and sensory target innervation after injury is dependent on the capacity of Schwann cells to switch into and maintain a repair phenotype (Büngner cell)[5,33–39]. These repair Schwann cells support axonal growth and path-finding. However, we previously showed that after several population doublings *in vitro*, Schwann

cells lose their pro-regenerative phenotype, change their morphology and become senescent[40]. This property reflects the *in vivo* situation: Although some Schwann cells remain their pro-regenerative capacities[14] after long periods of time after injury and extensive proliferation[41], the vast majority of Schwann cells become senescent and incapable of supporting axonal regeneration[42].

Besides inducing higher cell yields and reaching higher purity, we were able to prevent exhaustion of Schwann cells by treating the nerves with ESWT prior to Schwann cell isolation[40]. However, we investigated Schwann cells derived from the sciatic nerve in an attempt to explain effects previously found in an *in vivo* study on regeneration of sciatic nerves in rats after ESWT[27]. The sciatic nerve, being a mixed nerve, has several limitations in reflecting the clinical situation. The vast majority of nerve defects are repaired by autologous nerve transplantation using a sensory nerve. In 1994, Martini, Schachner and Brushart published preferential expression of the L2/HNK1 carbohydrate in motor Schwann cells[43]. Since then, a growing body of literature states differences between Schwann cells from different modalities such as motor nerves and purely sensory nerves regarding architecture[44], expression profile[13,35,39,45,46]and capacity to support axonal regeneration[18,47–49]. In this study we investigated effects of ESWT on phenotypically different Schwann cells using the motor and sensory branch of the femoral nerve of adult Sprague Dawley rats as representatives for motor and sensory nerves.

Isolation of Schwann cells from the motor branch resulted in less cell yield and less proliferating Schwann cells when compared to either the sciatic nerve or the sensory nerve (figure 2). However, in accordance with our previous study, we observed higher purity of

culture as well as higher cell yield after ESWT treatment in motor as well as sensory and sciatic nerve derived Schwann cells. The exact mechanisms of increased purity of primary SC cultures is not understood in detail, however, there is evidence for increased c-jun expression after ESWT treatment [40], indicating a fast switch from myelinating to repair phenotype and proliferation, respectively. Together with media selective for SC (by lacking the essential amino acid L-Valine), it is possible that in ESWT treated cultures, SC overgrow potentially present fibroblast, resulting in higher purity of the culture. Although cell yield was increased, phenotypic differences were conserved in passage 0. Possible explanations for this effect, are the higher prevalence of heavily myelinated large diameter axons in the motor nerve[44,50], the higher portion of Schwann cells expressing myelin protein zero (P0) in the cultures derived from motor nerves (figure 4), and the anti-proliferative effect of myelin proteins. This is also reflected in the varying morphology between motor and sensory Schwann cells *in vitro*. We observed an average length of all cultured Schwann cells of 100 µm, which is the length of immature Schwann cells *in vivo* as described by Gomez-Sanchez et al.[37] (figure 3). One of the hallmarks for Schwann cells after injury is elongation, with accompanying reduction of cell body size. Sensory Schwann cells display significantly reduced cell body and, after ESWT, stronger elongation than motor and mixed Schwann cells, indicating an accelerated onset of the repair phenotype. Motor derived Schwann cells appear to take longer for down-regulation of myelin-associated genes and the induction of the repair phenotype as indicated by the initially higher expression of P0 and the reduced expression of the pro-regenerative marker p75 (figure 4). Also, Jesuraj et al. showed an increased initial expression of another myelin protein the myelin basic protein in motor derived Schwann cells[39]. However, in contrast to

their findings we did not observe an increase of myelin-associated gene expression in sensory derived Schwann cells over time. Jesuraj et al. used differing culture conditions from this study, investigating expression on the mRNA level and on different time points. The reduced expression of GFAP in motor derived Schwann cells is consistent with publications, stating that GFAP is a marker for non-myelinating and dedifferentiated Schwann cells *in vitro*[51].

A central finding of this study was the inferior capacity to induce neurite outgrowth by supernatant derived from sensory Schwann cells, when compared to motor Schwann cells (figure 5). Sciatic Schwann cells perform again somewhere between sensory and motor Schwann cells, reflecting their mixed population and showing their inferiority as a model for the situation after autologous nerve grafting in the clinic. Further proof for the phenotypical commitment of motor, sensory and mixed Schwann cells was observed after induction of myelination. Here, motor Schwann cells displayed the highest capacity for re-inducing myelination-associated proteins such as MAG and P0, whereas sensory Schwann cells showed only very limited induction of these proteins. Surprisingly, sciatic nerve derived Schwann cells -although a mixed population- closely resembled sensory Schwann cells in this regard after induction of myelination (figure 6). However, it is noteworthy that the myelination response may differ if axonal contact would have been provided *in vitro*.

Taken together, we show a strong phenotype commitment of sciatic, motor and sensory Schwann cells *in vitro*. Our results suggest that the commonly used *in vitro* model of Schwann cell derived from rat sciatic nerves is not ideal, as these cultures display a mixed phenotype between motor and sensory Schwann cells, thereby hindering the investigation of novel treatment options for pressing issues in the field of peripheral nerve regeneration such

as Schwann cell senescence after long gap repair. As the investigated Schwann cell cultures differed significantly, we hypothesize that there is not one common immature repair Schwann cell phenotype, but several, depending on their origin. We propose the use of Schwann cells from "purely" motor and sensory nerves to better mimic the situation after clinical autologous nerve transplantation, where mostly a purely sensory nerve (and its Schwann cells) is used to re-establish continuity of injured nerves.

Extracorporeal shockwave therapy has been used widely in the clinics for a variety of indications, ranging from non-union bone fractures to tendinopathies and erectile dysfunction. The efficacy on peripheral nerve regeneration has been shown before *in vitro* and *in vivo* [28,52]. We provide evidence for the efficacy of ESWT to enhance proliferative capacity and an active repair phenotype of phenotypically different Schwann cell cultures over extended periods of time. Additionally, we show that supernatant of ESWT-treated Schwann cell cultures is significantly more potent in inducing neurites *in vitro*. This could be explained by an increased and prolonged induction of c-jun[40] and subsequent secretion of neurotrophic factor production, as other groups have shown that c-jun activation results in a strong induction of pro-regenerative genes[6,53–57].

Limitations

As we used rat Schwann cells in this study, it remains to be elucidated, if the observed differences between motor and sensory Schwann cells as well as their response to ESWT holds true also for human Schwann cells e.g. derived from human sural nerves. However, as the rat is the predominantly used animal model in the field of peripheral nerve regeneration, we believe that our results are still of high value for the understanding of Schwann cell

phenotype and their effect on peripheral nerve injury. One aim of our study was to provide evidence that ESWT treated Schwann cells are – given the appropriate environment - able to switch from a proliferative to a pro-myelinating phenotype as this is mandatory for functional regeneration *in vivo*. We showed that Schwann cells regain their capacity to produce myelin associated genes *in vitro*. However, as ESWT treatment would be applied in the acute phase of nerve damage, the effect of ESWT at later stages of regeneration (e.g. remyelination) was not elucidated in this study. Future *in vitro* co-culture and *in vivo* experiments should tackle this question.

Conclusion

We could demonstrate a strong phenotypical commitment of sciatic, motor and sensory Schwann cells *in vitro,* revealing an inferior regenerative potential of sensory Schwann cells. After ESWT, phenotypical commitment is strongly diminished in all Schwann cell populations, resulting in an increase of regeneration-associated properties. This study provides strong arguments for pursuing further research on the use of ESWT after autologous nerve grafting as the capacity of sensory Schwann cells to induce neurite outgrowth as well as expression of myelination-associated factors is significantly increased.

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Author Disclosure statement

We confirm that all authors (DH, HR and CS) have read the journal's authorship agreement, and that the manuscript has been reviewed by and approved by all named authors. Furthermore, we confirm that all authors have read the journal's policy on disclosure of potential conflicts of interest. All authors (DH, HR, CS) declare no conflict of interest.

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Figure Legends

Figure 1: **Experimental layout for ESWT treatment**; Sprague Dawley sciatic nerve and motor/sensory branches of the femoral nerve were dissected and transferred to conical centrifuge tubes. Tubes containing whole nerves in PBS were placed centered inside a water container, 5 cm in front of the applicator and treated with 300 pulses at an energy level of 0.1 mJ/mm^2 and 3 Hz. Subsequently nerves were digested with collagenase and resulting Schwann cells were plated on poly-L-lysine coated plates.

Figure 2: **ESWT increases cell yield in motor, sensory and mixed Schwann cells cultures**; Comparison between sciatic, motor and sensory cultures +/- ESWT, **A)** counted after 19 days in culture and normalized on 100 mg nerve wet weight, n=8; data is presented as mean ± SD, statistical significance was tested with 1-way ANOVA and Tukey range test with * indicating significant differences between phenotypical different cultures within the same treatment group (+/- ESWT) and § indicating significant differences between ESWT treated and control group of the same Schwann cell phenotype; *P<0.05, **P<0.01, ***P<0.001; **B)** Population doublings per passage: population doublings were obtained by cell count in passage 2, 4, 6,

and 8; n=6; one passage represents the detachment, counting, and seeding of the cells with $4*10^4$ cells/cm^2 for 6 days; **C)** BrdU proliferation assay in passage 2, 4, 6 and 8; n=6; data is shown as mean ± SD; statistical significance was tested with 2-way ANOVA; *P<0.05, **P<0.01, ***P<0.001 with * indicating significant differences between ESWT and ctrl of the same Schwann cell phenotype; color of * represents associated group;

Figure 3**: Sciatic, motor and sensory Schwann cell bodies differ in size; A)** Phase contrast micrographs depicting mixed, motor and sensory Schwann cells in passage 4 of control and ESWT treated group; **B)** Average area of cell body in sciatic, motor and sensory culture, ctrl (-) and ESWT(+) respectively; **C)** Average Schwann cell length in sciatic, motor and sensory culture, ctrl (-) and ESWT(+) respectively; for **B** and **C**: five different cultures were analysed; data is presented as mean ± SD, statistical significance was tested with 1-way ANOVA and Tukey range test with * indicating significant differences between phenotypical different cultures within the same treatment group (+/- ESWT) and § indicating significant differences between ESWT treated and control group of the same Schwann cell phenotype; §/*P<0.05, **P<0.01;

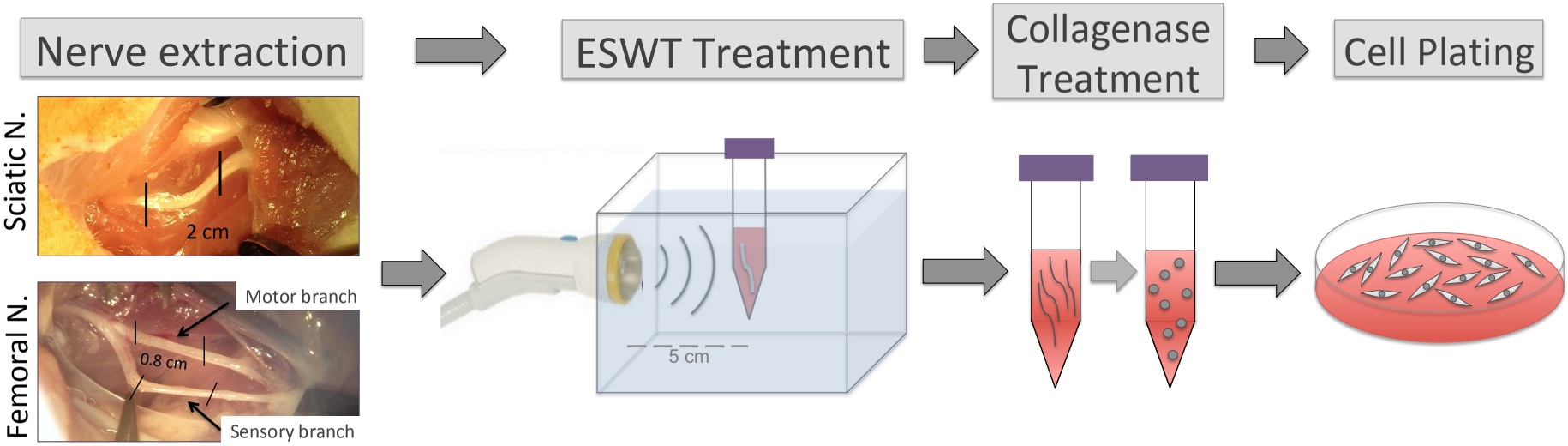
Figure 4: **Schwann cells of all phenotypes show an increased purity (S100b), along with increased expression of proliferation associated marker p75 and a decreased expression of myelin marker P0 treated with ESWT**; **A)** Flow cytometric immunophenotype analysis (S100b, p75, GFAP, P0) of Schwann cell cultures treated with ESWT (black), compared to control (grey) over 8 passages (P2-P9); one passage represents the detachment, counting, and seeding of the cells with $4*10^4$ cells/cm^2 for 6 days; n=7 nerves; data is shown as mean of percentage marker positive cells ± SD and significance was tested with student t test; *P <

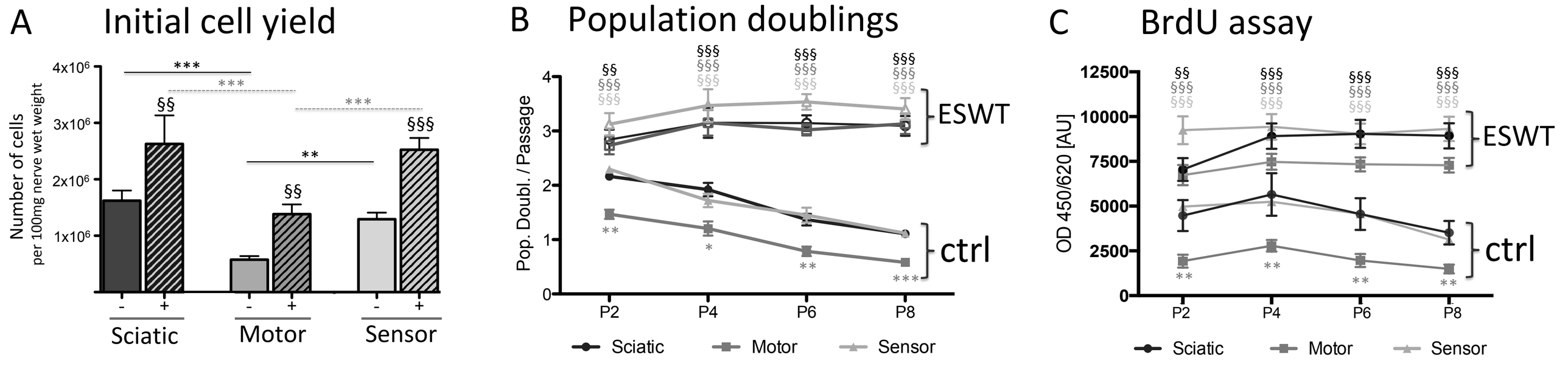
0.05; **P < 0.01; ***P < 0.001; **B)** Data obtained in passage 2 depicted in bar graphs. Data is shown as mean of percentage marker positive cells ± SD and statistical significance was tested with 1-way ANOVA and Tukey range test.; *P < 0.05; **P < 0.01; ***P < 0.001

Figure 5: **ESWT treated Schwann cells promote expression of neurites and increase neurite length in NG108-15 neuronal cells; A)** Phase contrast micrographs depicting NG108-15 cells treated for 48 hours with supernatants conditioned for 36 hours with mixed, motor and sensory Schwann cells treated with ESWT compared to control. Quantitative analysis of neurite outgrowth, measured in percentage of cells expressing neurites **(B)** and average neurite length **(C)**, after ESWT compared to untreated group as well as 20 ng/mL nerve growth factor (NGF), Schwann cell medium (SC medium) and NG108-15 expansion medium (Exp. medium); n=6; data is presented as mean ± SD, statistical significance was tested with 1-way ANOVA and Tukey range test with * indicating significant differences between phenotypical different cultures within the same treatment group (+/- ESWT) and § indicating significant differences between ESWT treated and control group of the same Schwann cell phenotype; *P<0.05, **P<0.01, ***P<0.001;

Figure 6: **ESWT reduces phenotypical differences of Schwann cells in specific expression profiles of activation state (regenerative and pro-myelinating)**. **A)** Flow cytometry analysis of expression of Schwann cell markers S100b, p75, MAG and P0 of sciatic, motor and sensory Schwann cells in proliferation medium (upper panel) and pro-myelination medium (lower panel), on day 5 after medium switch: **B)** BrdU assay of Schwann cells in activation medium and pro-myelination medium; n=6: Statistical significance was tested with 1-way ANOVA and Tukey range test. Data is presented as mean ± SD. Bars display

significant differences between indicated groups *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; § indicates significant differences between ESWT treated and control group of the same Schwann cell phenotype: §$P < 0.05$, §§$P < 0.01$, §§§$P < 0.001$.;


Nerve extraction
ESWT Treatment
Collagenase Treatment
Cell Plating
Sciatic N.
2 cm
Femoral N.
Motor branch
0.8 cm
Sensory branch
5 cm


A
Initial cell yield
Number of cells
per 100mg nerve wet weight
4x10^6
3x10^6
2x10^6
1x10^6
***
***
***
**
§§
§§
§§§
-
+
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+
-
+
Sciatic
Motor
Sensor
B
Population doublings
Pop. Doubl. / Passage
4
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ESWT
ctrl
P2
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P8
Sciatic
Motor
Sensor
C
BrdU assay
OD 450/620 [AU]
12500
10000
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5000
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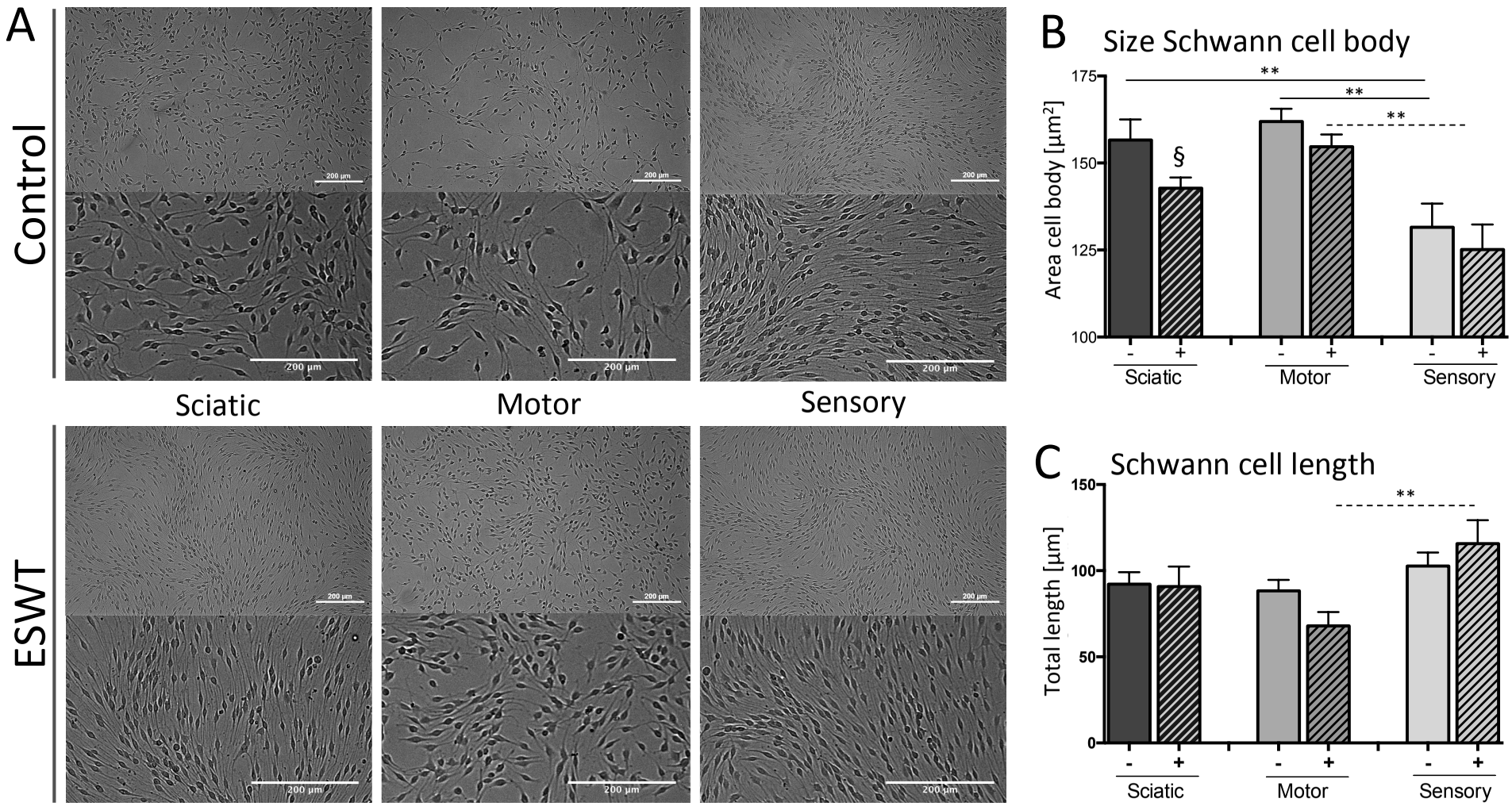
Sciatic
Motor
Sensor


A
Control
ESWT
Sciatic
Motor
Sensory
200 µm
B
Size Schwann cell body
Area cell body [µm²]
175
150
125
100
**
**
**
§
-
+
Sciatic
Motor
Sensory
C
Schwann cell length
Total length [µm]
150
100
50
0
**

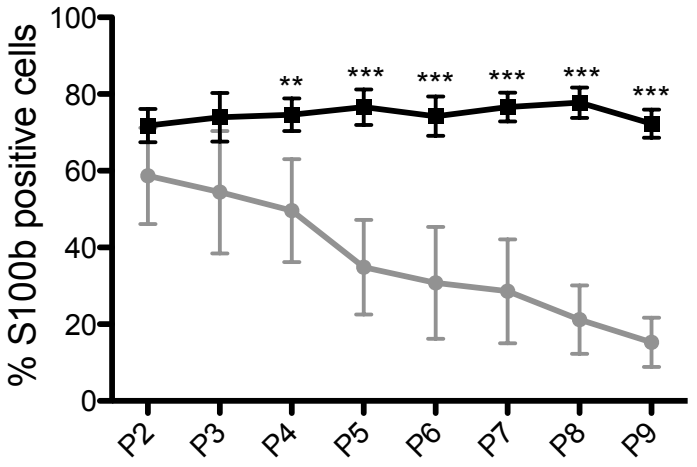
A

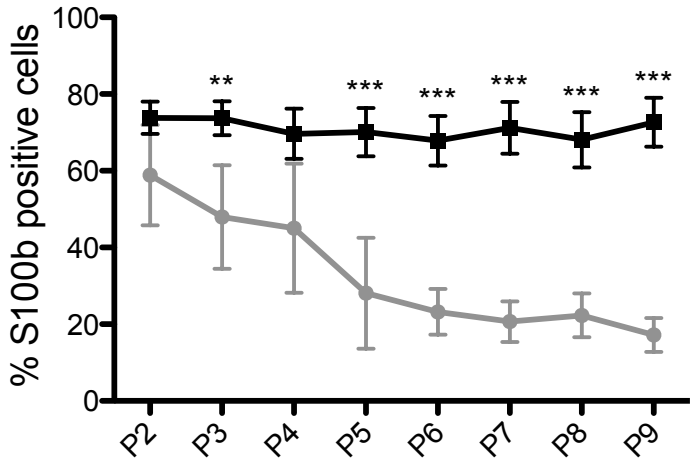
Sciatic

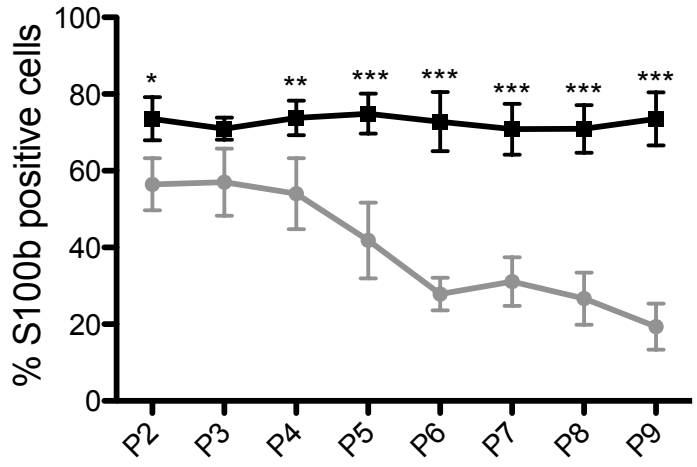
Motor

Sensory

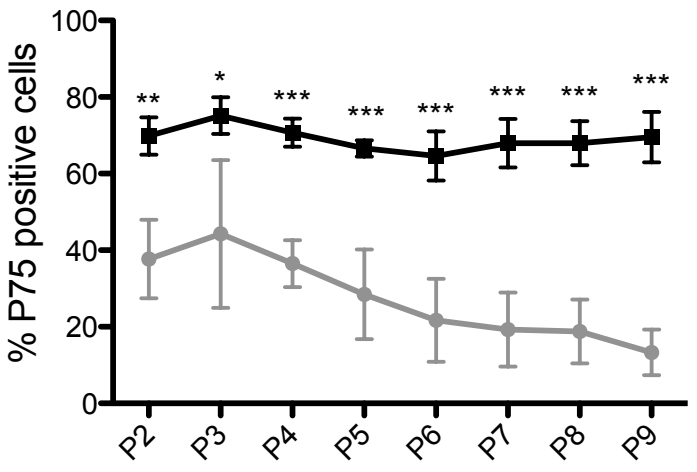
S100b

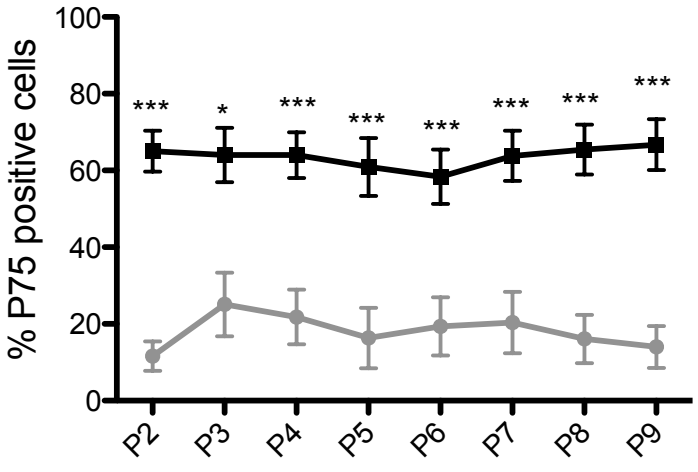


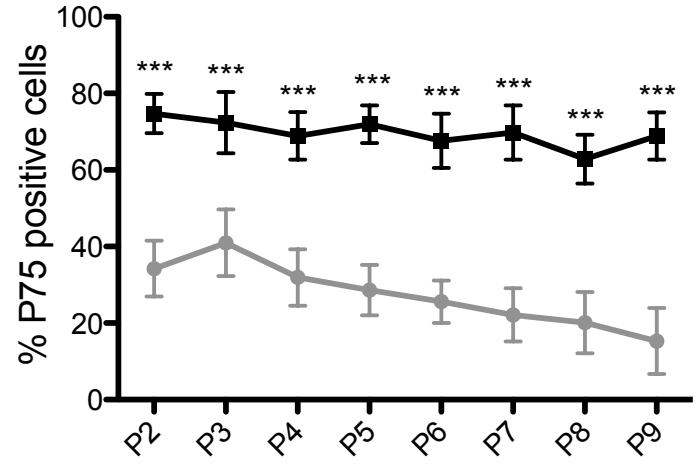




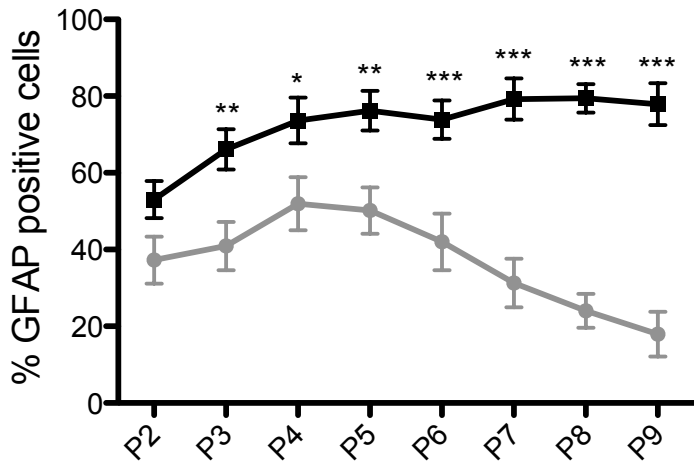
p75

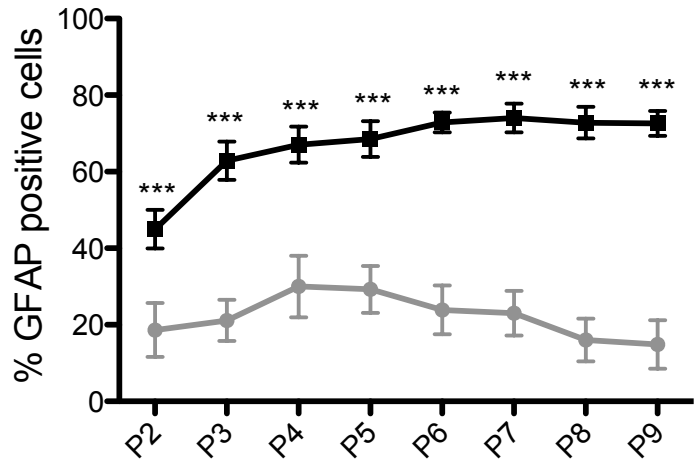


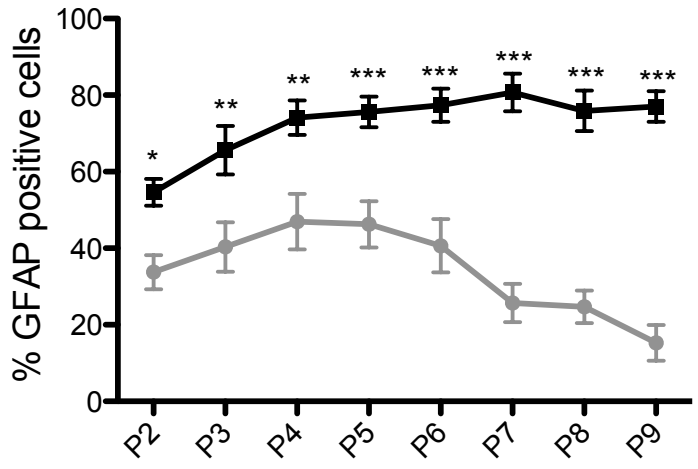




GFAP







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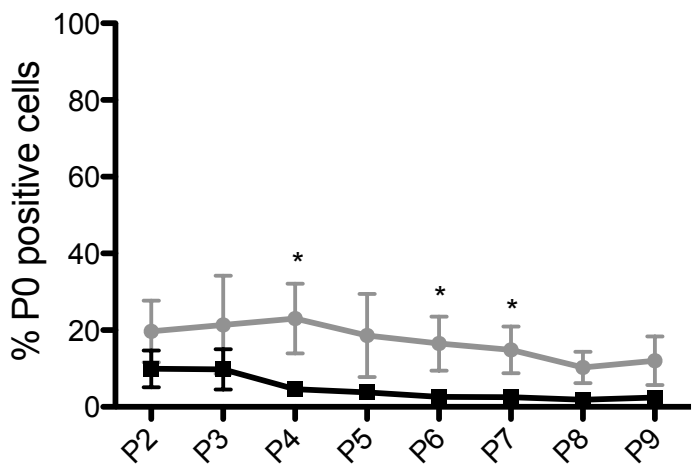
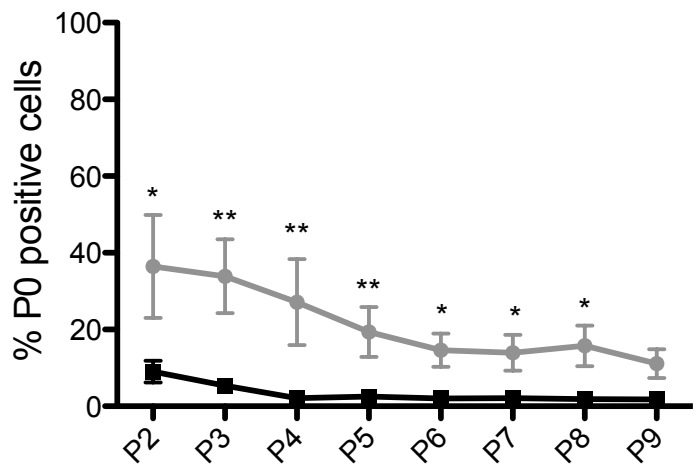
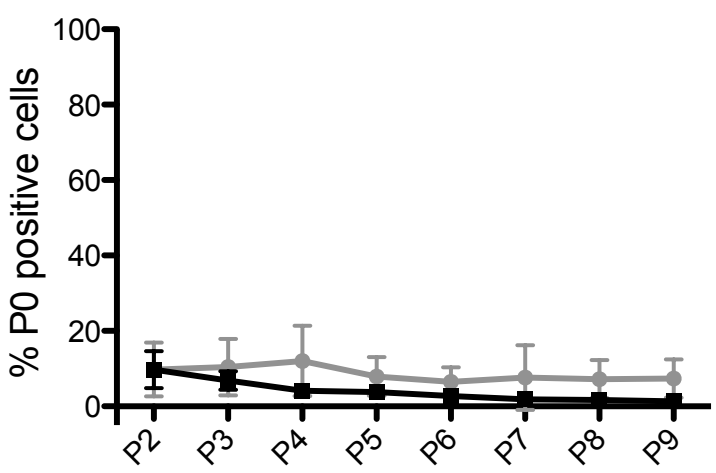


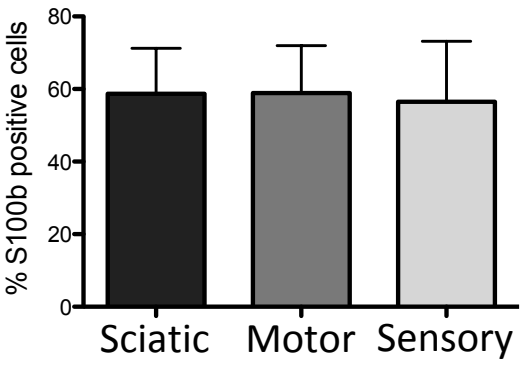




B

Passage 2

S100b



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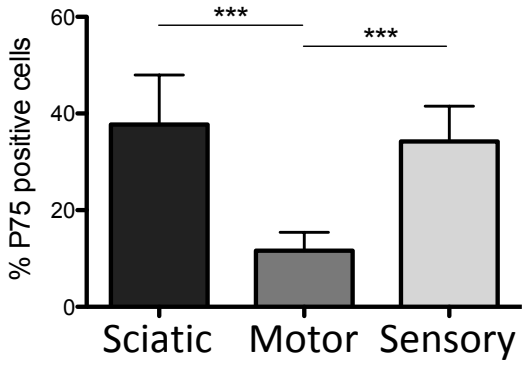

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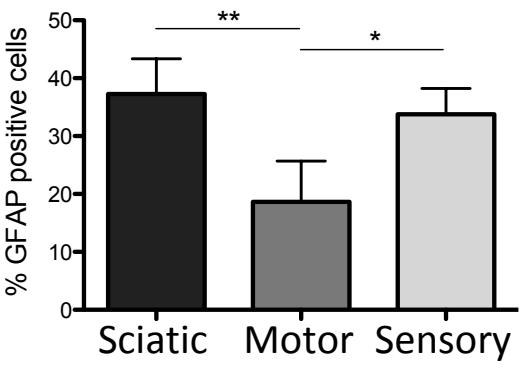
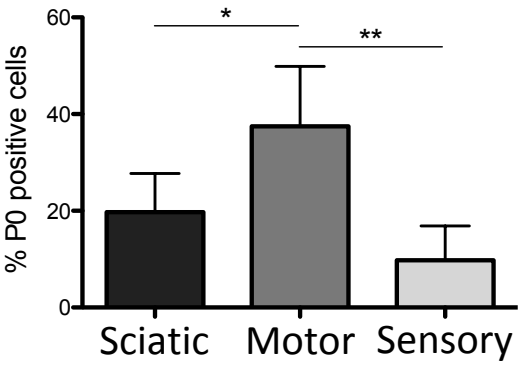
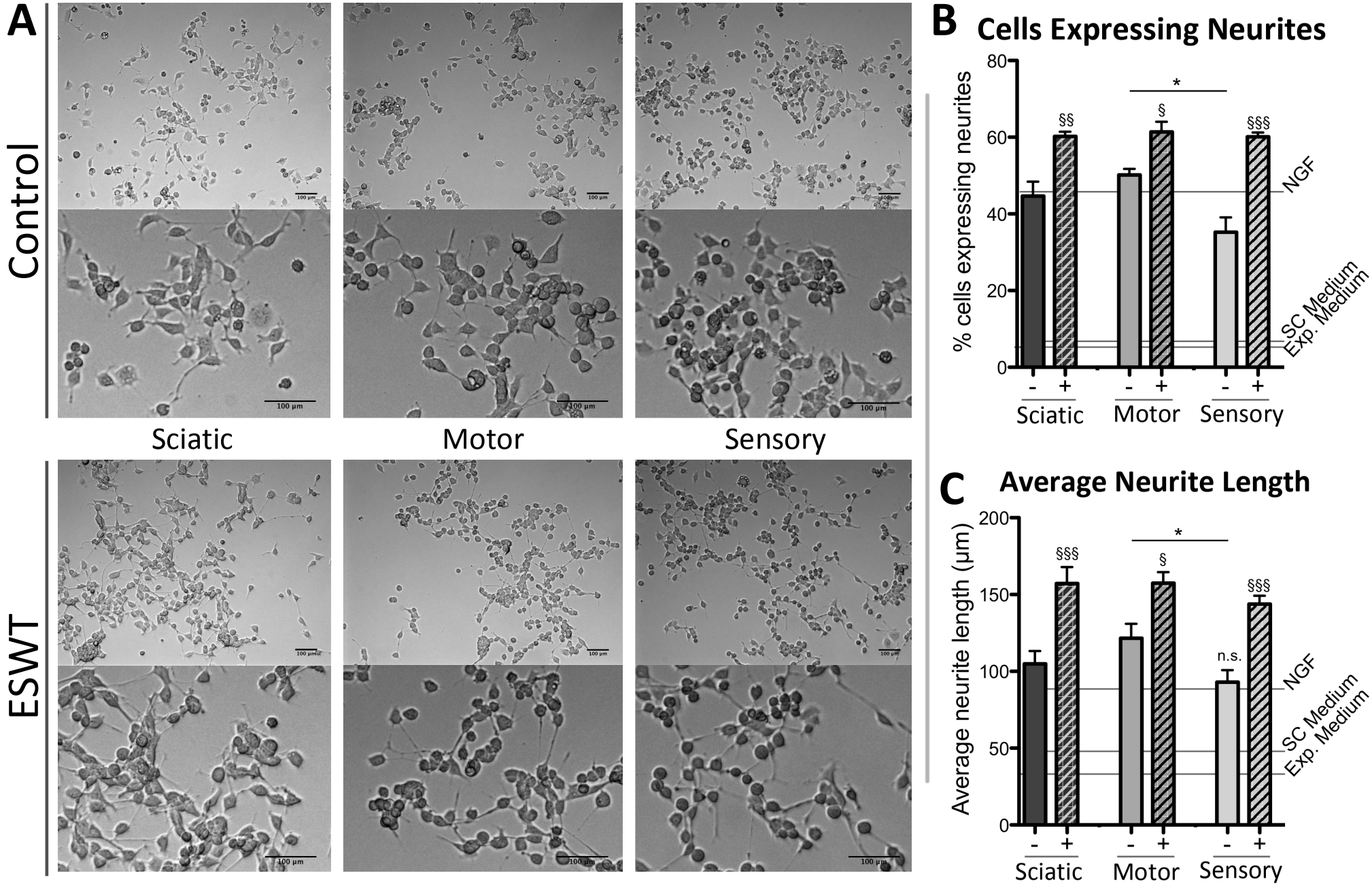
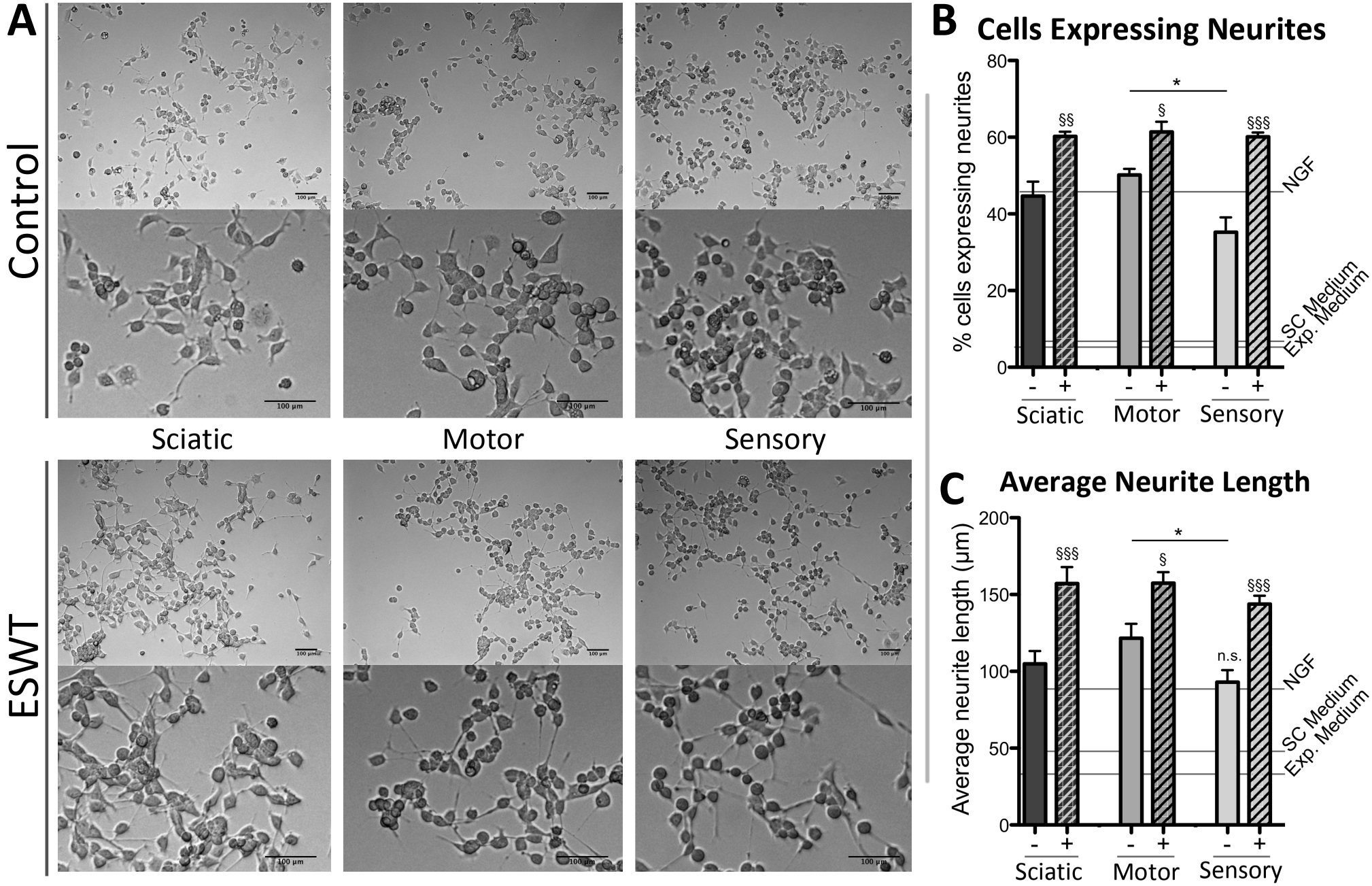


P0




A
Control
ESWT
Sciatic
Motor
Sensory
100 µm
B
Cells Expressing Neurites
% cells expressing neurites
80
60
40
20
0
*
§§
§
§§§
NGF
SC Medium
Exp. Medium
-
+
Sciatic
Motor
Sensory
C
Average Neurite Length
Average neurite length (µm)
200
150
100
50
0
§§§
§
n.s.


A
Control
ESWT
Sciatic
Motor
Sensory
100 µm
B
Cells Expressing Neurites
% cells expressing neurites
80
60
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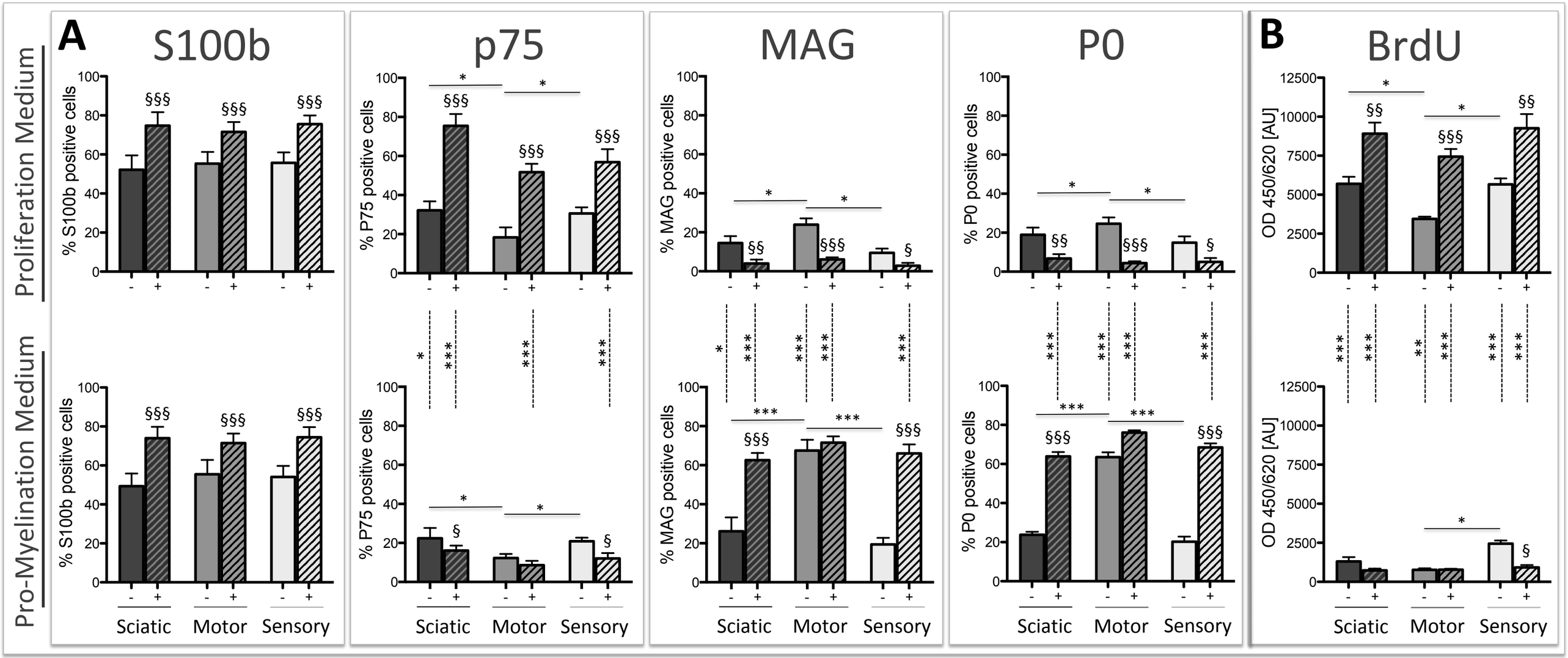
0
*
§§
§
§§§
NGF
SC Medium
Exp. Medium
-
+
Sciatic
Motor
Sensory
C
Average Neurite Length
Average neurite length (µm)
200
150
100
50
0
§§§
§
n.s.


A
S100b
p75
MAG
P0
B
BrdU
Proliferation Medium
Pro-Myelination Medium
% S100b positive cells
% P75 positive cells
% MAG positive cells
% P0 positive cells
OD 450/620 [AU]
Sciatic
Motor
Sensory