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2011 J. Neural Eng. 8 046023

(http://iopscience.iop.org/1741-2552/8/4/046023)

J. Neural Eng. **8** (2011) 046023 (13pp) doi:10.1088/1741-2560/8/4/046023

Neurite outgrowth is significantly increased by the simultaneous presentation of Schwann cells and moderate exogenous electric fields

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Received 22 October 2010
Accepted for publication 26 January 2011
Published 29 June 2011
Online at stacks.iop.org/JNE/8/046023

Abstract
Axonal extension is influenced by a variety of external guidance cues; therefore, the development and optimization of a multi-faceted approach is probably necessary to address the intricacy of functional regeneration following nerve injury. In this study, primary dissociated neonatal rat dorsal root ganglia neurons and Schwann cells were examined in response to an 8 h dc electrical stimulation (0–100 mV mm^{-1}). Stimulated samples were then fixed immediately, immunostained, imaged and analyzed to determine Schwann cell orientation and characterize neurite outgrowth relative to electric field strength and direction. Results indicate that Schwann cells are viable following electrical stimulation with 10–100 mV mm^{-1}, and retain a normal morphology relative to unstimulated cells; however, no directional bias is observed. Neurite outgrowth was significantly enhanced by twofold following exposure to either a 50 mV mm^{-1} electric field (EF) or co-culture with unstimulated Schwann cells by comparison to neurons cultured alone. Neurite outgrowth was further increased in the presence of simultaneously applied cues (Schwann cells + 50 mV mm^{-1} dc EF), exhibiting a 3.2-fold increase over unstimulated control neurons, and a 1.2-fold increase over either neurons cultured with unstimulated Schwann cells or the electrical stimulus alone. These results indicate that dc electric stimulation in combination with Schwann cells may provide synergistic guidance cues for improved axonal growth relevant to nerve injuries in the peripheral nervous system.

(Some figures in this article are in colour only in the electronic version)

1. Introduction

Following traumatic injury, regrowing axons must navigate through the injury site and restore connections with appropriate distal targets for functional recovery. However, this process is often complicated by scarring, inhibitory myelin, neuronal cell death and a lack of a permissive substrate at the injury site (Bresjanac and Sketelj 1989, Schmidt and Leach 2003, Chen *et al* 2007, Lu *et al* 2007). Axonal regrowth can be influenced by a variety of environmental factors such as participation of local glia, availability of a supportive substrate, neurotrophic factors, anisotropic topography and external biophysical forces (Clark *et al* 1993, Rajnicek *et al* 1997, Schmidt and Leach 2003, Lu *et al* 2007). *In vivo* wound healing and neural development studies suggest that neural cells and support cells (e.g. Schwann cells) are subjected to an endogenous constant voltage gradient due to leaky epithelium, estimated

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to range from 40 to 140 mV mm^{-1} (Barker 1982, McGinnis and Vanable 1986, Ard *et al* 1987, Hotary and Robinson 1990, 1994, Chiang *et al* 1992, Shi and Borgens 1995, McCaig *et al* 2002, 2005). Despite the potential importance of this biophysical cue to neuronal tissues, *in vitro* electrical stimulation on neural tissue has not been widely examined in mammalian models. Neurons exhibited a variable response to an applied electric field displaying cathodal preference (Jaffe and Poo 1979, McCaig 1990), anodal preference (Cork *et al* 1994), increased branching (Hinkle *et al* 1981), increased neurite outgrowth (Wood and Willits 2009) or no response (Cormie and Robinson 2007). Many of the aforementioned studies were conducted in chick (Jaffe and Poo 1979, Wood and Willits 2009), zebrafish (Cormie and Robinson 2007) or *Xenopus* models (Hinkle *et al* 1981, McCaig 1990); however, the regenerative properties of these species may not directly translate to mammalian or clinical models relevant to human injuries (Robinson and Cormie 2008). Therefore, characterization of mammalian cell behavior (neuron and glial) serves as an important step for the optimized application of electrical stimuli to promote axonal regrowth.

There have been limited studies of electrical stimulation on cultured mammalian neurons and these studies have resulted in conflicting responses, probably due to the varying range of stimulation parameters and the different developmental stages of the cells used. For example, E18 rat hippocampal neurons extended new outgrowth perpendicular to a constant dc electric field, in addition to a decrease in cathodal outgrowth (28, 80 or 219 mV mm^{-1}) (Rajnicek *et al* 1992). Similarly, mechanically dissociated cells isolated from postnatal (P0–P3) rat hippocampi were responsive to the electric stimulation but were found to preferentially divide parallel to the electric field when stimulated for 1 h in dc up to 300 mV mm^{-1} (Yao *et al* 2009). Davenport and McCaig (1993) also reported that embryonic rat hippocampal growth cones demonstrated a cathodal preference following brief and strong (<6 min) focal electric fields, significantly greater than physiologic levels (>580 mV mm^{-1}) (Davenport and McCaig 1993). In contrast, De Boni and Anderchek (1986) did not observe any changes in neurite outgrowth (dorsal root ganglia (DRG) isolated from E16–18 mice) following a 48 h stimulation with a physiologically relevant dc electric field (up to 50 mV mm^{-1}) (De Boni and Anderchek 1986). Due to the limited number of mammalian studies with varying parameters (cell type, field strength, duration) and variability in cell response, there is a need to investigate the response of neurons to electrical stimulation in a controlled environment.

It is known that glial support cells in the peripheral nervous system (Schwann cells) promote an increase in the rate of neurite outgrowth due to clearance of myelin debris, release of neurotrophic factors and expression of permissive surface ligands on the Schwann cells (Guenard *et al* 1992, Ramon-Cueto *et al* 1998, Hadlock *et al* 2000). Previous work *in vitro* has shown that oriented Schwann cell monolayers can also direct neurite outgrowth (Thompson and Buettner 2006, Seggio *et al* 2010) and the rate of Schwann cell migration influences the rate of axonal outgrowth (Guenard *et al* 1992, Han *et al* 2007). In the central nervous system (CNS), primary rat astrocytes (CNS glia) are responsive to high electric fields (500 mV mm^{-1}), aligning perpendicular to the electric field, and these aligned astrocytes can direct sensory neurite outgrowth (Borgens *et al* 1994, Alexander *et al* 2006). It is possible that Schwann cells (PNS glia) may reorganize or alter the rate of migration following electrical stimulation and this reorganization may direct or enhance axonal migration following injury.

Studying the effects of these physiological forces *in vitro* on a controlled and repeatable platform will provide valuable insighs into the sensitivity of mammalian sensory neurons and supporting glia to electrical stimuli. The external application of physiologically relevant electrical stimulation may orient and direct Schwann cell migration, due to the reported sensitivity of astrocytes to an electric field, which in turn may both enhance and direct axonal regrowth following injury in the peripheral nervous system. *In vivo* studies have demonstrated synergy when combining several cues within a single therapeutic intervention, and it is possible that the combination of both Schwann cells and electrical stimulation will enhance sensory neurite outgrowth (Ceballos *et al* 1999, Fouad *et al* 2005). In this study, a constant physiologic dc electrical stimulation (0–100 mV mm^{-1}) was applied for 8 h to primary dissociated DRG neurons, primary Schwann cells and Schwann cell–neuron co-cultures. Both Schwann cell and neuronal morphology were quantified to determine if the application of an electrical stimulus could promote and direct neurite outgrowth. Schwann cells and dissociated neurons were exposed to a constant current (0.1 mA) dc field to avoid changes in current density as an additional stimulus, and to observe any anodal or cathodal preference.

2. Materials and methods

2.1. Schwann cell isolation

Primary Schwann cells were isolated from neonatal rat sciatic nerves, as previously described (Assouline and Pantazis 1989, Seggio *et al* 2010). Sciatic nerves were dissected from Sprague Dawley postnatal day two (P2) neonatal rat pups (Taconic Farms, Inc., Germantown, NY) and placed in Ham's F-12 (Mediatech, Inc., Manassas, VA) on ice. Dissected sciatic nerves were minced with a scalpel and placed in six-well culture dishes in base medium containing Dulbecco's Modified Eagle Medium (DMEM; Mediatech, Inc.) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT), 2 mM L-glutamine (L-glut, Hyclone), and 50 U mL^{-1} penicillin/streptomycin (P/S, Mediatech, Inc.) under standard culture conditions (37 °C, 5% CO_2). Fibroblasts are the predominant cells to migrate out of the explants during days one–three, followed by Schwann cell migration as qualitatively determined by visual inspection. Upon visualization of Schwann cell migration, the explants were transferred to new dishes to collect a higher purity of Schwann cells (days three–seven). On day seven, the Schwann cells from the explants, together with any contaminating fibroblasts, were cultured in base medium supplemented with 10^{-5} M cytosine arabinoside (ARA-C; Sigma Aldrich, St Louis, MO) for 72 h

to remove highly mitotic fibroblasts. The contaminating fibroblasts were further targeted using a complement-mediated cell lysis (Morrissey *et al* 1991). First, cells were pelleted via centrifugation at 201 × *g* for 5 min and the supernatant was aspirated. Next, 1 mL of anti-CD90/Thy 1.1 (diluted 1:500 v/v in DMEM; Accurate Chemical, Westbury, NY) solution was mixed with the cells and incubated for 30 min at 37 °C to target contaminating fibroblasts. The treated cells were pelleted via centrifugation at 201 × *g* for 5 min, the supernatant was removed, and then the pellet was re-suspended in 1 mL of rabbit complement (Accurate Chemical) in DMEM for 30 min at 37 °C to selectively lyse the fibroblasts. Following the complement lysis, the cells were pelleted as described above and re-suspended in Schwann cell growth medium (base medium supplemented with 6.6 mM Forskolin (Sigma Chemical) and 10 μg mL^{-1} bovine pituitary extract (BPE; BD Biosciences, San Jose, CA)). The cells were seeded into a T25 tissue culture flask for expansion. The isolated, purified SC exhibited 97–99% purity as assessed by immunostaining with S100, a Schwann cell-specific marker. The complement lysis was repeated twice to reach this required high purity population of Schwann cells. Schwann cells from passages four to nine with purity >97% were used for all experiments in this study.

2.2. Neuron isolation

Neurons were isolated from P2 neonatal rat dorsal root ganglion (DRG) (Sprague Dawley, P2; Taconic Farms, Inc.), as previously described (Seggio *et al* 2008, 2010). The DRG were dissociated using both enzymatic digestion and mechanical trituration. The DRG were digested for 50 min in a 0.1% trypsin (MediaTech Inc.) and 1 mg mL^{-1} collagenase A (Sigma Aldrich) solution prepared in a 1× Hanks balanced salt solution (HBSS; Fisher Scientific, Pittsburgh, PA). The DRG were then further digested in a 0.1% trypsin in the HBSS solution for 10 min, centrifuged at 100× *g* and mechanically triturated 15 times using a flame-polished glass Pasteur pipette. The dissociated cells were cryopreserved at a density of 1 × 10^6 cells mL^{-1} in freezing medium (base medium supplemented with 10% dimethyl sulfoxide (Sigma Aldrich)). Aliquots were placed in the −80 °C freezer for 24 h before being transferred to liquid nitrogen for longer-term storage. Prior to seeding experiments, cryopreserved aliquots stored in liquid nitrogen were thawed in a water bath (37 °C) with gentle agitation.

2.3. Culture chamber design and construction

Culture chambers utilized in this work were modified from McCaig and Rajnicek (1991) and Pedrotty *et al* (2005). Sylgard 184 poly(dimethylsiloxane) base and curing agent (PDMS; Dow Corning, Midland, MI) were combined at a 10:1 wt/wt ratio and poured into rectangular Nuncleon plates to form a 1.5 mm thick layer (Thermo Fisher Scientific, Waltham, MA) (figure 1). Two identical chambers (7 mm × 30 mm × 1.5 mm) were excised from the PDMS sheet within the rectangular plate using a scalpel to form an experimental and a control. These chambers were covered with a 1 mm thick PDMS sheet to form the top of the chambers. A piece of 5 mm ID silicone tubing (Thermo Fisher Scientific) was placed at the end of each chamber and bonded with liquid PDMS to the top PDMS layer to form on-plate internal salt bridges. To avoid electrically induced reaction by-products or pH changes in the cell culture chambers, internal salt bridges were filled with 2% agarose (Fisher Scientific) in Steinberg's solution (Tanaka and Kirschner 1991). An external agar salt bridge was also constructed by filling 3 mm ID glass tubing with 2% agarose in Steinberg's solution (McCaig 1990). Steinberg's solution was then added above the internal salt bridges at both ends of the chamber to provide a conductive medium (figure 1).

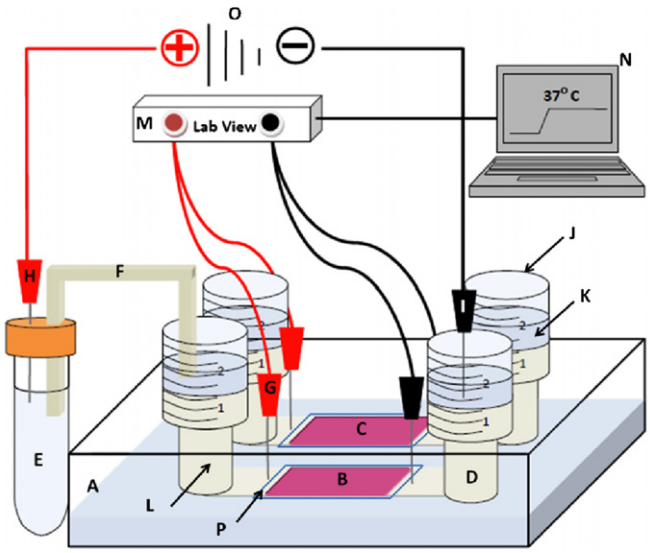

Figure 1. Electrical stimulation chamber. (A) Nunc plate housing both the experimental and control chambers excised from PDMS. (B) The experimental cell chamber (0–100 mV mm^{-1}). (C) Control cell chamber (0 mV mm^{-1}). (D) Internal salt bridges protect the cells from ionic by-products. (E) Steinberg's solution is added to allow electrical conduction. (F) An external salt bridge connects the positive electrode to the experimental chamber. (G) Reference platinum (Pt) electrodes are used to monitor the voltage drop across the chambers. (H) Positive platinum electrode. (I) Negative platinum electrode. (J) Modified syringes house internal salt bridges and create a well to hold Steinberg's solution (K). (L) Silicone tubing connects Steinberg's wells with internal salt bridges and culture chambers. (M) A LabView voltage pass-through and conditioning block are used to monitor the voltage drop across the chambers. (N) LabView is run on a PC. (O) Constant current dc power supply. (P) Glass microscope slides seal each chamber.

A biological grade platinum electrode (1 mm ID, Sigma Aldrich) connected to the power supply was inserted into a 15 mL centrifuge tube filled with Steinberg's solution to serve as the anode. The cathode was connected to the power supply and was inserted directly into the silicone tubing on the experimental chamber. Biological grade platinum wire (A–M systems, Inc., Sequim, WA) reference electrodes placed directly at the entrance and the exit of each chamber (just beyond the internal salt bridges) individually monitored the voltage drop using LabView and a PC. Constant currents (1 mA) were applied using a dc power supply (Marlin P. Jones & Assoc., Inc., Lake Park, FL) to electrically stimulate

the cells, generating electric fields (EF) ranging from 0 to 100 mV mm^{-1} (figure 1). The thin, rectangular chamber geometry generates a uniform electric field within the chamber.

2.4. Electrical stimulation of Schwann cells

Acid-etched glass coverslips (Belco Glass, Vineland, NJ) were coated with 100 μg mL^{-1} of poly-L-Lysine (Sigma Aldrich) for 30 min and rinsed three times with phosphate buffered saline (PBS; Cambrex, East Rutherford, NJ). Schwann cells were seeded at 2.5×10^4 cells cm^{-2} on the PLL-coated coverglass in six-well tissue culture plates and cultured overnight. This sub-confluent density allowed for any changes in the Schwann cell number to be visualized and measured as a result of electrical stimulation. Prior to electrical stimulation, the Schwann cell-seeded coverslips were rinsed with warm 1× PBS to remove any non-adherent cells and Schwann cell growth medium was added to both culture chambers. The cell-seeded coverslips were placed in each chamber (experimental and control) and incubated for 30 min at 37 °C (5% CO_2) prior to sealing the chambers with clean microscope slides (Fisher Scientific) to maintain a uniform field for the duration of the experiment.

A dc voltage was applied at a constant current of 0.1 mA to the experimental chamber to generate electrical fields of 0 (control), 10, 50 and 100 mV mm^{-1} for 8 h. An 8 h stimulation time was chosen based on preliminary studies within the range of reported stimulation times (data not shown) (De Boni and Anderchek 1986, Wood and Willits 2009). Using LabView software, an SC-2345 signal conditioning block and 6024E DAQcard™ (National Instruments, Austin, TX) continuously monitored both the voltage drop across the chambers (experimental and control) and the chamber temperature every 2 s for the duration of the experimental treatment. Both the temperature and voltage drop remained stable for the experimental treatment with no more than a 1% change detected. No voltage was detected in the unstimulated control chamber (data not shown). Following treatment, the chambers were unsealed and cells were fixed in a 4% paraformaldehyde, 4% sucrose (Sigma Aldrich) fixative prepared in a 2xPHEM buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 2 mM $MgSO_4$, pH 7.0 with KOH in DI H_2O; Sigma Aldrich) for 20 min.

2.5. Electrical stimulation of neurons

Cleaned, acid-etched coverglass was coated with 50 μg mL^{-1} of laminin (BD Biosciences, San Jose, CA) for 30 min and rinsed three times with PBS. Dissociated neurons were seeded at 7.1×10^3 cells cm^{-2} on the laminin-coated coverslips in SC growth medium supplemented with 25 ng mL^{-1} 2.5S nerve growth factor (NGF; Invitrogen). This serum-containing medium was used to maintain consistent conditions between the neuron-only and neuron–Schwann cell co-culture experiments. This low seeding density was chosen as it allows individual neurons to be imaged and quantitatively analyzed for morphometric properties to various stimuli without interfering neuron–neuron contact. The cells were incubated (37 °C, 5% CO_2) for 4 h to allow cell attachment without any visible neurite outgrowth. Prior to electric stimulation, the chambers were rinsed with warm PBS removing any non-adherent cells and fresh, warmed medium was added to the chambers. The chambers were incubated for 30 min prior to sealing the chambers with cleaned glass microscope slides, as described above. The experimental chamber was stimulated for 8 h by the application of a dc voltage at a constant current (0.1 mA) generating electric fields of 10, 50 or 100 mV mm^{-1}, while no voltage was applied to the unstimulated control chamber. Following treatment, the neurons were fixed for 20 min as previously described.

2.6. Electrical stimulation of neuron–Schwann cell co-cultures

Schwann cells were seeded onto poly-L-Lysine-coated acid-etched glass coverslips at 2.5×10^4 cells cm^{-2} and cultured overnight in six-well plates containing Schwann cell growth medium to generate a 70–80% confluent substrate. The Schwann cell-seeded coverglass was rinsed with 1× PBS to remove any non-adherent cells, and dissociated neurons were seeded at 7.1×10^3 cells cm^{-2} in Schwann cell growth medium supplemented with 25 μg mL^{-1} NGF. Neurons were co-cultured on the Schwann monolayers for 4 h allowing for neuronal attachment on top of the Schwann cells, as described above. Immediately prior to electrical stimulation, the co-cultures were rinsed to remove any non-adherent cells and placed within the experimental and control culture chambers. Warm medium (supplemented with NGF) was added to the chambers, and incubated (37 °C and 5% CO_2) for 30 min prior to sealing.

A dc voltage was applied to generate an electric field of 50 mV mm^{-1} across the experimental chamber for 8 h while the control chamber remained unstimulated (0 mV mm^{-1}). A 50 mV mm^{-1} electric field was selected as it generated the most neurite outgrowth and longest neurite (see section 3.2). Samples were immediately fixed post-stimulation.

2.7. Immunofluorescent staining

Fixed samples were rinsed three times with PBS and permeabilized for 5 min with 0.1% Triton X-100 (Sigma Chemical) prepared in PBS.

The Schwann cell samples were blocked for 1 h at room temperature in 2.5% goat serum in PBS and incubated with rabbit anti-S100 primary antibody (1:400 v:v in 2.5% goat serum (CellGrow); Dako North America, Inc., Carpinteria, CA) at room temperature for 30 min. The samples were rinsed three times for 5 min each and incubated with goat anti-rabbit AlexaFluor 546 secondary antibody (1:1000 v:v in 2.5% goat serum; Invitrogen) for 30 min.

The neuron samples were blocked for 1 h at room temperature in 2.5% goat serum in PBS and incubated with mouse anti-β-III-tubulin primary antibody (1:500 in 2.5% goat serum; Invitrogen) for 1 h at room temperature. The labeled samples were rinsed three times each in PBS for 5 min and then incubated for 1 h with goat-anti-mouse Alexa Fluor 488 IgG_{2b} secondary antibody (Invitrogen) at room temperature (1:1000 v:v in 2.5% goat serum).

Similarly, the neuron–Schwann cell co-cultures were blocked for 1 h in 2.5% goat serum. The samples were simultaneously incubated with both an anti-β-III-tubulin primary to label the neurons and a rabbit anti-S100 primary to label the Schwann cells for 1 h, as noted above. Following primary antibody incubation the co-culture sample was rinsed three times in PBS for a total of 15 min. Following the last rinse, goat-anti-rabbit IgG Alexa Fluor 546 (Invitrogen; 1: 400 v:v in 2.5% goat serum) and goat-anti-mouse IgG_{2b} Alexa Fluor 488 (Invitrogen; 1:1000 v:v in 2.5% goat serum) were applied simultaneously for 1 h at room temperature to visualize Schwann cells and neurons, respectively.

All labeled samples were rinsed three times for 5 min each in PBS and mounted on a glass slide with Prolong Gold anti-fade containing 4′,6-diamidino-2-phenylindol (DAPI; Invitrogen) to label all cell nuclei present. These sealed samples were stored at −20 °C prior to imaging.

2.8. Microscopy and image analysis

Samples were visualized using an Olympus IX81 inverted microscope (Olympus, Center Valley, PA) in fluorescence mode with a 10× dry objective. For the Schwann cell samples, five images were taken at evenly spaced, predetermined locations across the experimental and control chambers. For the neurons and neuron–Schwann cell co-cultures, every individual neuron (not in contact with adjacent neurons) was imaged using a 20× dry objective to obtain individual neuronal morphometric measurements. Images were acquired with Metamorph image acquisition software, which allowed for montages at 20× to be obtained and automatically stitched (Molecular Devices, Downingtown, PA).

Schwann cell orientation was approximated by measuring the orientation of every cell nuclei using NIH ImageJ 1.41 (National Institutes of Health), as previously described (Thompson and Buettner 2004, Seggio *et al* 2010). The fluorescent images of DAPI stained nuclei were inverted and thresholded, and best-fit ellipses were assigned to each nuclei automatically. The major and minor axes for each ellipse were determined and the resultant nuclear orientation (angle of the major axis relative to the horizontal plane) was calculated. To visualize the distribution of Schwann cell orientation, nuclear orientation angles were binned every 10° and the frequency distribution was plotted between 0° and 180° (Thompson 2001), with 90° being in the direction of the electric field. The frequency distributions for each image per sample were calculated and were then averaged to describe each treatment.

Neurons were semi-automatically traced using Neurolucida software (MBF Bioscience, Wilmington, VT) and quantitatively analyzed with respect to various morphometric features such as total neurite outgrowth, number of primary neurites, branching and orientation of neurite outgrowth (Seggio *et al* 2008). Total neurite outgrowth is the sum of the actual traced path length of each neurite for a given neuron. The number of primary neurites is defined as the number of neurites directly emerging from the soma. The number of branch points is normalized to total outgrowth to report the total number of branches per micron of neurite outgrowth, allowing ease of comparison between neurons of varying lengths. Neurite orientation was visualized by using a polar histogram to group all outgrowth on a point-by-point basis into 10° bins from 0 to 360° (approximately every 0.2 μm).

2.9. Statistics

Schwann cell electrical stimulation data were collected from three separate experiments ($n = 3$) for all treatment conditions (0, 10, 50, 100 mV mm^{-1}), with five images per sample, and three samples per experiment. Each image contained roughly 250 cells. Five images were chosen, evenly divided along the 20 mm long coverglass with two mm spacing between each image, avoiding edges. Neuron and neuron–Schwann cell co-culture electrical stimulation data were collected from three separate experiments ($n = 3$), with ten neurons per condition per experiment randomly selected from all the individual neurons imaged. Anderson–Darling normality tests were completed in Minitab (Minitab Inc., State College, PA) on the data sets and indicated that data were parametric. Statistical significance was determined using Minitab software to perform a two-tailed ANOVA. p-values < 0.05 were considered statistically significant. A minimum of ten neurons per group were calculated using a one-sample t-test (Minitab Inc.) in order for each experiment to have a 99% power to detect ($\alpha = 0.05$) a maximum difference of 251 μm within total outgrowth of control (unstimulated) neuron populations, assuming an average variability of 143 μm based on preliminary experiments.

3. Results

3.1. Schwann cell morphology is not altered by a persistent physiologic electrical stimulation (0–100 mV mm^{-1})

To evaluate Schwann cell responsiveness to low level electric fields, Schwann cells were electrically stimulated for 8 h in fields ranging from 0 to 100 mV mm^{-1}. The temperature and voltage drop across the experimental and control chambers were monitored and remained stable for the duration of the 8 h treatment. Following treatment, the cells were fixed and immunostained and no qualitative differences were observed between the unstimulated and electrically stimulated Schwann cells (0, 10, 50, 100 mV mm^{-1}) (figures 2(A)–(D)). All cells exhibited similar morphology, with a random, unbiased orientation and bipolar morphology (figures 2(A)–(D)). Quantitatively, the Schwann cells did not exhibit any significant bias relative to the generated electric field (figure 2(E); 90° is the direction of the electric field).

3.2. Neurite outgrowth is enhanced by moderate electrical stimulation

Neuron morphology was qualitatively examined following a similar 8 h electrical stimulation to visually determine if

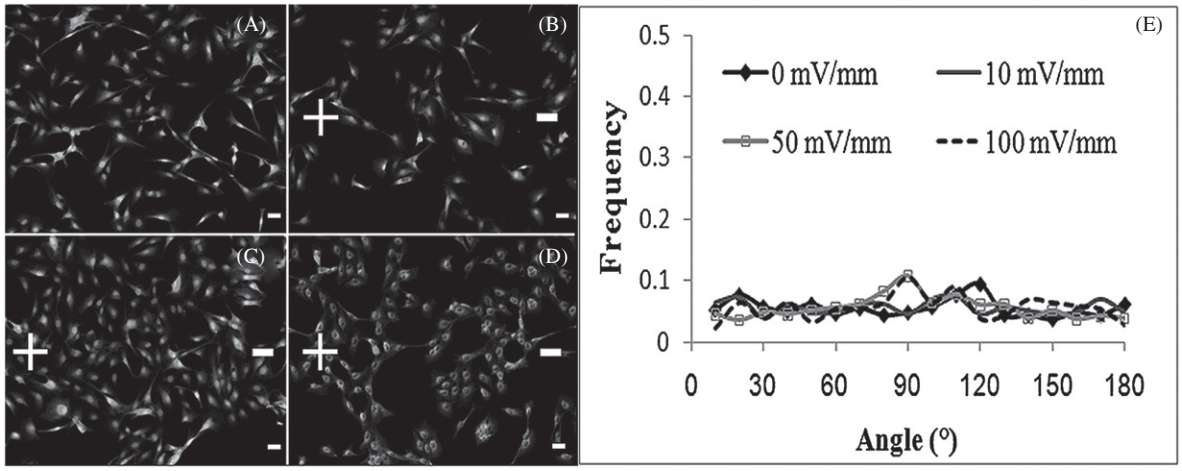

Figure 2. Schwann cells do not exhibit a directional bias in applied dc electric fields ranging from 0 to 100 mV mm^{-1}. Immunofluorescent images of Schwann cells were captured immediately following 8 h stimulation with (A) 0 mV mm^{-1}; (B) 10 mV mm^{-1}; (C) 50 mV mm^{-1}; and (D) 100 mV mm^{-1}. The direction of the generated electric field is labeled above (±). Samples were fixed immediately post-stimulation and stained for S100 to label the primary neonatal Schwann cell body and DAPI to identify all nuclei prior to analysis. Upon visual inspection, there was no change in Schwann cell alignment, indicating that electrical stimulation does not elicit a polar bias. Bar = 100 μm. (E) Similarly, the frequency distribution of Schwann cell orientation measured using ImageJ did not exhibit any bias toward or in opposition to the generated electric field. 90° indicates the direction of the electric field.

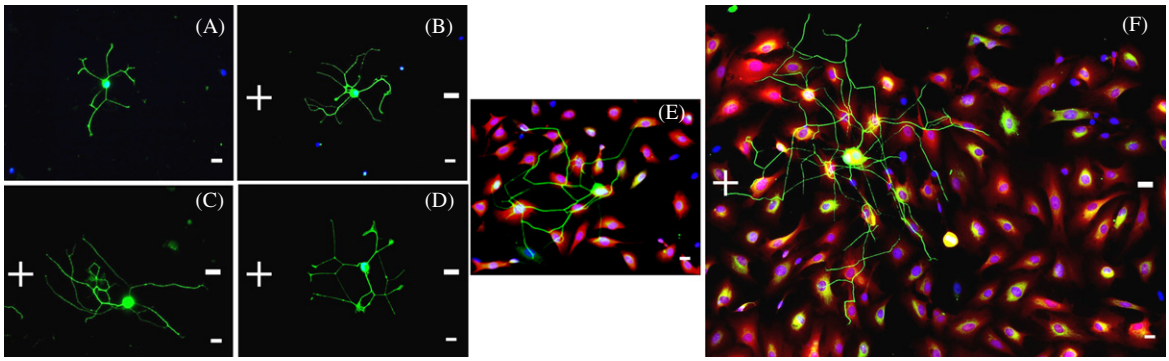

Figure 3. Neurite outgrowth increases with simultaneous presentation of electric stimulation and Schwann cells. Samples were stimulated for 8 h with a dc voltage at constant current. (A) Control, unstimulated dissociated neurons cultured on a permissive laminin substrate in 25 ng mL^{-1} of NGF exhibited only modest outgrowth (0 mV mm^{-1}). (B) Neurons stimulated with an electric field of 10 mV mm^{-1} or (D) 100 mV mm^{-1} exhibited growth similar to the control (A). (C) Neurons exposed to a moderate (50 mV mm^{-1}) electric field or (E) co-cultured with Schwann cells exhibited increased outgrowth relative to the unstimulated control. (F) The simultaneous co-presentation of electric stimulation in co-culture with Schwann cells resulted in the most extensive neurite outgrowth relative to either single stimuli (electrical stimulation or Schwann cell co-culture). The direction of the electrical field (±) is shown in (B)–(D) and (F). Green = anti-β-III-tubulin (neurons), red = anti-S100 (Schwann cells), blue = DAPI (nuclei); bar = 50 μm, $n = 3$.

any electrically induced changes in outgrowth and/or bias in outgrowth relative to the generated electric field were observed. Both the unstimulated control, as well as the 10 and 100 mV mm^{-1} electrically stimulated neurons, exhibited similar morphologies (figures 3(A), (B), (D)). However, when stimulated with a moderate dc electric field (50 mV mm^{-1}), neurite outgrowth appeared to significantly increase relative to all other treatments (0, 10 or 100 mV mm^{-1}; figure 3(C)).

As expected, neurite outgrowth is more extensive in co-culture with Schwann cells relative to neurons cultured alone (Ard *et al* 1987, Bunge 1987, Thompson and Buettner 2006, Seggio *et al* 2010). This increase in neurite outgrowth following co-culture (figure 3(E)) was visibly similar to the electrically stimulated outgrowth following a moderate 50 mV mm^{-1} stimulation in the absence of Schwann cells. The co-presentation of a moderate electrical stimulation (50 mV mm^{-1}) in co-culture with Schwann cells appeared to support robust outgrowth greater than each individual cue presented alone (50 mV mm^{-1} electrical stimulation or Schwann cell co-culture; figure 3(F)).

3.3. Total neurite outgrowth and the longest neurite significantly increase following a moderate electrical stimulation (50 mV mm^{-1})

Neuron morphology was quantitatively examined using Neurolucida software to quantify morphometric changes due to the electrical stimulation (figure 4). Physiologically

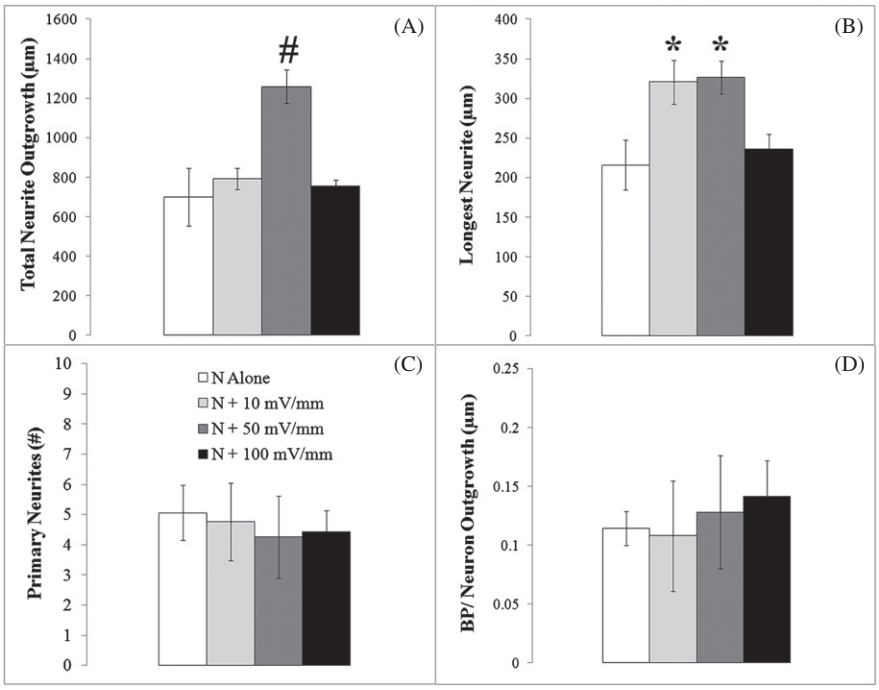

Figure 4. Both total neurite outgrowth and longest primary neurite increase in the presence of electrical stimulation. (A) The mean total neurite outgrowth significantly increased following 50 mV mm^{-1} stimulation ($p < 0.05$). $^{*}p < 0.05$ compared to control (N alone) or 10 mV mm^{-1} (N + 10 mV mm^{-1}), #$p < 0.05$ compared to all other conditions. (B) The average longest neurite increased following the 10 mV mm^{-1} or 50 mV mm^{-1} electric stimulation relative to unstimulated control neurons and neurons stimulated by 100 mV mm^{-1}. Regardless of the treatment, the number of primary neurites (C) and normalized branching (D) were not significantly different due to an 8 h electrical stimulation at field strengths of 0, 10, 50 or 100 mV mm^{-1} ($p > 0.05$). Branch points were normalized to total outgrowth per neuron. $n = 3$ for all conditions, error bars represent standard deviation.

relevant electric stimulation was applied to the cells from 0 to 100 mV mm^{-1}. Moderate stimulation (50 mV mm^{-1}) resulted in a significant 1.8-fold increase in neurite outgrowth (1260 μm; figure 4(A)), while both the low (792 μm; 10 mV mm^{-1}) and high (756 μm; 100 mV mm^{-1}) stimulation did not significantly influence neurite outgrowth relative to the unstimulated control (700 μm; $p < 0.008$). The longest neurite length following either a 10 or 50 mV mm^{-1} increased relative to the unstimulated control by roughly 1.5-fold, an increase in length from 215.6 to 320.8 μm and 326.4 μm, respectively ($p < 0.02$; figure 4(B)). Application of the higher stimulation (100 mV mm^{-1}) did not significantly change the average longest neurite length relative to control (235.6 μm; $p = 0.74$).

The increase in total neurite outgrowth following low or moderate electrical stimulation was not due to an increased number of primary neurites or more extensive branching. There were no significant differences in primary neurites following electrical stimulation (10–100 mV mm^{-1}; figure 4(C)). Neuron branching was normalized (the number of branch points per micron of total neurite outgrowth) to directly compare branching between treatment groups with varying amounts of outgrowth. Regardless of the treatment condition (0, 10, 50 or 100 mV mm^{-1}), the normalized neurite branching was not significantly different, ranging from ~0.14 to 0.48 branch points per micron of outgrowth ($p > 0.05$; figure 4(D)).

3.4. Co-stimulation with both a moderate electrical stimulation and Schwann cells results in a significant increase in neurite outgrowth

Application of a moderate electrical stimulation (50 mV mm^{-1}) maximized total neurite outgrowth (figure 4) and no changes in Schwann cell morphology were observed following stimulation ranging from 0 to 100 mV mm^{-1}. Therefore, a moderate electric field of 50 mV mm^{-1} was chosen for co-stimulation experiments to evaluate the potential synergy between these cues. Neurons co-cultured with Schwann cells (N + SC) or neurons exposed to moderate electrical stimulation (N + 50 mV mm^{-1}) both exhibited a roughly two-fold increase in neurite outgrowth (1423 μm, 1258 μm) relative to the unstimulated neuron controls (N; 700 μm; $p < 0.07$; figure 5(A)). Therefore, both of these cues (electrical stimulation or Schwann cell co-culture) produced increases in outgrowth that are roughly similar in magnitude.

Simultaneous co-stimulation with moderate electrical stimulation (50 mV mm^{-1}) and Schwann cell co-culture (N + SC + 50 mV mm^{-1}) further increased the total outgrowth 3.2 times greater than the unstimulated controls, resulting in 2229 μm of neurite outgrowth ($p < 0.01$; figure 5(A)). The simultaneous presentation of both cues (N + SC + 50 mV mm^{-1}) resulted in roughly 1.6 times more outgrowth than either unstimulated Schwann cell co-culture (N + SC) or electrically stimulated neurons alone (N + 50 mV mm^{-1}).

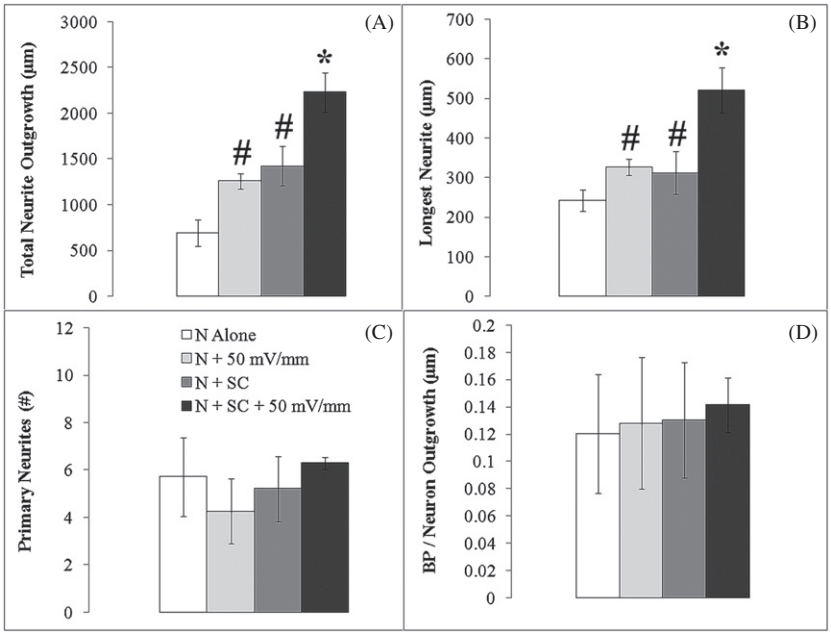

Figure 5. Both total neurite outgrowth and longest primary neurite increase with increasingly complex presentation of cues. (A) Neurite outgrowth significantly increased to a similar level following electrical stimulation with 50 mV mm^{-1} or SC co-culture. A further increase was reported following co-stimulation with both Schwann cells and an electrical field of 50 mV mm^{-1} ($p < 0.05$). $^{*}p < 0.05$ compared to all conditions, #$p < 0.05$ compared to control (N alone). (B) SC-EF co-stimulation (N + SC + 50 mV mm^{-1}) resulted in significantly longer average longest neurite across all conditions (#), while single cues (SC or 50 mV mm^{-1}) significantly increased relative to unstimulated controls (N alone; *). (C) The number of primary neurites and (D) the normalized branching across all conditions were not significantly different ($p > 0.05$). Branch points (BP) were normalized to the total outgrowth per neuron to allow the direct comparison of BP per micron of outgrowth. $n = 3$ for all conditions. Error bars represent the standard deviation.

All treatments significantly increased outgrowth over control unstimulated neurons (N).

Similarly, the mean length of the longest neurite was maximized by the co-presentation of a moderate electrical stimulus in co-culture with Schwann cells (N + SC + 50 mV mm^{-1} 520.2 μm), resulting in a roughly 2.4-fold greater increase in length relative to the unstimulated control (215.6 μm; $p < 0.006$; figure 5(B)). Presentation of individual cues of either Schwann cell co-culture (N + SC) or moderate electrical stimulation (N + 50 mV mm^{-1}) increased the mean length of the longest neurite 1.4-fold ($p > 0.05$) and 1.5-fold ($p < 0.02$) relative to the unstimulated control (figure 5(B)). The observed increase in neurite outgrowth can be attributed to longer neurites and is not due to an increase in either primary neurites (figure 5(C)) or branching (figure 5(D)). The number of primary neurites was not significantly different and ranged from 4.2 to 6.0 ($p > 0.05$; figure 5(C)). Branching for all treatment conditions was not altered relative to unstimulated neurons (control) and ranged from ~0.14 to 0.48 branch points per micron ($p > 0.05$; figure 5(D)).

3.5. Neurite outgrowth is not directionally biased in the applied electric field

Polar histograms of neurite outgrowth were plotted in Neurolucida Explorer to visualize any directional bias with respect to the applied electrical stimulation (0, 10, 50 or 100 mV mm^{-1}). While the plots confirm an increase radially in neurite outgrowth due to the presentation of one or more cues (electrical stimulation, Schwann cell co-culture or multiple cues presented in tandem), no directional bias was observed (figure 6).

4. Discussion

Neurite outgrowth was examined in the presence of two cues: co-culture with Schwann cells (cellular) and/or electrical stimulation (biophysical). Following 8 h of electrical stimulation (0–100 mV mm^{-1}), a moderate electric field of 50 mV mm^{-1} resulted in significantly greater neurite outgrowth than both the unstimulated controls and all other field magnitudes tested (10, 100 mV mm^{-1}; figures 3 and 4). This observed increase in neurite outgrowth was due to longer neurites as both the low (10 mV mm^{-1}) and moderate electric fields (50 mV mm^{-1}) had significantly longer neurite length and total neurite outgrowth compared to both unstimulated controls and the highest electric field of 100 mV mm^{-1}. During embryogenesis, strong and stable electric fields are known to occur and have been recorded to measure 21–140 mV mm^{-1} (Barker 1982, McGinnis and Vanable 1986, Hotary and Robinson 1990, 1994, Chiang *et al* 1992, Shi and Borgens 1995). Based on these reports, a range of 0–100 mV mm^{-1} was chosen due to its physiological relevance and low-level fields may be more readily tolerated for longer stimulation periods if clinically translated.

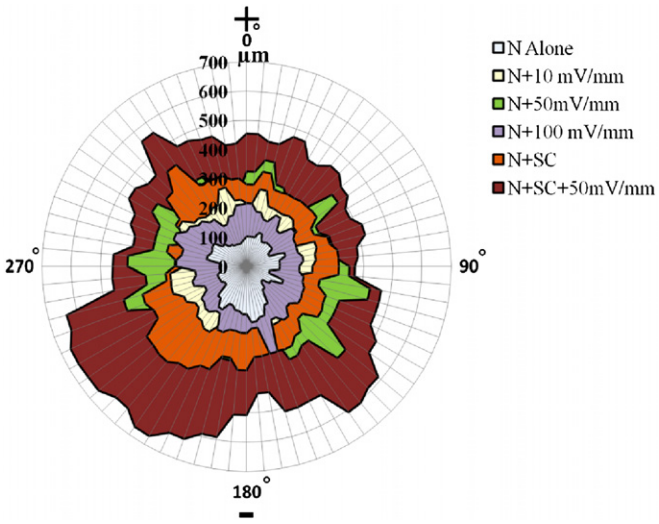

Figure 6. Polar histograms of the mean total neurite outgrowth illustrate that no directional bias relative to the EF stimulation was observed for various electric fields applied (0 (grey), 10 (cream), 50 (green), 100 (purple) mV mm^{-1}), in co-culture with Schwann cells (orange), or co-stimulation with Schwann cells and a moderate electric field (50 mV mm^{-1}; N + SC + 50 mV mm^{-1} (red)). Neurite outgrowth increased following a low or high electrical stimulation (10 or 100 mV mm^{-1}) relative to unstimulated neurons (control). Neurons in co-culture with Schwann cells or electrically stimulated by a moderate field of 50 mV mm^{-1} exhibited significantly more outgrowth than the lower or higher (10 or 100 mV mm^{-1}) fields tested. The co-presentation of both cues (Schwann cell co-culture + moderate electrical stimulation – 50 mV mm^{-1}) resulted in a dramatic 3.2-fold increase in neurite outgrowth relative to the unstimulated control and was significantly greater than the presentation of either individual cue. The orientation of the electric field is shown (0–180°), $n = 3$ for each condition.

Alternate studies with fish karatocytes, bovine corneal epithelial cells, rat corneal epithelial cells and P0-P3 rat hippocampal neurons have reported directional electrostatic migration due to applied or endogenous electric fields (Lois *et al*, Zhao *et al* 1996, 1999, 2006, Rajnicek *et al* 2007, Yao *et al* 2008, 2009). Similarly, cell organelles within dissociated day P0–P3 rat hippocampi asymmetrically align along an applied electric field ranging from 50 to 300 mV mm^{-1} over a 1–2 h stimulation, leading to oriented cellular division (Yao *et al* 2009). During development, electric fields present in the brain have been reported to transiently reach >300 mV mm^{-1} and cell sensitivity to these cues may be preserved beyond the developmental stages (McCaig *et al* 2005, Cormie and Robinson 2007). These studies provide evidence that electrical stimulation plays a role *in vivo* and cell behavior is influenced by this biophysical stimulation. Electrical gradients in the PNS have not been widely studied likely due to the difficulty in making measurements on the stromal side of the wound which is limited by the cornified layer of skin. However, investigating both neurons and Schwann cell responsiveness to electric field strength may provide new treatment parameters to apply following nerve injury.

Due to the ability of electric fields to orient glia of the CNS (astrocytes), Schwann cell response to physiologically relevant constant current dc electric fields (0–100 mV mm^{-1}) was examined for possible changes in Schwann cell alignment (Alexander *et al* 2006). Independent or co-culture stimulation of Schwann cells did not result in any distinct morphological changes or alignment with or in opposition to the generated electric field after 8 h of stimulation (figure 2). Preliminary experiments at higher electric fields (125–150 mV mm^{-1}) did not demonstrate any alignment relative to the electric field generated and electric fields greater than 200 mV mm^{-1} resulted in abnormal cell morphology and low viability (data not shown). While alignment of astrocytes perpendicular to an electric field has been reported, Schwann cells may not have retained sensitivity to electrical stimulation beyond the embryonic stage or, alternatively, longer stimulation times may be required to elicit morphological changes. Control of Schwann cell orientation is important as local Schwann cell orientation has been quantitatively shown to direct local neurite outgrowth (Seggio *et al* 2010). While no directional bias was observed in this study, McKasson *et al* in 2008 reported that electrical stimulation of embryonic chick glial cells exhibited a migratory bias toward the anode in fields ranging from 3 to 100 mV mm^{-1}. While avian glia were shown to be responsive to the induced biophysical forces generated by the electric field (McKasson *et al* 2008), perhaps primary neonatal rat Schwann cells are not. Future studies are planned to characterize any electrically induced changes in Schwann cell migration due to the application of electrical stimuli.

While no morphological changes to the electrically stimulated Schwann cells were observed, Huang *et al* (2010) reported an increase in the Schwann cell-mediated release of NGF in a calcium-dependent manner following a high but not constant electrical stimulation (1 Hz, 500 mV mm^{-1}) (Huang *et al* 2010). This stimulation resulted in a release of roughly 426 pg mL^{-1} of soluble NGF 12 h after stimulation (1 Hz, 500 mV mm^{-1}), which was 1.6-fold over unstimulated control Schwann cells. The addition of two times greater NGF to neuron culture media only resulted in neurite outgrowth comparable to the moderate electric stimulation (50 mV mm^{-1}) or Schwann cell co-culture alone (∼two-fold increase, data not shown). However, the simultaneous co-stimulation with Schwann cells and a moderate electric field of 50 mV mm^{-1} (N + SC + 50 mV mm^{-1}) had robust outgrowth that doubled outgrowth from neurons stimulated by single cues. Furthermore, we demonstrated that the application of moderate electrical stimulation was sufficient to promote neurite outgrowth to a similar level as neurons in co-culture with Schwann cells or neurons exposed to 2× more NGF (N + 50 ng mL^{-1} NGF). This provides some evidence that Schwann cell-mediated release of NGF is not wholly accountable for the robust increase in outgrowth during co-stimulation. While the Schwann cells alone are likely to undergo phenotypic changes by releasing an increased level of neurotrophic factors during electrical stimulation, as demonstrated by Huang *et al* 2010, electrical stimulation of neurons in the absence of Schwann cells resulted in increased outgrowth.

Directional increases in neurite outgrowth have been reported in constant dc electric fields where chick DRG explants grew faster toward the cathode than the anode ((Jaffe

and Poo 1979); 70–140 mV mm^{-1} for a 6.4 h stimulation). Similarly, several groups have reported faster growth toward the cathode in *Xenopus* cultures ((McCaig 1990) 100–150 mV mm^{-1} following a 5 h stimulation; (Patel and Poo 1982) 100 mV mm^{-1} following a 6 h stimulation; (Hinkle *et al* 1981) 7–190 mV mm^{-1} following a 20 h stimulation). However, our findings using a neonatal rat model did not demonstrate any directional response, but increased outgrowth was observed, which contradicted earlier reports from De Boni and Anderchek (1986), who found no response in neurite outgrowth from E16–18 mouse DRG subjected to a steady electric field ranging from 5 to 50 mV mm^{-1} for up to 48 h (De Boni and Anderchek 1986). Differences in neuronal responsiveness could be related to differences between age and/or species. Similarly, stimulation parameters required for a biological response may have not been sufficiently high to elicit enhanced outgrowth in the De Boni and Anderchek (1986) study. These results emphasize the need for further research in this area to characterize both the responsiveness of cells at the injury site and the mechanistic changes encountered due to the application of this biophysical stimulus.

While we reported enhanced neurite outgrowth, no directional bias was observed for both stimulated rat neonatal Schwann cells and neurons following 8 h of constant stimulation (0, 10, 50 or 100 mV m^{-1}; figure 5). This is in contrast to embryonic neurons from non-mammalian systems such as *Xenopus* (Patel and Poo 1982, McCaig 1990) or zebrafish (Cormie and Robinson 2007). The reported directionally biased neurite outgrowth may be explained in part to an enhanced responsiveness of embryonic amphibian cells to electrical stimulation relative to their mammalian cell counterparts, in part to their regenerative properties. DeBoni and Anderchek report perhaps the only examination of focal electric fields applied to human neuronal growth cones (20 from DRG, 3 from spinal explants) *in vitro*, with applied fields ranging from 0.2 to 42.4 mV mm^{-1} (0.5–100 nA) for durations up to 50 min (De Boni and Anderchek 1986). Results indicate that eight of these growth cones exhibited a cathodal preference, but only when the focal electrode acted as the current sink, which created a gradient, dissimilar to the uniform field applied in this study. Despite the growth cone response, the filopodia exhibited no anodal or cathodal preference in fields of 0–100 mV mm^{-1} for 2–6 h. The only change reported was an increase in the oscillatory behavior of the filopodia stimulated in a 5 mV mm^{-1} constant dc field. The increased filopodial activity indicates responsiveness to the applied electric stimulation, but perhaps directional bias or increased outgrowth may only occur at a narrow range of field strengths and durations. It is also possible that field strength may only serve to stimulate outgrowth, rather than directionally direct outgrowth.

While no directional bias was observed following electrical stimulation, the resultant increase in neurite outgrowth was significant. Application of single cues (Schwann cells co-culture or moderate (50 mV mm^{-1}) electrical stimulation) resulted in a significant increase in both the total outgrowth and longest neurite length in comparison to the unstimulated control (figure 5). Electrical stimulation of neurons co-cultured with Schwann cells resulted in a 114% increase in total outgrowth relative to any of the single cues (10 or 100 mV mm^{-1} or Schwann cell co-culture (figure 5)) with no directional bias observed (figures 5 and 6). This electrically induced increase in neurite length is a desirable effect and may serve to efficiently promote regrowth following injury. Furthermore, electrical stimulation at the injury site in conjunction with an aligned scaffold or pre-aligned glia may serve to both direct and promote robust axonal growth more efficiently. Further work is required to elucidate the exact mechanisms by which electrical stimulation affects both neurons and Schwann cells and determine optimal combinations for both cellular (Schwann cells) and biophysical (electric stimulation) cues to promote functional repair of nerve injuries.

In vivo application of electrical stimulation has demonstrated a mild response overall, and therefore optimizing stimulation parameters *in vitro* may improve clinical results. The incorporation of an oscillating electric field within a canine spinal cord injury model demonstrated promise; however, the observed increase in ambulation was not prominent upon translation to human clinical trials. This may be due to the complexity of the injury site where the local field strength (biophysical cue) present is not well characterized (Borgens 1999, Shapiro *et al* 2005). Furthermore, brief electrical stimulation (100 μs, 3 V, 20 Hz, 1 h duration) has been shown to accelerate motor neuron regrowth and target accuracy in adult rat femoral nerves. Electrodes were placed proximal to the wound site immediately after repair with a silastic guidance channel and results indicate that stimulation increased the release of neuro-supportive growth factors such as brain-derived neurotrophic factor (BNDF) and respective receptor tyrosine receptor kinase B (trkB) (Al-Majed *et al* 2000a, 2000b). Similar electrical stimulation regimes (100 ms, 3–5 V, 20 Hz, 1 h duration) in transected and repaired adult rat femoral trunks increased specificity of target reinnervation from 40% in unstimulated animals to 75% in stimulated rats, therefore enhancing selective regeneration (Brushart *et al* 2005). Application of ac electrical stimulation (20 Hz, 1 h) prior to surgical repair of the femoral nerve resulted in an increased rate of recovery over 6–8 months post-surgery (near max achieved 6 weeks earlier relative to unstimulated control) (Ahlborn *et al* 2007, Gordon *et al* 2007). Despite the observed increased rate of regrowth in this work, the short-term stimulation did not increase the total amount of regrowth illustrating a variable response (Ahlborn *et al* 2007).

Investigating the effects of stimulation duration (1 h, 3 h, one day, seven days, 14 days) with the same field parameters and animal model (100 μs, 3 V, 20 Hz) found that 1 h of stimulation had the most benefit for sensory axon regeneration compared to sham or longer stimulation times (Geremia *et al* 2007). Additionally, this 1 h stimulation regime was found to increase growth-associated protein 43 (GAP-43) and BDNF two days post-surgery in repaired adult rat femoral trunks. A similar release of supportive soluble factors following electrical stimulation may be

contributing to the increased regeneration reported in this study. While these studies offer important and exciting results in small animal models, the exact stimulation protocols (mode, duration, strength and frequency) for optimized repair in wounds of varying geometries and species remain unknown. The field strength present at the cellular level induced by the electrical stimulation in these *in vivo* studies may vary depending on signal attenuation in the tissue, and stimulation parameters effective in smaller animal models may not translate when moved to larger animals (Shapiro *et al* 2005). Also the authors mention that un-optimized stimulation may result in desensitization of sensory neurons to the elevated soluble factors, which may hinder long-term regenerative properties (Geremia *et al* 2007). Therefore, conducting *in vitro* studies to determine the necessary stimulation strengths at a cellular level for both neuronal and non-neuronal support cells will aid in a mechanistic understanding of electrical stimulation for neurons and glia relevant for clinical translation.

To summarize, co-stimulation of neurons and Schwann cells with applied electric fields *in vitro* creates a controllable platform to quantitatively examine cellular response. Results demonstrate that while Schwann cells do not exhibit a directional bias or changes in morphology following electrical stimulation (0–100 mV mm^{-1}), neurons in co-culture with Schwann cells exposed to a moderate stimulation (50 mV mm^{-1}) had robust neurite outgrowth relative to neurons singly presented with a range of electrical stimuli (0–100 mV mm^{-1}) or co-cultured with unstimulated Schwann cells. This observed increase in neurite outgrowth may be in part due to the release of Schwann cell-mediated factors (e.g. NGF) as well as changes in surface ligands following stimulation (Nieke and Schachner 1985, Seilheimer and Schachner 1987, 1988). This paper provides the first report of electrically induced increases in total outgrowth and length in mammalian neonatal sensory neurons co-cultured with electrically stimulated Schwann cells. Further understanding of the mechanistic changes induced in the neurons and Schwann cells by the application of the biophysical forces generated during the electrical stimulation will allow for the appropriate application of this stimulus *in vivo* for neural engineering solutions relevant to peripheral and central nervous system injuries.

Acknowledgments

This work was funded by New York State Spinal Cord Injury Research Board # C022067 (DMT). Abigail Koppes was supported by the Nanoscale Science and Engineering Initiative of the National Science Foundation under NSF Award Number DMR-0642573. The authors thank Dr Jonathan C Newell, PhD, in the Department of Biomedical Engineering and Dr Sheppard J Salon, PhD, PE, in the Department of Electrical, Computer, and Systems Engineering at Rensselaer for their helpful discussions regarding the chamber design. The authors further acknowledge the involvement of undergraduate researchers in the Biomedical Engineering Department (Ms Haley Darwish, Mr Kevin Keating, Ms Lindsay Minott, Ms Andrea Nordberg, Ms Gina Paolillo and Mr Darren Smith) for their assistance in chamber fabrication and neuronal analysis and High School student Mr Jake Cohen for the schematic diagram of the electric stimulation chamber.

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