Neurite Outgrowth is Additively Increased by Co-Stimulation with Physiologic Electric Fields and Schwann Cells

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Statement of Purpose: Previous work has separately demonstrated that neurons in co-culture with SC or electrically stimulated neurons exhibit increased neurite outgrowth[1,2]. This study investigates the potential synergistic response of co-stimulating dissociated neonatal DRG neurons with both small electric fields (EFs) and Schwann cells for neural engineering applications. Following peripheral nerve or spinal cord injury, regenerating axons must navigate through an injury site to restore connections with appropriate targets. This process is often hindered by factors such as scarring, inhibitory myelin, and cell death. Schwann cells, peripheral nerve glia, are closely associated with and support regenerating axons. Co-stimulation may further enhance regrowth following injury[1]. Our previous results have shown that SC orient in the presence of EF (results not shown) using physiologic electric fields (0-200 mV/mm) with maximal alignment at 50 mV/mm. Electric fields of 50 mV/mm were applied to primary neonatal rat neurons for 8 hr while both temperature and voltage were continuously monitored. Results indicate that neurons respond and increase total outgrowth following 8 hours of EF stimulation, and this is further increased in an additive manner when co-stimulated with primary Schwann cells.

Methods: *Cell Isolation:* Primary DRG neurons and Schwann Cells (SC) were isolated from Sprague Dawley neonatal rats (postnatal day 2 (P2) (Taconic Farms, Inc.)) using methods modified from Morrisey et al.[3] SC purity was >95% by immunostaining with S100, a SC marker (data not shown). Neurons were grown in supplemented SC media containing 25 ng/mL neuronal growth factor.[4] *Chamber Design and Electric Field Exposure:* Culture chambers utilized were modified from McCaig et al[4]. Sylgard 184 poly(dimethylsiloxane) (PDMS; Dow Corning) chambers were cut with dimensions of 7 x 30 x 2 mm. Agar bridges were built on plate and filled with 2% agar in Steinberg's Solution. One chamber was exposed to EF, and the other chamber served as the control (0 mV/mm). Neuron chambers were deposited with 50 µg/mL LN (Invitrogen) prior to seeding. Pt electrodes (1 mm ID) allowed electrical contact between the Steinberg's wells and culture chambers containing media. Pt wire (A-M systems, Inc.) reference electrodes were used measure the voltage drop across the chamber during stimulation. Constant DC currents were applied using DC power supplies (MPJA) providing EFs of 50 mV/mm. EFs were applied for 8 hours while both voltage and temperature were monitored in real time using LabView. *Fixing and Immunostaining, Cell Imaging, and Data Analysis:* Following exposure to EF, cells were fixed with 4% paraformaldehyde (Sigma Chemical) and labeled with FITC-phalloidin (Invitrogen) in 1% BSA (1:500 v:v) to visualize the actin cytoskeleton (SC) or anti-βIII-tubulin (neurons) (Invitrogen). Samples were mounted with Prolong Gold Antifade with DAPI (Invitrogen) and sealed. Samples were imaged in fluorescence mode on an Olympus IX 81 inverted microscope with a 10x objective and Metamorph software (Molecular Devices). Neurite outgrowth was characterized with Neurolucida (MBF).

Results: Following 8 hours of stimulation with a 50 mV/mm DC EF, neurites do not exhibit a directional bias (Fig. 1). However, EF stimulated neurons have roughly 2x more outgrowth than control neurons. SC co-culture continues to increase neurite outgrowth over EF alone and when neurons are co-cultured with SC and simultaneous EF stimulation they exhibit more outgrowth than with SC or EF alone (Fig. 2).

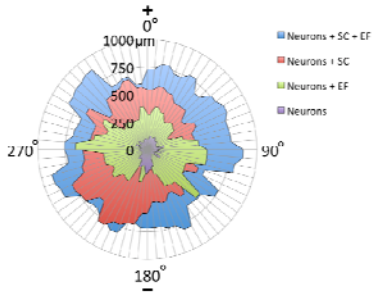

Figure 1. Polar histogram of total neurite outgrowth EF-SC co-stimulation shows no directional bias. Direction of EF is shown.

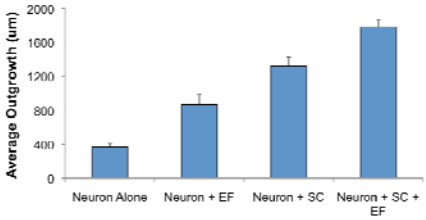

Figure 2. Average neurite outgrowth increases following co-stimulation of SC and EF in an additive manner. n=2.

Conclusions: Our preliminary results indicate that EF-stimulated neurons with or without SC do not exhibit an EF bias as previously described with Xenopus embryonic neurons[6]. SC and DC EFs increased total neurite outgrowth in an additive manner following 8 hours of co-stimulation in comparison to neurons singly stimulated by EF or SC. Due to the lack of directional bias with co-stimulation, co-cultures will be stimulated in sequence to examine the effect of multiple EF applications on SC orientation and neurite outgrowth. Furthermore, the use of oriented scaffolds can be incorporated in future work to direct neurite outgrowth while co-stimulation of EF in the presence of SC enhances the amount of neurite outgrowth. Neuronal responsiveness to DC EF ranging from 0-150 mV/mm will be investigated in this work.

References: (1) Lee, Bashur, Goldstein, Schmidt. *Biomaterials* 2009; 30: 4325. (2) Bunge. *J Exp Biol* 1987; 132:21. (3) Morrissey, Kleitman, Bunge. *J Neurosci* 1991;11(8):2433. (4) Dewitt, Kaszuba, Thompson, Stegemann . *J Tiss Eng A*. 2009; 15(10): 2785. (5) McCaig & Rajincek. *Exp Phys* 1991; 76: 473. (6) Rajnicek, Robinson, McCaig. *Dev Biol* 1998; 203:412.