2006

Short-duration, DC electrical stimulation increases chick embryo DRG neurite outgrowth

Matthew Wood, *Saint Louis University*
Rebecca K. Willits

Short-duration, DC electrical stimulation increases chick embryo DRG neurite outgrowth

Matthew Wood[1] and Rebecca Kuntz Willits[1,*]

[1] Department of Biomedical Engineering, Saint Louis University, St. Louis, MO

This is the accepted version of the following article: Wood M, Willits RK. Short-duration, DC electrical stimulation increases chick embryo DRG neurite outgrowth. Bioelectromagnetics. 2006;27(4):328-31, which has been published in final form at doi: 10.1002/bem.20214.

Citation: Wood M, Willits RK. Short-duration, DC electrical stimulation increases chick embryo DRG neurite outgrowth. Bioelectromagnetics. 2006;27(4):328-31. doi: 10.1002/bem.20214

*Correspondence and reprint address: Department of Biomedical Engineering, Saint Louis University, 3507 Lindell Blvd., St. Louis, MO 63103; Office: (314) 977-8273; Fax: (314) 977-8288; Email: willitsr@slu.edu

Short running title: Electrical Stimulation of DRG

Funding provided by: Sigma-Xi Grant in Aid of Research ID# 12030294

Abstract

This study aimed to reveal the influence of brief DC electric stimulation on neurite outgrowth and outgrowth rates after application. Chick embryo dorsal root ganglia neurite outgrowth, rates, and overall alignment to EF were measured before stimulation and at two time points after stimulation. The presence of a 25 V/m EF for 10 min increased overall neurite outgrowth over controls for up to 48 h after stimulation and all growth was symmetric. These results demonstrate that even 10 min of stimulation, which is approximately 80% shorter than previous studies, promotes enhanced nerve growth.

Key Words

Electric field, growth rate, nerve growth, platinum, preferential growth

Damaged nervous tissue results in injuries ranging from loss of feeling to complete paralysis. Various methods of treating nerve injury include the application of electric and magnetic guidance cues to encourage and direct regeneration. Electrical fields (EFs) and pulsed electromagnetic fields (PEMFs) have been found to improve repair of nerve injuries *in vivo* [Al-Majed *et al* 2000; Borgens 1988; Borgens *et al* 1999; Sisken *et al* 1989] and enhance neuronal development by aligning and increasing neurite outgrowth *in vitro* [Erskine and McCaig 1995; Greenebaum *et al* 1996a; Jaffe and Poo 1979; Macias *et al* 2000; McCaig *et al* 2000; Patel and Poo 1982; Schmidt *et al* 1997; Zhang *et al* 2005]. Although this enhanced neural behavior is encouraging, further work is needed to fully understand and characterize the effects of field stimulation on neural tissue. In particular, more on the long-term effects of stimulation need to be revealed to better understand the potential benefits *in vivo*.

While *in vitro* studies apply fields for extended times (2-18 h), *in vivo* studies have seen similar enhanced neural benefits from shorter applications. Stimulation of rat sciatic nerves with PEMF for as short as 1 h/day for 3 days increased nerve regeneration rates [Sisken *et al* 1989] or 1 h reduced axonal regeneration time from 9 weeks to 3 weeks [Al-Majed *et al* 2000]. To determine long-term effects *in vitro*, neurite outgrowth and outgrowth rates were examined for up to 48 h after a 10 min, direct current-EF application. In order to provide this short duration stimulation, a chamber was constructed to supply a uniform EF. Neurite outgrowth was measured at 3 time points to characterize outgrowth after stimulation, revealing sustained effects in growth rates but no directed outgrowth.

A stimulation chamber was fabricated to provide a suitable controlled and sterile environment for cell growth. The chamber consisted of a milled ultra high molecular weight

polyethylene block (ACI Plastic, (110 mm x 60 mm x 25 mm with 5 mm thick walls)) with the bottom cut out where a borosilicate glass window was glued with silicone adhesive (Loctite Corporation). The side walls were lined with platinum (Alfa Aesar (99.99% pure), ~200 mm^2/ electrode contacting the solution) glued flat with instant adhesive and primer (Loctite Corporation), providing a uniform EF. Platinum was chosen as it is a suitable potential implantable electrode due to the mild tissue and allergenic response [Geddes and Roeder 2003]. Stainless steel wire was woven into the top of the platinum and led out of the chamber. A constant-voltage power supply was utilized during stimulation and disconnected after stimulation; the actual voltage between the platinum sheets was utilized to calculate the EF magnitude. Each chamber was sterilized by autoclaving (121°C, 25 min.) before stimulation.

Circular 25 mm glass cover slips were acid etched with 9:1 H_2SO_4: H_2O_2 and the underside of the slip marked with a diamond tipped pen to indicate field alignment. Collagen (rat type I at 150 μg/mL) was adsorbed to the surface overnight at 2-8°C. The following day the slips were washed in PBS, sterilized by absolute alcohol treatment for 30 min, and stored in PBS.

DRG were harvested from 8-day-old chicken embryos (Specific Pathogen Free, Charles River Laboratories), cleaned of excess tissue, and incubated at 5% CO_2 and 37°C for 4 h with minimal fluid (~500-700 μL) on collagen-coated slips to encourage attachment. After initial attachment period, up to 1 ml of supplemented media (F12K with 20% fetal bovine serum and 50 ng/ml nerve growth factor) was added. Approximately 22 h after seeding (designated as day 1), DRG were imaged prior to stimulation. To stimulate, up to 3 cover slips were placed into a PBS-filled chamber and oriented to the field. Stimulations with a DC electric field for 10 min were performed at 25 V/m (0.40-0.50 mA), which is similar in magnitude as previous studies (for

review, see [McCaig *et al* 2002]). Two controls were examined: sham-exposed samples were treated identically (i.e., placed in the PBS-filled chamber) except no field was applied and control slips were incubated in PBS-filled Petri dishes for 10 min. All slips were then re-supplemented with media and incubated at 37˚C, 5% CO_2. Additional images were taken 24 h (day 2) and 48 h (day 3) after stimulation by briefly removing the cultures from the incubator. Time points used throughout the study are referred to as day 1, 2, or 3 for conciseness.

Images were analyzed to determine the neurite length and overall alignment to the electric field, similar to a previous study [Macias *et al* 2000], for all time intervals with the 10 trials performed. Briefly, a circles with four quadrants (cathode facing (C), anode facing (A), and perpendicular facing (B, D)) were created from the orientation of the DRG in the field and centered on where the slips faced the cathode in order to achieve equal quadrants sizes with 90° angles. Each quadrant was further divided into 9 equal sections, where the longest neurite was measured by tracing a path from the start of the neurite at the DRG body to the tip of the neurite. All sections were averaged to obtain the quadrant neurite length. Growth rates were determined by dividing the neurite outgrowth by the corresponding time point (22, 48, or 72 h). Statistical analysis was performed using single factor ANOVA (Microsoft Excel) where $p < 0.05$ was considered significant. Approximately 60 DRG per group (Stimulated, Sham, and Control) were imaged at day 1 and 2, and 40 DRG per group at day 3. The mean ± standard error of the mean (SE) was reported.

Tests investigating the chamber design, including spectra of the stimulated media, pH of the stimulated media, and controls of neurite growth in pre-stimulated media, indicated that any byproducts of the stimulation were minimal and did not affect length (data not reported). At day

1, DRG had no significant differences in average neurite length, ~180 μm, between controls and those to be stimulated, imparting no bias to pick presumably healthier DRG. All DRG for all time points had symmetric neurite outgrowth with no quadrant favoring outgrowth, even after stimulation (Table 1). Since neurite outgrowth was symmetrical, all quadrants (A-D) were pooled to obtain an overall measurement for all the days (Fig. 1a). Electrically stimulated DRG had significantly increased overall neurite lengths at day 2 and day 3 compared to the respective controls. At day 2 and day 3, the overall neurite outgrowth for those exposed to the EF was 524 ± 5 μm and 746 ± 10 μm while the controls were 450 ± 10 μm and 638 ± 19 μm, respectively, a ~17% increase each day. The growth rates were assessed from the pooled overall neurite outgrowth measurements at each time point. While growth rates of control neurites were relatively similar over the 3-day period (~8.8 ± 0.6 μm/h), they were statistically increased for both controls from day 1 compared to day 2 (Fig. 1b). Stimulated DRG exhibited increased growth rates that were sustained over the 2-day period after stimulation. The growth rates of

Table 1: DRG neurite outgrowth[a]

Sample	Timepoint	# of DRG analyzed	Average Outgrowth ± SE (μm)			
			Quadrant A	Quadrant B	Quadrant C	Quadrant D
Stimulated	Day 1	65	168 ± 9	185 ± 8	181 ± 11	167 ± 8
	Day 2	64	524 ± 16	532 ± 15	519 ± 15	520 ± 13
	Day 3	43	752 ± 31	714 ± 33	759 ± 34	759 ± 32
Sham	Day 1	58	186 ± 11	191 ± 11	192 ± 10	185 ± 11
	Day 2	58	469 ± 21	465 ± 21	452 ± 19	458 ± 20
	Day 3	39	672 ± 36	685 ± 42	665 ± 39	616 ± 41
Control	Day 1	56	170 ± 11	165 ± 10	182 ± 12	172 ± 10
	Day 2	51	436 ± 22	440 ± 18	444 ± 22	432 ± 19
	Day 3	37	604 ± 36	612 ± 39	632 ± 40	619 ± 37

[a]DRG exposed to a 25 V/m electric field for 10 min did not exhibit asymmetric or preferential growth in any quadrant after exposure. In addition, the sham and control did not demonstrate any preferential growth.

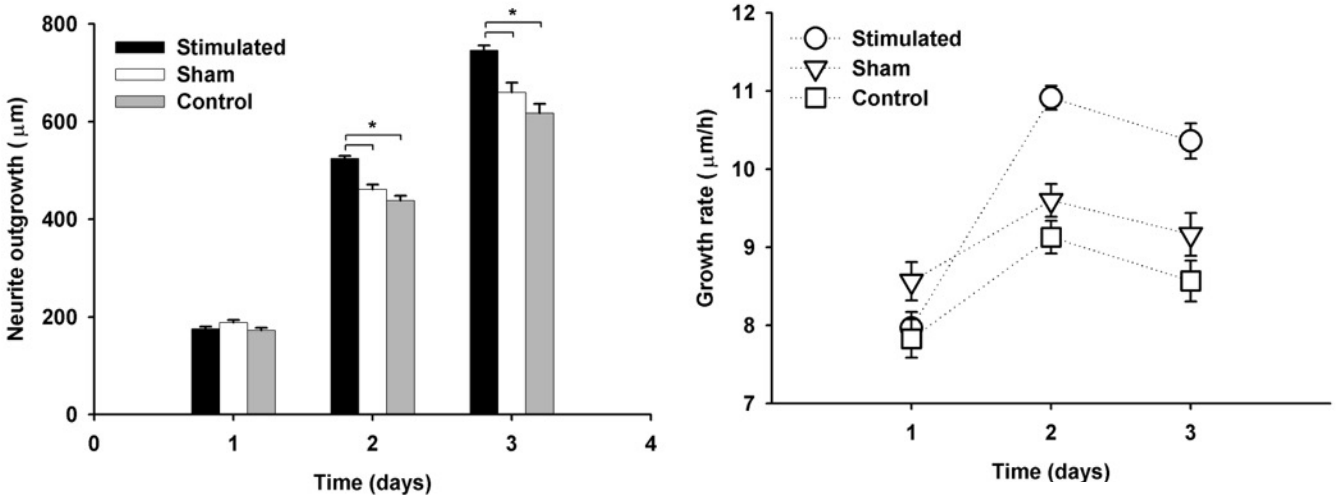

Fig. 1 Data from each quadrant was pooled to obtain an overall neurite outgrowth measurement and growth rate. (a) All DRG before exposure (day 1) had approximately the same neurite outgrowth while DRG exposed to a 25 V/m electric field for 10 min exhibited statistically increased neurite outgrowth at days 2 and 3 compared to each control. (b) Growth rates were increased over controls after stimulation, and the increase was sustained for up to two days. At each time point, rates of stimulated neurites were significantly increased over both controls. Control growth rates were similar over the course of three days, although some differences were noted. Data represented by mean ± SE. * indicates statistical significance ($p < 0.05$) between stimulated DRG and controls.

those exposed to the EF at day 2 and 3 were 10.9 ± 0.2 μm/h and 10.4 ± 0.2 μm/h, respectively, both being significantly increased from day 1, 8.0 ± 0.2 μm/h. The growth rates of day 2 and day 3 were statistically different from each other as well.

In vitro, EF and PEMF has increased overall neurite outgrowth during and after stimulation [Sisken and Smith 1975] [Erskine and McCaig 1997; Greenebaum *et al* 1996a; Jaffe

and Poo 1979; Macias *et al* 2000; Patel and Poo 1982; Schmidt *et al* 1997], but less is known about how long these beneficial effects persist and few studies have investigated shorter duration stimulations that have been used for *in vivo* studies. In mimicking *in vivo* durations, this study demonstrated that short duration stimulation of 10 min increased overall neurite outgrowth. This time is substantially shorter than the stimulation times (2-18 h) applied in other similar *in vitro* studies [Greenebaum *et al* 1996a; Macias *et al* 2000; Schmidt *et al* 1997]. Additionally, enhanced neurite outgrowth was maintained up to 48 h after stimulation. Although some differences in growth rates were noted between days 1 and 2, a delay in attachment after seeding may account for these differences in growth rates. Others have noted a problem with DRG attaching themselves to a surface [Greenebaum *et al* 1996b], potentially delaying the initiation of neurite extension. Any delay in attachment may potentially explain the differences seen in the growth rates of controls between day 1 and 2.

Although it had been established that an EF influences neurite outgrowth direction during the field application, usually with growth preferential to the cathode with suppressed growth to the anode [Erskine and McCaig 1995; Erskine and McCaig 1997; Jaffe and Poo 1979; McCaig *et al* 2000; Patel and Poo 1982], preferential growth was not noted here. However, it is not well understood if directional preference can persist or occur after EF is removed. For example, dissociated Xenopus neurons became increasingly aligned to the EF over the course of 5 h, with only minimal alignment after the first hour [Erskine and McCaig 1997], but lose their orientation a few hours after stimulation [Patel and Poo 1982]. In contrast, neurite alignment was noted up to 18 h after long duration PEMF stimulation (18 h) [Greenebaum *et al* 1996a; Macias *et al* 2000]. Therefore, the amount of time the field is applied seems to influence the ability for neurites to

align, with shorter times, such as the 10 min in this study or 2 h in a previous study [Schmidt *et al* 1997], not altering the direction of growth 24 h after stimulation.

In conclusion, application of a 25 V/m DC EF for 10 min increased and sustained DRG neurite outgrowth and growth rates for up to 2 days following simulation. No neurite guiding behavior was found in this study, since all quadrants exhibited approximately the same length of neurite outgrowth 24 and 48 h after stimulation. However, this result does not eliminate the possibility of guidance during electrical stimulation.

Acknowledgments

Both authors would like to thank Sigma Xi and the Saint Louis University Biomedical Engineering Department for funding, Dr. J. Gary Bledsoe, Frank Coffey, Christina Obert, and Pete Geisen for assistance in chamber construction.

References

Al-Majed AA, Neumann CM, Brushart TM, Gordon T. 2000. Brief electrical stimulation promotes the speed and accuracy of motor axonal regeneration. Journal of Neuroscience 20(7): 2602-8.

Borgens RB. 1988. *Stimulation of Neuronal Regeneration and Development by Steady Electrical Fields*. Waxman SG (ed.). New York: Raven Press. 547-561 p.

Borgens RB, Toombs JP, Breur G, Widmer WR, Waters D, Harbath AM, March P, Adams LG. 1999. An imposed oscillating electrical field improves the recovery of function in neurologically complete paraplegic dogs. J Neurotrauma. 16(7):639-57.

Erskine L, McCaig CD. 1995. Growth cone neurotransmitter receptor activation modulates electric field-guided nerve growth. Developmental Biology 171(2):330-9.

Erskine L, McCaig CD. 1997. Integrated interactions between chondroitin sulphate proteoglycans and weak dc electric fields regulate nerve growth cone guidance in vitro. J Cell Sci. 110(Pt 16):1957-65.

Geddes LA, Roeder R. 2003. Criteria for the selection of materials for implanted electrodes. Ann Biomed Eng. 31(7):879-90.

Greenebaum B, Sutton CH, Vadula MS, Battocletti JH, Swiontek T, DeKeyser J, Sisken BF. 1996a. Effects of pulsed magnetic fields on neurite outgrowth from chick embryo dorsal root ganglia. Bioelectromagnetics 17(4):293-302.

Greenebaum B, Sutton CH, Vadula MS, Battocletti JH, Swiontek T, DeKeyser J, Sisken BF. 1996b. Effects of pulsed magnetic fields on neurite outgrowth from chick embryo dorsal root ganglia. Bioelectromagnetics. 17(4):293-302.

Jaffe LF, Poo MM. 1979. Neurites grow faster towards the cathode than the anode in a steady field. Journal of Experimental Zoology 209(1):115-28.

Macias MY, Battocletti JH, Sutton CH, Pintar FA, Maiman DJ. 2000. Directed and enhanced neurite growth with pulsed magnetic field stimulation. Bioelectromagnetics 21(4):272-286.

McCaig CD, Rajnicek AM, Song B, Zhao M. 2002. Has electrical growth cone guidance found its potential? Trends Neurosci. 25(7):354-9.

McCaig CD, Sangster L, Stewart R. 2000. Neurotrophins enhance electric field-directed growth cone guidance and directed nerve branching. Developmental Dynamics 217(3):299-308.

Patel N, Poo MM. 1982. Orientation of neurite growth by extracellular electric fields. Journal of Neuroscience 2(4):483-96.

Schmidt CE, Shastri VR, Vacanti JP, Langer R. 1997. Stimulation of neurite outgrowth using an electrically conducting polymer. Proceedings of the National Academy of Sciences of the United States of America 94(17):8948-8953.

Sisken BF, Kanje M, Lundborg G, Herbst E, Kurtz W. 1989. Stimulation of rat sciatic nerve regeneration with pulsed electromagnetic fields. Brain Research 485(2):309-16.

Sisken BF, Smith SD. 1975. The effects of minute direct electrical currents on cultured chick embryo trigeminal ganglia. J Embryol Exp Morphol. 33(1):29-41.

Zhang Y, Ding J, Duan W, Fan W. 2005. Influence of pulsed electromagnetic field with different pulse duty cycles on neurite outgrowth in PC12 rat pheochromocytoma cells. Bioelectromagnetics. 26(5):406-11.