31st Annual International Conference of the IEEE EMBS
Minneapolis, Minnesota, USA, September 2-6, 2009

Adhesion Molecules Promote Chronic Neural Interfaces Following Neurotrophin Withdrawal

Jessica O. Winter, *Member IEEE,* Ning Han, Ralph Jensen, Stuart F. Cogan, *Member IEEE,* Joseph F. Rizzo, III

Abstract—**Neural prostheses and recording devices have been successfully interfaced with the nervous system; however, substantial integration issues exist at the biomaterial-tissue interface. In particular, the loss of neurons at the implantation site and the formation of a gliotic scar capsule diminish device performance. We have investigated the potential of a tissue-engineered coating, consisting of adhesion molecule-modified surfaces (i.e., polylysine and collagen) in combination with neurotrophin application (i.e., brain derived neurotrophic factor, BDNF), to enhance the electrode-host interface. Neurite length and density were examined in a retinal explant model. In the presence of BDNF for 7 days, we found no synergistic effect of BDNF and adhesion molecule-modified surfaces on neurite length, although there was a possible increase in neurite density for collagen-coated surfaces. After BDNF withdrawal (7 days BDNF+/7 days BDNF- medium), we found that both polylysine and collagen treated surfaces displayed increases in neurite length and density over negative, untreated control surfaces. These results suggest that adhesion molecules may be used to support chronic neuron-electrode interfaces induced by neurotrophin exposure.**

I. Introduction

NEUROTROPHIC comprise a family of proteins that promote neuron survival and neurite extension [1], and also influence glial activation [2]. As a result, several groups [3, 4], including ours [5], are investigating the potential of neurotrophins to improve integration of neural prostheses or recording devices with host tissue. However, it is also well-known that neurotrophic factors have short half-lives, on the order of minutes to hours [6], limiting their efficacy. To address these difficulties, our group [5, 7] and others [8-10] have developed drug delivery vehicles to provide neurotrophins at a predetermined rate for a limited period of time. We recently reported that these effects have a limited time course *in vitro* [7]; diminishing after the supply of neurotrophic factor is exhausted. Although the native *in vivo* environment will provide additional support cues, additional biological signals will be required to sustain *chronic* electrode-host integration produced in response to delivered neurotrophins.

Peripheral nerve regeneration, which in contrast to central nervous system regeneration occurs readily, can serve as a guide for these signals. In the peripheral nervous system, nerve regeneration is supported not only by soluble factors, but also by non-soluble adhesion molecules largely composed of extracellular matrix (ECM) proteins (e.g., collagen, laminin, and fibronectin). *In vitro,* adhesion molecules have been shown to promote neuronal attachment, survival, and neurite extension [11, 12]. Therefore, adhesion molecules represent one possible class of molecules to promote chronic neural interfaces.

We examined the combination of soluble neurotrophic factors and adhesion molecules as a means to induce chronic neurite extension in a retinal explant model. Recently, a similar report examined the ability of this combination to promote neurite extension and persistence in PC12 cells [13], a neural precursor cell that exhibits a neural phenotype in the presence of nerve growth factor (NGF) neurotrophin [14]. However, it was not possible to conclusively establish the ability of neurotrophins and adhesion molecules to support chronic interfaces with regenerating neurons because PC12 cells revert to an undifferentiated precursor phenotype in the absence of NGF. Here, we show that the addition of adhesion molecules to neurotrophin therapy increases neurite extension following exhaustion of neurotrophin supply. These results suggest that the combination of adhesion molecules and neurotrophins is a powerful tool to support neural integration with prosthetic devices.

II. Materials and Methods

A. Preparation of Cell Culture Substrates

Collagen (Type I, Rat Tail, BD Biosciences) and polylysine (Sigma, 30-70 kDA MW) adhesion molecules were deposited on polyester Transwell insert membranes (Corning, 6 well size), which served as the culture surfaces

Manuscript received April 7, 2009. This work was supported by grants from the Department of Veterans Affairs, Veterans Health Administration, Rehabilitation Research and Development Service and from the Ohio State University.

J. O. Winter is with the William G. Lowrie Department of Chemical Engineering and the Department of Biomedical Engineering, the Ohio State University, Columbus, OH, 43210 USA (corresponding author, phone: 614-247-7558; fax: 614-292-3769; email: winter.63@osu.edu).

N. Han is with the William G. Lowrie Department of Chemical Engineering and the Department of Biomedical Engineering, the Ohio State University, Columbus, OH, 43210 USA (email: han.234@osu.edu).

R. Jensen is with the Center for Innovative Visual Rehabilitation, VA Boston Healthcare System, Boston, MA 02130 USA (email: ralph.jensen@med.va.gov).

S. F. Cogan is with EIC Laboratories, Norwood, MA 02062 USA (email: scogan@eiclabs.com).

J. F. Rizzo, III is with the Center for Innovative Visual Rehabilitation, VA Boston Healthcare System, Boston, MA 02130 USA and the Massachusetts Eye and Ear Infirmary, Harvard Medical School, Boston, MA 02114 USA (email: joseph_rizzo@meei.harvard.edu).

for retinal explants. Briefly, collagen surfaces were created by incubating surfaces with a 5 μg/cm^2 solution in 0.02 N, sterile-filtered, acetic acid (Fisher) for 1 hour. Surfaces were rinsed in distilled, deionized H_2O to remove traces of acid. Polylysine surfaces were prepared by applying a 2-4 μg/cm^2 solution in distilled, deionized H_2O for 1 hour, followed by 3X washing with sterile distilled, deionized H_2O, air drying, and UV treatment overnight. As a control, we also examined bare surfaces consisting of polyester membranes.

B. Animals

Methods for animal care and use, retinal isolation and culture, and neurite counting and analysis are similar to those described previously [7]. Retinal explants were obtained from three adult New Zealand White rabbits (Millbrook, Amherst, MA). Explants from a single, distinct animal were used for each experimental surface examined (i.e., collagen, polylysine, control). Animals selected weighed between 2.5-3 kg and were maintained in a 12 h light (<300 lux)/12 h dark environment. Animals were fed high fiber rabbit chow (Purina 5326) and water, which were available *ad libitum*. All experimental methods and animal care procedures adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, were approved by the Boston VA Institutional Animal Care and Use Committee, and followed NIH guidelines for the care and use of laboratory animals.

C. Retinal Explant and Dissection

Animals were initially placed under general anesthesia using ketamine (35-50 mg/kg, i.m.) and xylazine (3-5 mg/kg, i.m.). Anesthesia was sustained using nose-mask administered 2-3% isoflurane. Local anesthesia consisting of lidocaine hydrochloride (20 mg/ml) was administered to the eyelids and surrounding tissue prior to enucleation. Eyes were then enucleated, and the anterior portion was discarded. Vitreous humor was removed using gentle suction provided by a Pasteur pipette. The retina was then dissected from the choroid using fine tweezers to produce thin retinal sections from which retinal explants were isolated using a 2 mm diameter trephine (Millennium Surgical). Sections were taken only from the inferior region of the retina.

Explants (N = 18) were placed on cell culture substrates consisting of uncoated polyester Transwell inserts or inserts coated with collagen or polylysine. Explants were cultured in Dulbecco's modified essential medium (DMEM, Sigma) supplemented with 1.5 g/L sodium bicarbonate (Sigma), 10% fetal bovine serum (FBS, Sigma) and 1% penicillin-streptomycin (Invitrogen), which was exchanged every 2-3 days. To evaluate the influence of adhesion molecules on neurite persistence, for the first 7 days of the experiment brain derived neurotrophic factor (BDNF, Peprotech) was present in medium at a therapeutic concentration of 100 ng/ml. For the remaining 7 days (Day 8-14), no BDNF was present in medium. Given the rapid degradation rate of BDNF, it is anticipated that BDNF not removed by medium exchange was rapidly dissipated (< 24 hours).

D. Evaluation of Neurite Extension

Neurites were identified using neurofilament staining (anti-neurofilament, 200kDa segment) at day 7 and day 14. Three independent samples taken from the same animal (18 explants distributed across 3 experimental conditions and two time points) were analyzed for each sample condition. Each explant was rinsed twice with phosphate buffered saline (PBS, without Ca^{2+} and Mg^{2+}, pH = 7.4, Sigma) before fixation in 4% (wt/v) formaldehyde (prepared freshly from p-formaldehyde (Sigma) in PBS with 4% (wt/v) sucrose (Sigma)). Samples were incubated in formaldehyde solution for 15 minutes and then rinsed twice with PBS. Following fixation, samples were placed for 30 minutes in blocking medium consisting of 1% (wt/v) BSA and 0.2% (v/v) Triton X-100 in PBS. Samples were then incubated overnight with mouse anti-neurofilament 200 kDa monoclonal antibody (Chemicon) in blocking buffer [1:500], which identifies neurites of retinal ganglion cells. Samples were rinsed three times with PBS before a 1 hour incubation with goat anti-mouse IgG (H+L) FITC conjugate (Jackson Immunochem) in blocking buffer [1:100]. Finally, samples were rinsed three times with PBS and protected with slow fade gold mounting medium (Invitrogen).

Mounted samples were examined using an Olympus CKX41 phase contrast/fluorescence optical microscope and a Sony iCY-shot DXC-S500 color digital camera. Sequential images of explant segments were constructed into composites using Adobe Photoshop, which were evaluated for neurite length and number using Image J image analysis software (NIH) (see Fig. 1). Neurite length was assessed as the length of the neurite from its base to the terminus of each branch point. Only neurites extending from the perimeter of the explant were assessed. The number of neurites was normalized to the perimeter of the explant within each well

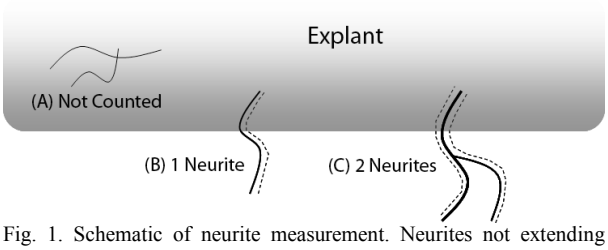

Fig. 1. Schematic of neurite measurement. Neurites not extending beyond the explant perimeter (A) were not counted. Neurites extending beyond the perimeter were traced from the base to the tip (B) for each branch (C).

(number/mm).

Neurite length and density (number/mm perimeter) were analyzed with Sigmastat (SYSTAT) statistical analysis software. For all tests, a p value < 0.05 was used to establish significance. Resultant neurite length distributions were non-Gaussian (skewed), making ANOVA tests or comparisons of the average and standard deviation not meaningful. Data were therefore analyzed using the Kruskal-Wallis test with Dunn's modification, which compares non-parametric data with unequal number of data points (35 < N < 618, pooled

data set from three samples for each experimental condition). Data for neurite number/mm were analyzed using one-way ANOVA or the Kruskal-Wallis test with Tukey's modification; however, the small sample size (N = 3 for each experimental condition), did not provide sufficient statistical power to identify all statistical significances; therefore, conclusions are primarily qualitative comparisons.

III. Results

The combination of soluble neurotrophic factors and adhesion molecules to promote neurite extension was examined using a retinal explant model. Three different experimental surfaces were examined: (1) polylysine and (2) collagen, both of which were deposited on polyester Transwell insert membranes, and a (3) negative control consisting of a polyester Transwell insert membrane only. A retinal explant was cultured on each of these surfaces, in BDNF+ medium for 7 days (Fig. 2A-C, representative images Day 7), and then in BDNF- medium for the remaining 7 days (Fig. 2D-F, representative images Day 14).

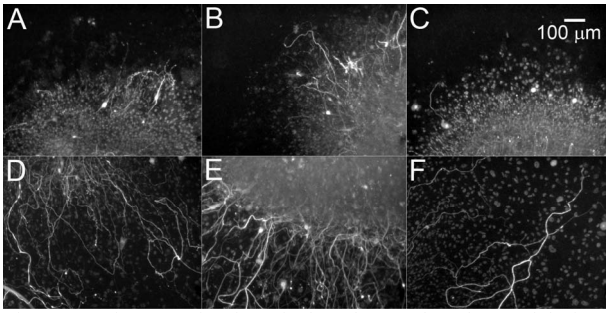

Fig. 2. Neurite extension in retinal explants cultured on polylysine- (A, D), collagen- (B, E) coated polyester Transwell insert membranes and polyester Transwell insert membrane only (C, F) after 7 (A-C) and 14 (D-F) days *in vitro*, consisting of 7 days in BDNF+ medium followed by 7 days in BDNF- medium. Scale Bar is 100 μm for all figures.

A. Neurite Extension in the Presence of Neurotrophins and Adhesion Molecules

After 7 days in BDNF+ medium, no statistically significant difference in neurite length was observed for any sample surface investigated (Fig. 3A), although samples cultured on polylysine coated surfaces displayed the longest neurites (200 ± 147 μm vs. 170 ± 111 μm for collagen and 135 ± 62.3 μm for the negative control surface). Neurite density (number/mm explant perimeter) was significantly increased for collagen coated surfaces ($p = 0.015$, 11 ± 4.5 neurites/mm vs. 5.0 ± 2.2 neurites/mm for polylysine and 1.4 ± 0.55 neurites/mm for the negative control surface) (Fig. 3B). However, the statistical power was too low to detect all differences, and additional experiments would need to be performed to confirm these results. Thus, when BDNF application was combined with adhesion molecule-modified surfaces, polylysine$^+$ and collagen$^+$ samples showed a slight (but not significant) increase in the neurite length and an increase in number density over uncoated, negative control surfaces.

Together, these data support an interpretation that at the concentrations used and over the time frame investigated (7 days), adhesion molecules do not substantially increase neurite length beyond that achieved with neurotrophin application. Thus, no synergistic effect for neurite length was observed. However, the combination of adhesion molecules and neurotrophin application did have an impact on neurite density (neurites/mm explant perimeter), particularly for collagen coated surfaces, indicating a possible synergistic effect for neurite density.

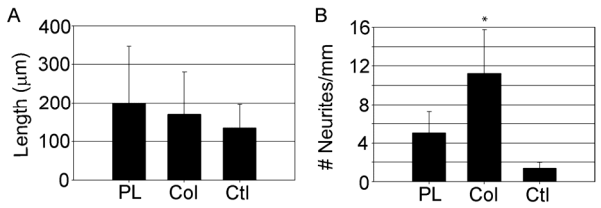

Fig. 3. Comparison of neurite lengths and density (number/mm) after 7 days of culture in BDNF+ medium. (A) Average neurite length. (N, number of neurites presented, > 35) (B) Average number of neurites normalized to explant perimeter (mm). (N = 3). PL = Polylysine, Col = Collagen, Ctl = Control. [Error bars = S.D., * = statistically significant difference, $p < 0.05$]

B. Neurite Extension Following Neurotrophin Withdrawal in the Presence of Adhesion Molecules

After 14 days consisting of 7 days in BDNF+ medium followed by 7 days in BDNF- medium, all of the samples showed a statistically significant increase in neurite length from day 7 to day 14 ($p < 0.005$) (Fig. 3A vs. 4A). Both adhesion molecule coated surfaces (i.e., collagen and polylysine) displayed significantly increases in neurite length ($p < 0.05$) when compared to control, uncoated surfaces. Similar to the 7 day data, polylysine samples displayed the longest neurites (513 ± 314 μm vs. 488 ± 307 μm for collagen and 345 ± 251 μm for untreated control surfaces), but these data were statistically indistinguishable from collagen samples.

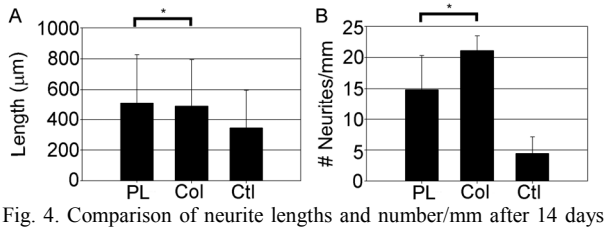

Fig. 4. Comparison of neurite lengths and number/mm after 14 days of culture, 7 days in BDNF+ medium and 7 days in BDNF- medium. (A) Average neurite length. (N, number of neurites presented, > 142) (B) Average number of neurites normalized to explant perimeter (mm). PL = Polylysine, Col = Collagen, Ctl = Control. (N = 3) [Error bars = S.D., * = statistically insignificant difference, $p > 0.05$.]

For neurite density, there was a statistically significant increase from day 7 to day 14 for collagen and polylysine coated ($p < 0.05$), but not control ($p = 0.124$), surfaces (Fig. 3B vs. 4B). Similar to day 7 data, collagen samples exhibited the largest neurite density (21 ± 2.3 neurites/mm vs. 15 ± 5.5 neurites/mm for polylysine coated and 4.4 ± 2.7 neurites/mm

for untreated control surfaces), but this difference was not statistically distinguishable from polylysine.

IV. Discussion

The primary focus of this study was to examine the *combination* of adhesion molecules and neurotrophic factors as a strategy to encourage neural regeneration and enhanced interfacing with implanted prostheses. One goal was to identify any possible synergistic effects. Data at Day 7 (in the presence of both adhesion molecules and BDNF), clearly demonstrate no synergistic effect for neurite length at the concentrations used and time points investigated. However, a possible synergistic effect for neurite density was observed, suggesting that biomaterials presenting both adhesion molecules and BDNF at tissue interfaces may increase the number of sprouting neurites, which is important in synapse formation. These materials may therefore enhance treatment of neurodegenerative diseases.

A second goal of this study was to determine whether adhesion molecules could support neurite extension following BDNF removal. Data at Day 14 (consisting of 7 days in BDNF+ medium followed by 7 days in BDNF- medium) clearly show that culture on adhesion molecule-modified surfaces improves neurite length and density following BDNF removal when compared to untreated control surfaces. These data demonstrate that adhesion molecules, in the absence of neurotrophins, have a positive effect on neurite extension length and density, as has been reported [13]. Additionally, these results suggest that culture on adhesion molecule-modified surfaces may prevent or slow neurite retraction, which occurred upon BDNF withdrawal in our previous studies using retinal explants [7]. Attiah et al. [13] reported similar neurite sustaining effects of adhesion molecule-modified surfaces in their analyses of PC12 cells using nerve growth factor and FGF-2 (fibroblast growth factor-2) neurotrophins. In their study, collagen-coated surfaces produced a greater sustaining effect than laminin-coated surfaces. (Polylysine was not evaluated). Our results confirm their findings in a primary culture model.

One notable difference in this study from our previous work [5, 7] is that neurite retraction did not occur following BDNF withdrawal, although the extent of neurite extension was significantly lower for unmodified surfaces than for samples cultured on adhesion molecule-modified surfaces. This difference from our previous work is most likely the result of animal to animal variation, which is a well-known variable in animal studies. Samples in this study and in our prior work are isolated from distinct animals. It is possible that neurite extension was sustained from endogenous neurotrophins, which may be produced by many of the cells present in explant culture [15, 16]. Levels of endogenous expression may vary from animal to animal. It is therefore important that these preliminary results be confirmed with in vivo studies, which we are currently planning.

V. Conclusion

This work provides strong evidence that adhesion molecules can enhance neurite extension and density following withdrawal of neurotrophic factors. Thus, soluble neurotrophins may be used for acute nerve regeneration, and, after their supply is exhausted, adhesion molecules may be used to encourage a chronic neural interface. These results suggest a possible design for improved interfacing between neural biomaterials and target tissue.

VI. References

[1] G. R. Lewin and Y. A. Barde, "Physiology of the neurotrophins," *Annu Rev Neurosci*, vol. 19, pp. 289-317, 1996.

[2] G. P. Lewis, K. A. Linberg, S. F. Geller, C. J. Guerin, and S. K. Fisher, "Effects of the neurotrophin brain-derived neurotrophic factor in an experimental model of retinal detachment," *Invest Ophthalmol Vis Sci*, vol. 40, pp. 1530-44, 1999.

[3] P. R. Kennedy, "The cone electrode: a long-term electrode that records from neurites grown onto its recording surface," *J Neurosci Methods*, vol. 29, pp. 181-93, 1989.

[4] S. J. Jhaveri, M. R. Hynd, N. Dowell-Mesfin, J. N. Turner, W. Shain, and C. K. Ober, "Release of nerve growth factor from HEMA hydrogel-coated substrates and its effect on the differentiation of neural cells," *Biomacromolecules*, vol. 10, pp. 174-83, 2009.

[5] J. O. Winter, S. F. Cogan, and J. F. Rizzo III, "Neurotrophin-Eluting Hydrogel Coatings for Neural Stimulating Electrodes," *J Biomed Mater Res B*, vol. 81B, pp. 551-563, 2007.

[6] A. Kishino, N. Katayama, Y. Ishige, Y. Yamamoto, H. Ogo, T. Tatsuno, T. Mine, H. Noguchi, and C. Nakayama, "Analysis of effects and pharmacokinetics of subcutaneously administered BDNF," *Neuroreport*, vol. 12, pp. 1067-72, 2001.

[7] J. O. Winter, M. Gokhale, R. J. Jensen, S. F. Cogan, and J. F. Rizzo III, "Tissue engineering applied to the retinal prosthesis: Neurotrophin-eluting polymeric hydrogel coatings," *Materials Science and Engineering C*, vol. 28, pp. 448-453, 2008.

[8] J. A. Burdick, M. Ward, E. Liang, M. J. Young, and R. Langer, "Stimulation of neurite outgrowth by neurotrophins delivered from degradable hydrogels," *Biomaterials*, vol. 27, pp. 452-9, 2006.

[9] S. J. Taylor and S. E. Sakiyama-Elbert, "Effect of controlled delivery of neurotrophin-3 from fibrin on spinal cord injury in a long term model," *J Control Release*, vol. 116, pp. 204-10, 2006.

[10] A. Piotrowicz and M. S. Shoichet, "Nerve guidance channels as drug delivery vehicles," *Biomaterials*, vol. 27, pp. 2018-27, 2006.

[11] R. Milner and I. L. Campbell, "The integrin family of cell adhesion molecules has multiple functions within the CNS," *Journal of Neuroscience Research*, vol. 69, pp. 286 - 291, 2002.

[12] T. Sakisaka and Y. Takai, "Cell adhesion molecules in the CNS," *J Cell Sci*, vol. 118, pp. 5407-5410, 2005.

[13] D. G. Attiah, R. A. Kopher, and T. A. Desai, "Characterization of PC12 cell proliferation and differentiation-stimulated by ECM adhesion proteins and neurotrophic factors," *J Mater Sci Mater Med*, vol. 14, pp. 1005-9, 2003.

[14] L. A. Greene and A. S. Tischler, "Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor," *Proc Natl Acad Sci U S A*, vol. 73, pp. 2424-8, 1976.

[15] N. J. Meilander, X. Yu, N. P. Ziats, and R. V. Bellamkonda, "Lipid-based microtubular drug delivery vehicles," *J Control Release*, vol. 71, pp. 141-52, 2001.

[16] J. Keshmirian, G. Bray, and S. Carbonetto, "The extracellular matrix modulates the response of PC12 cells to nerve growth factor: cell aggregation versus neurite outgrowth on 3-dimensional laminin substrata," *J Neurocytol*, vol. 18, pp. 491-504, 1989.