33rd Annual International Conference of the IEEE EMBS
Boston, Massachusetts USA, August 30 - September 3, 2011

Control of Neural Interfacing in Peripheral Nerves through Regenerative Molecular Guidance

Parisa Lotfi, Mario I. Romero-Ortega

Bioengineering Department, University of Texas at Arlington, Arlington, TX, USA

Abstract — **Regenerative peripheral nerve interfaces have been proposed as viable alternatives for the natural control of robotic prosthetic devices. However, random axonal pathfinding during peripheral nerve regeneration leads to mixed populations of sensory and motor neurons at the electrode interfaces preventing the precise identification of the modality nature of the recorded action potentials; motor or specific sensory sub-modalities. This study present evidence that supports the notion that type-specific neurotrophins can be used to preferentially entice and segregate the growth of defined axonal populations from transected peripheral nerves. Segregation of mixed sensory fibers from dorsal root ganglion neurons was evaluated in vitro by compartmentalized diffusion delivery of nerve growth factor (NGF) and neurotrophin-3 (NT-3), to preferentially entice the growth of trkA+ nociceptive and trkC+ proprioceptive subsets of sensory neurons, respectively.**

Index Terms — **Peripheral nerve segregation, nerve regeneration, neurotrophins, pain, proprioception.**

I. Introduction

In the peripheral nerve, where different modality axons are in close proximity, selectively electrical recording or stimulation is particularly challenging. This is even further complicated by the fact that large myelinated axons (i.e., proprioceptive) are depolarized with smaller currents, while smaller diameter neurons (i.e., pain fibers) require larger stimuli. Thus when stimulating the small caliber fibers, one can expect to non-specifically recruit large-size axons as well [1]. Intraneural electrode arrays have been reported to enable selective electrical stimulation [2]. However, chronically implanted intrafascicular electrodes resulted in endoneurial • brosis, edema, loss of nerve • bers, and variable shifts in threshold [3].

Regenerative sieve electrodes were proposed more than three decades ago as a viable alternative to interface motor and sensory nerve fibers [4] [5]. Sieve electrodes have been shown to obtain neural recordings after long-term (i.e., 2-6 months) implantation [6]. Unfortunately, neural activity was recorded only from a fraction of the animals tested [7]. We recently obtained single and multiunit recordings using a non-obstructive regenerative multi-electrode interface placed between the transected ends of an end-to-end repaired nerve, or in chronically amputated nerves [8].

Using this experimental paradigm we observed axonal regeneration in close proximity to the recording sites, with minimal inflammation at the bio-abio interface. We recorded action potentials as early as 8 days post-implantation with high signal-to-noise ratio, and for as long as 3 months in some animals. We have also previously reported that proprioceptive sensory neurons respond to trk receptor activation after injury [9] and that NGF stimulates the specific functional regeneration of trkA-positive nociceptive neurons [10]. In this study we reasoned that mixed axons form a regenerated nerve can be enticed to grow preferentially towards a compartmentalized electrode array through neuron-type specific growth factors. Such segregated or enriched neural interfaces would predictably increase the probability of specifically recording from motor neurons, and the modality-specific stimulation of sensory axons. This study demonstrates that specific neurotrophic factors can be used to selectively entice the regeneration of specific sensory modality neurons.

I. Materials and Methods

Dorsal root ganglion (DRG) explant cultures were obtained from neonate (P0-P3) rats. The animals were anesthetized by hypothermia and sacrificed. The spinal cord was exposed and the DRGs dissected into Hank's buffered salt solution, and cleaned from dorsal and ventral roots using tungsten needles. Individual DRGs were placed in the main well of the choice assay using ECM (Matrigel; BD Biosciences). After polymerization, neurobasal medium supplemented with L-glutamine, B-27 and penicillin/streptomycin was added and the explants were cultured at 5% CO^2 at 37°C. Animal care and surgical procedures were performed in accordance to the Institutional Animal Care and Use Committee at UT Southwestern Medical Center.

In vitro Y-shape Assay. Polydimethylsiloxane (PDMS) was used to fabricate a choice assay for DRG explants cultures. The "Y" shaped template contained a circular well for DRG placement in the common arm, After two days in culture, pieces of gelfoam pre-soaked in saline NGF (mNGF, 100ng/ml) or NT-3 (hNT-3, 5ng/ml) were placed in the "target" wells. A piece of glass coverslip was then placed over the entire PDMS template with openings at the DRG and gelfoam ends. The coverslip served as a ceiling to the microchannels, prevented floating of the gelfoam and delayed neurotrophin dilution into the media.

Gradient formation. To demonstrate that the choice assay provides independent gradients of neurotrophins from the separate "target" compartments to the DRG, we placed a piece of gelfoam with Cy2 (green) and Cy3 (red) labeled antibodies in the two separated "target" compartments and determine the fluorescence diffusion into the microfluidic channels at 4, 6, 8 and 12 hours. We used a Zeiss Pascal laser confocal microscope equipped with an environmental chamber to maintain the cultures at 37°C and 70% humidity, and evaluated the optical densitometry of the Cy2 and Cy3 fluorescence over time. The diffusion rate was confirmed in separate experiments using gelfoam loaded with bovine serum albumin-alexafluor 594, as its molecular weight approximates that of neurotrophins.

Immunocytochemistry. Explant cultures were fixed 2 days after the neurotropic treatment using 4% PFA. Cultures were permeabilized in 0.25% triton-X and blocked in 1% normal donkey serum. Regenerated axons from the DRG explants were labeled with β-tubulin (mouse anti-b-tub, 1:500; Sigma, St. Louis, MO). Double labeling studies were done using the monoclonal N52 clone of neurofilament 200 (mouse anti-N52, 1:3000; Sigma, St. Louis, MO) a marker of large diameter fibers and CGRP (rabbit anti-CGRP, 1:2000; Chemicon, Temecula, CA) a marker for trkA+ unmyelinated pain fibers. For immunofluorsecence studies, the tissue sections were blocked with 5% normal goat serum incubated overnight with the primary antibodies, and reacted with Cy2 Goat anti-Rabbit 1:250, Cy3 & Cy2 Goat anti-Mouse 1:500; Cy3 Goat anti-Rat 1:400 (Jackson labs) for visualization. Sections mounted using Vectashield containing the nuclear label DAPI (Molecular Probes).

Image analysis and quantification. Digital images were obtained using a confocal microscope (Zeiss LSM 510). The number of branches for each axon in a given field was quantified. All the branches per axon present in the digital image were traced and their lengths were measured to calculate mean branch length. Only neurite processes longer that 20 μm were counted.

Statistical analysis. All data were reported as the mean and the standard error of the mean [11]. An unpaired Student's t-test was used to determine statistical differences. In multiple group comparisons, one-way ANOVA was used followed by Neuman-Keuls multiple comparison post hoc evaluation (Prism 4, GraphPad). P values • 0.5 were considered significant

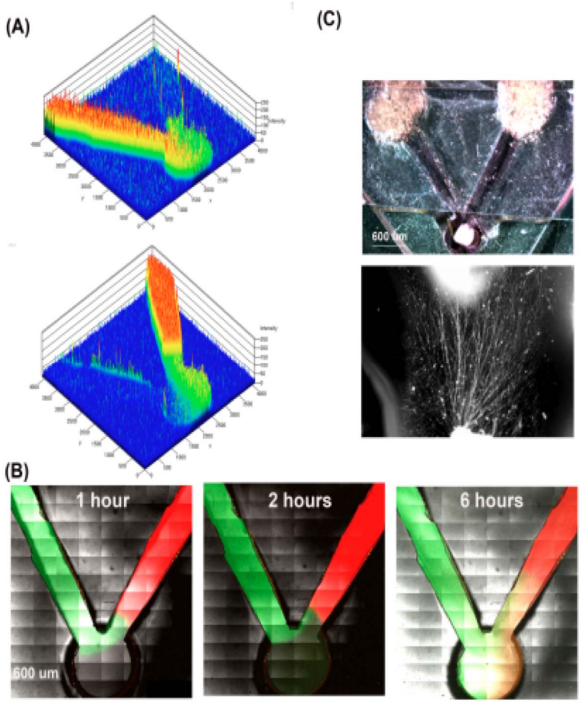

Fig. 1. Y-shaped PDMS assay for axonal segregation. A) Optical density graphs of Cy-2 (green) and Cy3 (red) antibodies diffusion. B) Fluorescent intensity was evaluated in each compartment at 1, 2 and 6 hours. C) Y-shaped assay fabricated from a PDMS scaffold after placement of a DRG and NGF or NT-3 soaked gelfoam. Light micrograph of the axons growing towards the NGF and NT-3 soaked gelfoam (top) and areas at higher magnification from A (bottom).

III. RESULTS

The *in vitro* "Y" shape assay was developed to determine if separate delivery of NGF or NT-3 can differentially entice the axonal regeneration of the mixed population of DRG sensory neurons. The assay consisted in a PDMS scaffold where early postnatal DRG were placed at a 1mm distance from the bifurcation of 5mm "Y" arms that end in a separate compartment where gelfoam soaked with either no growth factor (control), NGF or NT-3 was presented 48 hours after DRG plating. Six hours after adding a Cy2 (green) or Cy3 (red)-labeled antibody to each compartment. The antibodies were observed to separately diffuse in each arm and to provide a distinctive and measurable gradient signal at the cell-seeding compartment (Fig. 1A, B). The gradient was relatively stable for up to 8-12 hours.

In the control experiments in which no growth factors were added, mixed axons of variable length and number of branches were observed. In contrast, Y-assays with NGF in one branch and collagen only in the other branch revealed that axons growing towards NGF were long and lacked branches a characteristic trait prevalent amongst the nociceptive sensory axons. Conversely, Y-assays with NT-3 in one branch and collagen only in the other branch showed that axons growing in NT-3 conduit were short and highly branched, a characteristic property of the proprioceptive sensory axons (data not shown).

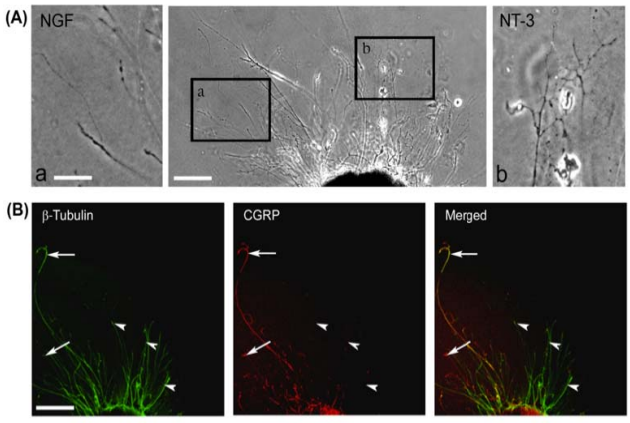

Fig. 2. Axonal differential enticement by NGF and NT-3. A) Light micrograph of the axons growing towards the NGF and NT-3, a and b depict areas at higher magnification in A (rectangles) indicating a distinct morphological difference between the NGF (long unbranched) and NT-3 (short branched) sensory axons. B) Visualization of ß-tubulin (green) and CGRP (red) demonstrated that axons growing towards NGF are CGRP positive. Scale bar= A left 50 μm, A center 250 μm, B 500 μm

The response of DRG cultures with simultaneous presentation of NGF and NT-3 demonstrated that axons growing towards NGF tended to be long and relatively unbranched (Fig. 2Aa), while the axons attracted towards NT-3 branched and were much shorter in length and with more branches (Fig. 2Ab). To further corroborate that these NGF and NT-3 attracted sensory axons are functionally different, dual labeling for ß-tubulin (axonal marker) and CGRP (specific marker for nociceptive sensory axons) was done. As is evident from figure 2B, CGRP-positive fibers are predominantly attracted towards NGF. In contrast, CGRP-negative axons were attracted towards NT-3. Quantitative analysis of this study (Fig. 3) showed that the average axon length in the NGF channel increased 2.5 fold compared to that in saline or NT-3 ($p \bullet 0.001$), whereas the number of branches increased 3 fold in the NT-3 channels compared to that in the saline groups ($p \bullet 0.01$). These results suggested the possibility that different sensory axon subtypes in a mixed in the DRG can be selectively enticed to grow into separate compartments using neuron-specific guidance cues.

IV. DISCUSSION AND CONCLUSION

Gradients of both substrate-bound and diffusible factors have been shown to play critical roles in directing axon outgrowth. Developmental neuronal extension and connectivity depend on a multitude of guidance cues including tropic factors, cytokines, Ephrins, Netrins, Semaphorins and Slits, detected by the neural growth cone through receptor classes such as cell adhesion molecules (CAMs), DCC and Neuropilins, and tyrosine kinases and receptor protein phosphatases (RPTPs) [12] Exogenous expression of developmental guidance cues can be used successfully to entice axonal sprouting or regeneration [13]. In order to increase the sensitivity of the neural interfaces by attracting neurons to the electrically active site, several growth factors and adhesion molecules have been incorporated to conductive substrates. NGF has been attached to polypyrrole [14] or combined with laminin and applied to polymer poly(ethylene dioxythiophene) (PEDOT) [15]. In both cases, the entrapped NGF was able to induce the differentiation of PC12 cells, but diminished the electrical and mechanical stability of the conductive polymers. Alternatively, neurotrophins like brain-

derived neurotrophic factor (BDNF) and NGF have been entrapped in hydrogels polymerized over the electrodes or in nanopore membranes [16].

This study demonstrates that neurotrophins can be used to entice axons towards specific targets, the guidance of specific neuron subtypes in to multi-electrode arrays, is expected to provide a more sophisticated and selective peripheral neuro-interface. Ultimately, such an arrangement would reduce the burden of data extraction from mixed signals from electrodes embedded in a mixed neuron population, and achieve selective recording and stimulation of the regenerative peripheral neurointerfaces, needed to accomplish more precise control of robotic prosthetic devices

ACKNOWLEDGEMENTS

This work was supported by the Defense Advanced Research Projects Agency and the Crowely-Carter Foundation. We thank Russ Daniel, Kshitija Garde and Ebrahim Bengali for assistance.

REFERENCES

1. Grill, W.M., S.E. Norman, and R.V. Bellamkonda, *Implanted neural interfaces: biochallenges and engineered solutions.* Annu Rev Biomed Eng, 2009. **11**: p. 1-24.
2. Yoshida, K. and K. Horch, *Selective stimulation of peripheral nerve fibers using dual intrafascicular electrodes.* IEEE Trans Biomed Eng, 1993. **40**(5): p. 492-4.
3. Lefurge, T., et al., *Chronically implanted intrafascicular recording electrodes.* Ann Biomed Eng, 1991. **19**(2): p. 197-207.
4. Edell, D.J., J.N. Churchill, and I.M. Gourley, *Biocompatibility of a silicon based peripheral nerve electrode.* Biomater Med Devices Artif Organs, 1982. **10**(2): p. 103-22.
5. Dario, P., et al., *Neural interfaces for regenerated nerve stimulation and recording.* IEEE Trans Rehabil Eng, 1998. **6**(4): p. 353-63.
6. Lago, N., et al., *Neurobiological assessment of regenerative electrodes for bidirectional interfacing injured peripheral nerves.* IEEE Trans Biomed Eng, 2007. **54**(6 Pt 1): p. 1129-37.
7. Lago, N., et al., *Long term assessment of axonal regeneration through polyimide regenerative electrodes to interface the peripheral nerve.* Biomaterials, 2005. **26**(14): p. 2021-31.
8. Garde, K., et al., *Early interfaced neural activity from chronic amputated nerves.* Front Neuroengineering, 2009. **2**: p. 5.
9. Romero, M.I., et al., *Deletion of Nf1 in neurons induces increased axon collateral branching after dorsal root injury.* J Neurosci, 2007. **27**(8): p. 2124-34.
10. Romero, M.I., et al., *Functional regeneration of chronically injured sensory afferents into adult spinal cord after neurotrophin gene therapy.* J Neurosci, 2001. **21**(21): p. 8408-16.
11. Litwack, E.D., et al., *Identification and characterization of two novel brain-derived immunoglobulin superfamily members with a unique structural organization.* Mol Cell Neurosci, 2004. **25**(2): p. 263-74.
12. Flanagan, J.G., *Neural map specification by gradients.* Curr Opin Neurobiol, 2006. **16**(1): p. 59-66.
13. Curinga, G. and G.M. Smith, *Molecular/genetic manipulation of extrinsic axon guidance factors for CNS repair and regeneration.* Exp Neurol, 2008. **209**(2): p. 333-42.
14. Gomez, N. and C.E. Schmidt, *Nerve growth factor-immobilized polypyrrole: bioactive electrically conducting polymer for enhanced neurite extension.* J Biomed Mater Res A, 2007. **81**(1): p. 135-49.
15. Green, R.A., N.H. Lovell, and L.A. Poole-Warren, *Impact of co-incorporating laminin peptide dopants and neurotrophic growth factors on conducting polymer properties.* Acta Biomater, 2009.
16. Jun, S.B., et al., *Modulation of cultured neural networks using neurotrophin release from hydrogel-coated microelectrode arrays.* J Neural Eng, 2008. **5**(2): p. 203-13.