ENORMOUS STRETCH-INDUCED GROWTH POTENTIAL OF AXONS UNDER THE APPLICATION OF CONTINUOUS TENSION

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INTRODUCTION

Axonal growth in nervous system development and repair has received significant attention over the years however; this research effort has primarily focused on axonal outgrowth and the chemotaxis theory of axonal growth cone pathfinding. Unfortunately, this research has offered little to the understanding of how developing and regenerating axonal fibers can transverse over great distances in the body, specifically the mechanisms of growth after axons have integrated with their targets. (Weiss 1941). A second, impressive and virtually ignored form of nervous system development is stretch-induced axonal growth. As the body grows, stretches and elongates, integrated axons are routinely forced to grow just as rapidly. Thus, there are two distinct forms of axonal growth: growth cone mediated growth and stretch induced axonal growth.

Stretch induced growth of nerves is perhaps the one important and useful feature of nerve growth and regeneration that has not yet been considered. Exploration into this new growth potential of axons will add to the fundamental knowledge of developmental neurobiology and may offer new insights into nervous system regeneration and repair. To investigate how axons can grow by stretch induction we have developed a innovative technique to mimic this process of axonal growth. We recently demonstrated that central nervous system axons could be grown to 1cm in ten days by applying continuous mechanical tension (Smith, Wolf et al. 2001). Due to the robust and regenerative nature of the peripheral nervous system and their potential for nervous system repair we thought they might improve upon our stretch induced growth technique. We used neurons from the dorsal root ganglion (DRG) of both the embryo and adult to evaluate their elongation potential and found them superior to CNS neurons.

MATERIALS AND METHODS

Cell Culture

Sensory neurons were isolated from the dorsal root ganglia (DRG) of E15 rat embryos using the method described by Kleitman et.al. (Kleitman, Wood et al. 1998). Both dissociated and whole (non-dissociated) embryonic DRG cultures were used. Adult DRG neurons were dissected from adult Sprague-Dawley rats of at least 8 weeks of age as described by Scott (Scott 1977). Adult DRGs were dissociated and purified by centrifuging though a BSA gradient.

Axon Elongation Device

DRG axons were grown by tension-induced elongation using a previously proven technique (Smith, Wolf et al. 2001). The device was created to gradually separate two adjoining substrates on which neural cells are cultured (see figure 2). The adjoining substrates were designed such that axons growing in culture can grow across the interface between the overlapping substrates easily. The bottom substrate made of optically transparent Aclar 33C film, covers the entire bottom of the elongation device on which a stationary population of neurons is cultured. An overlapping Aclar substrate, the towing substrate, is placed on top of the bottom Aclar substrate and serves as the moving population of cells. Once the neurons and their axons have matured and integrated across the bottom and towing substrate interface, the two substrates are separated using a microstepper motor system, see figure 1. The result is two populations of cell bodies connected together via elongated fascicular axon tracts.

FIGURE 1

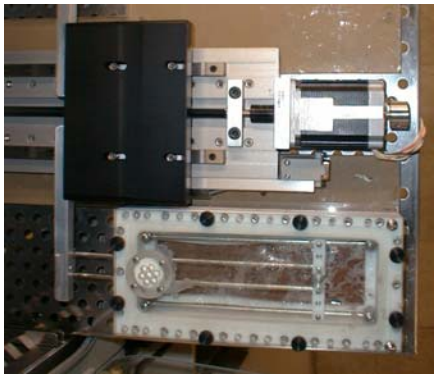
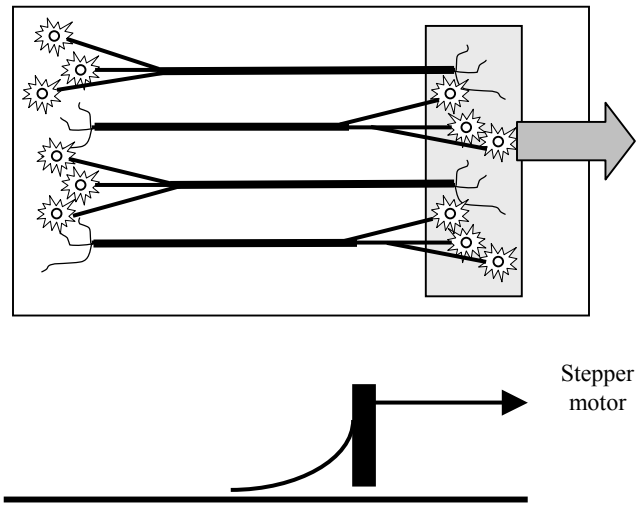


FIGURE 2



Axon Elongation Scheme

Elongation is controlled by displacement of the towing membrane. The stretch induced growth is initially strain limited therefore the elongation rate must begin slow and then ramped up to the desired growth rate. Stretch rate was programmed into the motion control device by choosing a displacement, and a resting time in a stepwise fashion. For example, 1mm/day is programmed as 1μm displacements every 86.4 seconds. Each elongation program starts at 1mm/day for the first 24 hours during which the system's connections tighten and the axons begin elongating. At the end of 24 hours, the axons are approximately 100-200μm in length. The elongation rate was then increased by 1mm/day every 6 to 12 hours until the maximum elongation rate is achieved. In the case of a maximal rate of 8mm/day, the ramping began 72 hours after starting and was ramped slower every 12 to 24 hours to allow the axons to increase in length and thereby minimizing strain induced damage.

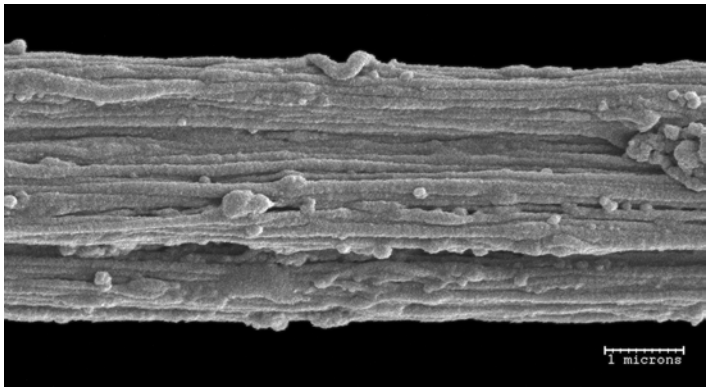
RESULTS

Stretch Induced Growth of DRG Axons

We have demonstrated that embryonic dorsal root ganglion (DRG) axons can be successfully and reliably grown by continuous mechanical tension. Indeed, we found that DRG axons were hardier and more compliant than the central nervous system cultures studied previously (Smith, Wolf et al. 2001). Our biomechanical studies demonstrate that DRG axons can continuously sustain elongation rates of 4mm/day for 10 days or more and up to 8mm/day for over 24 hours. Thus far we have been able to induce elongated growth to DRG axons up to 4cm in length in a period of 10 days whereas only 1 cm had been accomplished with CNS cells. The axons in these experiments did not show signs of excess damage or axonal tearing and suggests that these lengths and rates are not maximal. We confirmed that the elongating fibers were axons by using anti-bodies against MAP-2 and NF-H to distinguish between the axons and dendrites.

As DRG axons elongate, small groups coalesced and formed larger fascicles as their lengths were mechanically elongated. Once the axons reached about 1cm in length the fasciculation slowed and they continued to grow parallel to each other. Each fascicle consists of hundreds to thousands of axons adhering to one another. Figure 2 is a scanning electron micrograph illustrating a typical fascicle comprised of axons approximately 100-250 nm in diameter.

FIGURE 3



DRG Axons Sustain High Rates of Stretched Induced Growth

We found that the best elongation results were obtained when the rate of stretch was ramped slowly to the desired rate after the initial elongation begins. Our biomechanical studies have also shown that smaller and more frequent step sizes lead to less axonal damage and higher growth potential. For example, in our previous experience with cortical cells, the rate of 1mm/day was implemented by 3.5μm steps every 5 minutes. We found better results implementing this 1mm/day rate by 1μm steps every 86 seconds. This new elongation strategy has allowed us to reliably grow DRG axons at a rate of 4mm/day for several days without pause. While a slow ramp worked extremely well for our long-term experiments where stretch induced growth was sustained at 4mm/day for several days, this paradigm did not work well for the high growth rate experiments. Axons that were grown for one day at a rate of 1mm/day and then immediately ramped to 8mm/day showed significant sign of axonal breakage. However, if the axons were allowed to elongate for a period of 3 days at 1mm/day then slowly ramped to 6mm/day and even 8mm/day, there was no evidence of axonal breakage.

Stretch Grown Axons are Thriving

Immunocytochemical studies against the axon's cytoskeleton shows that these proteins are being transported throughout these stretch-grown axons. We labeled elongated axons against the phosphorylated heavy neurofilament (NF-H), type 3 beta tubulin, and tau. All stretch-grown axons exhibited positive immunoreactivity all throughout their lengths. In addition, transmission electron micrographs of axonal cross sections suggest these cytoskeletal components are present and assembled. To our surprise, we found that the axonal caliber in elongated axons significantly increased over non-stretch grown controls.

REFERENCES

Kleitman, N., P. M. Wood, et al. (1998). Tissue Culture Methods for the Study of Myelination. Culturing Nerve Cells. Banker, G. and Goslin, K. London, England, The MIT Press**:** 545-594.

Scott, B. S. (1977). "Adult mouse dorsal root ganglia neurons in cell culture." J Neurobiol **8**(5): 417-27.

Smith, D. H., J. A. Wolf, et al. (2001). "A new strategy to produce sustained growth of central nervous system axons: continuous mechanical tension." Tissue Eng **7**(2): 131-9.

Weiss, P. (1941). "Nerve patterns: The mechanics of nerve growth." Growth, Third Growth Symposium **5**: 163-203.