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Stretch growth of integrated axon tracts: Extremes and exploitations

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Abstract

Although virtually ignored in the literature until recently, the process of ‘stretch growth of integrated axon tracts’ is perhaps the most remarkable axon growth mechanism of all. This process can extend axons at seemingly impossible rates without the aid of chemical cues or even growth cones. As animals grow, the organization and extremely rapid expansion of the nervous system appears to be directed purely by mechanical forces on axon tracts. This review provides the first glimpse of the astonishing features of axon tracts undergoing stretch growth and how this natural process can be exploited to facilitate repair of the damaged nervous system.

Keywords

Axon; Axonal growth; Stretch growth; Peripheral nerve injury; Spinal cord injury; Brain machine interface; Neuron cytom mechanics; Nervous system development; White matter development

1. Introduction

There is an almost secret form of axonal growth that has been hiding in plain sight, yet it is perhaps the most remarkable growth mechanism of all: extending axons at rates thought to be impossible. The process of ‘stretch growth of integrated axon tracts’ is in marked contrast to well-studied forms of axon growth, such as sprouting and regeneration, since it does not require chemical cues, physical guides, or even growth cones. Rather, for axons that have already formed synapses, rapid growth can be triggered solely by mechanical forces, as first proposed by Weiss (1941) (Fig. 1A). This process can be observed throughout nature, where the growth of animals supplies continuous mechanical tension on nerves and white matter tracts.

Stretch growth of integrated axons likely begins during embryogenesis after axons have sprouted from the cell body and traveled relatively short distances to synapse with their target cells. As the animal’s body grows, the distance between most neuronal somata and target cells increases, thereby exerting tensile forces on the axons. Presumably, these forces stimulate the axons to add cytoskeleton, axolemma, and other cellular building materials somewhere along their central length to minimize strain. Otherwise, the axons would be stretched to the point of rupture. Since this general process has only just recently been demonstrated experimentally, many mysteries remain regarding how and where axon tracts can achieve such extreme rates of growth. Nonetheless, stretch growth of integrated axon tracts is currently being exploited as a promising new approach to repair the nervous system.

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2. The extremes of stretch growth of integrated axon tracts in nature

One of the most extreme examples of axon stretch growth can be inferred from blue whale spinal cord development (Fig. 1B). As the size of the whale vertebrae grow in length, spinal axons are presumably placed under continuous mechanical tension. These integrated axons have no growth cones, but nonetheless undergo enormous growth, reaching an unimaginable 30 m for some tracts.¹ This developmental growth process represents a truly unique form of tissue expansion. For almost all other cell types, division is a key mechanism to increase tissue volume during development. Neurons, however, are non-mitotic and rely on a unique process to contribute to expansion of nervous tissue. Indeed, progressive extension of axons increases individual neuron cell volume through progressively extending the length of axons. This exceptional cellular geometry must be particularly daunting for the blue whale, where the volume of spinal axons could be over a thousand times greater than their cell bodies.² Even for a small mammal, neurons with long axons may represent some of the largest cells by volume in the body.

In addition to creating axons of exceptional length, the pace of stretch growth can far exceed what has been well established for axon sprouting and regeneration. From a conventional understanding, axon growth is limited by the rate of slow axonal transport. For example, the key cytoskeletal elements of axons, neurofilament proteins, have been clocked at an average speed of up to only a few millimeters of transport down the axon per day (Brown, 2000, 2003; Nixon, 1998a,b; Roy et al., 2000; Shah and Cleveland, 2002). This rate appears very much in concert with the limiting length that sprouting axons from neurons in culture can extend each day. Likewise, axons regenerating from sites of peripheral nerve damage only grow up to a few millimeters each day on average (Burnett and Zager, 2004; Evans, 2001). However, these limitations clearly do not apply to stretch growth of integrated axon tracts during development. Indeed, spinal axons in the blue whale must increase in length over an estimated 3 cm each day³ to keep pace with the peak growth of the whale's body.

Although it is not known where new material is added onto axon tracts undergoing stretch growth, any great distance would necessitate a remarkable transport mechanism. For example, slow axonal transport of neurofilament proteins sent from the neuronal somata to the terminus of the longest whale axons would take decades.⁴ Even if these proteins were ferried at fast axonal transport rates of approximately 300 mm/day (Grafstein and Forman, 1980), the trip would still take months to complete.⁵ Accordingly, several unidentified mechanisms of greatly accelerated axonal transport may be required for extreme stretch growth of integrated axons. Potentially, the very process of elongating may help in this regard by rapidly redistributing building materials and already formed structures along the axon cylinder, as has been suggested (Abe et al., 2004; Miller and Heidemann, 2008). However, this alone does not seem to account for the massive requirements of new material needed for extreme growth of axons. Furthermore, once the growth of very long axons has finished, rapid transport of materials is still needed for the general maintenance or repair of

¹Calculation: The maximum length of adult blue whales is 34 m (American Cetacean Society Fact Sheet: <http://www.acsonline.org/factpack/bluewhl.htm>) with the estimated maximum length of spinal axons spanning the brainstem to the end of the spine at 30 m (rounded number for clarity).

²Calculation: The diameters of blue whale brainstem neurons and their long axons are unknown. Therefore, two estimations were used to determine volume ratios for a 30-m long structure: (1) Using a diameter of the cell body at 50 μ m with the diameter of axon at 2 μ m, the axon-to-cell body ratio is 1400:1. (2) Alternatively, an estimate using a cell body diameter of 30 μ m with a 1 μ m diameter axon yields a remarkable axon-to-cell body ratio of 1600:1.

³Calculation: With peak body length growth of blue whales at 3.8 cm/day (American Cetacean Society Fact Sheet: <http://www.acsonline.org/factpack/bluewhl.htm>), the corresponding peak growth of spinal axons is conservatively estimated at least 3 cm/day (rounded number for clarity).

⁴Calculation: Using a slow transport rate of 2 mm/day, it would take neurofilament proteins 15,000 days (or 41 years) to travel 30 m.

⁵Calculation: At a fast axonal transport rate of 300 mm/day, it would take neurofilament proteins 100 days (or over 3 months) to travel 30 m.

the distal regions. One suggested mechanism that might overcome the challenges of long-range transport is for protein synthesis to occur within axons (Alvarez et al., 2000; Brittis et al., 2002). If true, this process could provide a local supply of cytoskeletal elements as needed rather than relying on transport.

The rapid daily increase in axon length during extreme stretch growth must be mirrored by a high rate that cell volume is added. As non-mitotic cells, neurons are not generally thought to be highly active with regards to expanding their cytoskeleton. However, neurons with axons undergoing extreme stretch growth may rival the capacity of aggressive cancerous cells to increase cell volume. For example, blue whale spinal axons growing at 3 cm/day represent an increase in volume that is likely more than double the volume of the entire neuron cell body—*each day*.⁶ This rapid volume increase for neurons is akin to the peak cellular growth rate observed for rapidly dividing cancerous cells (Clarkson et al., 1965). Yet, managing the rapid volume increase in growing axons on neurons may be even more challenging than cell doubling for cancer cells. In particular, consider the critical challenges of cell transport with an increasingly elongated structure of axons.

3. Limited historic notice of stretch growth of integrated axon tracts

Perhaps one reason that stretch growth of axon tracts has not been well studied is the lack of an identifiable pathology linked with its disruption. Defects in the axonal stretch growth mechanism would likely be uniformly lethal very early in embryogenesis, leaving little evidence to attract attention. Nonetheless, the general concept of axon stretch growth has not been completely ignored. A paper from Paul Weiss appeared in a publication of a symposium on growth in 1941, in which he primarily detailed much of his seminal work examining developmental morphogenesis, axon sprouting and nerve repair (Weiss, 1941). However, in one short passage, he hinted at a mechanical basis of nerve growth after axons reach their targets and the growth cone is lost. He suggested that during this phase of growth “the nerve is drawn out by the growth and dislocations of its terminal tissues” (Fig. 1A). Yet, the first experimental examination on the cytomechanics of axon growth demonstrated an earlier phase of mechanically induced axon growth *prior* to synaptogenesis and the formation of tracts.

4. Experimental evidence of mechanical influences on axon sprouting

Based on experimental evidence, two distinct phases of mechanically induced axon growth are emerging, with the first occurring during axon sprouting. Although it has been well characterized that axon growth cones extending out from the neuron body are directionally guided by chemotactic and haptotactic cues (Dickson, 2002; Tessier-Lavigne and Goodman, 1996; Yu and Bargmann, 2001), the actual growth of the axon also appears to be dependent on mechanical stimuli. In pioneering studies first reported in 1984, Bray attached a microelectrode to the growth cones of individual chick sensory axons to examine their response to mechanical elongation (Bray, 1984). He found that stretching the axons up to 100 μm over a few hours did not result in disconnection. Rather than thinning down due to stretch, he also found that the diameter of ‘towed’ chick axons remained approximately the same and that the cytoskeleton appeared normal. Thus, he concluded that mechanical forces from the towed growth cone had induced axon growth *in vitro*. Furthermore, he hypothesized that this mechanical process of growth occurs naturally during development via the locomotion of the growth cone creating tensile forces on the trailing axon.

⁶Calculation: Ratios of the volume of 3 cm of new axon/day to the volume of the neuron cell body are based on two estimated sizes: Using a diameter of the cell body at 50 μm with the diameter of axon at 2 μm and 3 cm long, the axon-to-cell body ratio is 1.4:1. (2) Alternatively, an estimate using a cell body diameter of 30 μm with a 1 μm diameter axon yields an axon-to-cell body ratio of 1.6:1.

Accordingly, while chemical cues may stimulate axons to sprout and control their direction of growth, Bray posited that mechanically induced forces from the mobile growth cone may provide a mechanism that drives addition of new axon material.

The observations by Bray were soon substantiated and expanded in a series of elegant studies by Heidemann and Buxbaum (1994) and Heidemann et al. (1990, 1995) using a very similar experimental model. Glass pipettes were attached to the growth cones of single axons to tow the axons at precise increments, increasing tension as steps of constant force. As opposed to previous studies showing membrane being added just behind the growth cone during natural sprouting, mechanical towing resulted in the addition of new membrane all along the axon cylinder during elongation. In addition, it was shown that new microtubule assembly was essential within towed axons; otherwise, the axons would break. Through sampling a range of increasing rates, the investigators characterized the limitations for mechanically induced growth of individual neurites over hours of elongation in culture. It was confirmed that axons could be towed at the remarkable rate observed previously by Bray, with a maximal limit of 1 mm total extension over the course of 1 day. They also found that towed axons would retract when tension was suddenly diminished, demonstrating residual stress. More recently, some of these investigators have suggested that migrating growth cones themselves provide mechanical tension that induces growth (O'Toole and Miller, 2008). Notably, for axons that are fixed to a substrate, this growth appears to be restricted the region of the growth cone. Collectively, these *in vitro* studies demonstrated that mechanical forces can influence the first phase of axon growth during sprouting from the cell body in search of a target.

5. Experimental evidence of extreme stretch growth of integrated axon tracts

The second phase of mechanically induced axon growth –elongation of integrated axon tracts – was not experimentally demonstrated until 2001, notably 60 years after Paul Weiss first suggested that this process occurs naturally during development. *In vitro* studies in our laboratory confirmed that integrated axon tracts spanning two populations of mammalian neurons could undergo stretch growth even at seemingly impossibly extreme rates, and maintained at these rates for at least several weeks in culture (Pfister et al., 2004, 2006a,b; Smith et al., 2001). Ironically, however, our original goals had nothing to do with Weiss' hypothesis. Indeed, we set out to engineer transplantable nervous tissue constructs to repair large lesions in the central and peripheral nervous systems. Only when a literature search to determine mechanical parameters necessary to induce growth of axon tracts was performed was the striking paucity of information on this important natural process revealed.

The experimental approach to develop stretch growth of integrated axon tracts evolved from previous studies in our laboratory examining traumatic axonal injury using an *in vitro* model (Wolf et al., 2001). For the injury model, dynamic stretch was induced to axons spanning two populations of neurons at high strains and strain rates. Finding that the integrated axons had a remarkable resilience to rapid stretch, we sought to exploit this nature to create lab-grown nervous tissue via continuous mechanical tension. We developed an axon stretch growth model whereby two large populations of neurons were seeded on adjacent membranes within a bioreactor (Fig. 2). In initial studies, we used rat primary cortical neurons and human neurons differentiated from the N-tera 2 cell line. Over a few days, up to one million sprouting axons crossed the approximately 50–100 μ m division between the membranes and integrated with the opposing neuron population. A programmable microstepper motor system was then initiated, which progressively separated the two membranes away from each other at set rates, placing mechanical tension on the spanning axons.

In initial studies, a simple elongation paradigm was used to extend the bridging axons 1 mm/day with steps of at the set rate of 3.5 μ m every 5 min. In the expanding gap the axons responded to this tensile elongation by growing 1 cm in length by 10 days of stretch (Smith et al., 2001) (Fig. 3). Thus, these studies experimentally demonstrated the capacity of integrated axons to undergo substantial growth via continuous mechanical tension, supporting Weiss' hypothesis of nerves being forced to grow while being "drawn out" due to the growth of an animal.

These initial studies also potentially revealed another natural process. The somewhat random orientation of individual axons and small fascicles that crossed between the two plating membranes prior to stretch became transformed during stretch growth. As the gap expanded, all individual spanning axons joined with neighboring fascicles, taking on highly organized parallel orientations resembling harp strings. In turn, these parallel fascicles also gradually coalesced into larger tracts (Fig. 3). We posit that this *in vitro* process recapitulates natural morphogenesis during development that drives the organization of nerves and white matter into highly anisotropic structures.

With an eye on developing a transplantable nervous tissue construct to bridge nervous system damage, we subsequently chose to examine dorsal root ganglia (DRG) neurons as a cell source. Both animal and human DRG neurons have been shown to be robust in culture, surviving for months while maintaining normal electrophysiological function (Pfister et al., 2006a,b). Accordingly, these neurons appear ideal to examine the limits of stretch growth of integrated axon tracts, as well as to optimize tissue-engineering techniques to develop transplantable nervous tissue constructs.

From the previous studies of Heidemann and Buxbaum (1994) and Heidemann et al. (1990, 1995) towing individual axon growth cones, it was found that elongation rates of the single axons exceeding 100 μ m/h led to disconnection within a few hours (Pfister et al., 2004, 2006a,b). Likewise, using DRG neurons, we found that if high elongation rates of axon tracts were applied early to integrated axon tracts, the axons would rupture within the first day (Pfister et al., 2004). As such, we identified two sequential factors defined the boundaries of long-term stretch growth of integrated axons: strain and acclimation (Fig. 4A). Only by employing a gradual accelerating stretch rate could the axon tracts be conditioned to tolerate continuing expansion. The boundaries of this stretch-growth process follow a surprisingly steep curve of escalating elongation rates, reaching a remarkable 1 cm of growth per day after only a few days of stretch (Pfister et al., 2004). Notably, much higher rates and lengths may be possible using this approach, potentially rivaling the rapid peak growth of blue whale axons of over 3 cm/day. Regardless, even 1 cm/day far exceeds what has been conventionally thought to be the limits of axon growth.

By optimizing the stretch growth paradigm, DRG axon tracts initially only 100 μ m in length were extended up to an unparalleled 10 cm in less than 2 weeks of stretch (Pfister et al., 2004, 2006b) (5 cm in length shown in Fig. 4B). Yet, despite this rapid stretching, there was clear evidence that the integrated axons underwent equally fast growth. Scanning electron micrography demonstrated that stretch grown axons maintained a normal lengthwise morphological appearance, while transmission electron micrography of cross-sections demonstrated a completely normal ultrastructure. In particular, a normal complement of microtubules and neurofilaments was found per cross-section area for axons grown to 5 cm. Surprisingly, however, the average diameter of these axons was found to be 30% greater than non-stretched (static) axons in sister cultures (Pfister, 2004) (Fig. 4C). This increase in girth accompanying an increase in length due to stretch growth may reflect a natural adaptive process of development, potentially to facilitate structural integrity, transport and electrical signaling.

As for function, stretch growth was found not to alter sodium channel activation, inactivation, or recovery or potassium channel activation of the neuronal somata. As such, normal action potentials could be relayed from one end of the stretch grown culture across the long axon fascicles and be recorded from neurons on the other end (Pfister, 2006a) (Fig. 4D). Nonetheless, there appeared to be an increased density of sodium and potassium channels on stretch grown axons (Pfister et al., 2006a,b). This may also represent a natural adaptation of development designed to preserve the fidelity of neuronal signaling for axons undergoing rapid growth in length and diameter. Regardless, the functionality with the unique geometry of the stretch grown cultures creates a sort of mini-nervous system, with two separated neuronal nuclei communicating via bridging axon tracts.

6. The paradox of brain morphogenesis and stretch growth of axon tracts

The collective *in vitro* data demonstrate the natural phenomenon of long-term stretch growth of integrated axon tracts. Clearly, this process can occur at rates that defy our current understanding of axon growth. The experimental mechanical stretch of axon tracts is likely akin to natural developmental growth where relatively supple nervous tissue spanning growing rigid bony structures must either grow in kind or fail, as Weiss postulated. However, there is one very notable exception to this process. Specifically, white matter tracts in the brain are not oriented along bony scaffolds, yet they substantially increase in length during development. Much in contrast to the concept of weakling axon tracts being pulled along for the ride as the body grows, in 1997 Van Essen and Drury proposed that brain white matter itself exerts key mechanical forces driving brain morphogenesis (Van Essen and Drury, 1997). In particular, they posited that white matter tracts in the brain act as stabilizing cables, pulling against the cortex during development, inducing the formation of gyri. However, tissue properties studies of spinal cord have shown that gray matter is stiffer or at least the same stiffness as the white matter (Ichihara et al., 2001; Ozawa et al., 2001). If the same holds true for the brain, it could be argued that white and gray matter exert mechanical forces on each other, a type of tug-o-war, that influences the shaping of the brain. For example, expansion of the gray matter may stretch the bridging white matter, stimulating axon growth. In tandem, residual stress in the stretched white matter may provide mechanical forces that help shape the gyri, as suggested by Van Essen and Drury. Overall, there are likely many mechanical factors involved in brain morphogenesis. In particular, based on the stretch-growth process shown *in vitro*, it can be argued that the highly organized straight trajectories of white matter tracts in the brain is a result of uniaxial tension that molded them into their final orientation.

7. Exploiting stretch growth of integrated axon tracts to repair the nervous system

Understanding the mechanisms of axon regeneration and guidance has been one of the top priorities in the field of neuroscience. Indeed, the primary strategy to repair the damaged spinal cord and other nerve injuries is to bridge the lesions by promoting axon regeneration. However, coaxing a sufficient number of axons to grow substantial distances has posed a significant challenge. This is particularly exemplified in human spinal cord injury, where lesions commonly extend several centimeters in an environment that is normally non-permissive for axon growth (McDonald, 1999). Likewise for peripheral nerve transection – although axons do sprout out of the proximal nerve stump, their capacity to restore function to distant regions is limited due to the gradual loss of a labeled pathway (i.e., degeneration of the distal nerve stump) (Grafstein and Forman, 1980). To overcome the obstacles of the length and number of axons needed to bridge nervous system injuries, the axon stretch growth process has been exploited to engineer transplantable living nervous tissue constructs (Pfister et al., 2006b). While early in development, these constructs have shown promise in

repairing the nervous system. Notably, these constructs can be grown to bridge even extensive nervous system lesions.

Development of a nervous tissue construct

Possibly as impressive as the ability of axon tracts from DRG neurons to undergo extreme stretch growth is the capacity of stretch grown cultures to remain intact once removed from the *in vitro* environment. Typically, neuron cultures are discarded as dead when the neurons are no longer attached to bottom of the culturing container. Nonetheless, entire stretch grown cultures of millions of neurons and axons can be uprooted en masse, coaxed off of their attachment membranes in the elongation bioreactor while still maintaining their engineered geometry as well as their viability (Pfister et al., 2006a,b). It may actually be the high organization of the stretch grown cultures that promotes survival despite this harsh treatment. Regardless, their robustness to be removed from culture provides a unique opportunity to develop transplantable nervous tissue constructs to repair even extensive nervous system damage. With a geometry almost like a 'mini-nervous system,' stretch grown cultures appear to have the capacity to integrate with host nervous tissue at both sides of a lesion, creating a living bridge (Huang et al., 2006, 2008, 2009; Iwata et al., 2006; Pfister et al., 2004, 2006a,b).

To form nervous tissue constructs, elongated cultures are embedded in a hydrogel such as collagen. Once polymerized, the gel provides the structural support to lift the cultures off the underlying bioreactor membranes by carefully rolling the gel longitudinally to the axis of the axon tracts. With the geometry of the culture preserved, the rolled structure can be placed on a glass sheet or another support for transportation. The culture and the hydrogel essentially form the core components of a three-dimensional nervous tissue construct. These constructs can be used in this core form for CNS transplantation. Alternatively, this core construct can be placed in a synthetic tube, composed of materials such as polyglycolic acid or collagen, to provide further support and protection of the culture for peripheral nerve repair (Pfister et al., 2006b). Three avenues of restoring function using nervous tissue constructs have recently been initiated, focusing on repairing the spinal cord, peripheral nerves and creating a brain machine interface.

Transplantation of nervous tissue constructs

For spinal cord repair, the nervous tissue constructs were utilized to bridge a 1-cm long cavity spanning three vertebrae in the rat (modified lateral polyglycolic model). One month after transplantation, histopathologic analyses demonstrated that the transplanted constructs consistently survived completely intact in the injured spinal cord (Iwata et al., 2006). Specifically, the transplanted DRG neurons were found surviving at each end of the lesion with the associated tracts spanning the center of the lesion. Perhaps more importantly, axons from each end of the transplanted construct had extended out through the collagen, penetrating into the host tissue and host axons were found entering the construct. While early in the course of study, these results demonstrate the feasibility transplanting nervous tissue constructs as an alternative or supplemental approach to facilitate the formation of functional relays across extensive spinal cord lesions.

Nervous tissue constructs of stretch grown axon tracts have also been used to repair the peripheral nerve (Huang et al., 2009). In this case, the constructs were encased in tubes composed of polyglycolic acid to provide physical support. In addition, the neurons used in the constructs were derived from transgenic rats expressing green fluorescent protein (GFP +) to later identify their graft origin. To identify the host tissue, transgenic rats expressing alkaline phosphatase (AP+) were used as the recipients. In these rats, a 1.2–1.5-cm excision of the sciatic nerve was repaired by constructs of the same length. The constructs were sutured

or glued end to end with the proximal and distal nerve stumps shortly following excision of the nerve segment. Four months after transplantation, gross examination of the transplanted region revealed an almost normal appearing nerve and a compound action potential could be transplanted across this grafted region. Microscopically, the transplanted GFP+ neurons at each end of the graft were found completely intact, as were the spanning GFP+ axon tracts. Remarkably, AP+ regenerated host axons were observed intertwined with the graft axons and numerous axons throughout the graft region demonstrated extensive myelination. Thus, the living nervous tissue construct also appears to be a suitable scaffold to promote regeneration of transected peripheral nerves (Fig. 5).

Nervous tissue constructs as a brain machine interface

Another therapeutic use of nervous tissue constructs has been to develop a brain machine interface. Notably, there is neither convention nor consensus regarding the best approach to interface the brain with assistive machines such as prostheses. The challenge is for prosthetics to perform naturally, relaying two-way communication with the patient's brain. For example, the patient's thoughts could convert nerve signals into movements of a prosthetic, while sensory stimuli, such as temperature or pressure, provides feedback to adapt the movements and promote proprioception. The promising capacity of nervous tissue constructs to bridge nerve damage also lends itself to creating a bioelectric interface. Tapping into mixed (motor and sensory) peripheral nerves, such as those surviving in an amputated limb stump, provides two-way access to the brain. Accordingly, transplantable nervous tissue constructs have been created with one end coupled to a multiple electrode array (MEA) (Kameswaran et al., 2008; Pfister et al., 2007) (Fig. 6). Like an extension cord of sorts, the non-electrode end of the lab-grown nervous tissue could integrate with a patient's nerve relaying the signals to and from the electrode side, in turn connected to an electronic device. In this iteration, one of the two populations of neurons inside the elongation bioreactor is grown on a MEA. Following elongation, removal from the cultures from the bioreactor is performed while keeping the MEA affixed to the neurons. This allows for a construct to be transplanted with the non-MEA end of a construct free to integrate with a host nerve, while the MEA side can be used to record efferent signals from the host nerve and transmit afferent to the host nerve. This design allows for a bioelectric interface with a device that has both motor and sensory capabilities.

Capacity of human neurons to be engineered into nervous tissue constructs

To demonstrate the clinical feasibility of using axon stretch growth to repair the nervous system, adult human DRG neurons have also been engineered into nervous tissue constructs (Huang et al., 2008). Harvested adult human DRG neurons have been isolated from patients undergoing elective ganglionectomies and from organ donors. Under optimized culturing conditions, these human neurons can survive in culture for at least 3 months. Moreover, axon tracts from these adult human neurons have been stretch grown in the elongation bioreactors up to 10 mm in length with a remarkably similar appearance to the elongated rat DRG cultures (Fig. 7). Accordingly, even adult human DRGs demonstrate a capacity to be engineered into living nervous tissue constructs. Potentially, patients could donate their own neurons to create autologous grafts, or neurons could be recovered from organ donors to make allografts.

8. Conclusion

Ultimately, it is no longer a stretch to suggest that tension can actually be good for your nerves. Yet, there remain many secrets as to how the blue whale, or any large animal, can expand its nervous system so rapidly during development. It is certain that many as yet unidentified processes must occur to accomplish such unprecedented growth. It is

anticipated that an enhanced understanding of mechanisms governing the important natural process of stretch growth of integrated axons will reveal new approaches to repair the damaged nervous system and/or restore function.

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References

- Abe ION, Ichimura H, Tsujino A, Sun J, Hara Y. Internodes can nearly double in length with gradual elongation of the adult rat sciatic nerve. *J Orthop Res* 2004;22:571–577. [PubMed: 15099637]
- Alvarez J, Giuditta A, Koenig E. Protein synthesis in axons and terminals: significance for maintenance, plasticity and regulation of phenotype with a critique of slow transport theory. *Prog Neurobiol* 2000 September;62(1):1–62. [PubMed: 10821981]
- Bray D. Axonal growth in response to experimentally applied mechanical tension. *Dev Biol* 1984 April;102(2):379–389. [PubMed: 6706005]
- Brittis PA, Lu Q, Flanagan JG. Axonal protein synthesis provides a mechanism for localized regulation at an intermediate target. *Cell* 2002 July;110(2):223–235. [PubMed: 12150930]
- Brown A. Slow axonal transport: stop and go traffic in the axon. *Nat Rev Mol Cell Biol* 2000 November;1(2):153–156. [PubMed: 11253369]
- Brown A. Axonal transport of membranous and nonmembranous cargoes: a unified perspective. *J Cell Biol* 2003 March;160(6):817–821. [PubMed: 12642609]
- Burnett MG, Zager EL. Pathophysiology of peripheral nerve injury: a brief review. *Neurosurg Focus* 2004 May;16(5):E1. [PubMed: 15174821]
- Clarkson B, Ota K, Ohkita T, O'Connor A. Kinetics of proliferation of cancer cells in neoplastic effusions in man. *Cancer* 1965 October;18(10):1189–1213. [PubMed: 5890960]
- Dickson BJ. Molecular mechanisms of axon guidance. *Science* 2002 December;298(5600):1959–1964. [PubMed: 12471249]
- Evans GR. Peripheral nerve injury: a review and approach to tissue engineered constructs. *Anat Rec* 2001 August;263(4):396–404. [PubMed: 11500817]
- Grafstein B, Forman DS. Intracellular transport in neurons. *Physiol Rev* 1980 October;60(4):1167–1283. [PubMed: 6159657]
- Heidemann SR, Buxbaum RE. Mechanical tension as a regulator of axonal development. *Neurotoxicology* 1994 Spring;15(1):95–107. [PubMed: 8090366]
- Heidemann SR, Lamoureux P, Buxbaum RE. Growth cone behavior and production of traction force. *J Cell Biol* 1990 November;111(5 Pt 1):1949–1957. [PubMed: 2229183]
- Heidemann SR, Lamoureux P, Buxbaum RE. Cytomechanics of axonal development. *Cell Biochem Biophys* 1995;27 (3):135–155. [PubMed: 9279454]
- Huang JH, Cullen DK, Browne KD, Groff R, Zhang J, Pfister BJ, Zager EL, Smith DH. Long-term survival and integration of transplanted engineered nervous tissue constructs promotes peripheral nerve regeneration. *Tissue Eng Part A* 2009 July;15(7):1677–1685. [PubMed: 19231968]
- Huang JH, Zager EL, Zhang J, et al. Harvested human neurons engineered as live nervous tissue constructs: implications for transplantation laboratory investigation. *J Neurosurg* 2008 February;108(2):343–347. [PubMed: 18240932]
- Ichihara K, Taguchi T, Shimada Y, Sakuramoto I, Kawano S, Kawai S. Gray matter of the bovine cervical spinal cord is mechanically more rigid and fragile than the white matter. *J Neurotrauma* 2001 March;18(3):361–367. [PubMed: 11284555]

- Iwata A, Browne KD, Pfister BJ, Gruner JA, Smith DH. Long-term survival and outgrowth of mechanically engineered nervous tissue constructs implanted into spinal cord lesions. *Tissue Eng* 2006 January;12(1):101–110. [PubMed: 16499447]
- Kameswaran N, Cullen DK, Pfister BJ, et al. A novel neuroprosthetic interface with the peripheral nervous system using artificially engineered axonal tracts. *Neurol Res* 2008 December;30(10):1063–1067. [PubMed: 19079981]
- McDonald JW. Repairing the damaged spinal cord. *Sci Am* 1999 September;281(3):64–73. [PubMed: 10467750]
- Miller KE, Heidemann SR. What is slow axonal transport? *Exp Cell Res* 2008 June;314(10):1981–1990. [PubMed: 18410924]
- Nixon RA. The slow axonal transport debate. *Trends Cell Biol* 1998a March;8(3):100. [PubMed: 9722407]
- Nixon RA. The slow axonal transport of cytoskeletal proteins. *Curr Opin Cell Biol* 1998b February;10(1):87–92. [PubMed: 9484599]
- O'Toole MLP, Miller KE. A physical model of axonal elongation: force, viscosity, and adhesions govern the mode of outgrowth. *Biophys J* 2008;94:2610–2620. [PubMed: 18178646]
- Ozawa H, Matsumoto T, Ohashi T, Sato M, Kokubun S. Comparison of spinal cord gray matter and white matter softness: measurement by pipette aspiration method. *J Neurosurg* 2001 October;95(2 Suppl):221–224. [PubMed: 11599840]
- Pfister BJ, Bonislawski DP, Smith DH, Cohen AS. Stretch-grown axons retain the ability to transmit active electrical signals. *FEBS Lett* 2006a June;580(14):3525–3531. [PubMed: 16730003]
- Pfister BJ, Huang JH, Kameswaran N, Zager EL, Smith DH. Neural engineering to produce in vitro nerve constructs and neurointerface. *Neurosurgery* 2007 January;60(1):132–141. 137–141. discussion.
- Pfister BJ, Iwata A, Meaney DF, Smith DH. Extreme stretch growth of integrated axons. *J Neurosci* 2004 September;24(36):7978–7983. [PubMed: 15356212]
- Pfister BJ, Iwata A, Taylor AG, Wolf JA, Meaney DF, Smith DH. Development of transplantable nervous tissue constructs comprised of stretch-grown axons. *J Neurosci Methods* 2006b May;153(1):95–103. [PubMed: 16337007]
- Roy S, Coffee P, Smith G, Liem RK, Brady ST, Black MM. Neurofilaments are transported rapidly but intermittently in axons: implications for slow axonal transport. *J Neurosci* 2000 September;20(18):6849–6861. [PubMed: 10995829]
- Shah JV, Cleveland DW. Slow axonal transport: fast motors in the slow lane. *Curr Opin Cell Biol* 2002 February;14(1):58–62. [PubMed: 11792545]
- Smith DH, Wolf JA, Meaney DF. A new strategy to produce sustained growth of central nervous system axons: continuous mechanical tension. *Tissue Eng* 2001 April;7(2):131–139. [PubMed: 11304449]
- Tessier-Lavigne M, Goodman CS. The molecular biology of axon guidance. *Science* 1996 November;274(5290):1123–1133. [PubMed: 8895455]
- Van Essen DC, Drury HA. Structural and functional analyses of human cerebral cortex using a surface-based atlas. *J Neurosci* 1997 September;17(18):7079–7102. [PubMed: 9278543]
- Weiss P. Nerve patterns: the mechanics of nerve growth. *Growth, Third Growth Symposium* 1941;5:163–203.
- Wolf JA, Stys PK, Lusardi T, Meaney D, Smith DH. Traumatic axonal injury induces calcium influx modulated by tetrodotoxin-sensitive sodium channels. *J Neurosci* 2001 March;21(6):1923–1930. [PubMed: 11245677]
- Yu TW, Bargmann CI. Dynamic regulation of axon guidance. *Nat Neurosci* 2001 November;4(Suppl):1169–1176. [PubMed: 11687826]

Fig. 1.

(A) A 1941 diagram originally entitled, “Group of neuroplasts in three successive stages,” in the first published suggestion of a mechanical mechanism of nerve growth. Paul Weiss posited that “*the nerve is drawn out by the growth and dislocations of its terminal tissues,*” in a paper entitled, “Nerve patterns: the mechanics of nerve growth” in *Growth, Third Growth Symposium* 5:163–203. Note the initial phase of axon growth of a migrating growth cone navigating to a target. (B) Proposed second phase of axonal growth includes stretch growth of nerves and white matter tracts following synaptogenesis. As Weiss postulated, this axon growth is due to mechanical tension as the body grows. One of the most extreme examples is the growth of whale spinal axons during development as the vertebrae grow in length, reaching at least 3 cm of growth per day.

Fig. 2.

(A) Schematic illustration of the experimental process of stretch growth in integrated axon tracts. Top: A short membrane (yellow) attached to a plastic block is placed on top of a long rectangular membrane (blue). A chamber is formed by the plastic block, in which neurons are plated and allowed to integrate. A neural network (red) is formed, including axon fascicles that grow across the border between the top and bottom membranes. Bottom: Movement of the plastic block via a computer controlled microstepper motor system divides the culture and progressively separates the opposing halves by sliding the top membrane across the bottom membrane. This technique results in the creation of long fascicular tracts of axons spanning the two membranes. (B) Components of elongation device: Left, cell culture bioreactor chamber capable of producing stretch grown axon fascicles to 15 cm. Right, two types of fully assembled elongator apparatus, including the bioreactors, linear tables and microstepper motor. Computer driving system not shown.

Fig. 3.

(A) Stretch-growth-induced organization of axon tracts. Phase photomicrographs of a live cortical neuron cultures demonstrating a region of stretch-grown axons at the border of the top membrane at successive days (2, 4, and 7) of elongation. Note the gradual coalescing of neighboring axon bundles and thickening of the bundles at the edge of top membrane. Bar = 50 μ m. (B) Recapitulation of a natural stretch growth process of axon tracts. Fluorescence confocal photomicrograph of axon fascicles from cortical neurons at 7 days of stretch-induced growth, elucidated by immunostained microtubule protein in fixed cultures. Left: Note the harpstring appearance of multiple long fascicular axon tracts arranged in parallel (Bar = 50 μ m). Right: Two large fascicular tracts composed of thousands of axons that were produced by stretch-induced growth *in vitro* (each approximately 50 μ m wide).

Fig. 4.

(A) Stretch growth boundaries of integrated axon tracts. Left: Graphic representation of stretching conditions that define the boundaries of axon growth or disconnection for DRG neurons. Each line represents individual paradigms of accelerating displacement (elongation) of integrated axon tracts in culture. X's in shaded area denote disconnection of axon tracts during stretching. Lines without X's represent successful growth of axon tracts in response to escalating stretch rates. Right: Photomicrograph of immuno-labeled disconnected axon. Disruption resulted from a too rapidly accelerated elongation program. (B) Not jellyfish; stretch grown axon tracts. Simple light photograph of axon tracts from DRG neurons stretch grown to 5-cm long (specimen and background colors are modified to highlight axon tracts). Axon tracts (middle) bridge two populations of neurons (top and bottom). Prior to the initiation of stretch growth, the two populations of neurons were adjacent and the bridging axons were only approximately 100- μ m long. Progressively separating the neuron populations induced mechanical tension on the axon tracts resulting in enormous and rapid growth. (C) Normal ultrastructure of axons despite extreme stretch growth reaching 5-cm lengths in 14 days. Left: Scanning electron micrographs (boxed area on top expanded below) illustrating a small fascicle of stretch grown DRG axons. Right: Transmission electron micrograph of cross-sections near the center length of stretch grown axon fascicles shows a normal complement of cytoskeletal structures. Notably, however, the diameter of stretch grown axons is actually greater than that of short, non-stretch grown axons. Scale bar = 500 nm. (D) Schematic illustration at top showing communication across stretch grown axons. Application of KCl on neuron cell bodies at one end results in action potential transmission that can be recorded from neurons at the other side with recording trace shown at bottom.

Fig. 5.

Left: Survival and integration of transplanted living nervous tissue constructs for peripheral nerve repair. Top, grossly normal appearing 1.5-cm long region of nerve bridged by a nervous tissue construct 6 weeks post-transplantation; Middle, Surviving transplanted neuron cell bodies (red) and axons (green) at one end of the transplanted region; Bottom, confocal projection in the same region depicts host axons (red) and graft axons (red/yellow) assembled in a network extending through the transplanted region from the neuron clusters. Right: Top two panels show the center of the transplanted region, where the axons from host (red) have penetrated and are remarkably intertwined with transplanted axons (green). A cross-section of this region shows myelination (red) circumscribing axons (green).

Fig. 6.

A new approach for a brain machine interface. A microelectrode array (gray, right) serves as the plating membrane under one population of neuron cell bodies. An adjacent population is pulled away inducing stretch growth of the connecting axon tracts (bottom, right), creating a transplantable neuro-electric construct. This process creates a conceptual interface with the nervous system. For example, transplanting the non-electrode end onto a peripheral nerve may allow for bi-directional communication. Stimuli from a device (e.g., upper limb prosthetic) passed to an electrode (bottom right) could be transferred through the transplanted construct into the integrated nerve and onto the brain for sensory detection. Likewise, motor signals from the brain through the nerve to the electrodes could be detected to drive functions of the device.

Fig. 7. Tissue-engineering adult human neurons. Top: Fluorescent micrographs using multiple stains to identify harvested individual DRG neurons that survive for months in culture. Bottom: The axons from these neurons can be stretch grown to create long living nervous tissue constructs—a type of mini human nervous system that can be transplanted.