Brief Communication

Extreme Stretch Growth of Integrated Axons

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Large animals can undergo enormous growth during development, suggesting that axons in nerves and white matter tracts rapidly expand as well. Because integrated axons have no growth cones to extend from, it has been postulated that mechanical forces may stimulate axon elongation matching the growth of the animal. However, this distinct form of rapid and sustained growth of integrated axons has never been demonstrated. Here, we used a microstepper motor system to evaluate the effects of escalating rates of stretch on integrated axon tracts over days to weeks in culture. We found that axon tracts could be stretch grown at rates of 8 mm/d and reach lengths of 10 cm without disconnection. Despite dynamic and long-term elongation, stretched axons increased in caliber by 35%, while the morphology and density of cytoskeletal constituents and organelles were maintained. These data provide the first evidence that mechanical stimuli can induce extreme "stretch growth" of integrated axon tracts, far exceeding any previously observed limits of axon growth.

Key words: **axon; growth; development; stretch; elongation; cytoskeleton**

Introduction

Axon growth-cone extension and guidance toward a target during embryogenesis has been extensively examined (Tessier-Lavigne and Goodman, 1996; Yu and Bargmann, 2001; Dickson, 2002). During this process, it is believed that axon structure is built within the extending growth cone (Gallo and Letourneau, 2000; Dent and Gertler, 2003). However, little is known about a different form of growth that ensues after the axon reaches its target. It has been postulated that, as an animal grows, integrated axons are forced to increase in length (Weiss, 1941; Bray, 1984). Indeed, tension applied to growth cones of single axons in culture has been shown to induce transient lengthening (Dennerll et al., 1989; Zheng et al., 1991; Heidemann et al., 1995; Chada et al., 1997). However, integrated axons have no growth cones to extend from, suggesting that the addition of structure occurs by an alternative mechanism in response to stretch, or the axon would rupture. This distinct process, which we refer to as axon "stretch growth," likely represents an extreme phase of rapid and long-term axon growth that drives the formation of long nerves and white matter tracts.

Anecdotal evidence of extreme axon stretch growth is found throughout nature. It can be inferred that white matter tracts undergo rapid expansion in the blue whale's spine, which grows at a rate of >3 cm/d, or in the giraffe's neck, which increases in length by 2 cm/d at peak growth (Dagg and Foster, 1982; Bannister et al., 1996). Despite these observations, no current evidence demonstrates that integrated axons can sustain rapid growth for long periods of time. Furthermore, it is not known whether axons can maintain an intact structure during extreme stretch-growth conditions.

Here, we explored the stretch-growth potential and tolerances of integrated axon tracts *in vitro*. Using a specially designed microstepper motor system, axons spanning two populations of neurons were elongated at varying rates over days to weeks. Subsequently, the ultrastructure of mechanically elongated axons was examined to distinguish actual growth from a redistribution of axonal components.

Materials and Methods

Cell culture. Dorsal root ganglion (DRG) explants from embryonic day 15 rat embryos (Charles River Laboratories, Wilmington, MA) were isolated as described by Kleitman et al. (1998). Cultures were maintained in complete medium consisting of Neurobasal medium (Invitrogen, Carlsbad, CA) supplemented with B-27 (Invitrogen), 1% FBS (Hyclone, Logan, UT), 0.4 mM L-glutamine (Invitrogen), 2.5 gm/l glucose, and 10 ng/ml 2.5S nerve growth factor (Becton Dickinson, Bedford, MA). All of the cells were allowed to attach for 4 hr before the elongation-device chamber was filled with complete medium containing the mitotic inhibitors [mitotic inhibitor 1 (MI1), 5 μM cytosine β-D-arabinofuranoside (AraC; Sigma, St. Louis, MO), 20 μM 5-fluoro-2′-deoxyuridine (5FdU; Sigma), and 20 μM uridine (Sigma)]. After 2 d, the medium was exchanged with complete medium plus the mitotic inhibitors (MI2, 20 μM 5FdU, and 20 μM uridine) for 3 d. Thereafter, the medium was changed every 2–3 d, and MI2 was reapplied once a week.

At all times, we strictly adhered to the principles enumerated in the *Guide for the Care and Use of Laboratory Animals*, prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Service, National Research Council. The University of Pennsylvania Institutional Animal Care and Use Committee approved our protocols.

Elongation device. The axon stretch-growth system is composed of a custom-designed axon-expansion chamber, linear-motion table, and controller (supplemental Fig. 1, part of supplemental material, available at www.jneurosci.org). The expansion chamber is a sealed enclosure with a gas-exchange port that fits within a typical CO_2 incubator. It contains a removable axon-stretching frame that arranges two adjoining membranes in an overlapping manner, on which neurons can be cultured. The

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bottom membrane (198 μm thick), made of optically transparent Aclar 33C film (Structure Probe, West Chester, PA), covers the entire bottom of the elongation device on which the stationary population of neurons is cultured. An overlapping movable Aclar membrane (~10 μm thick) is placed on top of the bottom membrane and serves as the moving population of cells. Axons developing in culture can easily grow across the interface between the overlapping membranes interconnecting the neurons on both sides (supplemental Fig. 2, part of supplemental material, available at www.jneurosci.org). Axons bridging the two membranes were stretched by displacing the top membrane across the lower stationary membrane using a microstepper motor system (Servo Systems, Montville, NJ). Before assembly, Aclar was washed, treated in 1 M NaOH for 24 hr, and then sterilized in 100% ethanol for 10 min. Aclar membranes were attached using medical-grade RTV silicone (NuSil, Carpenteria, CA) and then treated with 10 μg/ml poly-L-lysine. Type 1 rat-tail collagen (Becton Dickinson, Franklin Lakes, NJ) as supplied was spread over the surface (10–20 μl/cm^2) and polymerized by exposure to ammonia vapors for 2 min and then allowed to dry completely before plating cells. The hydrophobic collagen surface allows for cells to be plated within any desired arrangement by applying the cell suspension in a defined puddle. DRG explants were plated and maintained as described above.

Axon stretch growth. To stretch isolated axons, rat DRG explants were plated, approximately one-half on each of the two adjacent membranes within an elongation chamber. Routinely, DRG neurons were given 5 d after plating for axons to extend between the two membranes by growth cone-mediated elongation. This growth formed a bridge of axons ~100 μm long, integrating the neuron populations on each membrane. To maximize the number of bridging axons available for stretch growth, DRG explants from 8 to 10 spinal cords were used in each experiment; however, smaller density cultures can also be successfully elongated. Bridging axons were stretched by slowly separating the two membranes via a microstepper motor (supplemental Figs. 1 and 2, supplemental material, available at www.jneurosci.org). The stretch rate was programmed into the motion-control system by implementing a displacement step and a resting time in a stepwise manner. Here, the net rate of axon elongation is expressed in units of millimeters per day. For example, the initial stretch rate of 1 mm/d was the net result of 2 μm displacements every 172 sec. Subsequent increases in the net stretch rate were applied by increasing the size of the displacement step or reducing the resting time.

To determine the optimal stretch-growth parameters during the initial 24 hr of elongation, the displacement steps and resting times were varied in a stepwise manner. In each experiment, cultures were examined after 24 hr of elongation for evidence of axon rupture. Based on initial studies, our experimental matrix maintained a net rate of elongation of 1 mm/d over the first day and began with displacement steps of 3.5 μm every 300 sec. With these parameters, stretch growth occurred, but with some evidence of axon disconnection. In each subsequent experiment, the displacement step was reduced in 0.5 μm increments (resting time adjusted to maintain a net elongation rate of 1 mm/d) until there was no additional evidence of axon disconnection. We then investigated whether the maximum net rate of elongation for the initiation of stretch growth could exceed 1 mm/d. The displacement step was reduced to the minimum resolution of the elongation system (0.5 μm per step). The net elongation rate was gradually increased above 1 mm/d by reducing the resting time until axon disconnection was observed.

After the initial 24 hr of growth, the net rate of axon elongation was increased by 1 mm/d every 48 hr until the desired plateau growth rate was reached. The cultures were examined each day, and maximal rate increases were determined according to whether the axon tracts disconnected or continued elongating. If the paradigm was successful, the stretch rate was increased more frequently (every 24, 12, 6 hr, and so on) until axon rupture was observed. If visual damage to the axons was noted, the rate increase strategy was reformulated over a longer time period. The desired rate of stretch growth was considered successful if axons could sustain that rate for a minimum of 24 hr without evidence of axon disconnection. In addition, we investigated whether rate accelerations >1 mm/d could be tolerated. Net elongation rates up to 4 mm/d were limited to increases of 1 mm/d, but subsequently the rate could be escalated by steps of 2–4 mm/d.

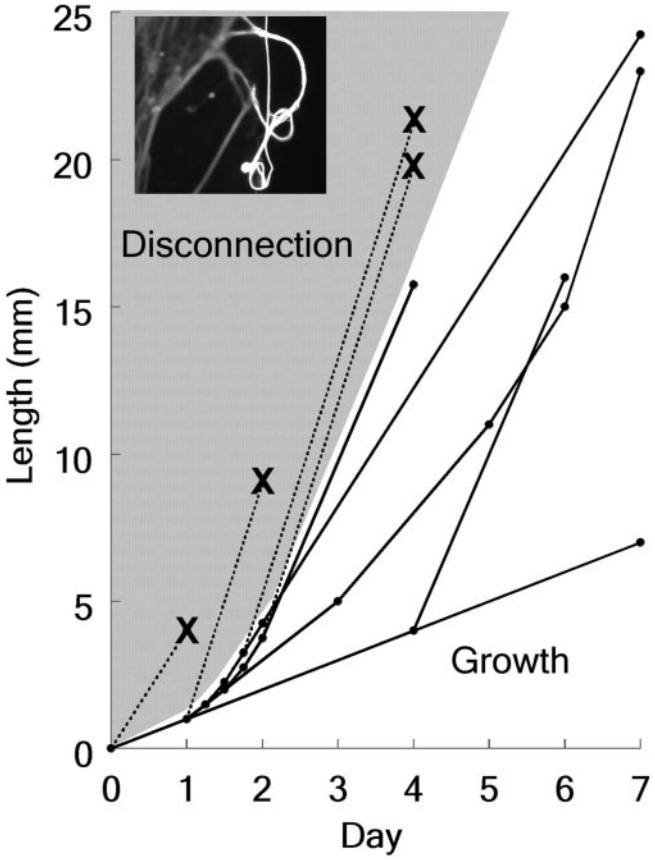

Figure 1. Graphic representation of stretching conditions that define the boundaries of axon growth or disconnection. Each line represents an individual paradigm of accelerating displacement (elongation) of integrated axon tracts in culture. Lines with an X in shaded area denote disconnection of axon tracts during stretching. Lines without an X represent successful growth of axon tracts in response to escalating stretch rates up to 8 mm/d. Inset figure shows a disconnected axon labeled with an antibody against phosphorylated neurofilament protein.

Specimen preparation and analysis. For transmission electron microscopy (TEM), elongated axons were fixed in 4% paraformaldehyde and 2% glutaraldehyde in 0.1 M sodium cacodylate buffer overnight at 4°C. To prevent damage to axons during their removal, the tissue was supported within 2% agar and then cut loose from the elongation device using a scalpel. The tissue was postfixed in 1% osmium tetroxide for 1 hr at 4°C and dehydrated in a graded ethanol series before infiltration and embedding in epoxy resin (EMbed-812; Electron Microscopy Sciences, Fort Washington, PA). After staining with uranyl acetate and lead citrate, sections were examined with a Jeol (Peabody, MA) 100CX transmission electron microscope. For scanning electron microscopy (SEM), elongated axons were fixed in 4% paraformaldehyde and 2% glutaraldehyde in 0.1 M sodium cacodylate buffer overnight at 4°C, postfixed in 1% osmium tetroxide for 30 min, and dehydrated in a graded ethanol series followed by a drying step of two 10 min applications of hexamethyldisilazane (Electron Microscopy Sciences). For image analysis, cross-sectional areas were measured for each axon using Scion (Frederick, MD) Image.

Results

We found that axon tracts can undergo extreme stretch growth under conditions of rapid as well as long-term mechanical elongation. However, there were clear boundaries that led to either growth or disconnection (Fig. 1). The thresholds of axon stretch growth were determined by performing >100 individual experiments evaluating a range of stretch rates escalated over several

Accelerating growth rate to 8mm per day

Day	0	1	3	5	6	7
Axon Length (mm)	0	1	5	11	15	23
Displacement step (mm)	0.002	0.002	0.002	0.004	0.004	
Step interval (s)	172	86.4	57.6	86.4	43.2	
Growth Rate (mm/day)	1	2	3	4	8	

Axon stretch-growth to 5cm in length

Day	0	1	1.5	2	14
Axon Length (mm)	0	1	2	3.5	50.5
Displacement step (mm)	0.002	0.002	0.002	0.004	
Step interval (s)	172	86.4	57.6	86.4	
Growth Rate (mm/day)	1	2	3	4	

Figure 2. Flowchart detailing two individual axon stretch-growth paradigms. Top, Scheme to reach an axon growth rate of 8 mm/d. Bottom, Scheme to produce 5-cm-long axon tracts.

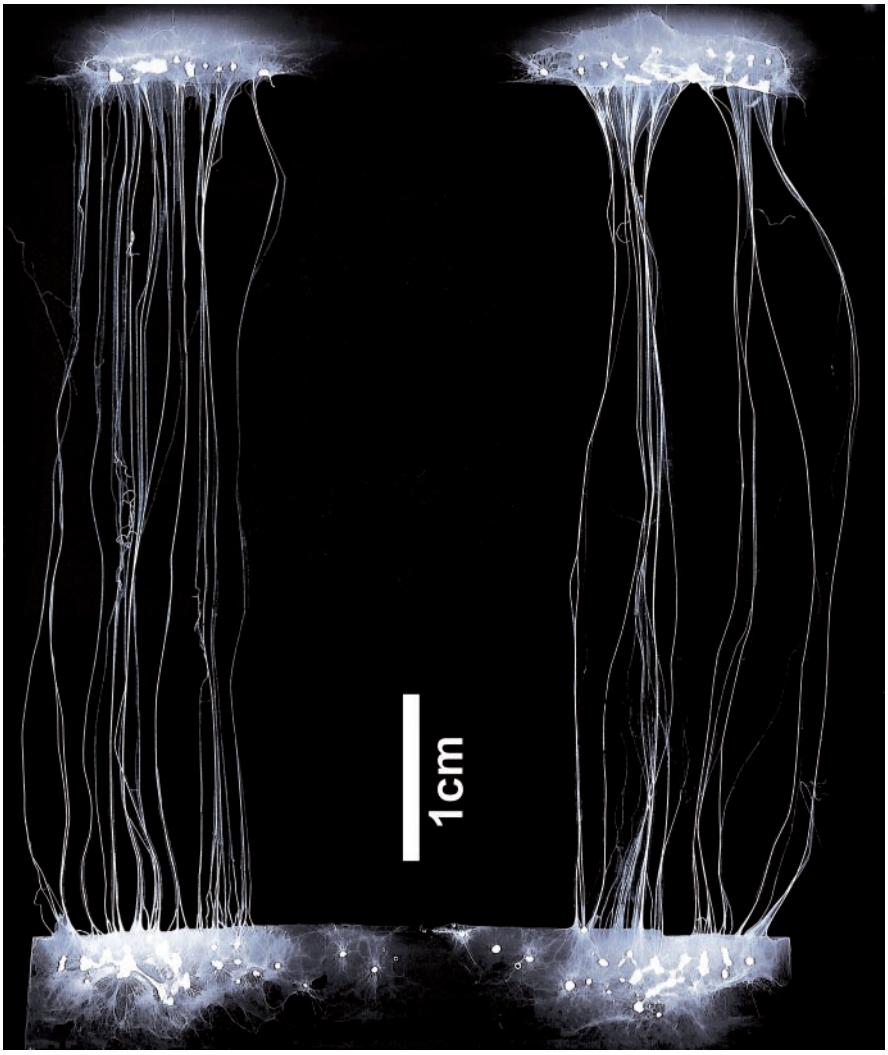

Figure 3. Axon tracts stretch grown to 5 cm long. Axon tracts (middle) bridge two populations of neurons (top and bottom). Before the initiation of stretch growth, the two populations of neurons were adjacent and the bridging axons were only ~100 μm long. Progressively separating the neuron populations induced mechanical tension on the axon tracts, resulting in enormous and rapid growth (colors are inverted to highlight axon tracts).

days and sustained elongation over a few weeks. Stretch-growth paradigms were considered successful if axons continued to elongate and there was no evidence of disconnection. Optimal conditions were found to be dependent on two parameters: 1) the size and frequency of displacement steps at a fixed stretch rate and 2) the time period for acclimation to each increase in the net rate of elongation.

Over the first 24 hr of stretch growth, axon tracts could be expanded without disconnection at a maximum elongation rate of 1 mm/d, representing an ~10-fold increase over their original length. However, disconnection was closely associated with the strain applied to axons, and growth could be achieved only through a gradual motion consisting of small and frequent displacement steps. Through a stepwise analysis, the optimal stretch parameters for the net elongation rate of 1 mm/d were found to be 2 μm displacements every 172 sec. This represents ~2% of applied strain at the start of elongation when axons measured ~100 μm. Stretch rates equivalent to 1 mm/d consisting of larger displacement steps and longer resting times led to rupture of some axon tracts, whereas reducing displacements smaller than 2 μm provided no additional benefit. In addition, regardless of the size and frequency of the displacement steps, stretch rates beyond 1 mm/d resulted in some axon disconnection during the first day of elongation.

After the 24 hr initiation period, axons tolerated progressively greater rates of elongation as long as sufficient acclimation time was allowed between increases in the net rate. As a result of the substantial growth in axon length after 24 hr of stretch, applied strain had rapidly decreased from 2 to 0.2%. However, axons would readily disconnect if elongation rates were stepped up too rapidly, even though the associated increase in strain was small. It was found that net increases of 1 mm/d required 12 hr or more of acclimation until a rate of 4 mm/d was reached. Thereafter, the stretch rate could be increased by a step of up to 4 mm/d (Fig. 2). This approach resulted in an exceptional growth rate of 8 mm/d, which was sustained for 24 hr ($n = 10$) to 48 hr ($n = 2$) without evidence of disconnection (Figs. 1, 2). This was the highest rate evaluated, and there was no evidence that even greater rates could not be achieved.

Using the above rate escalation scheme, it was also observed that stretch growth of axons could be maintained over several weeks in culture, producing axon tracts of unprecedented lengths. Axons were continuously extended at the rate of 4 mm/d over 14 d, resulting in 5-cm-long axon tracts ($n = 5$) (Fig. 3), or over 28 d, reaching a length of 10 cm ($n = 1$). In each preparation, the total number of elongating axons was estimated to be 10^5–10^6, with fascicles large enough to be seen easily with the naked eye.

Phase-contrast microscopy examination confirmed that the axon terminals integrated with the opposing cell population before elongation began. At initiation of elongation, these integrated regions appeared adherent to the Aclar membranes and displayed no motion with agitation of the expansion chamber. In addition, the topographic morphology of cell bodies and pro-

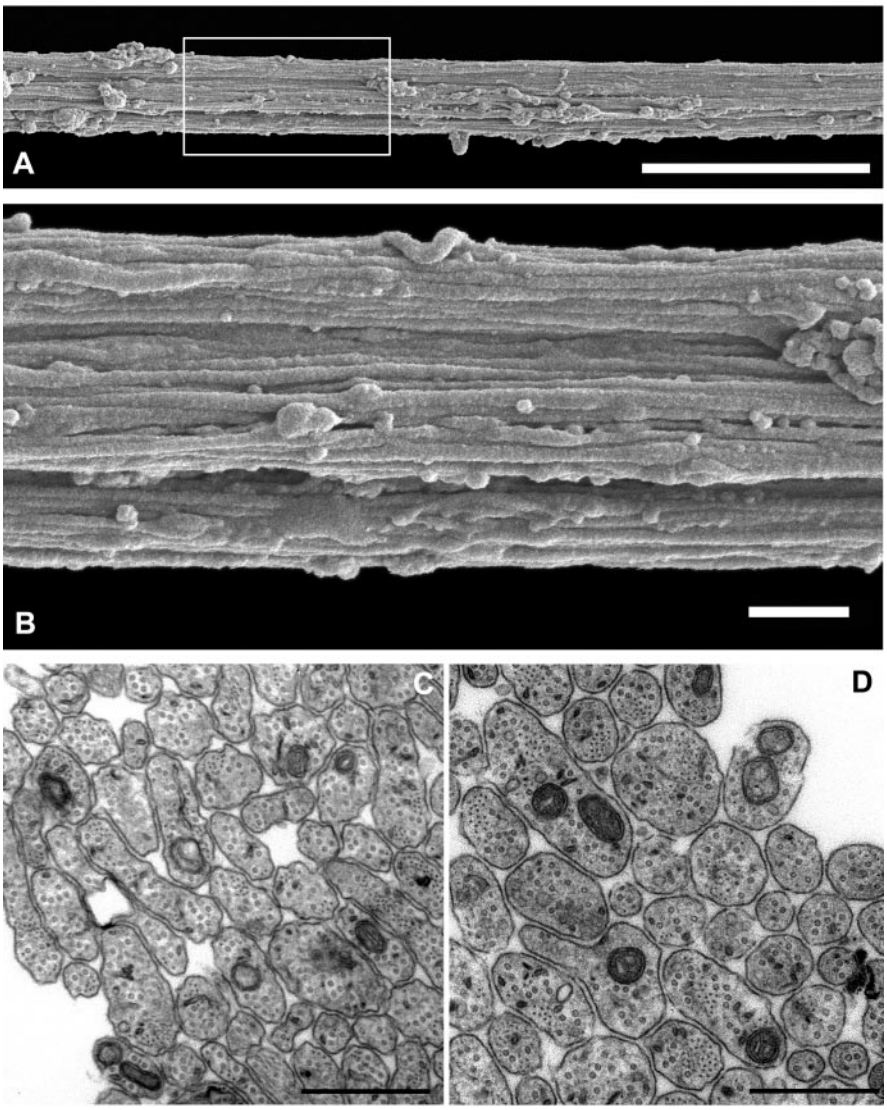

Figure 4. Electron microscopy of stretch-grown axons. Scanning electron micrographs illustrating a small fascicle composed of axons ~100–250 nm in diameter (*A*, *B*). Fasciculation of axons occurs during the elongation process as smaller bundles and individual axons coalesce and adhere to one another, forming larger bundles similar to the one depicted here. Transmission electron micrograph of cross sections near the center of axon fascicles in nonstretch conditions (*C*) and axons stretched to a length of 5 cm in 14 d (*D*), showing no change in axon cytoskeletal structures. Scale bars: *A*, 10 μm; *B*, 1 μm; *C*, *D*, 500 nm.

cesses in these two membrane regions remained completely static over time during elongation, demonstrating that they were not modified by stretch. In contrast, axon bundles spanning the two membranes were clearly nonadherent and would undergo free lateral motion with agitation of the chamber. Thus, the applied stretch and growth were conveyed only to the nonadherent central portion of the axons spanning the two membranes and not to the axon terminals.

Actual growth of the elongated axon tracts was confirmed by analyzing their structure with electron microcopy. SEM examination of the longitudinal aspect of elongated cultures demonstrated that the axons were highly organized in large tracts arranged in parallel to each other. Initially, axons are oriented in a web of free-floating small fascicles, which coalesce into larger tracts as they elongate (Fig. 4*A*,*B*). Axons appeared uniform along their lengths, and there was no evidence that the axons were thinning out or redistributing axonal constituents because of stretch. To examine the axon ultrastructure, TEM images were acquired from cross sections of axons stretch grown to 5 cm and "static" nonstretched axons taken near the center of the axon length [the farthest distance from the soma (Fig. 4*C*,*D*)]. Cross-sectional areas of ~2000 individual axons were measured from 50 images taken from four individual regions. Surprisingly, the average cross-sectional area was 35% larger for the elongated axons (0.58 μm^2) compared with nonelongated axons from a static culture (0.43 μm^2; $p < 0.001$). Axonal ultrastructure displayed the typical morphology and density of key structural elements and organelles found in static controls. Microtubule density remained constant at 153/μm^2 for static controls and 158/μm^2 for stretch-grown axons. In addition, neurofilament organization appeared unchanged, mitochondrial morphology was normal, and there was no change in the number of mitochondria. Furthermore, there was no evidence that stretch growth induced axonal pathology such as loss or interruption of cytoskeletal structure or the accumulation of proteins because of disruption of axonal transport.

Discussion

Large animals can undergo enormous growth during development, suggesting that their nervous systems are forced to rapidly expand as well. However, integrated axons no longer have growth cones from which to extend. Accordingly, integrated axon tracts may undergo a form of rapid and sustained growth that is not consistent with the current understanding of axon assembly within the growth cone (Gallo and Letourneau, 2000; Dent and Gertler, 2003). Here, mechanotransduction resulting from the growth of an organism may be the key mechanism that initiates and maintains stretch growth of the axon cylinder. By mapping out stretch conditions that lead to either disconnection or elongation of axon tracts *in vitro*, we found a defined range of parameters that are favorable for growth. The boundaries of this stretch-growth process follow a surprisingly steep curve of escalating elongation rates, reaching a remarkable 8 mm/d growth within a few days of stretching. In addition, this process rapidly induced 100-μm-long axons to extend up to 10 cm while maintaining a normal cytoskeletal structure. Unexpectedly, these mechanically elongated axons were found to increase in caliber as well as length despite such a high rate of extension. These *in vitro* observations support axon stretch growth as a primary *in vivo* mechanism driving the formation of long nerves and white matter tracts even under extreme growth conditions.

Surprisingly, few studies have examined the effects of mechanical elongation of axons. In a series of reports, the growth cones of single axons were attached to needles and towed over a few hours to explore the relationship between tension and neurite outgrowth (Bray, 1984; Dennerll et al., 1989; Zheng et al., 1991;

Heidemann et al., 1995; Chada et al., 1997). From these observations, it was proposed that tension from locomotor activity of growth cones is an important regulator of axon outgrowth. Thus, tension may be a key modulator for two distinct forms of axonal growth that act in continuum; initially, growth cones slowly pull individual axons to reach their targets (Bray, 1979; Lamoureux et al., 1989; Heidemann et al., 1990), followed by stretch growth, which forces large populations of integrated axons to grow in unison at enormous rates over long periods of time (Weiss, 1941; Bray, 1984).

From the previous studies of towed growth cones, it was found that short neurites could only be mechanically elongated at high rates for up to ~43 min and that rates exceeding 100 μm/hr led to disconnection within a few hours (Bray, 1984; Lamoureux et al., 1998). Likewise, our results reveal that if high elongation rates are applied early, growth cannot be sustained, and axons will rupture within the first 24 hr. In all, we found two sequential factors that defined the boundaries of long-term stretch growth of integrated axons: strain and acclimation. At the initiation of mechanical elongation, the axons were short and extremely vulnerable to disconnection relative to the strain applied in each displacement step. Thus, over the first day of stretch, growth without disconnection could be induced only with small steps and a limited net elongation rate. Subsequently, the applied strain in each step became negligible as the axons grew in length. Nonetheless, longer axons would rupture if the net elongation rate was accelerated too quickly, despite relatively small changes in strain. By providing a metered period of time to acclimate to each incremental increase in elongation rate, the axons could ultimately tolerate at least 8 mm of expansion per day. This suggests a cellular conditioning process that gradually increases the rate at which the cell can accommodate axonal growth. If the stretch rate increased faster than the neuron could accelerate the growth of axons, tension would accumulate, resulting in rupture and disconnection. Because the high rate of stretch growth of 8 mm/d was well tolerated, it is likely that even greater growth rates could be achieved with appropriate conditioning.

It is remarkable to consider the transport rate and quantity of cellular material necessary to expand axons at 8 mm/d or to sustain high rates of growth to 10 cm lengths. Even so, this extreme process is in considerable contrast to the ~1 mm/d maximal growth rate measured for growth-cone extension (Bray, 1991; Lamoureux et al., 1992; Buettner et al., 1994). Likewise, this rapid growth is not consistent with current understanding of axon transport, which has been shown to be limited to an average speed of ~1 mm/d for important structural elements such as neurofilament proteins (Nixon, 1998a,b; Brown, 2000; Roy et al., 2000; Shah and Cleveland, 2002). The rapid and sustained stretch growth demonstrated here clearly shows that the physiological capacity of axons to rapidly and continuously expand is not limited by protein synthesis, transport rates, or the availability of structural constituents (Lamoureux et al., 1998; Alvarez et al., 2000; Brown, 2000; Roy et al., 2000; Brittis et al., 2002). Because of the increased demand to add structure during stretch growth, an alternative axon growth process such as intercalated addition of the membrane and cytoskeleton may occur, as has previously been suggested (Khanin et al., 1998; Nixon, 1998c). Although the cellular processes of extreme stretch growth have yet to be elucidated, the current data demonstrate that an initiating mechanism is mechanical transduction.

Although we previously suggested that mechanical tension could induce the slow extension of axon tracts (Smith et al., 2001), our current data provide the first experimental evidence of extreme stretch growth of axons *in vitro*. Specifically, the growth rates and lengths achieved by stretch growth far exceed any previously identified limits of axon growth. Furthermore, SEM and TEM revealed that, even for extreme growth conditions, such as those that may occur in large animals, the axonal architecture is maintained during elongation, while the axon caliber actually increases. The normal structure of the stretched axons confirms that continuous mechanical stimulation can induce long-term and accelerating stretch growth of integrated axons responding with growth in both length and caliber. Accordingly, these data support the long-held hypothesis that the mechanical forces resulting from the growth of an organism can induce the growth of axon tracts (Weiss, 1941; Bray, 1984).

In addition to providing insight into the mechanisms of axon growth during development, it is important to consider that extreme axonal stretch growth in culture may be exploited for nerve repair. By recapitulating a natural growth process, long nerve tracts can be rapidly produced and potentially used as transplant material to bridge even extensive nerve damage.

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