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A New Strategy to Produce Sustained Growth of Central Nervous System Axons: Continuous Mechanical Tension

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ABSTRACT

Although a primary strategy to repair spinal cord and other nerve injuries is to bridge the damage with axons, producing axons of sufficient length and number has posed a significant challenge. Here, we explored the ability of integrated central nervous system (CNS) axons to grow long distances in response to continuous mechanical tension. Neurons were plated on adjacent membranes and allowed to integrate, including the growth of axons across a 50-μm border between the two membranes. Using a microstepper motor system, we then progressively separated the two membranes further apart from each other at the rate of 3.5 μm every 5 min. In the expanding gap, we found thick bundles comprised of thousands of axons that responded to this tensile elongation by growing a remarkable 1 cm in length by 10 days of stretch. This is the first evidence that the center portion of synapsed CNS axons can exhibit sustained "stretch-induced growth." This may represent an important growth mechanism for the elongation of established white matter tracts during development. We also found by doubling the stretch rate to 7 μm/5 min that the axon bundles could not maintain growth and disconnected in the center of the gap by 3 days of stretch, demonstrating a tolerance limit for the rate of axonal growth. We propose that this newfound stretch-induced growth ability of integrated CNS axons may be exploited to produce transplant materials to bridge extensive nerve damage.

INTRODUCTION

Recently, there has been enormous attention on treatment strategies for spinal cord injury (SCI), including development of tissue and cell transplant techniques. The basic premise of most of these transplant efforts is to create a bridge that can span the injured portion of the spinal cord. Ideally, this bridge would reestablish bidirectional communication in the spinal cord that is normally carried via axons in the white matter. One of the most vigorously pursued bridging strategies is to transplant a synthetic scaffolding seeded with chemoattractant molecules and/or cells into a damaged spinal cord that would entice axons to grow out from one end of the lesion and guide them to synapse with viable nervous tissue on the other side (for review, see ref. 1). However, a major obstacle of this approach is the enormous length that the axons would have to grow across SCI lesions in an environment that is normally nonpermissive for

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axon growth. This challenge is particularly acute in humans where SCI lesions commonly extend several centimeters.

Other SCI bridging techniques include transplanting peripheral nerves, intact fetal spinal cords, dissociated nervous tissue cells, stem cells, and glial cells (for review, see refs. 1–4); recent examples in refs. 5–8). Although some of these transplant techniques have improved functional outcome in animal models, this benefit is thought to be due to physical and biochemical support for the host tissue surrounding the lesion rather than through the formation of an axon bridge.[3,4] Despite enormous efforts, no technique has come close to the goal of reestablishing robust axonal connections, even through relatively small SCI lesions in mature animals.

Here, we used a fundamentally novel strategy to grow numerous CNS axons across relatively long distances, up to 1 cm. In contrast to techniques such as chemical guidance to create long axons, we explored the ability of cultured CNS axons to grow progressively in response to continuous mechanical tension. Previous studies have demonstrated that the short-term tension on single axon growth cones from chick sensory neurons resulted in "towed growth."[9–11] Although poorly understood, we believe that this growth mechanism also commonly occurs for integrated central nervous system (CNS) white matter tracts during embryogenesis and development. Because axons in these tracts are synapsed and have no growth cones from which to extend to match the growth of an organism, axonal elongation growth must occur by structural reorganization and extension of the central length of the axon. We hypothesize that continuous tensile forces along axons trigger this growth in length. On the basis of this premise, we developed a model system to evaluate mechanical "stretch-induced growth" of axons by applying continuous mechanical tension on tracts of axons spanning two populations of CNS neurons. We designed a device to physically split integrated neuronal cultures into two halves and separate the halves progressively further apart using a microstepper motor system. Accordingly, bundles of axons that crossed the border between the two halves prior to separation would be elongated progressively. We used this technique to evaluate both the ability of axon bundles to grow in response to mechanical tension and the tolerances of these axons for long-term growth.

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METHODS

Axonal elongation apparatus

The axon elongation apparatus consisted of a custom-built Plexiglas box with a gas-exchange port and removable top. Sterilization of the chamber was achieved by ethanol washing followed by ultraviolet irradiation. As depicted in Fig. 1, inside this box were rectangular overlapping flexible plastic membranes. The plastic membranes, made of Aclar (Allied Signal Plastics, Morristown, NJ), served as the substrate for the neuronal cultures. The top membrane (50 μm thick) was polished on one edge to create gradual slope to the border of the exposed underlying membrane. The other side of the top membrane was fixed to a machined plastic block that could be moved over the bottom membrane by a microstepper motor system. This system consisted of a motor/table assembly (Servo Systems, Inc., Montville NJ) and a miscrostepper motor (Pacific Scientific, Rockford, IL). Control of the movements was computer driven using a linear table (Aerotech, Irvine, CA), an encoder (Remco Encodors, Inc., Goleta, CA), and an indexer/driver (Panther, Intelligent Motor Systems, Marlborough, CT; QuickStep II driver software). The plastic block created a chamber in which neurons could be plated over the overlapping membranes.

Neuron cultures

We used integrated neuronal cultures of primary rat cortical neurons or differentiated human neurons from the N-tera2 (NT2) cell line[12,13] to test the principle of stretch-induced growth. Rat primary cortical cells were obtained from the cortices of day-18 rat embryos. The tissue was tryspsinized, dissociated, and then filtered to remove nonneuronal cells. The cells were plated in the elongator device at a density of 650,000/cm^2 and maintained in supplemented media (10% F12 (Bio-Whitaker), 5% fetal bovine serum (FBS; Hyclone, Logan, UT), B-27 (Gibco-BRL, Gaithersberg, MD), 2 mM *L*-glutamine (Gibco-BRL). Al-

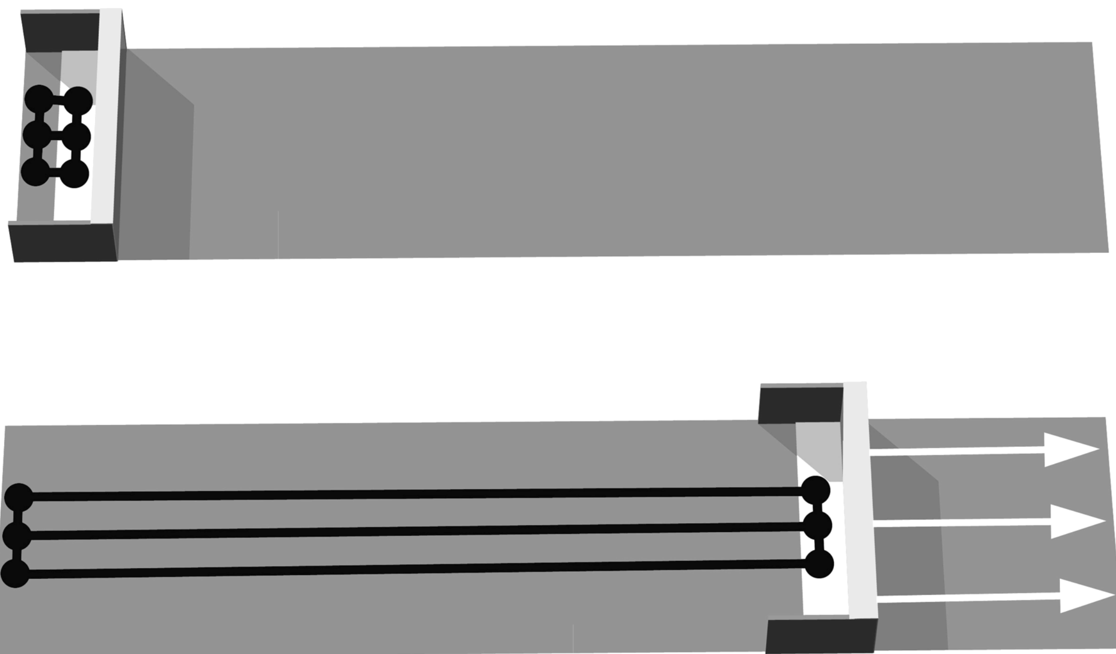

FIG. 1. Schematic illustration of the process of axonal "stretch-induced growth". (**Top**) A short membrane (white, 0.5 × 2.5 cm) is attached to a three-sided plastic block and placed on top of a long rectangular membrane (gray, 7 × 2.5 cm). In the chamber formed by the block, mammalian CNS neurons are plated and allowed to integrate over 3 days. A neural network (black) is formed, including axons 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 at a step-rate of 3.5 μm every 5 min. This technique results in the stretch-induced growth of fascicular tracts of axons spanning the two membranes.

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ternatively, differentiated human neurons from the NT2 cell line were plated in the elongation device at a similar density. The NT2 cells were maintained in culture with OptiMEM (Gibco, Grand Island, NY) media supplemented with 5% FBS (Hyclone, Logan, UT) and 1% penicillin-streptomycin (Gibco). To differentiate the NT2 cells into neurons (NT2N), NT2 cells were cultured for 5 weeks in DMEM supplemented with 10% FBS (Hylone), and 10 μM retinoic acid (Sigma, St. Louis, MO). To isolate neurons in the culture, the cells were trypsinized, triturated with a fire-polished Pasteur pipette, and replated in DMEM supplemented with 5% FBS and mitotic inhibitors (10 μM 5-flouro-2′-deoxyuridine, 10 μM uridine, 1 μM cytosine β-arabinofuranoside) (Sigma) for 9 days. These cultures were maintained in conditioned media (50% media from the first replate, 50% DMEM with 5% FBS) for 3 weeks prior to the study.

Mechanical elongation of cultured axons

As depicted in Fig. 1, movement of the plastic block in the elongation device via the microstepper motor system divided the integrated culture into two halves and progressively separated the opposing halves by sliding the top membrane across the bottom membrane. Precision machining ensured that the moving top membrane did not separate vertically from the lower membrane, thus eliminating a vertical gap from developing over time during the growth period. As the microstepper motor moved the top membrane, the portion of the axonal bundles crossing between the top and bottom membranes was stretched to a new displacement, inducing tension of the bundles. In consideration of potential deleterious effects of continuous tension on stretched axons, we calculated that steps in length in less than 1 sec could be compensated by 5-min 'rest' intervals between steps. This stretch and rest cycle was designed to minimize the residual stress in the cultured axons over extended periods of time. We based the average rate of stretch on the estimated

maximum *in vivo* growth of mammalian white matter tracts during development (less than 100 μm/h). Our goal was to establish conditions that minimized the peak force applied to the cultured tracts during incremental elongation. Accordingly, in this study, we evaluated stretch rates of 3.5 μm and 7.0 μm every 5 min (42 and 84 μm/h, or 1 mm and 2 mm/day, respectively), corresponding to an estimated peak force on the axons of 45 and 90 μdynes. The 5-min rest period between the step changes in length was estimated to decrease the force generated in the axon to less than 10% of its initial post-step value.

Upon activation of the microstepper motors, axonal elongation was performed in over 20 separate studies. Most of the studies were terminated at 7 days of stretch, whereas in three studies, axonal elongation was allowed to proceed for 10 days of stretch. Phase microscopic examination was periodically performed and phase photomicrographs were taken of the live cultures during elongation. In addition, some elongated cultures were prepared for confocal microscopic examination.

Fixation of stretched cultures and immunocytochemistry

Following elongation, some of the cultures were fixed in 0.2% paraformaldehyde and 0.05% gluteraldehyde for 30 min, extracted with 0.5% TritonX-100 for 1 min, and then labeled with monoclonal antibodies against the α subunit of the tubulin dimer (Clone Z-022, 1:400, Zymed, San Francisco, CA). Bound antibodies were elucidated with fluorescent label (Alexa 488, Molecular Probes, Oregon) conjugated to anti-mouse immunoglobulin G (IgG) and illuminated for photomicroscopy with epiflourescence. Images of the stained axons were acquired using a Bio-Rad Radiance 2000 confocal microscope (Hercules, CA).

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RESULTS

In over 20 separate studies of mechanical elongation at the step rate of 3.5 μm every 5 min, we found that few or no neuronal somata were present in the expanding center region between the two populations of neurons. Remarkably, however, bridging the expanding gap between the two populations of neurons were numerous large bundles of axons, with each bundle ranging from 5–70 μm in diameter (Figs. 2–4). These bundles originated from fascicular tracts of axons that had crossed the dividing line between the top and bottom membranes prior to separation. Although these tracts had random directional orientations prior to stretch, the axon bundles crossing the expanding gap gradually assumed parallel orientations similar to the arrangement of harp strings (Fig. 4). These bridging axons adapted to the stretch by increasing their length from no more than 50–200 μm to become 7 mm long over 7 days of stretch-growth and 1 cm long by 10 days of stretch. Surprisingly, the bridging axon fascicles grew in girth as well as in length. In particular the 'hillocks' of the axon bundles at the edges of the neuronal populations became much wider during elongation (Figs. 3 and 4). This thickening resulted, in part, from the joining together of neighboring axon bundles during stretch-induced growth. Thus, there were progressively fewer, but much broader fascicular tracts of axons bridging the two populations of neurons. With the typical diameter of the axons at less than 1 μm, we estimate that the larger bundles contained much greater than 1,000 axons. Furthermore, for each stretch preparation, 100–300 axon bundles traversed the gap, suggesting that the total number of stretched axons far exceeded 10^5.

In contrast to the results of the 3.5 μm/5 min elongation speed, doubling this rate to 7 μm/5 min led to the almost complete obliteration of the axon bundles, with only a few intact fascicles that remained spanning the gap by 3 days of stretch. The terminal ends of some disconnected bundles appeared 'frayed,' with individual axons trailing out. These observations were consistent across repeated tests at these applied growth rates, showing that the critical sustainable rate of these cultures was 1 mm/day.

DISCUSSION

These experimental data are the first to demonstrate long-term sustainable growth of large tracts of integrated CNS axons in response to continuous mechanical tension *in vitro*. In addition, we found that large bundles of thousands of axons readily adapted to mechanical elongation and that these bundles gradually

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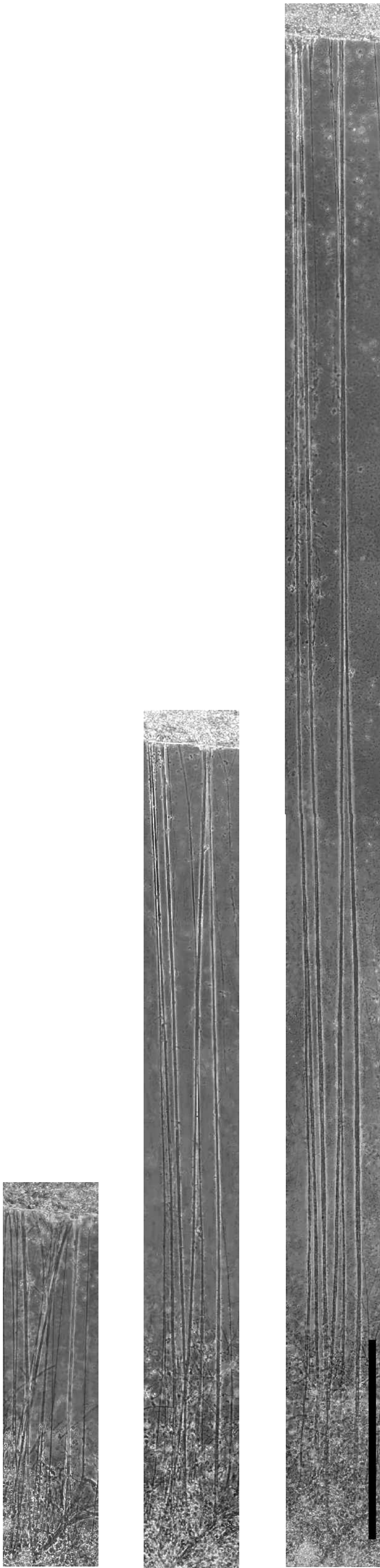

FIG. 2. Representative phase photomicrographs demonstrating stretch-induced growth of integrated CNS axons. The same region of a progressively expanding live culture is shown at 2 days (**left**), 4 days (**middle**), and 7 days (**right**) of elongation. At each end are the parent and target neurons adhering to the bottom membrane (**bottom**) and top membrane (**top**). Spanning these neurons are large bundles of progressively elongated axon tracts. Bar = 1 mm.

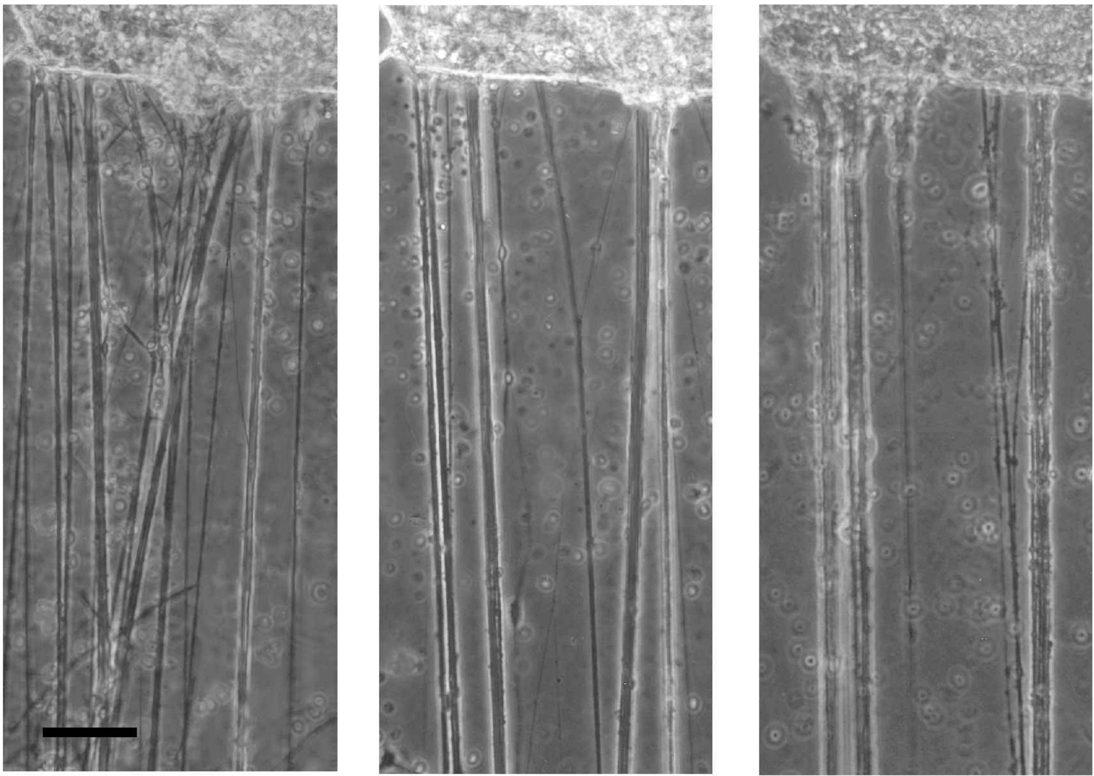

FIG. 3. Representative phase photomicrographs of one region of stretch-grown axons at the border of the top membrane at 2 days (**left**), 4 days (**middle**), and 7 days (**right**) of elongation. Note the gradual joining (coalescing) of neighboring axon bundles and thickening of the bundles at the edge of top membrane. Bar = 50 μm.

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consolidated into larger tracts. Furthermore, the elongated axon/neuron cultures appeared sufficiently viable to be used for transplant material. Although we terminated our studies at 7–10 days of stretch-growth, with the axon bundles reaching a remarkable 0.7–1.0 cm in length, there was no indication that further axonal elongation growth could not be achieved.

Although many studies have evaluated axonal elongation via extension of growth cones, the process of growth of the center portion of integrated axons is not well understood. The results of the present study suggest that stretch-induced growth may be a primary mechanism involved in the elongation of established CNS white matter tracts during growth of an organism. In addition, the present results suggest a potential threshold for long-term elongation growth of integrated CNS axons. The limiting factors for producing stretch-induced growth are the tensile strength of axons and their ability continually to add cytoskeletal elements for growth in response to stretch. Previously, we observed that CNS axons have very high tolerances for a single rapid stretch, with no disconnection occurring even at strains exceeding 65% increase in length induced over 20 msec.[12] Nonetheless, this high strain rate does induce cytoskeletal damage. Other previous studies have shown that much slower towing of single chick sensory axonal growth cones resulted in an increase in length of 100–400 μm/h over a few hours without obvious damage.[9,11] In the present study, we found that the axon bundles readily accommodated the stretch rate of 3.5-μm steps every 5 min (42 μm/h, 1 mm/day), demonstrating long-term growth. However, by only doubling this rate to 7 μm every 5 min (84 μm/h, 2 mm/day), most axon bundles appeared as if they were torn apart in the center regions by 3 days of stretch. The failure to continue to elongate at this rate of stretch suggests that the growth capacity of axons was gradually reduced to the point of exhaustion. It is worth noting that this growth rate limit of axonal bundles over days is substantially lower than that previously reported for elongation of single axons towed from their growth cones over hours.[9,11] Accordingly, both the rate of mechanical elongation and the duration of the growth period appear to be important limiting factors in the growth of axons.

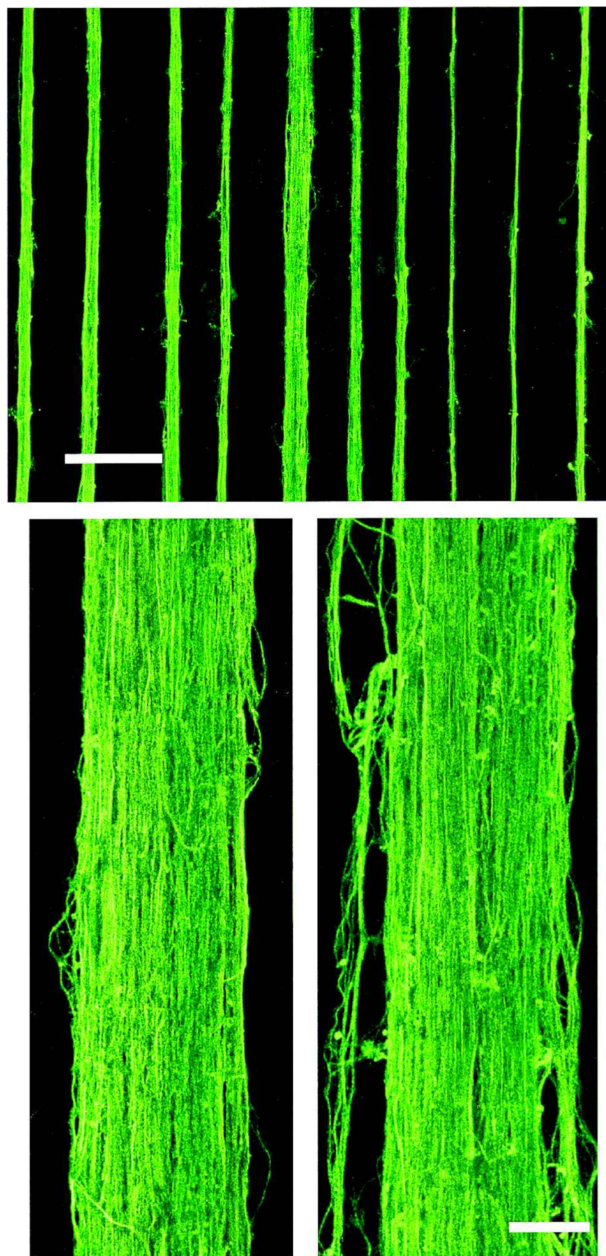

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FIG. 4. Representative fluorescence confocal photomicrographs of axon tracts at 7 days of stretch-induced growth, elucidated by immunostained microtubule protein in fixed cultures. (**Top**) Multiple long fascicular axon tracts arranged in parallel produced by stretch-induced growth (bar = 50 μm). (**Bottom**) Two huge fascicular axon tracts (each approximately 50 μm wide) demonstrating the stretch-induced coalescing of thousands of axons into large bundles (bar = 25 μm).

In consideration of transplanting stretch-grown axons, we have also found that neuron cultures readily grow on membranes made of biologically absorbable material (Lactosorb, BioMet, Inc., Warsaw, IN, data not shown), which may provide a more permissive and practical transplant material to promote host integration. With specific regard to SCI in humans, in the present study, we found that human neurons from the N-Tera2 cell line[12,13] work well in our axon elongation system. Importantly, these human neurons are currently being evaluated for transplantation into the brain in human stroke studies.[14] However, it remains

unknown if any axon bridge across a SCI lesion would be sufficient to initiate propagation of spinal cord signals through the transplanted axons or restore function to regions below the lesion. Nonetheless, as with all strategies to bridge nerve damage, it is hoped that an axon bridge may facilitate the body's remarkable plasticity in 'rewiring' the damaged spinal cord.

In addition to spinal cord repair, it is important to consider that the transplant material of elongated axon cultures could be used as a bridge for other types of neural injuries affecting long axon tracts, including optic nerve damage and peripheral nerve damage. Transplant of elongated axons for peripheral nerve repair damage may be most feasible due to the more permissive neural growth environment in the peripheral nervous system compared with the CNS.

Examinations of mechanical elongation of integrated CNS axons may also prove useful for the study of sustained axonal growth during development. Little is known of the structural rearrangement of established white matter tracts during embryogenesis and normal developmental growth of the organism. Driving cultured neurons to maintain considerable growth and consolidation of their integrated axonal tracts may allow for the temporal examination of cellular and molecular events of long-term sustained growth, including the regulation of cytoskeletal protein production, transport, and assembly. Thus, this technique may represent 'reverse engineering' of growth and regeneration in the CNS. Studies of this nature may be of great value in determining mechanisms of white matter degeneration during development, such as occurs in Canavan's and other diseases.

In summary, we have developed a fundamentally novel tissue engineering technique to elongate integrated CNS axons mechanically in culture. By continuously stretching large bundles of axons, we discovered the first evidence of the remarkable tolerance of integrated CNS axons for long-term, stretch-induced growth. Moreover, using this technique, we have produced the first potential transplant material consisting of thousands of elongated CNS axons of sufficient length to bridge many SCI lesions. Transplant studies are currently underway to evaluate the potential of mechanically elongated axons for nerve repair.

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