TISSUE ENGINEERING: Part A
Volume 14, Number 00, 2008
© Mary Ann Liebert, Inc.
DOI: 10.1089/ten.tea.2008.0294

Long-Term Survival and Integration of Transplanted Engineered Nervous Tissue Constructs Promotes Peripheral Nerve Regeneration

Jason H. Huang, M.D.,[1,*] D. Kacy Cullen, Ph.D.,[2,*] Kevin D. Browne, B.A.,[2] Robert Groff, B.S.,[2] Jun Zhang, M.D.,[3] Bryan J. Pfister, Ph.D.,[4] Eric L. Zager, M.D.,[2] and Douglas H. Smith, M.D.[2]

Although peripheral nerve injury is a common consequence of trauma or surgery, there are insufficient means for repair. In particular, there is a critical need for improved methods to facilitate regeneration of axons across major nerve lesions. Here, we engineered transplantable living nervous tissue constructs to provide a labeled pathway to guide host axonal regeneration. These constructs consisted of stretch-grown, longitudinally aligned living axonal tracts inserted into poly(glycolic acid) tubes. The constructs (allogenic) were transplanted to bridge an excised segment of sciatic nerve in the rat, and histological analyses were performed at 6 and 16 weeks posttransplantation to determine graft survival, integration, and host regeneration. At both time points, the transplanted constructs were found to have maintained their pretransplant geometry, with surviving clusters of graft neuronal somata at the extremities of the constructs spanned by tracts of axons. Throughout the transplanted region, there was an intertwining plexus of host and graft axons, suggesting that the transplanted axons mediated host axonal regeneration across the lesion. By 16 weeks posttransplant, extensive myelination of axons was observed throughout the transplant region. Further, graft neurons had extended axons beyond the margins of the transplanted region, penetrating into the host nerve. Notably, this survival and integration of the allogenic constructs occurred in the absence of immunosuppression therapy. These findings demonstrate the promise of living tissue-engineered axonal constructs to bridge major nerve lesions and promote host regeneration, potentially by providing axon-mediated axonal outgrowth and guidance.

Introduction

Although axonal regeneration occurs after peripheral nerve injury (PNI), there are currently insufficient means for major nerve reconstruction, commonly resulting in incomplete functional recovery. Autologous nerve grafts are generally utilized to repair severe lesions; however, this results in permanent loss of donor nerve function and is inadequate to repair extensive nerve damage due to limited availability.[1,2] While alternative clinical approaches include synthetic tubes, such conduits are only effective for relatively small peripheral nerve (PN) gaps.[3,4] Moreover, no current strategy addresses gradual degeneration of support cells in the nerve segment distal to the injury site, which severely limits recovery of function due to the loss of cues to guide axonal regeneration beyond the lesion.

To address these shortcomings, there has been great interest in developing alternative or complementary approaches to facilitate regeneration across PN lesions. A successful nerve graft must efficiently guide axonal regeneration from the proximal nerve stump to the remaining distal nerve segment by providing physical and/or neurochemical cues. Thus, experimental strategies have focused on creating environments that promote axonal outgrowth, including combinations of permissive scaffolds (e.g., decellularized grafts or hydrogels), extracellular matrix, trophic factors, and glial or stem cells.[5–14] Anisotropic properties are important to direct axon growth, and are typically achieved via gradients (e.g., neurotrophic or extracellular matrix), longitudinally aligned fibers, or tailored porosity.[15–18] In practice, axonal guidance cues and anisotropy are typically provided by a choreographed organization of Schwann cells

[1]Department of Neurosurgery & Center for Neural Development and Disease, University of Rochester Medical Center, Rochester, New York.
[2]Department of Neurosurgery, School of Medicine, Center for Brain Injury and Repair, University of Pennsylvania, Philadelphia, Pennsylvania.
[3]Neurosurgical Department, PLA General Hospital, Fu Xing Road 28, 100853, Beijing, PR China.
[4]New Jersey Institute of Technology, 323 Martin Luther King Jr. Blvd., University Heights, Newark, New Jersy.
*The first two authors contributed equally to this work.

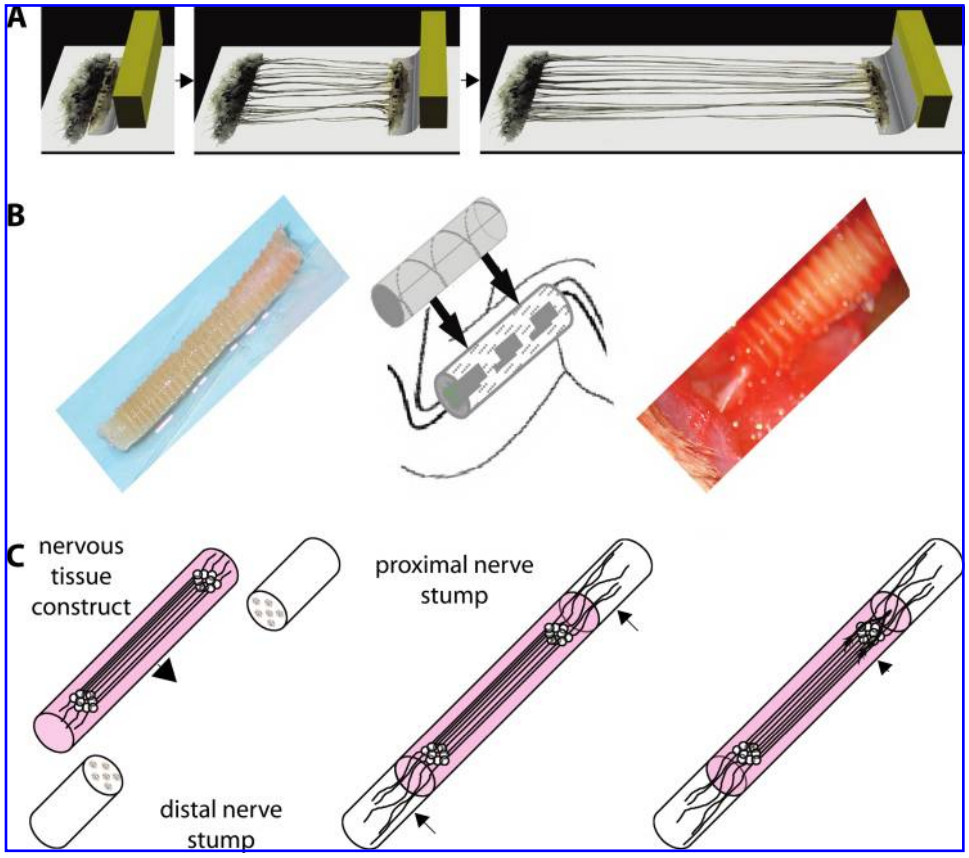
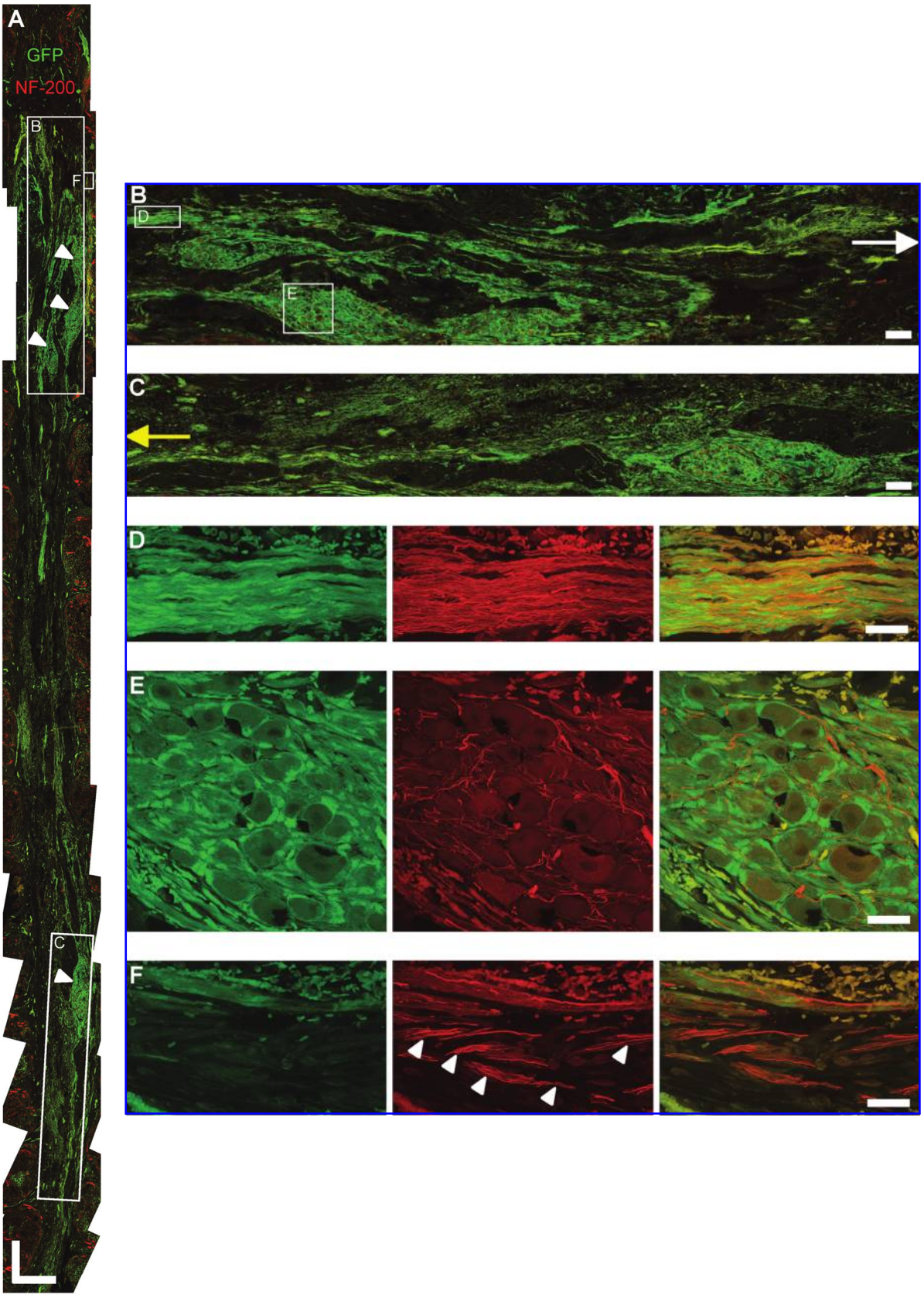


FIG. 1. Our approach for PN repair using living engineered nervous tissue constructs. Constructs consisted of longitudinally aligned axonal tracts spanning two neuronal populations. Neurons were plated on adjacent membranes that were gradually displaced to induce stretch-growth in the spanning axonal tracts (**A**). Immediately before implantation, stretch-grown cultures were embedded in a collagenous matrix and inserted into a PGA tube. The constructs were then surgically implanted by suturing to the proximal and distal stumps (**B**). The constructs were used to bridge excised segments of nerve (arrows) (**C**). After implantation, neurites from the construct may extend into the host nerve (arrows) and/or the construct may serve as a living labeled pathway to guide sprouting axons from the host proximal nerve stump (small arrowhead) across the lesion. Color images available online at www.liebertonline.com/ten.

(SCs), either delivered or endogenous, which form aligned regenerative columns (i.e., Bands of Bungner). These columnar-aligned SCs provide neurotrophic support and contact guidance to direct axonal regeneration toward target tissue, with axonal functional maturation occurring after elimination of extraneous or erroneous connections.[19] An alternative, yet largely unexplored, mechanism of regeneration is axon-mediated axonal outgrowth. Here, axonal regeneration occurs directly along living axons engineered to bridge a PN lesion. This combination of anisotropic contact guidance and neurotrophic support would provide a labeled pathway for axonal outgrowth, potentially facilitating expeditious, targeted regeneration.

The ability to directly exploit axon-mediated axonal regeneration may significantly add to the repertoire of tissue-engineered strategies for PNI repair. Accordingly, we have recently developed a novel tissue engineering approach to create transplantable living nervous tissue constructs composed of parallel tracts/fascicles of axons spanning two neuronal populations.[20] This technique is based on the ability of integrated axons to respond to continuous mechanical tension by exhibiting "stretch-growth," which produces progressively longer axons that gradually coalesce into large nerve tracts.[21] These engineered nervous tissue constructs consisting of living axonal tracts mimics the uniaxial geometry of axons in the missing nerve segment, and thus may facilitate axonal regeneration (Fig. 1). The current study was a first-order assessment of the overall feasibility of this approach based on the repair of a 1.2–1.3 cm nerve gap, suitable for an initial evaluation of graft survival, host regeneration, and any potential relationships between these outcomes. Therefore, the objectives of this study were to (1) determine

FIG. 2. Neuronal survival and maintenance of architecture in engineered nervous tissue constructs. Representative confocal reconstructions of transplanted GFP^+ engineered nervous tissue constructs used to bridge an excised segment of sciatic nerve (6 weeks postimplantation). Continuous proximal (top) and distal portions (bottom) from a GFP^+ construct immunolabeled for NF-200 (red) (scale bars = 0.5 mm) (**A**). Multiple transplanted ganglia were evident on the proximal and distal ends (arrow heads) with aligned axonal tracts spanning these neuronal populations. Remnants of the PGA tube were observed bordering the transplant at this time point (note arced border material autofluorescing red). Higher magnification regions from (**A**) rotated 90° (scale bars = 100 μm) (**B, C**). Major bundles of neurites projected from the proximal ganglia across the constructs as well as into host nerve toward the spinal cord (white arrow) (**B**). Similarly, neuritic bundles also projected from the distal ganglia across the constructs and distally into the distal nerve segment (yellow arrow) (**C**). Increased magnification from specified regions: GFP^+ (left column), $NF\text{-}200^+$ (center column), with overlay (right column); scale bars = 20 μm (**D–F**). Central axonal tracts colabeled for GFP and NF-200 (**D**). Transplanted ganglia became dense, three-dimensional clusters of neurons (**E**). Neurites growing from the host into the proximal end of the constructs were observed as $NF\text{-}200^+$ axons that were not colocalized with GFP (arrows) (**F**).


A
GFP
NF-200
B
F
C
B
D
E
C
D
E
F

posttransplantation survival and architecture of the nervous tissue constructs and (2) assess host axonal regeneration across these constructs.

Materials and Methods

The Institutional Animal Care and Use Committee of the University of Pennsylvania approved all procedures involving animals. All components were from Invitrogen (Carlsbad, CA) or BD Biosciences (San Jose, CA) unless noted.

Engineered nervous tissue constructs

Dorsal root ganglia (DRG) neurons were isolated from embryonic day 15 fetuses from timed-pregnant dames using either Sprague-Dawley (Charles River, Wilmington, MA) or transgenic rats expressing green fluorescent protein (GFP; strain TgN(act-EGFP)OsbCZ-004). DRG explants were suspended at 5×10^6 cells/mL in Neurobasal© medium supplemented with 2% B-27, 0.4 mM L-glutamine, 1% penicillin/streptomycin, 2 mg/mL glucose (Sigma-Aldrich, St. Louis, MO), 10 ng/mL 2.5S nerve growth factor (NGF), 1% fetal bovine serum (FBS) (HyClone, Logan, UT), 10 μM 5-fluoro-2′-deoxyuridine (FdU) (Sigma-Aldrich), and 10 μM uridine (Sigma-Aldrich). Mitotic inhibitors in the medium limited glial proliferation and promoted nonneuronal elimination. Cultures were maintained within custom-built mechanical elongation devices. Explants were plated in two populations along the margin of two overlapping collagen-coated (rat tail type 1, 3.67 mg/mL) aclar membranes (50–100 μm apart). Over 5 days *in vitro*, axonal outgrowth integrated these two neuronal populations. Next, a computerized micro-stepper motor system (motor: HT23401D; programmable driver: Si3540; Applied Motion Systems, Watsonville, CA) was engaged to progressively separate the membranes. Over the subsequent 7 days *in vitro*, this continuous mechanical tension induced stretch-growth in the axons spanning the neurons[21,22] (Fig. 1). Stretch-growth was terminated once the axonal tracts reached 12–13 mm in length. The cultures were then embedded in a collagen-based matrix (3.0 mg/mL) in Dulbecco's modified Eagle's medium supplemented with NGF (2.5S, 10 ng/mL). After gelation at 37°C, embedded cultures were gently removed from the substrate and placed within a premeasured absorbable poly(glycolic acid) (PGA) Neurotube™ (Neuroregen, Bel Air, MD). Before receiving the construct, the Neurotube was cut lengthwise and held open to permit construct placement along the center, and once released, the tube reassumed a closed tubular shape. These constructs consisted of a nearly pure neuronal phenotype.[20]

Peripheral nerve surgery and implantation of tissue-engineered nerve constructs

Experimental subjects were adult male Sprague-Dawley rats (Charles River) ($n=6$) or adult male transgenic rats expressing alkaline phosphatase (AP; strain R26-hPAP) ($n=3$). Animals were anesthetized using intraperitoneal injections of sodium pentobarbital (60 mg/kg). The left sciatic nerve was exposed and separated from surrounding fascia, and a 1.0 cm segment was completely excised. Constructs were implanted by inserting the proximal and distal nerve stumps into the ends of the PGA tube (~3 mm overlap), which was then sutured to the epineurium using four 10–0 silk sutures. The incision was closed, and the wound site was disinfected with Betadine (Purdue Products, Stamford, CT). AP^+ transgenic rats were implanted with constructs containing GFP^+ DRG and were permitted to survive 6 weeks (i.e., GFP^+ constructs into AP^+ rats). Nontransgenic Sprague-Dawley rats were implanted with constructs containing nontransgenic DRG and were permitted to survive 16 weeks (i.e., GFP^- constructs into AP^- rats).

Histological analyses

Rats were anesthetized with sodium pentobarbital (60 mg/kg), and the left sciatic nerve was exposed. A 3.0 cm segment of the nerve containing the repair site was excised. Animals were then sacrificed by pentobarbital overdose. Nerve segments were fixed for 48 h in cold 4% paraformaldehyde, and were then dehydrated, paraffin-embedded, cut in serial sections (6 μm thickness), and mounted on glass slides. For the 6-week time point, all samples were sectioned longitudinally, and for the 16-week time point, half of the samples were sectioned longitudinally and half axially. Immunohistochemistry was performed using the following primary antibodies: (1) neurofilament-200 kDa (NF-200; N0142, 1:400; Sigma-Aldrich), (2) myelin basic protein (MBP; SMI-94R, 1:1000; Covance Research Products, Princeton, NJ), (3) calcitonin gene-related peptide (CGRP; C8198, 1:500; Sigma-Aldrich), or (4) alkaline phosphatase (AP; A2951, 1:500; Sigma-Aldrich). The appropriate secondary fluorophore-conjugated antibodies (FITC- or TRITC-conjugated IgG; Jackson ImmunoResearch, West Grove, PA) were used. Adjacent sections were stained for hematoxylin and eosin (H&E) (Thermo Shandon, Pittsburgh, PA). Sections were examined using an epifluorescent microscope (Eclipse E600; Nikon, Melville, NY) with images digitally captured (Spot RT Color; Diagnostic Instruments, Sterling Heights, MI). Alternatively, sections were fluorescently imaged using a confocal laser scanning microscope consisting of a BioRad Radiance 2000-MP system (Zeiss, Oberkochen, Germany) on an Eclipse TE-300 (Nikon).

Results

Neuronal survival and maintenance of aligned axonal architecture in engineered nervous tissue constructs

Engineered nervous tissue constructs consisted of DRG neurons with stretch-grown axonal tracts embedded in a collagenous matrix within PGA tubes. These allogeneic constructs were sutured to the proximal and distal stumps after excision of a segment of sciatic nerve in the rat (Fig. 1). To discern transplanted neurons from host cells, several constructs were generated using GFP^+ stretch-grown DRG. After implantation, the survival of transplanted neurons and maintenance of construct architecture were readily observed in all animals receiving GFP^+ engineered nerve constructs (Fig. 2). Specifically, multiple GFP^+ ganglia were identified on both the proximal and distal ends of the implants, demonstrating survival of the transplanted neurons. There were GFP^+ tracts spanning these ganglia, indicating preservation of the basic construct architecture consisting of longitudinally aligned axonal tracts. Neuronal phenotype within the constructs was verified based on immunoreactivity for the neuronal/neuritic cytoskeletal protein NF-200. This demonstrated that the proximal and dis-

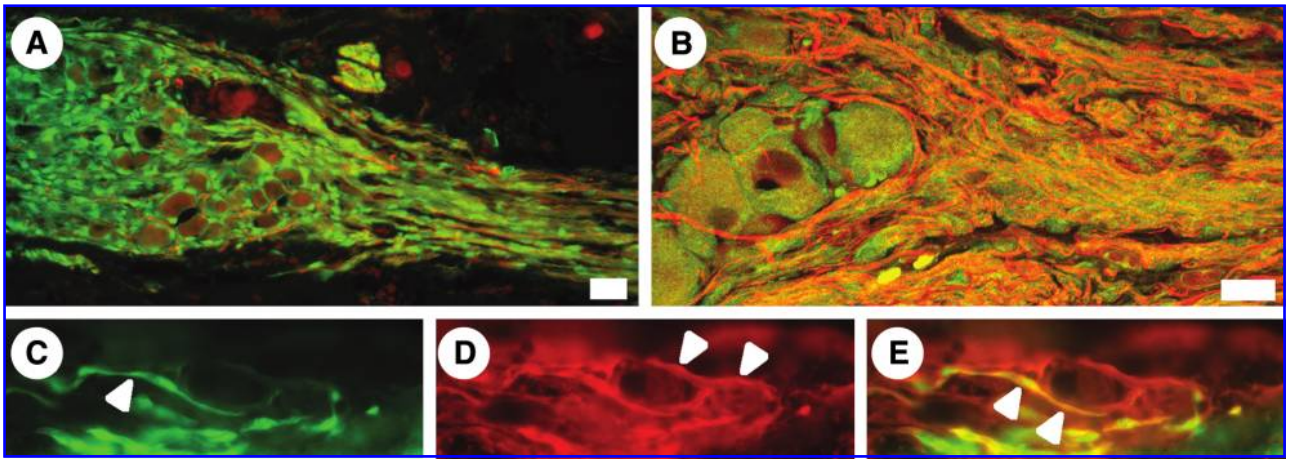


FIG. 3. Ganglia morphology and integration of transplanted neurons with host. Representative micrographs from transplanted tissue-engineered axonal constructs (6 weeks postimplantation). Elongated neurites coalesced into tightly packed tracts of axons departing from the transplanted ganglia (GFP^+; NF-200 [red]) (**A**). In some cases, robust NF-200 (red) labeling was observed indicating host infiltration across the ganglia of the nervous tissue constructs (**B**). Additionally, GFP^+ axonal constructs were transplanted across the excised sciatic nerve in transgenic rats expressing AP (red) (**C–E**). Neurites from transplanted neurons (GFP^+) (**C**) were observed in intimate contact with neurites from the host (AP^+) (**D**). Host axons intertwined with transplanted neurites, further indicating neurite outgrowth from the host into the elongated axonal constructs (overlay) (**E**). Such ingrowth along transplanted axonal tracts may provide a labeled pathway for host regeneration. Scale bars = 20 μm.

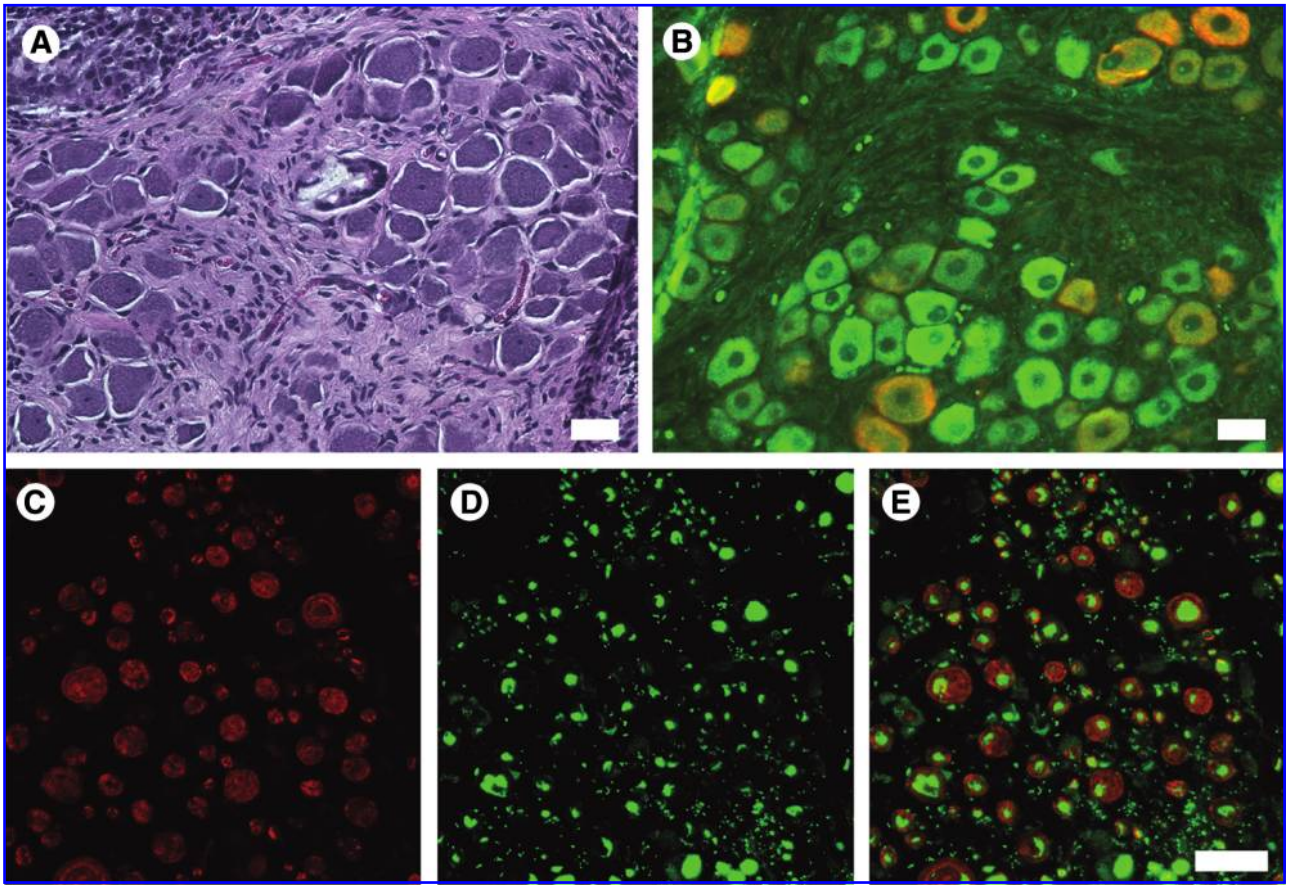


FIG. 4. Long-term survival and integration of transplanted nervous tissue constructs. Representative micrographs showing long-term neuronal survival after implantation to facilitate sciatic nerve regeneration (16 weeks postimplantation). Surviving neurons were readily identifiable based on ganglia morphology (H&E stain) (**A**). Within these transplanted ganglia, there were neurons immunolabeled for the DRG neuron-specific marker CGRP (green) and NF-200 (red) (**B**). Regenerated and/or transplanted axons became myelinated across the nervous tissue constructs (**C–E**). Representative fluorescent confocal micrographs from the central region (axial section) of a transplanted elongated axonal construct immunolabeled for MBP (**C**), NF-200 (**D**), with overlay (**E**); note numerous myelinated axons. Scale bars = 20 μm.

tal transplanted ganglia had coalesced into dense, three-dimensional neuronal clusters. Additionally, there were tightly packed, continuous neuritic tracts projecting across the constructs to span these neuronal populations.

Integration of neurons in engineered nervous tissue constructs with host nerve

Nervous tissue constructs demonstrated physical integration with the host based on outgrowth from transplanted neurons into host nerve. In nerves with implanted constructs containing GFP^+ stretch-grown DRG, GFP^+ processes were found to have extended beyond the margins of the graft into host nerve, penetrating at least 5 mm (Fig. 2). These projections, which consisted of major bundles of tightly packed GFP^+ processes, occurred from the proximal ganglia toward the spinal cord and from the distal ganglia into the distal nerve segment. Thus, in addition to the proximal and distal transplanted ganglia being connected via GFP^+ axonal tracts, neurites also projected outward into the host.

Host regeneration across nervous tissue constructs

Regeneration of host axons into and across the nervous tissue constructs was observed in all cases. Host axons growing into the proximal end of the constructs were identified as NF-200^+ axons that were not colocalized with GFP (Fig. 2). NF-200^+/GFP^- neurites were also observed within the ganglia and along the axonal tracts spanning the ganglia. In some cases, there was robust host neuritic infiltration across neuronal somata within transplanted ganglia (Fig. 3). Additionally, these constructs consisting of GFP^+ stretch-grown DRG were transplanted into AP^+ rats. In these cases, regeneration across the constructs was confirmed by identifying AP^+ host processes throughout the constructs. These AP^+ processes were often observed in direct physical contact with GFP^+ neurites from transplanted neurons, which appeared to intertwine in many cases (Fig. 3). Thus, host axons were typically in intimate contact with transplanted neuronal somata/neurites, demonstrating host neuritic infiltration and integration throughout the constructs.

Long-term neuronal survival and axonal myelination within nervous tissue constructs

By 16 weeks postimplantation, the nerve segment bridging the gap appeared grossly normal in all cases. Survival and functional integration of the transplanted constructs were evaluated in nontransgenic rats at this time point. Based on immunoreactivity for NF-200 and CGRP, surviving clusters of neurons were identified in the proximal and distal regions of the constructs (Fig. 4). Examination of H&E-stained sections revealed no overt signs of an immunologic response to the transplant, such as infiltrating macrophages or neutrophils. Notably, these observations of transplant survival and lack of an overt immune response were surprising given that immunosuppressants were not used. Additionally, many NF-200^+ axons within the constructs between the proximal and distal ganglia were surrounded by MBP^+ cells, indicative of axonal myelination (Fig. 4). These myelinated axons were either host axons that regenerated, surviving axons from the transplanted constructs, or potentially some mixture of these populations. Further, because the transplant consisted of a nearly pure neuronal population, myelination was likely due to host SC infiltration.

Discussion

We exploited the ability of integrated axons to rapidly grow in length in response to continuous mechanical tension to create transplantable nervous tissue constructs for PN repair. Using these engineered constructs consisting of aligned axonal tracts to bridge an excised region of PN, we found (1) a grossly normal appearing nerve segment by 16 weeks post-transplantation, (2) long-term survival of the neurons and axonal tracts originally comprising the construct, (3) maintenance of the longitudinally aligned construct geometry, (4) facilitation of host axonal regeneration across the lesion with close interactions between graft and host axons, (5) myelination of axons crossing the center of the constructs, and (6) penetration of graft axons into the host nerve. This initial assessment was performed using a relatively short PN lesion to serve as a starting point to assess construct survival, integration, and host regeneration. However, our findings demonstrate the feasibility and promise of this new approach for PN repair. In particular, by providing a living bridge of axonal tracts, the transplanted constructs may have promoted a form of axon-mediated axonal growth and guidance, evidenced by the intertwining of graft and regenerating host axons throughout the repaired segment. Moreover, because axons from the constructs grew into the distal and proximal nerve segments, this labeled pathway may extend beyond the margins of the lesion.

To create long axonal tracts *in vitro*, we relied on a newfound mechanism of axon growth that occurs during development.[21] As an animal grows, mechanical forces are thought to induce stretch-growth of nerves and white matter tracts. Likewise, in the laboratory, continuous mechanical tension on integrated axons (i.e., in the absence of a growth cone) induces progressive stretch growth of the axonal tracts even at very high rates. Indeed, integrated axonal tracts can be stretch grown up to 1 cm/day reaching at least 10 cm in length. Despite this extremely rapid growth, the axons maintain a normal ultrastructure and gradually coalesce into progressively larger nerve tracts consisting of up to 10^6 axons.[22] These stretch-grown living axonal tracts were the backbone of our engineered nervous tissue constructs, which may have been advantageous in promoting PN regeneration by mimicking the uniaxial alignment of axons in the missing nerve segment. Notably, we have recently used this general approach to repair spinal cord lesions.[23]

For PNI repair, these nervous tissue constructs may have provided guidance for regenerating axons due to physical contact, geometric attributes, and potentially neurochemical support. By design, contact guidance for regenerating axons was provided via longitudinally aligned axon fascicles. These fascicles may interact with regenerating axons via direct cell–cell interactions (e.g., receptor-mediated). Additionally, the anisotropic fascicle alignment may provide regenerative benefits due to geometric features, such as axonal continuity and micron-scale radii. For instance, longitudinally aligned polymer fibers confer a directional bias for neurite outgrowth based on specific geometrical attributes.[24,25] Moreover, our nervous tissue constructs may recapitulate an axonal regeneration process that occurs in damaged nerves that are not

completely transected, where regenerating axons follow the path of intact axons. Additionally, this form of regeneration may not be dependent upon cellular infiltration, proliferation, or organization, and thus may occur more expeditiously than that reliant upon SC columnar alignment and matrix deposition. There may also be multiple neurochemical influences of axon-induced axon growth. Here, the transplanted neurons may supply an important sustained source of biological cues and trophic factors for regenerating axons. While we did not directly assess the specific mechanism(s) of axon-induced axonal growth observed in the present study, the remarkable intertwining of host and graft axons throughout the lesion site suggests a strong attraction that may be related to both physical and neurochemical cues from the transplanted axons.

Another interesting finding in this study was the high survival rate of allogeneic transplants in the absence of immunosuppressive therapy. In general, allogeneic nerve transplants elicit a strong immunogenic response and thus require immunosuppression.[26] However, delayed immunological responses after nervous system transplants have been reported.[27] It is noteworthy that in our transgenic studies, the graft neurons were harvested from an outbred strain of rats from the host strain. Yet, neurons within the constructs appeared intact and healthy, and there was no overt evidence of an immune response. Why the transplanted constructs were immuno-privileged is unclear. Potentially, pure allogeneic neurons in the construct (i.e., no nonneuronal cells) and/or the fetal origin of the cells did not elicit an immunogenic response after transplantation. Additionally or alternatively, the integration of the transplanted construct with host nerve, including graft and host axon contact, myelination of axons, and formation of a complete nerve sheath, may have attenuated immunological attack. Regardless, the observation of immuno-privilege of transplanted allogeneic neurons in the PNS challenges the conventional wisdom. Overall, the high degree of neuronal survival within our constructs supports the concept that the transplanted axons may be in place long enough to guide host axonal regeneration across even much longer lesions. Indeed, we have demonstrated that constructs can be prepared reaching 10 cm, with the potential to achieve even greater lengths.[20,22]

An additional factor governing survival of the transplanted constructs was their ability to successfully extract nutritional requirements from the host despite their elongated structure. Limitations in mass transport are of paramount concern for tissue-engineered strategies incorporating living cells, especially when used to bridge defects longer than a few centimeters.[28] When transplanted cells are homogeneously distributed throughout a construct, diffusion-based mass transport may fail to support cells at the construct center before the reestablishment of host vasculature. In contrast, the design of our constructs includes neuronal somata only at the transplant extremities, potentially in sufficient proximity to remaining host vasculature for adequate mass transport. In turn, the transplanted neuronal somata may provide the necessary sustenance to their axons spanning the graft, thus permitting time for host revascularization across the lesion. Notably, by 16 weeks posttransplant, the PGA tube was completely absorbed, leaving behind a relatively normal-appearing, well-vascularized nerve structure.

While regeneration of axons across a nerve gap is essential, restoration of function is equally dependent on the distance axons must subsequently travel down the distal nerve segment. Degeneration of the distal axonal segment is an inevitable consequence of axotomy, although the supporting cells in the distal nerve segment remain to guide regenerating axons once they cross the lesion. However, in a race against time, the distal nerve segment will gradually degenerate if axonal regeneration across the lesion site has been too protracted.[29] Thus, even for nerves that have been repaired, long distance regeneration and restoration of function is thwarted by the gradual loss of the pathway to target tissues. This scenario is commonly observed in brachial plexus injuries, where proximal limb function may be restored after nerve repair, yet there is typically no recovery of hand function due to lack of reinnervation. In these cases, the months it takes for regenerating axons to reach the hand at approximately 1 mm of growth/day is surpassed by the rate of degeneration of the distal segment.[30] It is not clear why the support cells in the distal segment degenerate because the vasculature should be intact. Possibly, the absence of axons in the nerve sheath eventually triggers programmed death of the supportive cells.

Potential approaches to facilitate long-distance axonal regeneration after nerve repair include preventing degeneration of the distal nerve segment and/or providing a long guide that maintains a labeled pathway. Notably, we found that the axons sprouting from the transplanted construct penetrated into the host nerve, raising the possibility that outgrowth from the construct may help maintain the distal pathway far beyond the margins of the lesion. If axons from the graft promote survival of the distal nerve segment, there would be more time for host regenerating axons to reach appropriate targets. Thus, transplanted living nervous tissue constructs could provide both a sustained pathway for regeneration, even over long distances, and potentially extend this pathway by sending axons into the distal nerve segment.

For clinical considerations, we have very recently demonstrated that adult human DRG neurons can also be tissue-engineered to form transplantable constructs similar to those used in the present study.[31] Neurons for these human nervous tissue constructs were harvested from both living patients undergoing therapeutic ganglionectomies and from organ donors, demonstrating the feasibility of creating either autologous or allogeneic grafts. Notably, the survival of the allogeneic transplants in the present animal study bodes well for clinical application using either cell source. After transplantation, the axons in our nervous tissue constructs are anticipated to survive at least long enough to guide host regeneration across the damaged region, thus providing a robust and persisting axonal pathway for regenerating axons to follow. This is much in contrast to the current gold standard of transplanting harvested segments of donor autologous nerves, where the axons have been cut off from the cell body and rapidly degenerate posttransplantation, leaving no axonal pathway. Clearly, many more preclinical studies must be performed before this new tissue engineering approach can be translated to humans. However, if successful, it could offer an alternative and potentially improved therapy, by providing laboratory-grown off-the-shelf living constructs with no limitations in supply.

Overall, this first-step study demonstrates the feasibility of a novel tissue engineering approach to repair PN damage. The data provide evidence that axon-mediated axonal

outgrowth and guidance may be utilized to enhance nerve regeneration. Using this technology, our long-term goal is to build constructs to repair even extensive PN lesions and to provide a mechanism to prevent degeneration of the support cells in the distal nerve segment. Future studies will examine the potential of these constructs to repair longer nerve lesions and the restoration of sensory and motor function compared to conventional approaches. With further development and implementation, this strategy may prove beneficial for major nerve reconstruction.

Acknowledgments

Financial support was provided by NIH Grants NS048949, NS38104, NS056202, and NRSA NS46170; the Sharpe Trust; and AANS/CNS Codman and Kline Awards. GFP^+ transgenic rats were provided by Japan SLC and Professor Masaru Okabe of Genome Research Center, Osaka University, Yamadaoka, Japan. The authors thank Dr. Eric Sandgren and Allyson Holler of the University of Wisconsin-Madison School of Veterinary Medicine for providing the AP^+ transgenic rats and for technical assistance.

Disclosure Statement

No competing financial interests exist.

References

1. Lundborg, G. Alternatives to autologous nerve grafts. Handchir Mikrochir Plast Chir **36,** 1, 2004.
2. Sinis, N., Haerle, M., Becker, S.T., Schulte-Eversum, C., Vonthein, R., Rosner, H., and Schaller, H.E. Neuroma formation in a rat median nerve model: influence of distal stump and muscular coating. Plast Reconstr Surg **119,** 960, 2007.
3. Dellon, A.L. Clinical results with the polyglycolic acid neurotube for nerve repair and reconstruction. In: Slutsky, D.J., and Hentz, V.R., eds. Peripheral Nerve Surgery: Practical Applications in the Upper Extremity. Philadelphia, PA: Churchill Livingstone Elsevier, 2006, pp. 129–140.
4. Trumble, T.E., Parisi, D., Archibald, S., and Allan, C.H. Synthetic nerve grafts. In: Slutsky, D.J., and Hentz, V.R., eds. Peripheral Nerve Surgery: Practical Applications in the Upper Extremity. Philadelphia, PA: Churchill Livingstone Elsevier, 2006, pp. 121–128.
5. Nie, X., Zhang, Y.J., Tian, W.D., Jiang, M., Dong, R., Chen, J.W., and Jin, Y. Improvement of peripheral nerve regeneration by a tissue-engineered nerve filled with ectomesenchymal stem cells. Int J Oral Maxillofac Surg **36,** 32, 2007.
6. Lee, A.C., Yu, V.M., Lowe, J.B., 3rd, Brenner, M.J., Hunter, D.A., Mackinnon, S.E., and Sakiyama-Elbert, S.E. Controlled release of nerve growth factor enhances sciatic nerve regeneration. Exp Neurol **184,** 295, 2003.
7. Evans, G.R., Brandt, K., Katz, S., Chauvin, P., Otto, L., Bogle, M., Wang, B., Meszlenyi, R.K., Lu, L., Mikos, A.G., and Patrick, C.W., Jr. Bioactive poly(L-lactic acid) conduits seeded with Schwann cells for peripheral nerve regeneration. Biomaterials **23,** 841, 2002.
8. Fansa, H., and Keilhoff, G. Comparison of different biogenic matrices seeded with cultured Schwann cells for bridging peripheral nerve defects. Neurol Res **26,** 167, 2004.
9. Frerichs, O., Fansa, H., Schicht, C., Wolf, G., Schneider, W., and Keilhoff, G. Reconstruction of peripheral nerves using acellular nerve grafts with implanted cultured Schwann cells. Microsurgery **22,** 311, 2002.
10. Hu, Y., Leaver, S.G., Plant, G.W., Hendriks, W.T., Niclou, S.P., Verhaagen, J., Harvey, A.R., and Cui, Q. Lentiviral-mediated transfer of CNTF to schwann cells within reconstructed peripheral nerve grafts enhances adult retinal ganglion cell survival and axonal regeneration. Mol Ther **11,** 906, 2005.
11. Keilhoff, G., Goihl, A., Stang, F., Wolf, G., and Fansa, H. Peripheral nerve tissue engineering: autologous Schwann cells vs. transdifferentiated mesenchymal stem cells. Tissue Eng **12,** 1451, 2006.
12. Stang, F., Fansa, H., Wolf, G., and Keilhoff, G. Collagen nerve conduits—assessment of biocompatibility and axonal regeneration. Biomed Mater Eng **15,** 3, 2005.
13. Yu, X., and Bellamkonda, R.V. Tissue-engineered scaffolds are effective alternatives to autografts for bridging peripheral nerve gaps. Tissue Eng **9,** 421, 2003.
14. Chalfoun, C.T., Wirth, G.A., and Evans, G.R. Tissue engineered nerve constructs: where do we stand? J Cell Mol Med **10,** 309, 2006.
15. Bellamkonda, R.V. Peripheral nerve regeneration: an opinion on channels, scaffolds and anisotropy. Biomaterials **27,** 3515, 2006.
16. Dodla, M.C., and Bellamkonda, R.V. Anisotropic scaffolds facilitate enhanced neurite extension *in vitro*. J Biomed Mater Res A **78,** 213, 2006.
17. Matsumoto, K., Ohnishi, K., Kiyotani, T., Sekine, T., Ueda, H., Nakamura, T., Endo, K., and Shimizu, Y. Peripheral nerve regeneration across an 80-mm gap bridged by a polyglycolic acid (PGA)-collagen tube filled with laminin-coated collagen fibers: a histological and electrophysiological evaluation of regenerated nerves. Brain Res **868,** 315, 2000.
18. Bozkurt, A., Brook, G.A., Moellers, S., Lassner, F., Sellhaus, B., Weis, J., Woeltje, M., Tank, J., Beckmann, C., Fuchs, P., Damink, L.O., Schugner, F., Heschel, I., and Pallua, N. *In vitro* assessment of axonal growth using dorsal root ganglia explants in a novel three-dimensional collagen matrix. Tissue Eng **13,** 2971, 2007.
19. Navarro, X., Vivo, M., and Valero-Cabre, A. Neural plasticity after peripheral nerve injury and regeneration. Prog Neurobiol **82,** 163, 2007.
20. Pfister, B.J., Iwata, A., Taylor, A.G., Wolf, J.A., Meaney, D.F., and Smith, D.H. Development of transplantable nervous tissue constructs comprised of stretch-grown axons. J Neurosci Methods **153,** 95, 2006.
21. Smith, D.H., Wolf, J.A., and Meaney, D.F. A new strategy to produce sustained growth of central nervous system axons: continuous mechanical tension. Tissue Eng **7,** 131, 2001.
22. Pfister, B.J., Iwata, A., Meaney, D.F., and Smith, D.H. Extreme stretch growth of integrated axons. J Neurosci **24,** 7978, 2004.
23. Iwata, A., Browne, K.D., Pfister, B.J., Gruner, J.A., and Smith, D.H. Long-term survival and outgrowth of mechanically engineered nervous tissue constructs implanted into spinal cord lesions. Tissue Eng **12,** 101, 2006.
24. Wen, X., and Tresco, P.A. Effect of filament diameter and extracellular matrix molecule precoating on neurite outgrowth and Schwann cell behavior on multifilament entubulation bridging device *in vitro*. J Biomed Mater Res A **76,** 626, 2006.
25. Kim, Y.T., Haftel, V.K., Kumar, S., and Bellamkonda, R.V. The role of aligned polymer fiber-based constructs in the

bridging of long peripheral nerve gaps. Biomaterials **29,** 3117, 2008.
26. Sen, S.K., Lowe, J.B., 3rd, Brenner, M.J., Hunter, D.A., and Mackinnon, S.E. Assessment of the immune response to dose of nerve allografts. Plast Reconstr Surg **115,** 823, 2005.
27. Hermanns, S., Wunderlich, G., Rosenbaum, C., Hanemann, C.O., Muller, H.W., and Stichel, C.C. Lack of immune responses to immediate or delayed implanted allogeneic and xenogeneic Schwann cell suspensions. Glia **21,** 299, 1997.
28. Fansa, H., Schneider, W., and Keilhoff, G. Revascularization of tissue-engineered nerve grafts and invasion of macrophages. Tissue Eng **7,** 519, 2001.
29. Burnett, M.G., and Zager, E.L. Pathophysiology of peripheral nerve injury: a brief review. Neurosurg Focus **16,** E1, 2004.
30. Kline, D.G. Clinical and electrical evaluation. In: Kline, D.G., Hudson, A.R., Kim, D.H., Midha, R., Murovic, J.A., and Spinner, R.J., eds. Nerve Injuries: Operative Results for Major Nerve Injuries, Entrapments, and Tumors. Second edition. Philadelphia, PA: Saunders Elsevier, 2008, pp. 43–63.
31. Huang, J.H., Zager, E.L., Zhang, J., Groff, R.F., Pfister, B.J., Cohen, A.S., Grady, M.S., Maloney-Wilensky, E., and Smith, D.H. Harvested human neurons engineered as live nervous tissue constructs: implications for transplantation. J Neurosurg **108,** 343, 2008.

Address reprint requests to:
Douglas H. Smith, M.D.
Department of Neurosurgery
University of Pennsylvania
3320 Smith Walk, 105 Hayden Hall
Philadelphia, PA 19104

E-mail: smithdou@mail.med.upenn.edu

Received: May 20, 2008
Accepted: October 20, 2008
Online Publication Date: February 10, 2009