

ORIGINAL ARTICLE

Rebuilding Brain Circuitry with Living Micro-Tissue Engineered Neural Networks

Laura A. Struzyna, BSE,¹⁻³ John A. Wolf, PhD,^{1,2} Constance J. Mietus, BA,¹ Dayo O. Adewole, BSE,¹⁻³ H. Isaac Chen, MD,^{1,2} Douglas H. Smith, MD,¹ and D. Kacy Cullen, PhD^{1,2}

Prominent neuropathology following trauma, stroke, and various neurodegenerative diseases includes neuronal degeneration as well as loss of long-distance axonal connections. While cell replacement and axonal pathfinding strategies are often explored independently, there is no strategy capable of simultaneously replacing lost neurons and re-establishing long-distance axonal connections in the central nervous system. Accordingly, we have created micro-tissue engineered neural networks (micro-TENNs), which are preformed constructs consisting of long integrated axonal tracts spanning discrete neuronal populations. These living micro-TENNs reconstitute the architecture of long-distance axonal tracts, and thus may serve as an effective substrate for targeted neurosurgical reconstruction of damaged pathways in the brain. Cerebral cortical neurons or dorsal root ganglia neurons were precisely delivered into the tubular constructs, and properties of the hydrogel exterior and extracellular matrix internal column (180–500 μm diameter) were optimized for robust neuronal survival and to promote axonal extensions across the 2.0 cm tube length. The very small diameter permits minimally invasive delivery into the brain. In this study, preformed micro-TENNs were stereotaxically injected into naive rats to bridge deep thalamic structures with the cerebral cortex to assess construct survival and integration. We found that micro-TENN neurons survived at least 1 month and maintained their long axonal architecture along the cortical–thalamic axis. Notably, we also found neurite penetration from micro-TENN neurons into the host cortex, with evidence of synapse formation. These micro-TENNs represent a new strategy to facilitate nervous system repair by recapitulating features of neural pathways to restore or modulate damaged brain circuitry.

Introduction

THE EXQUISITE CAPABILITIES of the human brain rely on a multitude of long-distance axonal connections between specialized neuroanatomical regions. Neuronal degeneration as well as the loss of axonal pathways frequently occur in many central nervous system (CNS) disorders, including traumatic injury, stroke, and neurodegenerative diseases.¹⁻⁵ Unfortunately, in the CNS, neurogenesis is restricted to a few distinct domains.⁶⁻⁸ Moreover, natural regeneration of these long axon pathways appears impossible due to endogenous inhibition of axon growth and absence of directed guidance to far distant targets.^{1-5,9,10} This lack of neuroregeneration has devastating and often lifelong effects on neurological function.

Current approaches to facilitate CNS repair include cell replacement strategies and approaches to promote axonal

outgrowth and guidance. Cell replacement strategies most commonly utilize stem cells either recruited endogenously or delivered from exogenous sources.¹¹⁻¹³ To date, benefits of stem cell therapies include secretion of neuroprotective factors, providing glia to remyelinate denuded axons, and in some cases providing new neurons to discrete regions.¹⁴⁻¹⁹ While cell replacement strategies have received great attention to repair the brain, transplantation of dissociated cells cannot restore the key anatomic features of damaged pathways, most notably long axon tracts. Alternatively, studies that attempt to restore long-distance axonal connections typically aim to create a permissive environment for axonal outgrowth²⁰⁻²³ and/or augment the intrinsic capacity of axons to regenerate.²⁴⁻²⁶ These strategies most commonly involve biomaterial or cellular scaffolds to increase growth-promoting cues, diminishing

¹Center for Brain Injury and Repair, Department of Neurosurgery, Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania.

²Philadelphia Veterans Affairs Medical Center, Philadelphia, Pennsylvania.

³Department of Bioengineering, School of Engineering and Applied Science, University of Pennsylvania, Philadelphia, Pennsylvania.

inhibitory factors, and/or enhance the regenerative capacity of individual axons.^{27–31} While these approaches have demonstrated an ability to promote and guide axonal regeneration,^{27–29,32–36} they do not address degeneration of source neuronal population(s). Overall, while promising studies have independently demonstrated the survival of transplanted neural cells and modest axonal outgrowth/guidance, neuronal replacement coupled with targeted axonal regeneration to appropriate targets remain a significant challenge.

Therefore, we have recently developed micro-tissue engineered neural networks (micro-TENNs) as a strategy to simultaneously replace lost neurons and physically restore their long-distance axonal connections.³⁷ Micro-TENNs consist of discrete neuronal populations with long integrated axonal tracts encased in miniature tubular hydrogels (roughly three times the diameter of a human hair and extending up to several centimeters). The exterior hydrogel shell contains an interior extracellular matrix (ECM) core optimized to support neuronal survival and neurite extension. Whereas prior studies have transplanted fetal grafts, single cell suspensions, or cells in three-dimensional (3-D) matrices, our method is considerably different in that it involves generating the final cytoarchitecture of the micro-TENN *in vitro* and transplanting it *en masse*.^{19,38–44} The general geometry of these micro-TENNs recapitulates the anatomy of long axonal tracts, and thus may serve as an effective substrate for targeted neurosurgical reconstruction of long-distance axonal pathways in the brain (Fig. 1).

In the current study, we advance our micro-tissue engineering techniques to generate bidirectional micro-TENNs using either rat cerebral cortical neurons or dorsal root ganglia (DRG) neurons, achieving lengths of integrated axonal tracts up to 2.0 cm. Moreover, in a first demonstration study, we stereotaxically delivered fully formed micro-TENNs to connect deep thalamic structures with the cerebral cortex in rats, revealing long-term micro-TENN neuronal survival, maintenance of axonal architecture along the cortical–thalamic axis, and evidence of neurite outgrowth and

synaptic integration with the brain. Overall, our promising neural micro-tissue engineering strategy represents the first approach capable of facilitating CNS repair by simultaneously replacing neurons and recreating long-distance axon connections in a miniature dimension form factor.

Materials and Methods

3-D Micro-TENN fabrication

All supplies were from Invitrogen, BD Biosciences, or Sigma-Aldrich unless otherwise noted. Micro-TENNs were comprised of an agarose ECM hydrogel molded into a cylinder through which axons could grow. The outer hydrogel structure consisted of 1–4% agarose in Dulbecco's phosphate-buffered saline (DPBS). The cylinder, with an outer diameter that ranged from 500 to 990 μm , was generated by drawing the heated agarose solution into a microliter glass capillary tube (Drummond Scientific) through capillary action. An acupuncture needle (diameter: 180–500 μm) (Seirin) was inserted into the center of the liquid agarose-filled capillary tube to produce an inner column. Once the capillary tubes had cooled to room temperature, they were dipped into a liquid collagen (rat tail type I collagen, 3.0 mg/mL), collagen–laminin (rat tail type I collagen, 1.0 mg/mL; mouse laminin, 1.0 mg/mL), or fibrin (salmon fibrin, 1.0 mg/mL fibrinogen with 0.5 units/mL thrombin [Reagent Proteins]) solution. A negative pressure gradient was created by slowly retracting the needle, which drew the ECM solution into a central column. These microcolumns, now comprised of an agarose tubular shell with a collagen, collagen–laminin, or fibrin core, were then incubated at 37°C for 30 min. The cured microcolumns were gently pushed out of the capillary tubes and placed in DPBS where they were cut to 4–35 mm in length and sterilized under UV light (15 min). Following sterilization, DRG neurons were plated in microcolumns with a collagen core and cerebral cortical neurons were plated in microcolumns with a fibrin or collagen–laminin core.

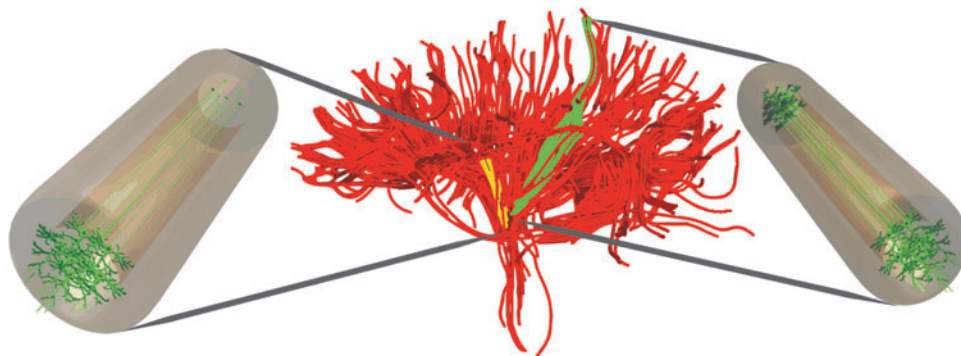


FIG. 1. Repair of the Connectome Using Micro-Tissue Engineering Neural Networks. A diffusion tensor imaging representation of the human brain demonstrating the exquisite connectome comprising of a multitude of long-distance axonal tracts (*red*) connecting functionally distinct populations of neurons. This conceptual rendition shows how fully formed micro-TENNs—consisting of discrete neuronal populations spanned by long integrated axonal tracts to recapitulate aspects of *gray–white* matter neuroanatomy—can be used to physically reconstruct lost axonal pathways. Here, a bidirectional micro-TENN is being used to repair a cortical–thalamic tract involved in sensory-motor processing (shown in *green*) whereas a unidirectional micro-TENNs is shown to repair the nigrostriatal pathway implicated in Parkinson's disease (shown in *yellow*). Micro-TENNS, micro-tissue engineered neural networks. Color images available online at www.liebertpub.com/tea

Neuronal cell culture

All procedures involving animals were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania and followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80–23; revised 2011).

Sprague Dawley rats (Charles River) were the source for primary DRG neurons (embryonic day 16) and cerebral cortical neurons (embryonic day 18). Briefly, carbon dioxide was used to euthanize timed-pregnant rats, following which the uterus was extracted through Caesarian section. Each fetus was removed from the amniotic sac and put in cold Hank's balanced salt solution (HBSS) or Leibovitz-15. To isolate DRG neurons, the spinal cords were dissected out and individual DRG were plucked using forceps. DRG explants were dissociated using prewarmed trypsin (0.25%) + EDTA (1 mM) at 37°C for 1 h. Following the addition of Neurobasal medium + 5% fetal bovine serum (FBS), the tissue was triturated and then centrifuged at 1000 rpm for 3 min. The supernatant was aspirated, and the cells were resuspended at 5×10^6 cells/mL in Neurobasal medium + 2% B-27 + 500 μ M L-glutamine + 1% FBS (Atlanta Biologicals) + 2.5 mg/mL glucose + 20 ng/mL 2.5S nerve growth factor + 10 mM 5FdU + 10 mM uridine. To isolate cerebral cortical neurons, the brains were removed and cerebrum was isolated. The cortices were dissociated in prewarmed trypsin (0.25%) + EDTA (1 mM) for 12 min at 37°C. The trypsin-EDTA was then removed and the tissue was triturated in HBSS containing DNase I (0.15 mg/mL). The cells were centrifuged at 1000 rpm for 3 min and resuspended at 30×10^6 cells/mL in Neurobasal medium + 2% B27 + 0.4 mM L-glutamine. To create micro-TENNs, 5–10 μ L of cell solution was precisely delivered to one or both ends of the micro-columns using a micropipette (total micro-TENNs created with DRG neurons: $n=144$; cortical neurons: $n=157$; see Experimental Design breakdown below). The cultures were placed in a humidified tissue culture incubator (37°C and 5% CO₂) for 50–75 min to allow cells to attach, after which, media were added to the culture vessel. Fresh, prewarmed media were used to replace the culture media every 2–3 days *in vitro* (DIV). In some instances, micro-TENNs were transduced with an adeno-associated virus viral vector (AAV2/1.hSynapsin.EGFP.WPRE.bGH, UPenn Vector Core) to produce green fluorescent protein (GFP) expression in the neurons. Here, at 5 DIV the micro-TENNs were incubated overnight in media containing the vector (3.2×10^{10} Genome copies/mL) and the cultures were rinsed with media the following day before being returned to the incubator.

Live/dead assay

Calcein AM (Sigma-Aldrich) and ethidium homodimer (Life Technologies) were used to perform Live/Dead assays on micro-TENNs and control cultures at 7 DIV. Cultures were rinsed with DPBS, after which they were incubated in a solution of 4 mM calcein AM and 2 mM ethidium homodimer for 30 min at 37°C. Following incubation, the cultures were rinsed three times with DPBS.

Immunocytochemistry

Micro-TENNs were fixed in 4% formaldehyde for 35 min, rinsed in phosphate-buffered saline (PBS), and permeabi-

lized using 0.3% Triton X-100 plus 4% horse serum for 60 min. Primary antibodies were added (in PBS + 4% serum) at 4°C for 12 h. The primary antibodies were the following markers: (1) MAP-2 (AB5622, 1:100; Millipore and AB5392, 1:100; Abcam) a microtubule-associated protein expressed primarily in neuronal somata and dendrites; (2) β -tubulin III (T8578, 1:500; Sigma-Aldrich), a microtubule element expressed primarily in neurons; and (3) glial fibrillary acidic protein (GFAP, AB5804, 1:500; Millipore), an intermediate filament protein expressed in astrocytes. After rinsing, appropriate fluorescent secondary antibodies (Alexa-488, -594 and/or -649 at 1:500 in PBS + 30 nM Hoechst + 4% serum) were added at 18–24°C for 2 h.

Transplantation of micro-TENNs

Adult male Sprague Dawley rats ($n=12$) were maintained under isoflurane anesthesia. Once anesthetized, rats were mounted in a stereotaxic frame (Kopf) and a craniotomy was performed lateral to the sagittal suture and between lambda and bregma to access the sensory cortex. Immediately before delivery, a 4 mm micro-TENN (7–10 DIV for either DRG neuron or cerebral cortical neuron micro-TENNs) was drawn into a 16-gauge needle in warmed 1 \times DPBS. The needle was centered 4.0 mm posterior to the bregma, and adjacent to the temporal ledge. The needle was then lowered 6.0 mm at an 11-degree angle over 2 min to target the S1 barrel cortex.⁴⁵ A glass plunger was engaged within the needle to secure the micro-TENN within the cortex while the needle was slowly retracted over 2 min. Once micro-TENN delivery was complete, the craniotomy sight was covered with sterile Teflon tape and secured with bone wax (Ethicon). The scalp was then sutured with 4-0 prolene sutures and the animals were recovered. Post-surgical analgesia included meloxicam (2.0 mg/kg) and bupivacane (2.0 mg/kg). Animals receiving DRG neuron micro-TENNs were survived for 3 days ($n=2$), while animals receiving cortical neuron micro-TENNs were survived for either 7 days ($n=4$) or 28 days ($n=4$). Control animals received acellular microcolumns ($n=2$). At the time of sacrifice, animals were anesthetized with Euthasol and underwent transcardial perfusion with 0.1% heparinized saline followed by 4% paraformaldehyde.

Immunohistochemistry

After 24 h postfix in 4% paraformaldehyde, brains were blocked from bregma –6 to bregma +3, and the blocked samples were placed in a 30% sucrose solution for a minimum of 2 days. Tissue samples were flash frozen in isopentane, frozen to a chuck with embedding medium, and sectioned at 20 μ m using a cryostat (CM1850; Lieca). Slides were rinsed with PBS, dipped in 70% ethanol, and blocked in 2% normal horse serum for 35 min. Primary antibodies were applied in OptiMax solution overnight at 4°C. The following primary antibodies were used: (1) calcitonin gene-related peptide (CGRP, C8198, 1:500; Sigma-Aldrich); (2) SMI31 (SMI-31R, 1:1500; Covance), a 200 kDa neurofilament protein; (3) MAP2 (AB5392, 1:50; Abcam); (4) synapsin I, (A6442, 1:50; Invitrogen), a phosphoprotein enriched in presynaptic terminals; and (5) GFAP (AB53554, 1:50; Abcam). After rinsing, appropriate fluorescent secondary antibodies (Alexa-568, -594,

and/or -649 at 1:500 in OptiMax solution) were added at 18–24°C for 1 h. Slides were cover slipped using ProLong Gold with DAPI (Life Technologies).

Microscopy and data acquisition

For *in vitro* analyses, micro-TENNs were imaged using phase contrast and fluorescence on a Nikon Eclipse Ti-S microscope with digital image acquisition using a QIClick camera interfaced with Nikon Elements BR 4.10.01. To determine the rate of neurite penetration, the longest observable neurite in each micro-TENN was measured at 7, 14, 21, 28, 35, and 42 DIV ($n=5$ each, repeated measurements). To quantify neurite health, micro-TENNs were imaged in phase contrast and 50 neurites per micro-TENN were randomly selected and scored as healthy (fully intact neurite with no beadings or other swellings) or unhealthy (neurite showing degenerative morphology, including varicosities, beading, and/or frank discontinuities). These counts were averaged to estimate neurite health for each micro-TENN. In this fashion, neurite health was assessed to test the impact of microcolumn (1) outer diameter (OD) \times inner diameter (ID): $630 \times 180 \mu\text{m}$, $n=4$; $630 \times 200 \mu\text{m}$, $n=5$; $630 \times 250 \mu\text{m}$, $n=5$; $630 \times 300 \mu\text{m}$, $n=6$; $701 \times 200 \mu\text{m}$, $n=5$; $701 \times 350 \mu\text{m}$, $n=5$; planar, $n=5$; and (2) agarose concentration: 1–2%, $n=6$; 3–4%, $n=6$; planar, $n=5$. Live/Dead assays were also performed to determine neuronal viability in the microcolumns. Here, the number of live and dead cells in each micro-TENN was counted and the percentage of viable cells was calculated. The Live/Dead assay was also used to determine the effects of microcolumn (1) OD \times ID: $630 \times 180 \mu\text{m}$, $n=4$; $630 \times 300 \mu\text{m}$, $n=4$; $630 \times 350 \mu\text{m}$, $n=4$; $701 \times 500 \mu\text{m}$, $n=4$; $701 \times 500 \mu\text{m}$, $n=4$; planar, $n=3$; and (2) agarose concentration: 1–2%, $n=9$; 3–4%, $n=10$; planar, $n=5$. All OD/ID studies were performed using 3% agarose microcolumns, while agarose concentration studies were performed using $701 \mu\text{m}$ OD \times $350 \mu\text{m}$ ID microcolumns (both analyses were performed at 7 DIV). For both neurite health and cell viability analyses, planar sister cultures grown on polystyrene served as positive controls. Representative images of the micro-TENNs used for the Live/Dead assays were taken using a Nikon A1RSI Laser Scanning Confocal microscope. For *in vitro* immunocytochemistry analyses, micro-TENNs were fluorescently imaged using a laser scanning confocal microscope (LSM 710; Zeiss) on a Zeiss Axio Observer microscope (Zeiss). Micro-TENN cytoarchitecture was qualitatively assessed at 7, 14, 21, 32, and 42 DIV for DRG and cortical neurons ($n=6$ each). For each *in vitro* micro-TENN analyzed through confocal microscopy, multiple z-stacks were digitally captured and analyzed. All confocal reconstructions were from full-thickness z-stacks ($\geq 180 \mu\text{m}$ when imaging neuronal somata or axons within the inner diameter). For analysis of micro-TENNs posttransplant into the brain, micro-TENNs were fluorescently imaged using the LSM 710 confocal microscope. For each $20 \mu\text{m}$ section analyzed through confocal microscopy, multiple z-stacks were digitally captured and analyzed to assess the presence, architecture, and outgrowth/integration of micro-TENN neurons/neurites.

Statistic analyses

Analysis of variance (ANOVA) was performed for the neuronal viability and neurite health studies with micro-

column agarose concentration and ID/OD as independent variables (with “ n ” as the number of distinct micro-TENNs per condition). When differences existed between groups, *post hoc* Tukey’s pair-wise comparisons were performed. For all statistical tests, $p < 0.05$ was required for significance. Data are presented as mean \pm standard deviation.

Results

Development of bidirectional micro-TENNs

We previously developed unidirectional micro-TENNs using DRG neurons with axonal projections extending up to 6 mm along the internal column.³⁷ In the current study, we advanced our micro-tissue engineering techniques to achieve longer lengths of axonal outgrowth while exploring bidirectional versus unidirectional architectures. Bidirectional micro-TENNs were created by seeding neuronal populations on both ends of the microcolumns, with extension of neurites through the interior of the tube. As with the previous unidirectional constructs, our biomaterial strategy encouraged DRG neuronal somata of the bidirectional constructs to form dense ganglia, predominantly restricted to the extremities. These ganglia projected long neurites into the interior, which, given sufficient time *in vitro*, overlapped and grew along each other (Fig. 2). The neurites primarily extended along the border between the ECM internal core and the agarose walls of the microcolumn. The length of neurite penetration in DRG neuron micro-TENNs was measured at weekly time points over 42 DIV. At 7 DIV, neurites extended 1.85 ± 0.62 mm into the microcolumn interior. At 21 DIV, neurites penetrated 4.90 ± 1.25 mm, and by 42 days, axonal projections from bidirectional neurons had crossed the microcolumn to form constructs measuring up to 2 cm (accurate growth measurements were not possible once axons from the two ends had crossed each other) (Fig. 3). In these micro-TENNs, axonal growth rate through the microcolumn interior remained fairly linear over the first 4 weeks *in vitro*, with an overall average rate of $0.23 \text{ mm/day} \pm 0.08$ during this period. However, in cases with optimal ECM and cell delivery (determined by visual inspection), robust growth was achieved as the axon fascicles extended to 1 cm by 14 DIV and 2 cm by 28 DIV, at which time the fascicles completely overlapped in the microcolumn center. Of note, interconnected axonal projections across microcolumns of lengths longer than 2 cm were not achieved and, interestingly, resulted in reduced axonal penetration compared to 2 cm microcolumns. These observations suggest that soluble neurotrophic/chemotactic factors from the target (i.e., opposite) neurons/axons in bidirectional micro-TENNs are necessary to sustain axonal growth over extreme distances and that the hydrogel shell may maintain gradients of such factors within the microcolumn internal core for lengths of 2 cm or less.

Cortical neuron micro-TENNs

Since our objective was to engineer the first transplantable micro-tissue approximating features of nervous system anatomy—specifically discrete neuronal populations spanned by long axonal tracts—we started with what is widely considered to be the most robust primary neuronal subtype: DRG neurons. However, since the ultimate application of

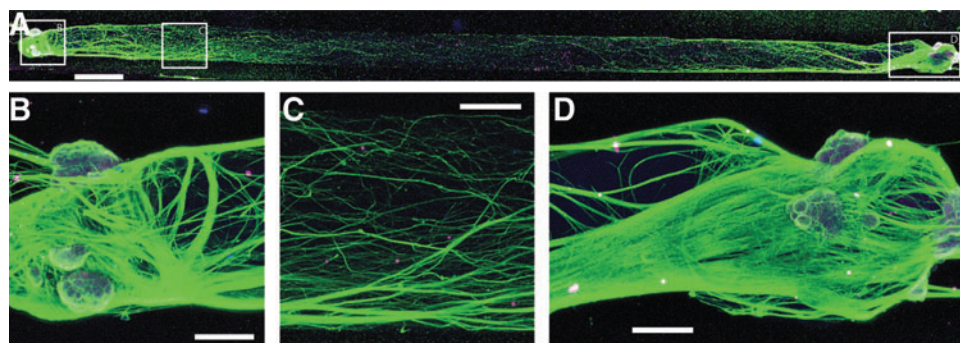


FIG. 2. Bidirectional Micro-TENNs. Representative confocal reconstructions of a bidirectional micro-TENN generated using primary DRG neurons stained through immunocytochemistry to denote neuronal somata/dendrites (MAP-2; *pink*), axons (β -tubulin III; *green*), and cell nuclei (Hoechst; *blue*) at 31 DIV (A). Neuronal somata were primarily restricted to dense ganglia on both ends of the micro-TENN (B, D), while the interior of the micro-TENN was comprised exclusively of long neurites (C). Scale bar (A) = 500 μ m; (B–D) = 100 μ m. DIV, days *in vitro*; DRG, dorsal root ganglia. Color images available online at www.liebertpub.com/tea

this technology is to restore brain pathways, once we demonstrated the ability to make healthy DRG neuron micro-TENNs, we made appropriate modifications to our protocols to create micro-TENNs using primary cerebral cortical neurons. Although these cortical neurons are a more suitable cell source for brain circuit reconstruction, they are notoriously more challenging to culture than DRG neurons, especially within the 3-D constructs at relatively low cell densities. Most fundamental aspects of the microcolumn fabrication and neuronal seeding remained consistent between the cortical neuron micro-TENNs and the DRG neuron micro-TENNs. However, we found that a collagen ECM alone in the microcolumn interior did not adequately support cortical neuron survival and axon extension, resulting in virtually complete neuronal death (data not shown). Through systematic optimization of the ECM constituents for the inner core, we found that both a salmon-derived fibrin matrix (1 mg/mL) and a laminin–collagen mixture (1 mg/mL LN and 1 mg/mL Col) supported the health and outgrowth of primary cortical neurons within the microcolumns.

Next, we investigated the relationship between various microcolumn parameters and cortical neuronal network growth and survival at 7 DIV within the micro-TENNs.

Specifically, we assessed the effects of microcolumn agarose concentration (1–4%; affecting hydrogel stiffness and pore size) and dimensions (OD/ID combination; affecting wall thickness and thus diffusional distances) on neuronal viability and neurite outgrowth and health (with laminin–collagen as the inner core ECM). This revealed that neuronal viability did not vary across the range of agarose concentrations ($p=0.076$) or OD/ID combinations ($p=0.154$) that were evaluated (Fig. 4). In contrast, micro-TENNs fabricated with 3–4% agarose microcolumns maintained healthier neurites than those fabricated with 1–2% agarose ($p<0.001$). However, neurite health did not vary across the various microcolumn dimensions that were tested ($p=0.131$) (Fig. 4). Of note, all micro-TENN parameters tested resulted in a reduced neurite health at this time point compared to planar polystyrene controls ($p<0.001$), underscoring the challenges in culturing primary cortical neurons within engineered 3-D microenvironments. Together, these analyses revealed that within the agarose microcolumns with an internal laminin–collagen ECM, the OD/ID combinations explored did not result in statistically significant changes in viability or neurite health; however, varying agarose concentrations statistically impacted neurite maintenance and health.

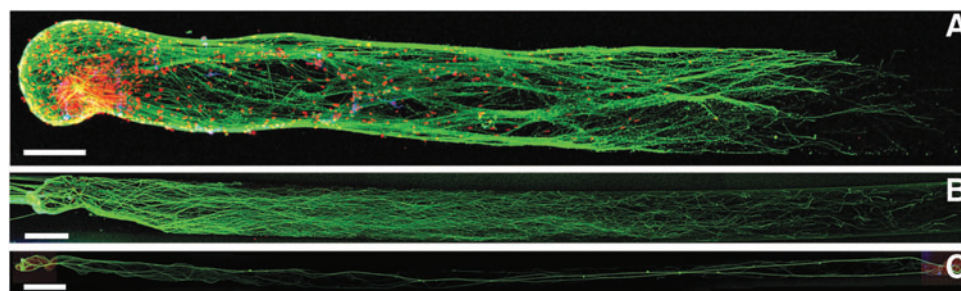
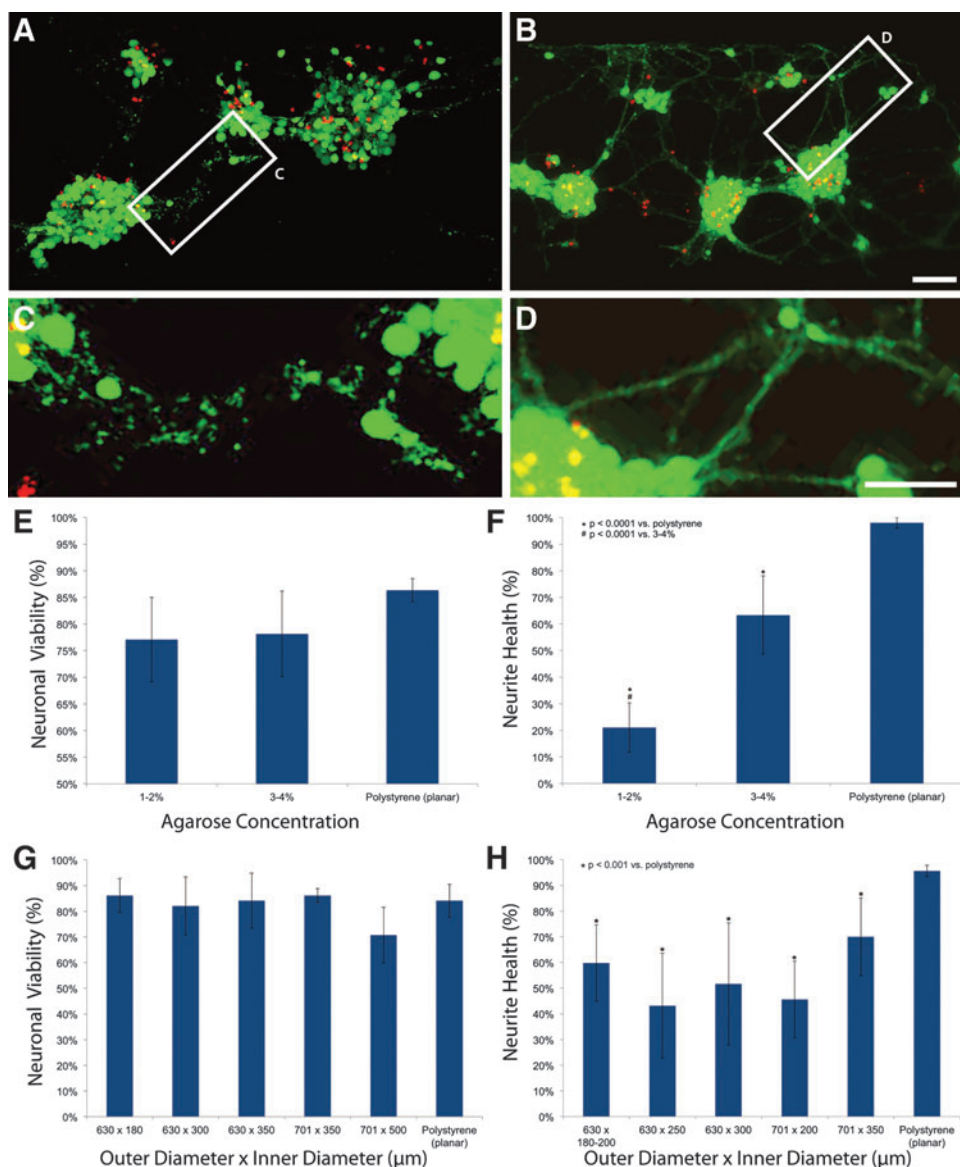


FIG. 3. Neurite Penetration Into Micro-TENNs. Confocal reconstructions of representative micro-TENNs generated using primary DRG neurons at 7, 21, and 42 DIV. Constructs were stained through immunocytochemistry to denote neuronal somata/dendrites (MAP-2; *red*), axons (β -tubulin III; *green*), and cell nuclei (Hoechst; *blue*). At 7 DIV, neurites extended approximately 3 mm into the microcolumn interior (A). At 21 DIV, neurites penetrated over 6 mm (B), and by 42 days, bidirectional axons had crossed to form a construct measuring up to 2 cm (C). Scale bar (A) = 200 μ m; (B) = 300 μ m; (C) = 800 μ m. Color images available online at www.liebertpub.com/tea



Based on these various insights, we demonstrated the creation of robust cortical neuron micro-TENNs exhibiting the desired cytoarchitecture. Specifically, cortical neurons were induced to form dense 3-D clusters consisting of discrete neuronal populations spanned by long fasciculated axonal tracts within the microcolumns (Fig. 5). Moreover, based on the amount of neurons seeded and the initial adhesion, both low-density and high-density cortical neuron micro-TENNs were generated. Immunocytochemistry and confocal microscopy revealed the presence of robust fasciculated and nonfasciculated axons (β -tubulin-III⁺) that were observed projecting between neuronal clusters (MAP-2⁺) as well as projecting along the length of the microcolumn interior to form neuronal networks at various spatial scales (Fig. 5). Moreover, a similar immunocytochemical analysis demonstrated a negligible astrocytic population within the micro-TENNs (near absence of GFAP⁺ cells/processes), which is consistent with our use of a defined (serum-free) culture media that is optimized for neuronal health, but is metabolically limiting for glial proliferation. In

general, we observed greater somatic infiltration and the formation of multiple cell clusters in the cortical neuron micro-TENNs rather than a singular dense ganglion at the microcolumn extreme as was observed in the DRG neuron micro-TENNs.

Micro-TENN transplantation

As a first demonstration study, we stereotaxically injected preformed living micro-TENNs into naive (nonlesioned) rats to mimic cortico-thalamic pathways. Even though we demonstrated the ability to generate long micro-TENNs (2.0 cm using DRG neurons and 1.0 cm using cerebral cortical neurons), the transplanted micro-TENNs only needed to be 4.0 mm in length to bridge the cerebral cortex to the thalamus in rats.⁴⁵ At terminal time points, rats were transcardially perfused with fixative and the brains processed for immunohistochemical analysis of transplant cell survival, maintenance of cyto and axonal architecture, and integration with host. Histological examination and confocal

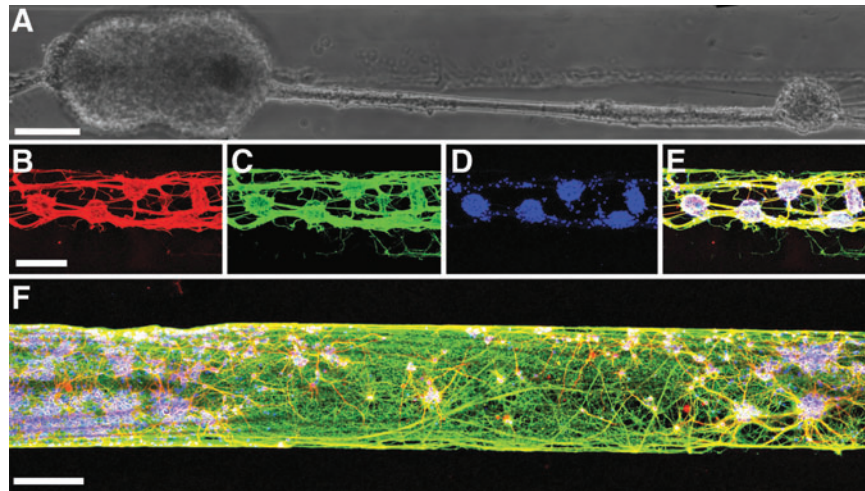


FIG. 5. Micro-TENN Cytoarchitecture Using Cerebral Cortical Neurons. Phase contrast and confocal reconstructions of micro-TENNs plated with primary cerebral cortical neurons at 7–14 DIV. Low-density cortical micro-TENNs formed discrete 3-D neuronal clusters spanned by fasciculated axonal tracts with additional fine neurite projections, which can be appreciated using phase contrast microscopy (A). Low- (B–E) and high-density (F) cortical micro-TENNs stained through immunocytochemistry to denote neuronal somata/dendrites (MAP-2; red), axons (β -tubulin III; green), and cell nuclei (Hoechst; blue). Discrete labeling for neuronal somata/dendrites (B), axons (C), cell nuclei (D), as well as their overlay (E) revealed distinct clusters of neuronal somata spanned by discrete axonal tracts in low-density micro-TENNs. High-density cortical neurons formed numerous clusters at the micro-TENN extremities, and extended dense neurites into the microcolumn interior at lengths spanning several millimeters (F). Scale bar (A) = 125 μ m; (B, F) = 200 μ m. 3-D, three-dimensional. Color images available online at www.liebertpub.com/tea

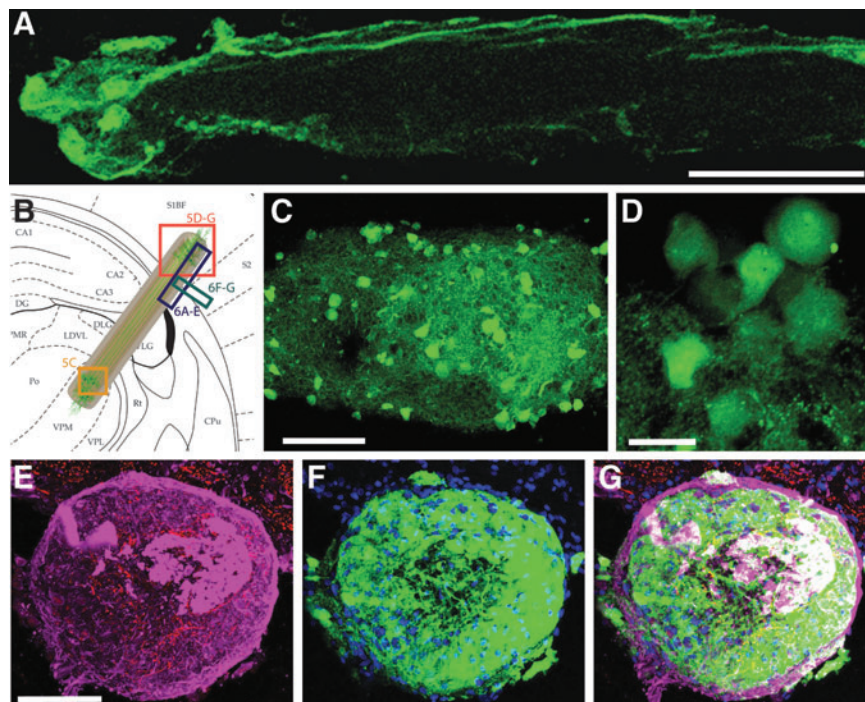


FIG. 6. Micro-TENN Transplant Survival. Representative confocal reconstructions of surviving transplanted neurons at multiple time points (from 20 μ m thick histological sections). Micro-TENNs generated using DRG neurons demonstrated acute survival and maintenance of axonal tract architecture (CGRP, green) at 3 days posttransplant along the cortical–thalamic axis (A). For reference, schematic depicting micro-TENN transplant location from a rat brain atlas with regions of interest in Figures 6 and 7 denoted by boxes (B). Micro-TENNs generated using cerebral cortical neurons were transduced *in vitro* to express GFP to permit identification of transplanted cells (C–G). Surviving clusters of micro-TENN neurons were found in the deep end of the microcolumn within the thalamus (C) and in the superficial end of the microcolumn in the cerebral cortex (D–G) at both 7 and 28 days posttransplant. Micro-TENN neurons survived out to 28 days posttransplant, the longest time point evaluated (D). Micro-TENNs were labeled through immunohistochemistry to denote neuronal somata/dendrites (MAP-2, purple) and axons (NF200, red) (E), cell nuclei (Hoechst, blue) (F), with overlay (G), confirming that surviving GFP⁺ cells consisted of neuronal somata/dendrites and axons (E–G). Scale bar (A) = 50 μ m; (C) = 100 μ m; (D) = 20 μ m; (E–G) = 100 μ m. Rat atlas adapted from Paxinos and Watson.⁴⁵ CGRP, calcitonin gene-related peptide; GFP, green fluorescent protein. Color images available online at www.liebertpub.com/tea

microscopy revealed that DRG neuron micro-TENNs survived within the microcolumn at 3 days postimplant (the only time point evaluated for DRG neuron micro-TENNs). Here, DRG neurons remained in a tight cluster with numerous axons projecting through the microcolumn interior along the cortical–thalamic axis (Fig. 6). Based on these encouraging proof-of-concept results, micro-TENNs generated using cerebral cortical neurons were also microinjected into naive rats along the cortical–thalamic axis. These preformed micro-TENNs consisted of GFP⁺ cortical neurons to permit identification of transplanted neurons/neurites *in vivo*. At both of the terminal time points evaluated (7 and 28 days postimplant), histological and confocal assessment demonstrated surviving GFP⁺ micro-TENN neurons contained within the microcolumn interior in both the cerebral cortex and the thalamus (Fig. 6). These transplanted neurons exhibited a healthy morphology and maintained a neurite-bearing cytoarchitecture. Immunohistochemistry verified that the dense clusters of surviving GFP⁺ cells within the micro-TENNs were neurons, as indicated through MAP-2

labeling for neuronal somata and dendrites, as well as neurofilament labeling for axons (Fig. 6).

In addition to transplant neuronal survival, we also found that the micro-TENNs maintained their architecture of long axonal tracts projecting along the cortical–thalamic axis. Here, histological examination revealed that deeper along the length of the microcolumns there were micro-TENN neurons and neurites aligned longitudinally along the interface between the hydrogel microcolumn and the host cortex (Fig. 7). Immunohistochemistry revealed that the GFP⁺ longitudinal projections within the micro-TENNs consisted of aligned axons (Fig. 7). Moreover, immunohistochemistry for the presynaptic protein synapsin revealed numerous synapses involving the neurons and neurites within the micro-TENNs, suggesting multiple synaptic relays along these projections. Of note, the agarose walls of the microcolumn appeared to be maintained at the 7 day time point, but had considerably thinned by 28 days postimplant. As this thinning occurred, the micro-TENN neurons and axons appeared to shift laterally toward the cortical tissue.

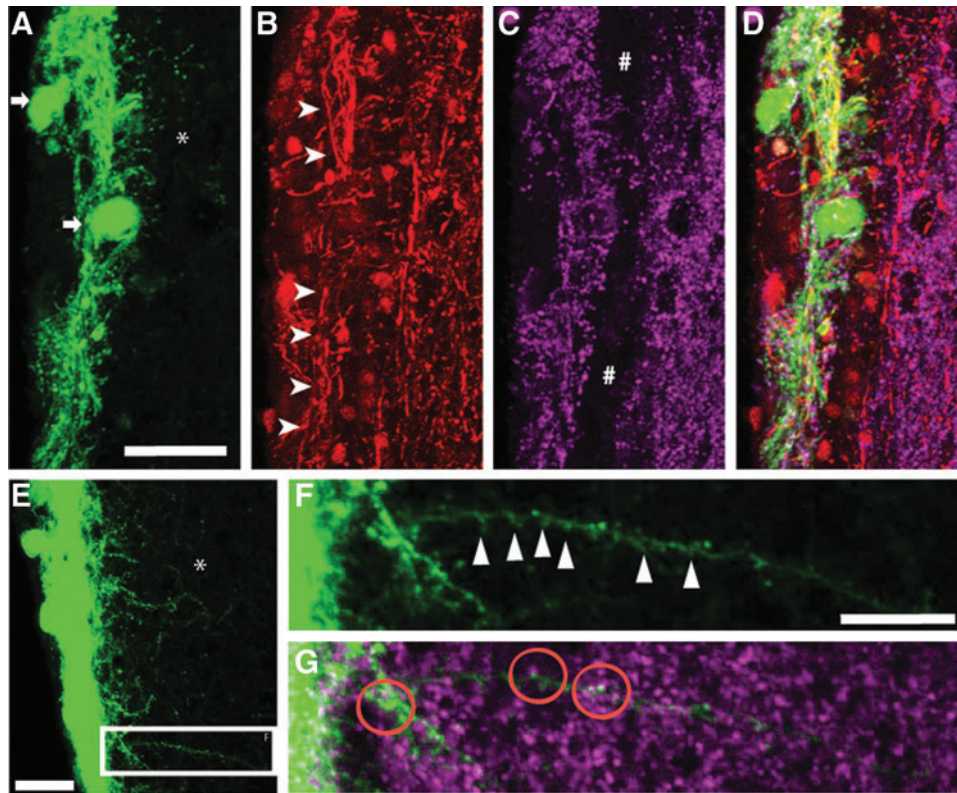


FIG. 7. Transplanted Micro-TENN Architecture and Integration. Representative confocal reconstructions of GFP⁺ cortical neuron micro-TENNs at 28 days posttransplant (20 μ m thick histological sections). As indicated by the blue rectangle in (Fig. 6B), GFP⁺ cells [arrows in (A)] and longitudinal projections [arrow heads in (B)] were observed deep along the cortical–thalamic axis within the remaining hydrogel microcolumn (A–D). Immunohistochemistry revealed that these GFP⁺-aligned processes were predominantly axonal (NF200, red) (B) with numerous synapses (synapsin, purple) (C) along the length as well as in the bordering host cortical tissue [denoted by * in (A) and (E)]; with overlay of all channels (D). The synapsin⁺ puncta along the radial projections suggests the presence of numerous synaptic relays along the transplanted neurons and neurites (C). The hydrogel border between the shaft of the micro-TENNs and the host tissue gradually thinned between 7 and 28 days postimplant, revealing a residual border at 28 days denoted by # in (C). In the region designated by the green rectangle in (Fig. 6B), by 28 days postimplant the transplanted neurons had extended neurites to penetrate into the host cortex (E). Higher magnification reconstructions from a demonstrative region in (E) show ingrowing neurites with putative dendritic spines [arrow heads in (F)] immediately opposed by synapsin⁺ puncta (G). Scale bar (A–E) = 40 μ m; (F, G) = 20 μ m. Color images available online at www.liebertpub.com/tea

Direct ingrowth/integration with host tissue is intended to occur at the micro-TENN extremities, as the microcolumn ends are deliberately free of hydrogel barrier. Here, histological examination determined that GFP⁺ micro-TENN neurons extended numerous neurites into the host cortex (Fig. 7). Interestingly, these neurites were predominantly projecting laterally from the micro-TENN extremity (orthogonal to the cortical–thalamic axis). Neurites from micro-TENN neurons penetrated deep into the host cortex, in general on the order of 50–150 microns, but in some cases reaching lengths up to several hundred microns. Moreover, morphological examination using confocal microscopy identified putative dendritic spines along GFP⁺ neurites projecting from the micro-TENNs. Immunohistochemistry suggested that these neurites projecting from the micro-TENNs formed synapses with host neurons in the cerebral cortex. In particular, there were synapsin⁺ puncta—indicative of presynaptic specializations—in immediate proximity to the morphologically identified dendritic spines along neurites projecting from the micro-TENNs (Fig. 7). Collectively, these results show the translation of the micro-TENN technology *in vivo*, with proof-of-concept data demonstrating our ability to deliver preformed living micro-TENNs into the brain as well as micro-TENN neuronal survival, maintenance of long axonal tract architecture, and integration with host neural tissue.

Discussion

The complex connectome of the human brain is distinguished by a multitude of long-distance connections between various specialized neuroanatomical regions. Disconnection of these axon connections is a common feature following traumatic injury, stroke, and neurodegenerative disease, with devastating effects on neurological function. Damaged pathways must be regenerated or replaced to restore function; however, there is currently no approach capable of repairing long-distance axonal pathways in the CNS. As a strategy to physically restore these axonal tracts, we have developed micro-TENNs that replicate the basic systems-level functional architecture of the CNS: discrete neuronal populations spanned by long integrated axonal tracts. In the current study, we created bidirectional micro-TENNs using multiple neuronal subtypes to serve as the basic neural systems building blocks to repair long-distance brain circuitry. Moreover, in proof of concept *in vivo* transplantations, we demonstrated that preformed micro-TENNs could successfully be delivered into the brain and that implanted neurons survived, maintained long-spanning axonal architecture, and putatively integrated with the host brain.

Building on our first-generation micro-TENNs,³⁷ in the current study we have advanced micro-tissue engineering techniques *in vitro* to generate discrete neuronal populations with long integrated axonal tracts encased in miniature hydrogel tubes. The exterior hydrogel shell provided mechanical stability for the microconstructs and had a low enough porosity to prevent lateral neurite outgrowth.^{46–48} The interior bioactive ECM (e.g., collagen, collagen–laminin, and/or fibrin) was designed to control the movement of the cell bodies, create distinct somatic and axonal regions, and support neuronal survival and neurite extension.^{49–51} Following precise delivery of dissociated embryonic rat neurons to one or both ends of the microcolumns, we demonstrated neuronal

survival in discrete populations and robust longitudinal axonal extensions out to at least 42 days in culture. Moreover, we assessed the influence of microcolumn agarose concentration, OD/ID, and ECM constituents on neuronal survival and neurite maintenance, establishing parameters necessary to form robust micro-TENNs with ideal cytoarchitecture on various length scales. These studies revealed that micro-TENNs built using DRG versus cortical neurons required different microenvironmental parameters to exhibit optimal health and network formation. However, the extension of robust fascicles through the 3-D microcolumns was achieved for both DRG and cortical neurons.

These micro-tissue engineering techniques are complementary to several different approaches used to pattern neural cells in specific 2-D or 3-D geometries. In particular, we also utilize controlled mechanical tension applied to integrated axonal tracts in custom mechanobioreactors to induce so-called axon stretch growth.^{52–55} Of note, this technique is currently only applied on a macroscale using cultures several millimeters to centimeters in width and generating axonal tracts measuring between 5 and 10 cm in length in 14–21 days. The current micro-TENN technology differs due to miniature dimensions and that neuronal/axonal growth occurs within self-contained transplantable biomaterial constructs. Additional methodologies are also used to create desired patterns of growth by seeding subregions with cells, selectively treating the culture surface with cell adhesive factors, and/or controlling gradients of structural or soluble cues. For instance, isolated neural populations on culture surfaces have been achieved during plating using silicon barriers, which when removed create distinct somatic and axonal regions.⁵⁶ The methods most commonly used to selectively treat surfaces include microstamping, photolithography, and microfluidic deposition. Microstamping involves loading an etched polymer stamp with adhesion molecules and bringing it into contact with the culture substrate.^{57,58} Photolithography uses light exposure through a mask followed by chemical treatment to immobilize proteins in precise geometries.⁵⁹ Lastly, microfluidic systems control the flow of small amounts of liquids to create gradients of signaling molecules.⁶⁰ Similar techniques have been applied in 3-D to create distinct somatic and neuritic regions. For instance, microfluidic technology has recently been advanced to produce 3-D multilayered scaffolds for neural cell culture where two cell–hydrogel layers were separated by two cell-free hydrogel layers.⁶¹ Other methods to direct outgrowth in 3-D include the use of structured anisotropic scaffolds for preferential neurite outgrowth, such as a micromesh that created patterned neurite growth along nylon fibers.⁴⁴ Our novel method to create distinct neuronal somatic and axonal regions shares features with these other 3-D approaches by controlling regions for cell seeding and axonal outgrowth.

To our knowledge, the creation of micro-TENNs exhibiting long axonal growth—2 cm for DRG neurons and 1 cm for cortical neurons—between discrete populations of neurons represents the longest targeted axonal extension for these neuronal subtypes within a 3-D engineered microenvironment *in vitro*. Of note, attempts to make micro-TENNs longer than these lengths resulted in reduced axonal penetration, which suggests that soluble factors released from the target cells and axons are critical to sustaining axonal

growth. Although the microcolumn and ECM structure likely support longitudinal diffusion of such factors, it is possible that appropriate concentration gradients are only maintained below a critical length threshold (per neuronal subtype) in this system. Thus, to generate constructs beyond these limits, we likely need to incorporate exogenous chemotactic/neurotrophic support into the micro-TENNs. Our current results generating bidirectional micro-TENNs *in vitro* represent an important advancement to our previous DRG neuron micro-TENNs. In consideration of transplant and repair, the microconstructs were optimized to extend to lengths feasible for bridging axon pathways for a range of disorders. In addition, techniques were developed to allow minimally invasive deep delivery of the constructs into the brain.

We propose a transplant strategy that differs significantly from previous approaches in that the final cytoarchitecture of the micro-TENNs—mimicking the architecture of brain pathways—is created *in vitro* and transplanted *en masse*. This approach offers an alternative to many other neural cell transplantation strategies proposed for neurodegenerative disorders, such as Huntington's disease and Parkinson's disease, as well as for stroke and traumatic brain injury (TBI). Previous studies have typically involved the transplantation of fetal grafts, single cell suspensions, or cells in 3-D matrices.^{19,38–44} Although these promising approaches have made impressive strides in improving transplant cell survival, integration, and axonal outgrowth, and thus may someday be able to replace target and/or source cells, there is currently no strategy capable of simultaneously restoring directed, long-distance axonal connections.

In the current study, we found that transplanted preformed micro-TENNs spanning the thalamus and cortex survived long-term while maintaining their engineered geometry. Moreover, the cortical neuronal construct transplants were found to extend dendrites into the brain and form synapses with both cortical and thalamic neurons. This included the observation of putative dendritic spines along the ingrowing neurites from the transplant cells and synapsin⁺ puncta in immediate proximity to these dendritic spines. These findings suggest that micro-TENNs may be a promising strategy to directly restore lost neural circuitry by replacing lost neurons and recreating long-distance axonal tracts. However, future studies are required to assess the electrophysiological assimilation of micro-TENN neurons to functionally integrate and relay information across the long axonal tracts, leading to useful participation in host neural networks. Key challenges that must be considered include neuronal/axonal phenotype, strength and number of synaptic connections, magnitude of fiber volleys, conduction velocity and myelination, and timing/frequency of inputs, among many others. Thus, a much more thorough assessment of micro-TENN structural and functional integration is required to assess whether these constructs can functionally assimilate into host neural networks and relay appropriate signals between functionally discrete nuclei in the brain.

Additional challenges related to the starting human cell source and microcolumn biomaterial scheme will need to be overcome to enable future clinical translation of micro-TENN technology. For instance, ultimately this approach will converge with stem cell-based methods (e.g., induced pluripotent stem cells [iPSCs]) as the source cells to generate micro-TENNs, allowing the creation of neuroanatomically inspired

microconstructs consisting of multiple neural cell types—neurons and glia—that should not provoke an immune response upon transplant. Importantly, this advancement may enable the engineering of autologous micro-TENNs on a per-patient basis that mimic the precise cytoarchitecture and extent of repair necessary due to patient-specific neurodegeneration or trauma. We also recognize the need to utilize alternative biomaterial microfabrication techniques that will enable the creation of more precisely manufactured micro-TENNs. Advanced strategies such as 3-D bio-printing and micromolding may enable the creation of microconstructs with improved resolution and reproducibility for scale up. Importantly, these methods may be performed sterilely, which would negate the need for sterilization through UV light, a process that can be damaging to certain biomaterials. In addition, the ultimate fate of the agarose microcolumns after transplantation will need to be addressed if agarose is to remain the major hydrogel constituent of micro-TENNs. Although degradation of agarose in the brain has not been characterized, in other systems it has broken down over weeks to months through nonspecific means likely involving phagocytosis by macrophages.^{62–64} This suggests that the agarose microcolumns may remain structurally viable on the order of at least weeks—long enough to chaperone micro-TENN neuronal delivery and integration—however, future longer-term implantation studies will need to be performed for confirmation. Addressing these challenges, among others, would significantly advance micro-TENN technology toward potentially broad therapeutic application.

An additional application of micro-TENN technology is to modulate the activity of dysfunctional neural circuitry (versus the primary purpose of reconstructing lost circuitry described thus far). In a phenomenon we describe as biological neuromodulation, tailored micro-TENNs may be precisely delivered to key locations to influence specific node(s) in a dysfunctional neural circuit through excitatory inputs, inhibition, or bulk neurotransmitter release. Such biologically based circuit modulation would be useful for a range of applications, many of which are current or experimental targets for deep brain stimulation (DBS). Here, micro-TENNs offer several advantages over DBS due to reduced size, organic composition, permanence, and lack of external power needs. Importantly, micro-TENNs used for such biological neuromodulation would be inherently capable of responding to feedback from the host by relaying input from one region to another region (or from the brain surface to deep regions).

In addition to directly replacing neurons and long-distance pathways, micro-TENNs may also facilitate nervous system repair by serving as a living scaffold to promote and direct host axon regeneration by exploiting axon-facilitated axonal regeneration.⁶⁵ Tissue-engineered living scaffolds are regenerative scaffolds comprised of living neural cells in a preformed, often anisotropic, 3-D architecture, generally consisting of aligned glial cells and/or longitudinal axonal tracts that have driven robust and targeted axonal regrowth and neural cell migration.^{52,65,66} Living scaffolds may facilitate targeted neural cell migration and axonal pathfinding by mimicking key developmental mechanisms. Indeed, directed axon growth and cell migration along pathways formed by other cells is a common tactic in nervous system development and is crucial to the proper formation of axonal connectivity and cellular localization.^{67–71} Although recent studies have

demonstrated the utility of axon-facilitated axon outgrowth for axon pathfinding in repair of peripheral nerve lesions, the utility of targeted axon-facilitated axon regeneration in the CNS has not been established. Taken together, micro-TENNs may function by the additional mechanism of providing a living pathway for directed long-distance axon regeneration in cases when source neuronal somata are preserved.

Conclusions

Although axonal loss is a prominent feature of many neurological diseases and trauma, there is currently no strategy capable of repairing long-distance axonal connections in the brain. We have created novel micro-TENNs consisting of preformed transplantable constructs that recapitulate the basic neural system level functional unit: discrete groupings of neurons spanned by long axonal tracts. These micro-TENNs present a new strategy for nervous system repair by simultaneously providing neuronal replacement and restoring long-distance axonal connections in the brain. Our promising findings demonstrate long-term survival of micro-TENN neurons *in vivo*, maintenance of their axonal architecture, as well as their capacity to integrate with the host cortex. Current efforts are focused on further assessing structural connectivity and electrophysiological integration of the micro-TENNs to utilize this technology to restore the connectome and facilitate functional restoration. We ultimately envision that this micro-tissue engineering approach will converge with iPSC-based methods as the source cells to generate micro-TENNs, potentially enabling autologous constructs on a per-patient basis that mimic the precise cytoarchitecture lost due to neurodegeneration or trauma. If successful, these novel micro-TENNs could revolutionize the treatment of a range of neurological disorders, including TBI, stroke, and neurodegenerative disease.

Acknowledgments

Financial support was provided by the U.S. Army Medical Research and Materiel Command through the Armed Forces Institute of Regenerative Medicine (#W81XWH-08-2-0034), the Congressionally Directed Medical Research Program (#W81XWH-10-1-0941), and the Joint Warfighter Medical Research Program (#W81XWH-13207004); the Department of Veterans Affairs through a RR&D Merit Review (#B1097-I); the National Institutes of Health/NINDS (T32-NS043126); Penn Medicine Neuroscience Center; and a Graduate Research Fellowship through the National Science Foundation (DGE-1321851). The authors would like to thank Andrew Jaye, Joseph Morand, and Ariana Barkley for technical assistance in these studies, and Dr. James Harris for assistance with figure preparation.

Disclosure Statement

No competing financial interests exist.

References

1. Tallantyre, E.C., *et al.* Clinico-pathological evidence that axonal loss underlies disability in progressive multiple sclerosis. *Mult Scler* **16**, 406, 2010.
2. Belal, A., and Ylikoski, J. Pathology as it relates to ear surgery II. Labyrinthectomy. *J Laryngol Otol* **97**, 1, 1983.
3. Levin, P.S., *et al.* A clinicopathologic study of optic neuropathies associated with intracranial mass lesions with quantification of remaining axons. *Am J Ophthalmol* **95**, 295, 1983.
4. Cheng, H.C., Ulane, C.M., and Burke, R.E. Clinical progression in Parkinson disease and the neurobiology of axons. *Ann Neurol* **67**, 715, 2010.
5. Marshall, V.G., *et al.* Deep white matter infarction: correlation of MR imaging and histopathologic findings. *Radiology* **167**, 517, 1988.
6. Gage, F.H. Mammalian neural stem cells. *Science* **287**, 1433, 2000.
7. Rakic, P. Adult neurogenesis in mammals: an identity crisis. *J Neurosci* **22**, 614, 2002.
8. Temple, S. The development of neural stem cells. *Nature* **414**, 112, 2001.
9. Curinga, G., and Smith, G.M. Molecular/genetic manipulation of extrinsic axon guidance factors for CNS repair and regeneration. *Exp Neurol* **209**, 333, 2008.
10. Huebner, E.A., and Strittmatter, S.M. Axon regeneration in the peripheral and central nervous systems. *Results Probl Cell Differ* **48**, 339, 2009.
11. Kim, S.U., and de Vellis, J. Stem cell-based cell therapy in neurological diseases: a review. *J Neurosci Res* **87**, 2183, 2009.
12. Trueman, R.C., *et al.* Repair of the CNS using endogenous and transplanted neural stem cells. *Curr Top Behav Neurosci* **15**, 357, 2013.
13. Horner, P.J., and Gage, F.H. Regenerating the damaged central nervous system. *Nature* **407**, 963, 2000.
14. Cummings, B.J., *et al.* Human neural stem cells differentiate and promote locomotor recovery in spinal cord-injured mice. *Proc Natl Acad Sci U S A* **102**, 14069, 2005.
15. Kim, H.J. Stem cell potential in Parkinson's disease and molecular factors for the generation of dopamine neurons. *Biochim Biophys Acta* **1812**, 1, 2011.
16. Orlacchio, A., Bernardi, G., and Martino, S. Stem cells: an overview of the current status of therapies for central and peripheral nervous system diseases. *Curr Med Chem* **17**, 595, 2010.
17. Shear, D.A., *et al.* Neural progenitor cell transplants promote long-term functional recovery after traumatic brain injury. *Brain Res* **1026**, 11, 2004.
18. Tate, M.C., *et al.* Specific beta1 integrins mediate adhesion, migration, and differentiation of neural progenitors derived from the embryonic striatum. *Mol Cell Neurosci* **27**, 22, 2004.
19. Tate, M.C., *et al.* Fibronectin promotes survival and migration of primary neural stem cells transplanted into the traumatically injured mouse brain. *Cell Transplant* **11**, 283, 2002.
20. Bradbury, E.J., *et al.* Chondroitinase ABC promotes functional recovery after spinal cord injury. *Nature* **416**, 636, 2002.
21. Stichel, C.C., *et al.* Inhibition of collagen IV deposition promotes regeneration of injured CNS axons. *Eur J Neurosci* **11**, 632, 1999.
22. Mingorance, A., *et al.* Regeneration of lesioned entorhino-hippocampal axons *in vitro* by combined degradation of inhibitory proteoglycans and blockade of Nogo-66/NGR signaling. *FASEB J* **20**, 491, 2006.
23. Tang, X.Q., *et al.* Targeting sensory axon regeneration in adult spinal cord. *J Neurosci* **27**, 6068, 2007.

24. Jain, A., Brady-Kalnay, S.M., and Bellamkonda, R.V. Modulation of Rho GTPase activity alleviates chondroitin sulfate proteoglycan-dependent inhibition of neurite extension. *J Neurosci Res* **77**, 299, 2004.
25. Liu, K., *et al.* PTEN deletion enhances the regenerative ability of adult corticospinal neurons. *Nat Neurosci* 2010.
26. Yip, P.K., *et al.* Cortical overexpression of neuronal calcium sensor-1 induces functional plasticity in spinal cord following unilateral pyramidal tract injury in rat. *PLoS Biol* **8**, e1000399, 2010.
27. Moore, M.J., *et al.* Multiple-channel scaffolds to promote spinal cord axon regeneration. *Biomaterials* **27**, 419, 2006.
28. Silva, N.A., *et al.* Development and characterization of a novel hybrid tissue engineering-based scaffold for spinal cord injury repair. *Tissue Eng Part A* **16**, 45, 2010.
29. Tsai, E.C., *et al.* Synthetic hydrogel guidance channels facilitate regeneration of adult rat brainstem motor axons after complete spinal cord transection. *J Neurotrauma* **21**, 789, 2004.
30. Filous, A.R., *et al.* Immature astrocytes promote CNS axonal regeneration when combined with chondroitinase ABC. *Dev Neurobiol* **70**, 826, 2010.
31. Borisoff, J.F., *et al.* Suppression of Rho-kinase activity promotes axonal growth on inhibitory CNS substrates. *Mol Cell Neurosci* **22**, 405, 2003.
32. 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.
33. Wen, X., and Tresco, P.A. Fabrication and characterization of permeable degradable poly(DL-lactide-co-glycolide) (PLGA) hollow fiber phase inversion membranes for use as nerve tract guidance channels. *Biomaterials* **27**, 3800, 2006.
34. Cai, J., *et al.* Permeable guidance channels containing microfilament scaffolds enhance axon growth and maturation. *J Biomed Mater Res A* **75**, 374, 2005.
35. Kim, Y.T., *et al.* The role of aligned polymer fiber-based constructs in the bridging of long peripheral nerve gaps. *Biomaterials* **29**, 3117, 2008.
36. Cullen, D.K., *et al.* Developing a tissue-engineered neural-electrical relay using encapsulated neuronal constructs on conducting polymer fibers. *J Neural Eng* **5**, 374, 2008.
37. Cullen, D.K., *et al.* Microtissue engineered constructs with living axons for targeted nervous system reconstruction. *Tissue Eng Part A* **18**, 2280, 2012.
38. Denham, M., *et al.* Neurons derived from human embryonic stem cells extend long-distance axonal projections through growth along host white matter tracts after intracerebral transplantation. *Front Cell Neurosci* **6**, 11, 2012.
39. Fawcett, J.W., Barker, R.A., and Dunnett, S.B. Dopaminergic neuronal survival and the effects of bFGF in explant, three dimensional and monolayer cultures of embryonic rat ventral mesencephalon. *Exp Brain Res* **106**, 275, 1995.
40. Mine, Y., *et al.* Grafted human neural stem cells enhance several steps of endogenous neurogenesis and improve behavioral recovery after middle cerebral artery occlusion in rats. *Neurobiol Dis* **52**, 191, 2013.
41. Ren, H., *et al.* Intracerebral neural stem cell transplantation improved the auditory of mice with presbycusis. *Int J Clin Exp Pathol* **6**, 230, 2013.
42. Sinclair, S.R., Fawcett, J.W., and Dunnett, S.B. Dopamine cells in nigral grafts differentiate prior to implantation. *Eur J Neurosci* **11**, 4341, 1999.
43. Tate, C.C., *et al.* Laminin and fibronectin scaffolds enhance neural stem cell transplantation into the injured brain. *J Tissue Eng Regen Med* **3**, 208, 2009.
44. Yoo, S.J., *et al.* Simple and novel three dimensional neuronal cell culture using a micro mesh scaffold. *Exp Neurol* **20**, 110, 2011.
45. Paxinos, G., Watson, C.; ebrary Inc. *The Rat Brain in Stereotaxic Coordinates*. Amsterdam; Boston: Elsevier, 2007, p. 1 v.
46. Balgude, A.P., *et al.* Agarose gel stiffness determines rate of DRG neurite extension in 3D cultures. *Biomaterials* **22**, 1077, 2001.
47. Bellamkonda, R., *et al.* Hydrogel-based three-dimensional matrix for neural cells. *J Biomed Mater Res* **29**, 663, 1995.
48. Cullen, D.K., Lessing, M.C., and LaPlaca, M.C. Collagen-dependent neurite outgrowth and response to dynamic deformation in three-dimensional neuronal cultures. *Ann Biomed Eng* **35**, 835, 2007.
49. Cullen, D.K., *et al.* In vitro neural injury model for optimization of tissue-engineered constructs. *J Neurosci Res* **85**, 3642, 2007.
50. Ju, Y.E., *et al.* Enhanced neurite growth from mammalian neurons in three-dimensional salmon fibrin gels. *Biomaterials* **28**, 2097, 2007.
51. Bellamkonda, R., Ranieri, J.P., and Aebischer, P. Laminin oligopeptide derivatized agarose gels allow three-dimensional neurite extension in vitro. *J Neurosci Res* **41**, 501, 1995.
52. Huang, J.H., *et al.* Long-term survival and integration of transplanted engineered nervous tissue constructs promotes peripheral nerve regeneration. *Tissue Eng Part A* **15**, 1677, 2009.
53. Pfister, B.J., *et al.* Extreme stretch growth of integrated axons. *J Neurosci* **24**, 7978, 2004.
54. 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.
55. Pfister, B.J., *et al.* Development of transplantable nervous tissue constructs comprised of stretch-grown axons. *J Neurosci Methods* **153**, 95, 2006.
56. Smith, D.H., *et al.* High tolerance and delayed elastic response of cultured axons to dynamic stretch injury. *J Neurosci* **19**, 4263, 1999.
57. Nam, Y., and Wheeler, B.C. Multichannel recording and stimulation of neuronal cultures grown on microstamped poly-D-lysine. *Conf Proc IEEE Eng Med Biol Soc* **6**, 4049, 2004.
58. Wheeler, B.C., Nam, Y., and Brewer, G.J. Patterning to influence in vitro neuronal interfaces. *Conf Proc IEEE Eng Med Biol Soc* **7**, 5337, 2004.
59. Sorribas, H., Padeste, C., and Tiefenauer, L. Photolithographic generation of protein micropatterns for neuron culture applications. *Biomaterials* **23**, 893, 2002.
60. Millet, L.J., *et al.* Guiding neuron development with planar surface gradients of substrate cues deposited using microfluidic devices. *Lab Chip* **10**, 1525, 2010.
61. Kunze, A., *et al.* Micropatterning neural cell cultures in 3D with a multi-layered scaffold. *Biomaterials* **32**, 2088, 2011.
62. Emans, P.J., *et al.* Autologous engineering of cartilage. *Proc Natl Acad Sci U S A* **107**, 3418, 2010.
63. Jain, A., *et al.* In situ gelling hydrogels for conformational repair of spinal cord defects, and local delivery of BDNF after spinal cord injury. *Biomaterials* **27**, 497, 2006.
64. Varoni, E., *et al.* Agarose gel as biomaterial or scaffold for implantation surgery: characterization, histological and

- histomorphometric study on soft tissue response. *Connect Tissue Res* **53**, 548, 2012.
65. Struzyna, L.A., Katiyar, K.S., and Cullen, D.K. Living scaffolds for neuroregeneration. *Curr Opin Solid State Mater Sci* **18**, 308, 2014.
66. East, E., *et al.* Alignment of astrocytes increases neuronal growth in three-dimensional collagen gels and is maintained following plastic compression to form a spinal cord repair conduit. *Tissue Eng Part A* **16**, 3173, 2010.
67. Jacobs, J.R., and Goodman, C.S. Embryonic development of axon pathways in the *Drosophila* CNS. I. A glial scaffold appears before the first growth cones. *J Neurosci* **9**, 2402, 1989.
68. Raper, J., and Mason, C. Cellular strategies of axonal pathfinding. *Cold Spring Harb Perspect Biol* **2**, a001933, 2010.
69. Raper, J.A., Bastiani, M., and Goodman, C.S. Pathfinding by neuronal growth cones in grasshopper embryos. II. Selective fasciculation onto specific axonal pathways. *J Neurosci* **3**, 31, 1983.
70. Sepp, K.J., Schulte, J., and Auld, V.J. Peripheral glia direct axon guidance across the CNS/PNS transition zone. *Dev Biol* **238**, 47, 2001.
71. Stiles, J., and Jernigan, T.L. The basics of brain development. *Neuropsychol Rev* **20**, 327, 2010.

Address correspondence to:
D. Kacy Cullen, PhD
Department of Neurosurgery
University of Pennsylvania
105E Hayden Hall
3320 Smith Walk
Philadelphia, PA 19104

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

Received: September 22, 2014

Accepted: August 28, 2015

Online Publication Date: October 20, 2015