ENGINEERED TISSUES DERIVED FROM INDUCED-PLURIPOTENT STEM CELLS (IPSCs) FOR DISEASE MODELING, DRUG DISCOVERY, AND REPLACEMENT THERAPIES*

Functional Cortical Axon Tracts Generated from Human Stem Cell-Derived Neurons

H. Isaac Chen, MD,^{1,2} Dennis Jgamadze, MD, PhD,¹ James Lim, BA,¹ Kobina Mensah-Brown, BA,¹ John A. Wolf, PhD,^{1,2} Jason A. Mills, PhD,³ and Douglas H. Smith, MD¹

Axon tracts support connectivity among different brain regions, which is crucial for the proper functioning of the brain. It is well known that axon regeneration in the central nervous system is poor, and thus, axonal injury often leads to persistent neurological and cognitive deficits. A novel approach to this problem is the transplantation of preformed axon tracts engineered in a laboratory setting. In this study, we describe a potential human substrate for this repair strategy: cortical axon tracts generated from human pluripotent stem cell-derived neurons using the technique of axon stretch growth. Human cortical neurons were differentiated from embryonic stem cell or induced pluripotent stem cell lines. Subjecting these neurons to a stretch growth protocol resulted in the formation of 1-cm-long axon tracts in less than 1 month. Stretch rates of up to 1 mm/day were achieved. These constructs consisted of two clusters of neuronal somata connected by dense intervening axons. The neuronal clusters were composed of primarily infragranular cortical neurons, although some supragranular neurons also were observed. Individual axons exhibited spontaneous calcium waves, illustrating their functionality. Our findings demonstrate the feasibility of engineering functional cortical axons from human stem cell sources, raising the possibility of an autologous approach for cerebral axon transplantation after injury. These constructs also provide a resource for *in vitro* studies into the dynamics of modular networks and for disease modeling purposes.

Keywords: axons, cortical neuron differentiation, neuronal network, stem cells, tissue engineering

Impact Statement

Axon regeneration is negligible in the adult mammalian brain, and thus, white matter damage often leads to permanent neurological deficits. A novel approach for axon repair is the generation of axon tracts in the laboratory setting followed by transplantation of these constructs. This article details a human substrate for this repair strategy. Using the technique of axon stretch growth, functional cortical axon tracts are generated from human pluripotent stem cells at rates of up to 1 mm/day. These results form the basis of a potential patient-specific protocol for cerebral axon transplantation after injury.

Introduction

OVER THE PAST DECADE, explanations of how the brain functions have increasingly relied on the concept of network connectivity, in which information processing occurs via the transfer of data among computational nodes.¹ The substrate that enables this dissemination of information is white matter, the bundles of axons that essentially act as the wiring of the brain. Clinical^{2–4} and computational⁵ studies suggest that the extent of white matter injury, more so than the total volume of brain damage, predicts the

natural history of patient outcomes and expected gains from rehabilitation and neuromodulatory therapies. Axon tracts within the brain are thus of vital importance for maintaining normal cerebral function. However, axonal regeneration in the adult mammalian central nervous system (CNS) is severely limited as a result of environmental factors related to glial scar formation and myelin breakdown products,⁶ as well as intrinsic restrictions on axonal growth in mature CNS neurons.^{7,8} As a result, white matter injury often is associated with persistent neurological and cognitive deficits.

¹Department of Neurosurgery, Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania.

²Corporal Michael J. Crescenz Veterans Affairs Medical Center, Philadelphia, Pennsylvania.

³Center for Advanced Retinal and Ocular Therapeutics, Department of Ophthalmology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania.

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Developing alternative methods for reconstructing cerebral circuitry and restoring brain function may be possible due to recent advances in neural tissue engineering and stem cell biology. Techniques have been explored to engineer axon tracts *in vitro* at different length scales between two populations of neurons.^{9–12} In particular, the obstacle of slow growth cone-mediated axon growth has been overcome using the novel process of axon stretch growth. Two clusters of neurons are allowed to integrate synaptically. These clusters are then gradually separated from each other, and the resulting mechanical tension induces active growth of the preformed axons. Using rat embryonic dorsal root ganglia (DRGs) neurons, axon tracts up to 10 cm in length have been created at elongation rates up to 1 cm/day.¹³ This process also has been applied successfully to rat embryonic cortical neurons, although at slower rates of elongation.^{10,14} Theoretically, such laboratory-grown axon tracts could be transplanted into the brain to replace lost connections among brain regions.^{15,16} The finding that stretch-grown axons can be transplanted successfully into animal models of spinal cord¹⁷ and peripheral nerve injury¹⁸ provides evidence of the feasibility of this approach.

In order for any engineered neural tissue to be translated into the clinical setting, an appropriate source of human neurons must be available. The advent of induced pluripotent stem (iPS) cells¹⁹ raises the possibility of generating autologous or donor-matched neuronal tissue. Various protocols have been developed to generate a wide variety of CNS neurons, including cortical projection neurons,^{20,21} interneurons,²² dopaminergic neurons,²³ and spinal motor neurons.²⁴ With regard to cortical projection neurons, prior studies have demonstrated that *in vitro* differentiation results in an impressive degree of layer subtype specification. This process produces all six cortical layers in the proper temporal sequence, thus recapitulating corticogenesis during fetal development.^{20,25}

In this study, we applied the technique of axon stretch growth to human cortical neurons derived from embryonic stem (ES) and iPS cell lines to generate robust human cortical axon tracts. Constructs up to 1 cm could be generated in less than 1 month. We describe this methodology and the phenotype of the resultant axon tracts. We further demonstrate the functionality of the axons using calcium-imaging techniques.

Materials and Methods

Stem cell maintenance

Stem cell lines used in this study included H9-GFP and PBWT2, which were provided by the Stem Cell Core of the Children's Hospital of Philadelphia. The PBWT2 iPS line was created using a lentiviral cassette overexpressing *Oct4*, *Sox2*, *c-Myc*, and *Klf4* in peripheral blood erythroblasts obtained from a healthy human volunteer.²⁶ These lines were maintained as colonies grown on a feeder layer of irradiated mouse embryonic fibroblasts (MEFs; MTI-GlobalStem). Daily media changes were performed using human embryonic stem (hES) cell media, which consisted of Dulbecco's modified Eagle's medium (DMEM)/F12 (Thermo Fisher Scientific) supplemented with 20% knockout serum replacement (Thermo Fisher Scientific), 100 U/mL penicillin, 100 µg/mL streptomycin (Thermo Fisher Scientific), 2 mM

GlutaMAX (Thermo Fisher Scientific), 1× nonessential amino acids (NEAA; Thermo Fisher Scientific), 100 µM β-mercaptoethanol (Thermo Fisher Scientific), and 6 ng/mL basic fibroblast growth factor (bFGF) (R&D Systems). At the desired time, colonies were dissociated from the underlying feeder layer using TrypLE (Thermo Fisher Scientific) or type IV collagenase (Thermo Fisher Scientific) and triturated to obtain clusters of ~30–50 cells. These clusters were then replated on fresh feeders at a dilution of 1:50 in hES media containing 10 µM Y-27632 (Tocris), a ROCK pathway inhibitor, which was removed after 24 h. All stem cell cultures were maintained under normoxic conditions.

Cortical neuron differentiation

Stem cell colonies were dissociated from the underlying feeders using TrypLE. Cell clusters were then transferred to feeder-free conditions by plating them on tissue culture plates coated with 5% Matrigel (Sigma) and fed with hES media containing 10% MEF-conditioned media. Conditioned media were obtained by incubating hES media without bFGF in the presence of MEFs (no older than 7 days *in vitro*) and removing it after 24 h. Once the cells had reached 70–80% confluency, they were dissociated with Accutase (Stem Cell Technologies) into a single-cell suspension, and 2.5×10^5 cells were plated in a 24-well plate coated with 5% Matrigel. The differentiation process, which combines features of two previous protocols,^{22,25} was started after the wells were 100% confluent, which usually took 1–2 days.

From differentiation day (dd) 0–10, the cells were fed with defined default medium (DDM), which contained DMEM/F12, 100 U/mL penicillin, 100 µg/mL streptomycin, 2 µM GlutaMAX, 1× NEAA, 100 µM β-mercaptoethanol, 1 mM sodium pyruvate, 500 µg/mL bovine serum albumin (Sigma), 1× N2 supplement (Invitrogen), and 1× B27 supplement without vitamin A (Thermo Fisher Scientific), plus 100 nM LDN-193189 (Stemgent), 5 µM SB431542 (Stemgent), and 2 µM XAV939 (Stemgent). In addition, 0.5 µM cyclopamine (Calbiochem) was added from dd2–10 to dorsalize the neural precursors. Media changes were performed daily during this initial period. From dd10–16, the cells were fed daily with DDM + 100 nM LDN-193189. From dd16–24, the cells were fed daily with DDM only. The cells were then removed from their plating surface using a 200 µL pipette tip on dd24, lightly triturated, and replated on 12-well plates coated with 5% Matrigel (1 well in a 24-well plate into 3 wells in a 12-well plate) in DDM with 10 µM Y-27632 for 24 h. Thereafter, the media were gradually transitioned from DDM to Neurobasal (NB) media containing 0.5 mM GlutaMAX and 1× B27 with vitamin A (Thermo Fisher Scientific). After dd33, ½ media changes with NB were performed every other day.

Axon stretch growth

The detailed protocol for axon stretch growth has been described previously.²⁷ In brief, a bioreactor chamber was assembled so that a movable towing membrane overlapped a base membrane. The growth surfaces were then coated with 0.05% w/v polyethylenimine (PIE) (Sigma) overnight at room temperature followed by a 100-µL bubble of 3.33 µg/mL laminin (Corning) at the interface between the towing and base membranes for 2 h at 37°C. Stem cell-derived neurons (dd55–101) were dissociated using 0.25% trypsin-

ethylenediaminetetraacetic acid (EDTA) (Invitrogen) for 10 min at 37°C, and DNA from dead cells was dissolved using 0.3 mg/mL DNase I (Sigma). A total of 3×10^5 cells were plated in the region of the laminin bubble after it had been aspirated in NB media supplemented with 5% fetal bovine serum (Atlanta Biologicals) and 10 μ M Y-27632 at 37°C. After 2 days, the media were replaced with NB alone, and 1/3 media changes with NB were performed twice weekly thereafter.

On day *in vitro* 10, the bioreactor chamber was connected to a microstepper motor, which gradually moved the towing membrane with respect to the base membrane. The microstepper motor was capable of moving in 1- μ m increments, and stretch rates were set by specifying the number of steps taken in a single cycle and the length of time the motor was paused between cycles. Stretch growth was started either immediately on microstepper motor attachment or delayed for 2–3 days. Axon stretch was initially performed at a rate of 0.288 mm/day. After 24 h, the rate was then increased to 0.5 mm/day. For stretch rates greater than 0.5 mm/day, the rate was increased by 0.5 mm/day increments every 24 h until the intended maximal stretch rate was achieved. For calcium activity experiments, stretch growth was terminated after 5–8 days. For stretch rate optimization experiments, the maximal stretch rate was applied for at least 3 days before the cultures were analyzed. No media changes were performed during the period of stretch growth.

Immunocytochemistry (monolayer cultures and stretch-grown axons)

Cells were fixed with 4% paraformaldehyde (Electron Microscopy Sciences) diluted in phosphate-buffered saline (PBS; Roche) for 15 min at room temperature and then rinsed three times with PBS. Blocking and permeabilization were performed with 5% normal goat serum (NGS; Vector Labs) and 0.3% Triton X-100 (Sigma) in PBS for 30 min at room temperature. Primary antibodies diluted in 5% NGS/PBS were incubated with cells overnight at 4°C. After three rinses with PBS the following day, cells were incubated with the appropriate Alexa Fluor secondary antibody (Thermo Fisher Scientific) diluted in 5% NGS/PBS for 2 h at room temperature. Samples were then rinsed another three times with PBS before coverslips were mounted with Aqua-Poly/Mount (Polysciences, Inc.). Imaging was performed using a Nikon Eclipse Ti-2 with a CoolSNAP DYNO camera or a Nikon A1R confocal system with NIS-Elements software (Nikon). Primary antibodies used in this study are listed in Supplementary Table S1.

Quantification of human cortical neuron phenotype

For samples fixed at dd6, cells were plated on coverslips coated with Matrigel (Sigma) on dd0. For samples fixed at dd30–33 and dd50–54, differentiating neurons were dissociated on dd24 with Accutase as a single-cell suspension and plated on coverslips coated with PEI and laminin at a plating density of 100,000 cells/cm². Cells were maintained in the appropriate media with 10 μ M Y-27632 overnight. Because high cell densities made counting at later differentiation dates difficult, neurons were redissociated on dd60–70 with 0.25% trypsin-EDTA (Thermo Fisher Scientific) for samples fixed at dd80–84. The cells were plated on coverslips

and maintained in a similar manner as dd30–33 and dd50–54 samples. After immunocytochemistry, 2–5 randomly chosen 10 $\times$ fields were counted until data for a total of at least 800 cells were obtained for each sample.

Calcium imaging and analysis

Calcium activity was assessed 1–4 days after stretch growth was stopped (dd92–98). Stretch-grown axons were incubated with 5 μ M Fluo-4 (Thermo Fisher Scientific) in NB for 30 min at 37°C and then washed once with NB before imaging. Calcium imaging was performed in NB at 37°C using a Nikon Eclipse TE3000 microscope with a 10 $\times$ objective, Hamamatsu C11440 camera, and NIS-Elements software. Videos were recorded at 5 frames per second.

Selection of regions of interest (ROIs) and extraction of mean gray values were performed using ImageJ software (NIH). Calcium responses were calculated using FluoroSNNAP software.²⁸ In brief, background was subtracted from the signals in each sample, and then, $\Delta F/F_0$ values were calculated for each ROI using the 10th percentile value of the complete raw fluorescence trace as F_0 . Identification of calcium events and calculation of synchronization indices (scale of 0–1) also were done with FluoroSNNAP. For each sample, at least five randomly selected neuronal somata or axonal ROIs were selected for these analyses. Default FluoroSNNAP settings, including the reference calcium waveform library, were used.

To assess the origin of axonal calcium activity, we used tetrodotoxin to block sodium channel-dependent action potentials. After Fluo-4 incubation, baseline calcium activity was recorded for a total of 2 min. Tetrodotoxin (0.5 μ M) then was added to the media, and another 2 min of calcium activity were recorded. Processing of the calcium signals was performed as described above.

Quantification of stretch growth quality

Samples were initially categorized as either having or lacking stretch-grown axons, based on the presence or absence of at least one intact axon spanning the two somatic regions, respectively. Subsequently, samples having stretch-grown axons were graded as either good or intermediate stretch using 10 $\times$ phase-contrast micrographs (Nikon Eclipse TE3000 with a Hamamatsu C11440 camera). Intermediate stretch growth was delineated in two ways. The first definition was fewer than 10 axons or fascicles in a 10 $\times$ field of view of the densest axonal region. The second was more than half of the axons having neuronal somata attached to at least 1/3 of the axon. This second definition took into account constructs in which there was significant contamination of the axonal segment with neuronal somata. Good stretch growth was defined as the inverse of intermediate stretch growth.

Statistical analyses

Descriptive statistics are reported as mean $\pm$ standard deviation. Comparison of neuronal somata versus axonal calcium event frequencies was performed using a two-sample Student's *t*-test. A *p*-value of 0.05 was used as the threshold for significance. Statistical analyses were performed using OriginPro (OriginLab).

Results

Differentiation of cortical neurons from human pluripotent stem cell sources

We generated cortical neurons from an ES (H9) and iPS (PBWT2) cell line using a protocol that combined features of previously published methods.^{22,25,29} Before dd24, neural progenitors (Pax6+) and immature neurons grew in a tightly packed monolayer (Fig. 1A, B). Although the number of Pax6+ cells dropped significantly at dd30, many of these cells remained present at all time points (Fig. 1I). Following replating of cultures on dd24, clusters of developing neurons began extending processes (Fig. 1C). The density of these processes progressively increased over months (Fig. 1D). By dd30, a large proportion of the cells in the cultures were neuronal (β -III tubulin+), a finding that was relatively stable up to nearly 3 months (Fig. 1I).

Additional immunocytochemical studies helped characterize the lineage commitment of these differentiated human neurons. The vast majority of the β -III tubulin+ cells expressed FoxG1, a telencephalic marker commonly found during cortical development (Fig. 1E). The percentage of FoxG1+/ β -III tubulin+ continued to increase over time. Markers of infragranular cortical layers, including Tbr1 (Fig. 1F) and CTIP2 (Fig. 1G), were found by dd30. While CTIP2 expression plateaued thereafter, Tbr1 expression continued to increase until dd50 before leveling off (Fig. 1I).

No expression of Satb2, a marker of supragranular cortical layers, was seen until dd80 (Fig. 1H, I), an observation that is in line with previous results for dissociated neurons differentiated from pluripotent stem cells²⁵ and some brain organoids.³⁰ These results show that the temporal course of normal cortical development is maintained (lower layers formed before upper layers), mirroring the findings of prior studies.^{20,25}

Growth of human cortical axon tracts using mechanical tension

We used the differentiated human cortical neurons to create axon tracts by applying mechanical tension (Fig. 2A).²⁷ Neurons were dissociated into single-cell suspensions and replated at the interface of two Aclar membranes (towing and base) in a custom stretch growth bioreactor. Cultures were maintained for 10 days to allow for local axon regrowth and synapse formation among neurons. Subsequently, the towing membrane was gradually moved using a microstepper motor to create two separate neuronal populations connecting by intervening stretch-grown axons.

Initial cultures of iPS cell-derived neurons were subjected to a maximal stretch rate of 0.5 mm/day, which resulted in dense, parallel processes spanning clusters of neuronal somata on either side (Fig. 2B). Similar findings were obtained using ES cell-derived neurons (Supplementary Fig. S1).

FIG. 1. Differentiation of cortical neurons from human pluripotent stem cell lines. Cortical neurons were differentiated from an ES (H9-GFP) and iPS (PBWT2) cell line. Results depicted in this figure are from the PBWT2 line. (A) At early time points, nearly all cells were positive for Pax6, a marker of cortical neural progenitors. (B) Cells were tightly packed during the early stages of the differentiation process. (C, D) After cells were replated on dd24, the axon network became increasingly dense. (E) The majority of differentiated neurons (B3T+; β -III tubulin) also stained positive for the telencephalic marker FoxG1. (F–H) Markers of infragranular cortical neurons (Tbr1, CTIP2) were more prominent than markers of supragranular cortical neurons (Satb2) at dd84. Scale bars: 100 μ m (A–H). (I) This graph summarizes the percentage of cells expressing different phenotypic markers as determined by immunocytochemistry at four time points. The data for CTIP2, Satb2, FoxG1, and Tbr1 are expressed as percentages of the neuronal (B3T+) population. Data are expressed as mean $\pm$ standard deviation ($n=3$ different differentiations per data point). ES, embryonic stem; iPS, induced pluripotent stem.

FIG. 2. Generation of stretch-grown cortical axon tracts from human iPS cell-derived neurons. **(A)** Schematic of axon stretch growth bioreactor and process. **(B)** Stretch growth of cortical neurons derived from an iPS cell line (PBWT2) resulted in dense processes aligned in a parallel orientation. These processes were axons rather than dendrites (Tau+/MAP2-). **(C, D)** Individual axons in the region near the neuronal somata merged to form thicker axon fascicles in the center of the construct. **(E)** Within the somatic region, GFAP+ astrocytes (green) were mixed with β -III tubulin+ neurons (red). Infragranular cortical neurons (CTIP2+, **F**) were more frequently observed in the somatic region than their supragranular counterparts (Satb2+, **G**). **(H)** Punctate synapsin staining was seen along the processes of the differentiated neurons, suggestive of synapse formation. Scale bars: 1 mm (**B**), 100 μ m (**C–E**), 50 μ m (**F, G**), and 10 μ m (**H**).

These processes stained for tau but not MAP2, indicating that they were axonal in nature rather than dendritic. Individual axons near the neuronal somata merged to form thicker fascicles (Fig. 2C, D), which dominated the center of the axon tracts. GFAP+ astrocytes were identified in the neuronal clusters (Fig. 2E) but not the axonal region. Similar to cultures not subjected to stretch growth, a significant number of infragranular CTIP2+ cells were found in the neuronal clusters (Fig. 2F). Supragranular Satb2+ cells were observed at a lower frequency (Fig. 2G). Synapsin+ puncta were found among the differentiated neurons (Fig. 2H).

Functionality of human cortical axons

We next interrogated the functionality of stretch-grown axon tracts generated from iPS cell-derived neurons by examining spontaneous calcium waves. Calcium activity was assessed 1–4 days after stretch growth was stopped. In five of seven samples, we observed evidence of calcium activity in neuronal somata. In four of the active samples, we also observed calcium waves in the axons (Fig. 3A, B). The frequency of calcium events in the axons was 0.042 ± 0.030 events/s, which trended lower than the frequency of calcium events in the neuronal somata (0.078 ± 0.063 ; $p = 0.078$, $n = 3$ samples in each group).

There was little evidence of synchronization of calcium activity in either the axons (0.19 ± 0.078 , $n = 3$ samples) or the neuronal somata (0.18 ± 0.059 , $n = 3$ samples). The addition of tetrodotoxin resulted in the elimination of calcium activity in the axons, suggesting that this activity is dependent on functional sodium channels (Supplementary Fig. S2).

Limits of axon stretch rates

Rat DRG neurons can achieve stretch rates up to 10 mm/day.¹³ We also have shown that rat embryonic cortical neurons can tolerate stretch rates of at least 1.7 mm/day without difficulty.¹⁴ Therefore, we next sought to determine the tolerance of human cortical axons over a range of stretch rates. To compare different stretch rates, we initially categorized samples as either having or lacking at least one continuous axon between the neuronal clusters. Because there was significant variability in the quality of the former group, we also categorized our stretch growth results using a semiquantitative 3-point scale: good stretch growth (2), intermediate stretch growth (1), and no stretch growth (0) (Fig. 4A–F). Good stretch growth was observed in more than half of the samples at a maximal stretch rate of 0.5 mm/day, and at least one continuous stretched axon was observed in 80% of

FIG. 3. Calcium activity of human stretch-grown cortical axon tracts. Calcium activity in the stretch-grown axon tracts was visualized with Fluo-4. (A) This fluorescence micrograph depicts 1 frame from a 5 frames per second video recording of calcium activity. Scale bar: 10 μ m. (B) Spontaneous calcium waves are shown for three axonal regions of interest in (A). (C) The frequency of calcium waves was compared between neuronal somata and axons ($p=0.078$, $n=3$). The box plots depict means and standard deviations, while the whiskers show the minimum and maximum values.

the samples. When the maximal stretch rate was increased to 1 mm/day, no samples yielded good stretch growth, and more than half resulted in no stretch growth (Fig. 4G). We hypothesized that connecting the stretch growth bioreactor to the microstepper motor could disrupt axons at the towing/base membrane interface. To overcome this potential problem, we introduced a 2–3-day delay between when the bioreactor was connected to the microstepper motor and when stretch growth was initiated to allow axons to recover and/or reform. This intervention resulted in more successful stretch growth at a maximal rate of 1 mm/day with nearly half of samples yielding good stretch growth and 85.7% of samples having at

least one continuous stretched axon (Fig. 4G). However, when the maximal stretch rate was increased to 1.5 mm/day, we again observed no good stretch growth with more than half of the samples resulting in no stretch growth.

Discussion

In this study, we demonstrated the feasibility of engineering functional human cortical axon tracts from ES and iPS cell lines using the technique of axon stretch growth. Using cultures of differentiated human cortical neurons as the initial substrate, we generated robust axon tracts up to

FIG. 4. Determining and optimizing maximal stretch rates. To compare outcomes across different stretch rates, constructs were graded as good (2), intermediate (1), or no stretch growth (0). See the Materials and Methods section for detailed definitions of these categories. (A, B) Two examples of good stretch growth are shown in these phase-contrast micrographs (maximal stretch rate of 1 mm/day for both). (C, D) These two examples of intermediate stretch growth include a construct with few stretch-grown axons (C) and one with many neuronal somata mixed with the axons (D) (maximal stretch of 1.5 mm/day for both). (E, F) Two examples of no stretch growth are shown [maximal stretch rates of 1.5 (E) and 1 mm/day (F)]. Scale bars: 100 μ m (A–F). (G) This graph summarizes the distribution of stretch quality grades for different maximal stretch rates. In addition to the 3-point grading scale shown in (A–F), constructs were also categorized in a binary manner as either having (hatched segments) or lacking (solid segments) stretch-grown axons. The two bars on the left represent trials in which stretch growth was initiated immediately after the microstepper motor was connected, while the two bars on the right are data from trials in which stretch growth was delayed for 2–3 days after the microstepper motor was connected.

1 cm in length in less than 1 month. We achieved growth rates up to 1 mm/day, which is comparable with the average rate of axon regeneration in the mammalian peripheral nervous system (PNS) and greatly exceeds the negligible regeneration rates in the mature mammalian CNS.⁷ Culture conditions within the stretch growth bioreactor supported a moderate degree of astrocyte growth and retained the predominance of infragranular over supragranular cortical neurons observed with the differentiation protocol. The engineered axons exhibited functional activity, as shown by the presence of spontaneous calcium waves. Our results raise the possibility of generating human cortical axon tracts as substrates for rebuilding cerebral circuitry. These constructs also could contribute as test beds for studying modular networks or for disease modeling.

White matter repair strategies

Given the importance of axon tracts to cerebral function, their repair has been the subject of intense interest. Leaving aside the activation of plasticity mechanisms such as latent pathways,^{31,32} two general approaches have been considered. The conventional strategy has focused on understanding and promoting axon regeneration beyond what normally occurs *in vivo*. These studies have provided significant insight into the mixed effects of the damaged CNS environment on axon regeneration^{6,33} and the limited intrinsic axon growth capacity of mature CNS neurons.^{7,8} Efforts to enhance CNS axon outgrowth have included recreating the more permissive environment of the PNS,^{17,34,35} reducing the inhibitory effects of the astrocytic scar,⁶ and upregulating regeneration-associated pathways using either genetic techniques or extrinsic factors.^{7,8}

Our results represent a different strategy, in which axon tracts are generated *in vitro* with the intent for subsequent transplantation *in vivo* to reconstruct CNS circuitry. Other axon engineering platforms for achieving this objective include prefabricated axon growth pathways such as hydrogel microcolumns.^{12,36,37} These *in vitro* approaches have the potential advantages of finer control over the growth characteristics and phenotype of the engineered axons and mitigation of the negative impact of the injured CNS milieu. Specifically for the axon stretch technology used in this study, the possibility also exists for creating centimeter-long axon tracts within relatively short periods of time. Ultimately, combining different aspects of axon regeneration *in vivo* and axon tract replacement may accelerate progress toward a clinically viable therapy.

More efficient generation of stretch-grown cortical axons

Optimization in several areas would facilitate the transition of stretch-grown human axon tracts into the *in vivo* setting. The first area pertains to their engineering efficiency. Our current protocol requires a 10–13-day period to allow the differentiated human neurons to form a sufficiently dense axon network to permit axon elongation, more than twice the time necessary for rat cortical neurons.¹⁴ Furthermore, the maximal stretch rate of human cortical neurons lags behind the rates attainable with rat cortical neurons (up to at least 1.7 mm/day).¹⁴ Both situations could be remedied by pro-

ducing more robust networks of axons in a shorter period of time before the initiation of stretch growth.

This objective could be achieved in a number of ways. Adjustments to the stretch growth bioreactor could be implemented to minimize disruption of the towing/base membrane interface associated with bioreactor connection to the microstepper motor. Neuronal aggregates could be plated instead of a monolayer of cells to promote axon growth and fasciculation.^{37,38} The use of more mature neurons also could lead to more robust axon networks. Finally, modulating intrinsic axon growth properties could shorten the time needed to create an axon tract of a given length or increase the length that could be produced in a given period of time. The addition of growth factors, such as brain-derived neurotrophic factor and basic fibroblast growth factor, could increase axon network density via effects on both axon length and branching.^{39–41} Although the specific cellular machinery responsible for axon stretch growth is not yet known, manipulation of intrinsic axon growth mechanisms, such as the nuclear factor kappa-light-chain-enhancer of activated B cells,⁴² mechanistic target of rapamycin,⁴³ and Kruppel-like factor^{44,45} signaling pathways, could enhance axonal tolerance to mechanical tension. Such gains would be of great benefit for translational and clinical applications to decrease the turnaround time for generating human cortical axon tracts.

Structural stabilization for in vivo delivery

The second area of optimization is the development of techniques for protecting stretch-grown axon tracts from the physical manipulation necessary for transplantation. Stretch-grown cortical axons are less sturdy than stretch-grown DRG axons and can be broken by fluid waves within the culture media. Even in the absence of frank disconnection, axonal strain can lead to functional pathology.^{46,47} Stabilization of stretch-grown cortical axons could be attained in two ways. Hydrogel columns^{11,12,36} and other scaffolding techniques⁴⁸ could provide the necessary structural support. These approaches have not yet been adapted for axon stretch growth, but a marriage of the two concepts could be a promising avenue of further inquiry. Alternatively, structural support could be derived from other cells, including hardier neurons whose axons could be elongated in parallel with the cortical neurons (e.g., DRGs¹³) or glial cells that normally coexist with the neurons.^{49,50} In the latter case, more glial cells than what we observed in the current iteration of the stretch-grown cortical axons would be needed to create a construct more akin to a true tissue. Inclusion of oligodendrocytes also could lead to axon myelination and increase the conduction velocity of the engineered axons.

Choice of human neurons

The third engineering consideration relates to the optimal cellular basis of human stretch-grown cortical axons. Cortical neurons differentiated from ES or iPS cells were utilized in this study. This class of stem cell-derived neurons has the benefits of cortical layer specificity, an embryonic-like state, and the potential for generating a nearly endless number of cells.²⁵ Axons from layer-specific cortical neurons may be necessary to reestablish efficient communication with host cortex. Future studies will need to test this hypothesis and, if correct, establish protocols for

producing axon tracts with more precise laminar phenotypes. In particular, differentiating supragranular cortical neurons in greater numbers would become more important.^{20,25} Brain organoids may provide additional insight into the process of producing these supragranular neurons, as they appear at earlier time points in some organoid protocols (dd52–56)^{51,52} than in cultures of dissociated neurons (dd72–80).²⁵ Compared with postnatal or aging cells, embryonic neurons exhibit a greater potential for plasticity, which should promote their survival and integration after transplantation. Finally, the ability to generate a large number of patient-specific or donor-matched neurons is an important factor in the practical translation of any regenerative medicine strategy. One of the translational hurdles for neuron differentiation protocols is the length of time needed to generate neurons, but recent screens of small-molecule inhibitors have led to the acceleration of these time lines.⁵³

The emerging technology of direct conversion, either from somatic cells⁵⁴ or stem cells,⁵⁵ also deserves consideration as a cell source for stretch-grown axons. One of the primary benefits of direct conversion techniques is the considerably shortened time frame for generating mature neurons. Current disadvantages include uncertain cellular identities, aged phenotypes in the case of conversion from somatic cells, and lower neuronal yields.^{55,56} One particular concern about the uncertain identity of these neurons is the observation that specific cortical phenotypes control the degree of integration of transplanted neurons.^{57,58}

Two issues that may affect both differentiation and direct conversion approaches are immunological complications and teratogenicity. The autologous nature of cellular reprogramming implies that no immunosuppressive regimens are necessary, but some studies suggest that at least a subset of cells derived from these techniques induces an immune response after syngeneic transplantation.^{59,60} Tumor formation from aberrant transgene integration or residual progenitors lingers as a concern. Recent transplantation studies of differentiated cortical neurons did not observe teratoma formation or oncologic transformation,^{25,58} but integration-free cellular reprogramming methods and built-in safety measures, such as apoptotic switches, warrant continued investigation.

Considerations for in vivo neural circuit reconstruction

Functional factors are likely to impact the effectiveness of stretch-grown cortical axons in rebuilding brain circuits. We demonstrated that our engineered axons exhibit spontaneous calcium waves, a common indicator of neural activity. These results are consistent with recent calcium-based analyses of the activity of human stem cell-derived neuron.^{61,62} While we have demonstrated that human stretch-grown cortical axons are likely functionally active, further work is needed to evaluate the firing of spontaneous action potentials, the maturity of the constituent networks, and the computational capacity of these constructs. We have previously examined the propagation of multichannel data across rat stretch-grown cortical axons using a combination of optogenetic stimulation and microelectrode array recordings.¹⁴ Similar studies would be useful in ascertaining the ability of engineered human axons to transmit information and serve as a model of modular network. The latter capacity could be

appropriated to investigate dysfunctional internodal communication in neuropsychiatric and neurocognitive conditions, trauma, and other brain disorders.

A fundamental question for neural circuit repair based on transplanted axon tracts is what parts of the brain need to be reconnected to restore function. This issue is not straightforward, especially in light of the shift in defining brain function from a localization-based model to a network perspective.¹ As such, simply reconnecting point A to point B may be too simplistic. Establishing the specific points of reconnection and the axon “dose” will likely require network analyses to determine the minimum set of edges necessary to restore efficient network function. Similar work has been done to predict the network-level impact of local brain stimulation⁶³ and the effect of hypothetical cortical resections on seizure networks.⁶⁴ At a more granular level, reconnection strategies may need to incorporate an understanding of cortical layer-specific connectivity and function. Thorough investigation of these factors will be crucial for translating the idea of neural circuit reconstruction into a viable clinical intervention.

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Disclosure Statement

H.I.C., D.J., J.L., K.M.-B., J.A.W., and J.A.M. have no competing financial interests. D.H.S. is the cofounder of Axonova Medical.

Supplementary Material

Supplementary Figure S1
Supplementary Figure S2
Supplementary Table S1

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Address correspondence to:

H. Isaac Chen, MD

Department of Neurosurgery

University of Pennsylvania

3rd Floor, Silverstein Pavilion

3400 Spruce Street

Philadelphia, PA 19104

E-mail: isaac.chen@uphs.upenn.edu

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