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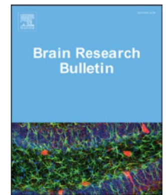

Restoring lost nigrostriatal fibers in Parkinson's disease based on clinically-inspired design criteria

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A B S T R A C T

Parkinson's disease is a neurodegenerative disease affecting around 10 million people worldwide. The death of dopaminergic neurons in the substantia nigra and the axonal fibers that constitute the nigrostriatal pathway leads to a loss of dopamine in the striatum that causes the motor symptoms of this disease. Traditional treatments have focused on reducing symptoms, while therapies with human fetal or stem cell-derived neurons have centered on implanting these cells in the striatum to restore its innervation. An alternative approach is pathway reconstruction, which aims to rebuild the entire structure of neurons and axonal fibers of the nigrostriatal pathway in a way that matches its anatomy and physiology. This type of repair could be more capable of reestablishing the signaling mechanisms that ensure proper dopamine release in the striatum and regulation of other motor circuit regions in the brain. In this manuscript, we conduct a review of the literature related to pathway reconstruction as a treatment for Parkinson's disease, delve into the limitations of these studies, and propose the requisite design criteria to achieve this goal at a human scale. We then present our tissue engineering-based platform to fabricate hydrogel-encased dopaminergic axon tracts *in vitro* for later implantation into the brain to replace and reconstruct the pathway. These tissue-engineered nigrostriatal pathways (TE-NSPs) can be characterized and optimized for cell number and phenotype, axon growth lengths and rates, and the capacity for synaptic connectivity and dopamine release. We then show original data of advances in creating these constructs matching clinical design criteria using human iPSC-derived dopaminergic neurons and a hyaluronic acid hydrogel. We conclude with a discussion of future steps that are needed to further optimize human-scale TE-NSPs and translate them into clinical products.

1. Introduction

Parkinson's disease (PD) is a disorder characterized by motor deficits, such as tremors, slowness of movement (bradykinesia), and rigidity, as well as non-motor symptoms including autonomic dysfunction, depression, and dementia (DeMaagd and Philip, 2015; Poewe et al.,

Abbreviations: BDNF, brain-derived neurotrophic factor; CSPG, chondroitin sulfate proteoglycans; ChABC, chondroitinase ABC; CT, computer tomography; CED, convection-enhanced delivery; DBS, deep brain stimulation; DTI, diffusion tensor imaging; DARPP-32, dopamine- and cyclic-AMP-regulated phosphoprotein of molecular weight 32000; ESC, embryonic stem cell; ECM, extracellular matrix; FSCV, fast-scan cyclic voltammetry; GABA, gamma aminobutyric acid; GDNF, glial-derived neurotrophic factor; GFRα1, GDNF family receptor α1; GFP, green fluorescent protein; HA, hyaluronic acid; iPSC, induced pluripotent stem cell; IND, investigational new drug; L-DOPA, L-34-dihydroxyphenylalanine; ^{18}F-DOPA, L-6-[^{18}F] fluoro-34-dihydroxyphenylalnine; MRI, magnetic resonance imaging; MFB, medial forebrain bundle; MSN, medium spiny neuron; MeHA, methacrylated hyaluronic acid; Micro-TENN, micro-tissue engineered neural network; OEC, olfactory ensheathing cell; PD, Parkinson's disease; PET, positron emission tomography; PLGA, poly(lactic-co-glycolic acid); SN, substantia nigra; SNpc, substantia nigra pars compacta; SNpr, substantia nigra pars reticulata; TE-NSP, tissue engineered nigrostriatal pathway; TGFα, transforming growth factor α; TH, tyrosine hydroxylase; VEGF, vascular endothelial growth factor.

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2017; Struzyna et al., 2018). PD is the second most common neurodegenerative disease, affecting the quality of life of 10 million people worldwide, including 1–2% of people 65 years or older (DeMaagd and Philip, 2015; Poewe et al., 2017). Projections indicate that the number of PD patients in the ten most populated countries will double by 2030 (Dorsey et al., 2007). This condition also represents a substantial economic burden, as the Parkinson's Foundation has estimated that the total cost of treatment in the United States is $52 billion/year, including $2,500/year for medications and $100,000 for surgery per patient. Existing therapies can mitigate symptoms for a limited time, but there are currently no approved treatments to prevent or repair the underlying pathology. The search for an effective treatment is thus a crucial endeavor.

A key aspect of PD pathology (Fig. 1A,B) is the loss of A9 dopaminergic neurons in the substantia nigra pars compacta (SNpc) and the degeneration of their axonal projections along the nigrostriatal pathway (DeMaagd and Philip, 2015; Grealish et al., 2010). These axons synapse with medium spiny neurons (MSNs) in the dorsolateral striatum, a region associated with motor movements, decision making, and learning (Freed et al., 2011; Tewari et al., 2016). In healthy patients, activated dopaminergic neurons release dopamine, which acts on striatal MSNs and modulates the gamma aminobutyric acid (GABA)-dependent inhibitory output of the basal ganglia. With the loss of dopamine released by nigrostriatal terminals in PD, the net effect is increased inhibition of the thalamus from the globus pallidus internus and reduced activation of the motor cortex (DeMaagd and Philip, 2015; Poewe et al., 2017). A loss of ~30–50% of dopaminergic neurons and a reduction of 50–60% in striatal dopamine has been estimated to occur by the time the motor deficits are detected and the disease is diagnosed (Freed et al., 2011; Padmanabhan and Burke, 2018). Moreover, axon degeneration on its own, years before the death of neuron bodies, has been interpreted as an early sign of PD (Kordower et al., 2013).

Current treatments focus on managing symptoms with drug therapy or deep brain stimulation (DBS). On the first front, the gold standard treatment has been administration of L-3,4-dihydroxyphenylalanine (L-DOPA), the immediate precursor to dopamine, which has a short half-life as well as variable absorption and capacity for transport across the blood-brain barrier (Poewe et al., 2017). Possibly because of this discontinuous drug delivery, patients on long-term L-DOPA therapy often develop secondary complications including motor fluctuations and dyskinesias (Poewe et al., 2017). Alternatively, dopamine agonists (*i.e.*, pramipexole, ropinirole, apomorphine, rotigotine) mimic the normal action of dopamine on receptors and tend to have a longer half-life and less pulsatile effects than L-DOPA, but with less potency and greater propensity for troublesome side effects (Poewe et al., 2017). On the other hand, DBS involves electrodes implanted in the subthalamic nucleus or globus pallidus internus, which can improve motor scores and reduce dependence on dopamine replacement therapies (Poewe et al.,

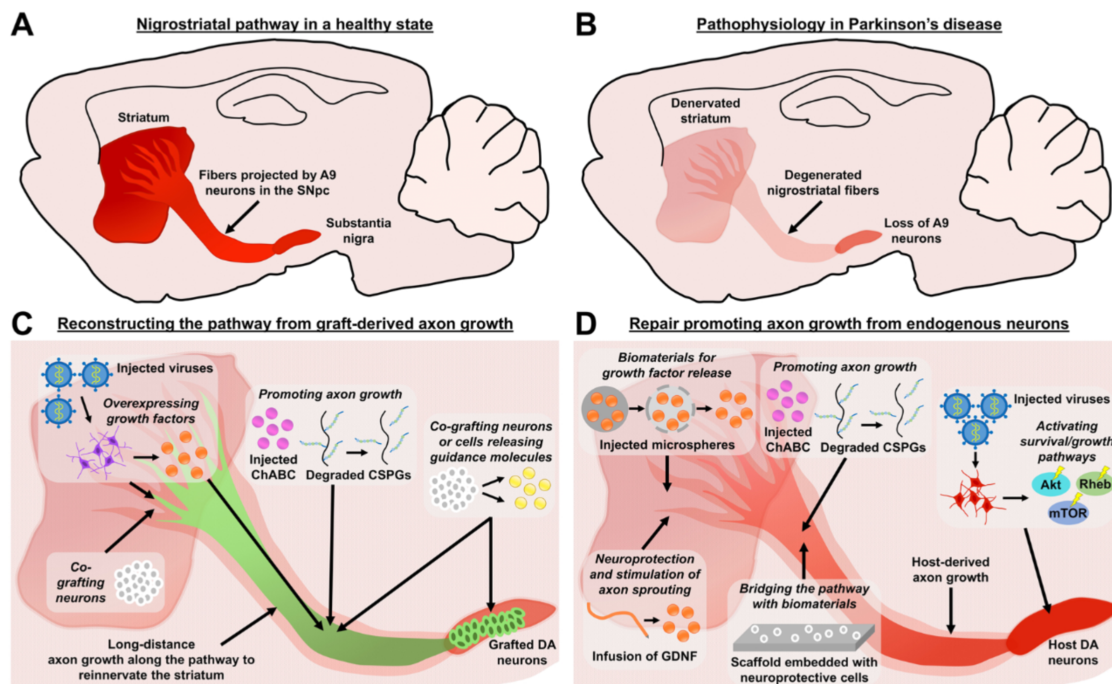


Fig. 1. The nigrostriatal pathway as a treatment target for Parkinson's disease. (A, B) The concept figures show the healthy and diseased appearance of the nigrostriatal pathway, respectively, in a rat brain section. In the healthy state, axonal fibers projected by A9 dopaminergic neurons in the substantia nigra innervate the striatum to provide the dopamine needed for proper regulation of the motor circuit. These axonal fibers spanning the nigra and striatum are known as the nigrostriatal pathway. In the parkinsonian state there is a significant loss of A9 neurons and degeneration of nigrostriatal fibers, leading to denervation of the striatum and depletion of its dopamine input. **(C)** One approach for the reconstruction of the nigrostriatal pathway involves promoting *in vivo*, long-distance growth from grafted DA neurons to reinnervate the striatum. Studies centered on this approach have included: implanting neurons in the substantia nigra alone (Adler et al., 2019; Cardoso et al., 2018; Gaillard et al., 2009; Thompson et al., 2009; Wictorin et al., 1992) or in combination with grafts along the pathway or in the striatum (Baker et al., 2000; Mendez et al., 2005; Mukhida et al., 2001; Sladek et al., 2008); simultaneous grafting of cells releasing guidance molecules (Díaz-Martínez et al., 2013; Weng et al., 2017; Zhou et al., 1996); injecting chondroitinase ABC (ChABC) to degrade chondroitin sulfate proteoglycans (CSPGs) that impede axon growth along the pathway (Kauhausen et al., 2015); overexpression of growth factors via viral delivery (Ghosh et al., 2019; Kauhausen et al., 2013; Redmond et al., 2009; Thompson et al., 2009). **(D)** Pathway reconstruction has also been attempted by promoting neuroprotection, axonal sprouting, and/or growth from host DA neurons in the SNpc through: delivery of growth factors in the striatum using biomaterials (Herrán et al., 2013; Jollivet et al., 2004; Ucar et al., 2021); continuous or intermittent infusion of GDNF through catheters in the striatum (Kells et al., 2010; Love et al., 2005; Whone et al., 2019); injection of ChABC to create a permissive environment for growth along the pathway (Moon et al., 2001, 2002); implantation of biomaterial bridges between the nigra and striatum (Gómez-Pinedo et al., 2019); and viral delivery for the expression of constitutively active versions of key proteins in neuroprotective signaling (Kim et al., 2012, 2011; Padmanabhan and Burke, 2018; Ries et al., 2006).

2017). Regardless, electrical stimulation is not highly specific, the mechanisms of DBS are unknown, and electrode effectiveness can be affected by foreign body responses (Adil et al., 2017; Poewe et al., 2017). Surgical risks of bleeding and infection and neuropsychiatric symptoms related to stimulation can also affect patients (Adil et al., 2017; Poewe et al., 2017). Importantly, both of these treatment strategies fail to intervene in the death of dopaminergic neurons and the loss of dopaminergic striatal innervation at the core of PD pathology and symptomology.

To remedy the deficiencies of conventional treatments, cell-based therapies have centered on implanting cells directly in the striatum to restore dopaminergic innervation to MSNs. The first attempts to do this in humans occurred around the 1980s and involved neurons sourced from the ventral midbrain of human embryos and grafted into the dorsal striatum (caudate/putamen) (Barker et al., 2015; Freed et al., 2011). These types of implants improved motor scores, increased striatal uptake of fluorodopa, and reduced L-DOPA dosage (Freed et al., 2011, 1992; Spencer et al., 1992; Widner et al., 1992). There have even been reports of maintained benefits for 10 years, total withdrawal of L-DOPA for 5 years, and graft survival for 24 years (Li et al., 2016). Other studies have shown no clinical benefit from these transplants (Freed et al., 2001; Olanow et al., 2003). These procedures have not been fully translated due to inconsistent protocols, study designs and results, the emergence of side effects, the low availability of fetal tissue, and ethical concerns with the use of human embryos (Abbott, 2014). An unforeseen effect of cell transplantation in the striatum has been runaway dyskinesia, possibly due to a lack of the regulatory influences present on dopaminergic neurons in the SNpc, their natural environment (Hagell et al., 2002). Tissue availability is particularly limiting, as optimal results were observed with cells pooled from at least 3–4 human embryos, given that ~95% of cells die after transplant (Abbott, 2014; Freed et al., 2011).

Pursuing a more feasible cell source, recent studies have sought to employ pluripotent stem cells such as human embryonic stem cells (ESCs), parthenogenetic embryonic stem cells (pESCs), and induced pluripotent stem cells (iPSCs). These cells can be obtained from pre-implantation or peri-implantation human embryos, parthenogenetically-activated unfertilized oocytes, and somatic cells from patients themselves, respectively, and may be differentiated indefinitely into dopaminergic precursors and neurons to provide an ample supply (Cyranoski, 2018; Freed et al., 2011; Turovets et al., 2011). These stem cell-derived neurons have been grafted in mice, rats and primates, and led to growth into the striatum, motor restoration, and evidence of dopamine metabolism with functional imaging (Grealish et al., 2014; Kikuchi et al., 2017; Kriks et al., 2011; Wang et al., 2018). Clinical trials with human pluripotent stem cells are ongoing and include the following: (1) an Australia-based open-label phase I trial (NCT02452723) that started in 2016 and was designed to test the implantation of human pESC-derived neural stem cells in the striatum and the SN (Garitaonandia et al., 2018, 2016); (2) an open-label phase I/II trial (NCT03119636) starting in 2017 in China, which is testing the safety and efficacy of a combination of L-DOPA and striatal grafting of human ESC-derived neural precursors (Wang et al., 2018); (3) a trial started in 2018 in Japan, which is evaluating the safety and efficacy of striatal implants of human allogeneic iPSC-derived dopaminergic progenitors (Doi et al., 2020). Other clinical studies are in the planning phase: (1) autologous iPSC-derived neurons currently in investigational new drug (IND)-enabling studies by Summit for Stem Cell/Aspen Neuroscience in the United States (Loring, 2018); (2) human ESC-derived dopaminergic neurons in an open-label phase I trial (NCT04802733) recently approved to start in the United States to test safety, tolerability, and preliminary efficacy, supported by Memorial Sloan Kettering Cancer Center, BlueRock Therapeutics, and NYSTEM (Kim et al., 2021; Piao et al., 2021); (3) human ESC-derived dopaminergic neurons as part of the European STEM-PD program that aims to start clinical trials in the near future (Barker et al., 2017; Jang et al., 2020; Kirkeby et al., 2017). Moreover, a 2020 report about an Individual Patient Expanded Access-IND study, in which neurons derived from autologous iPSCs were implanted in the putamen of a single patient, described graft survival and stabilization or improvement of symptoms after 18–24 months (Schweitzer et al., 2020).

Despite the accelerating pace of stem cell-based clinical trials, cell therapies have been limited by low survival rates of <1-5% of implanted cells (Adil et al., 2017), which may be due to stresses during injection, host immune responses, and a lack of protection during and after implant. Biomaterials and tissue engineering could be useful to address these issues, but these tools have not been widely applied for the treatment of PD. Crucially, the striatal implants commonly investigated to treat PD do not replace the dopaminergic neurons in the SNpc or the axonal fibers of the nigrostriatal pathway. Despite the positive therapeutic effects seen with striatal implants, addressing these unmet needs with a pathway reconstruction framework could be clinically relevant. Restoring the entire nigrostriatal circuitry and the ensuing natural mechanisms underlying the regulation of striatal dopamine may result in better and more sustainable outcomes and lessen the probability of side effects relative to traditional cell-based strategies.

In this manuscript, we review the state-of-the-art of nigrostriatal pathway reconstruction and present auspicious tissue engineering-based platforms. We first survey the literature in order to present the benefits of fiber repair, studies attempting long-distance axon growth from the SNpc to reinnervate the striatum, and remaining challenges particularly for reconstruction at the human scale. Subsequently, we shift to the potential of tissue engineering in this endeavor by giving an overview of the advantages and history of our tissue-engineered nigrostriatal pathway (TE-NSP), a construct of hydrogel-encased dopaminergic axons that is fabricated *in vitro* to recapitulate structural and functional aspects of the nigrostriatal pathway. The goal of these constructs is to be implanted fully grown to replace the neurons and fibers lost in PD according to the natural neuroanatomy without relying completely on long-distance growth *in vivo*. We have created TE-NSPs with rat embryonic and human stem cell-derived dopaminergic neurons, and we are currently characterizing their therapeutic effect in a rat model of PD. In this manuscript we highlight steps we have taken recently to optimize TE-NSPs for clinical settings. Specifically, we present original research data on TE-NSPs built with human iPSC-derived dopaminergic neurons and a hyaluronic acid hydrogel based on human-scale design criteria for cell numbers and axon lengths. The manuscript concludes with an assessment of future steps and outstanding challenges for the translation of TE-NSPs into clinical-grade products. We believe that reconstructing nigrostriatal fibers using tissue engineering, as done with TE-NSPs, will be at the forefront of PD treatments, as this method may repair the underlying pathology of PD with a degree of anatomical and circuit-level fidelity not found in other approaches.

2. Pathway reconstruction as a strategy for fiber repair in Parkinson's disease

2.1. Biological inspiration and benefits of pathway reconstruction

Nigrostriatal pathway reconstruction is an approach for PD treatment whereby endogenous or implanted dopaminergic neurons in the SNpc are directed to grow axonal fibers through the native pathway to reinnervate their striatal targets. In contrast, conventional cell transplants rely on an unnatural shortcut by implanting cells in the striatum to innervate and release dopamine directly where the target MSNs reside. While this method has been widely studied in animal models and humans and has shown promising results, the main distinction of pathway reconstruction is that it is anatomically- and biologically-inspired and seeks to restore both the neurons and axons that comprise the circuitry lost in PD. Preserving all the components of the pathway likely imparts advantages with biochemical, signaling, functional, and motor-control implications. For example, the location of individual dopaminergic neurons in the SNpc is tied to the regions of

innervation and arborization patterns of their axons within the striatum, which is related to the role of these fibers in regulating the activity of MSNs (Harris et al., 2020; Prensa and Parent, 2001). Grafting cells in the substantia nigra (SN) is essential for having a microenvironment best suited for their integration and with the trophic support needed for long-term health and function (Gaillard et al., 2009).

Host-mediated regulation of dopaminergic neurons is also a key characteristic emerging from the structure of the pathway. The literature on both animal and human studies suggests that dopaminergic neurons in the SNpc are themselves innervated by: (1) GABAergic fibers from the ipsilateral neostriatum, globus pallidus, and substantia nigra pars reticulata (SNpr) that comprise the majority of inputs; (2) glutamatergic fibers from the ipsilateral subthalamic nucleus and pedunculopontine nucleus; and (3) cholinergic fibers from the pedunculopontine nucleus (Harris et al., 2020; Lee and Tepper, 2009). These inputs regulate the firing rate of neurons in the SNpc, modulating it from a regular, pacemaker-like pattern into random or burst-like firing (Lee and Tepper, 2009). Furthermore, these GABAergic and glutamatergic fibers regulate the somatodendritic release of dopamine, a mechanism needed for autoinhibition and subsequent modulation of firing patterns and dopamine release in the striatum, as suggested by several mechanistic studies with animal brains (Rice and Patel, 2015). The importance of anatomy is also observed with projections from the tail of the nearby ventral tegmental area to the SNpc, which have been implicated in the inhibition of its neurons and with motor deficits in animal models (Bourdy et al., 2014). Choosing the nigra as the implant location may ensure that normal SNpc inputs reinnervate the replacement tissue and that grafted cells are functionally integrated with the motor circuit and thus release dopamine based on host feedback. In fact, having this properly regulated dopamine may prevent the emergence of dyskinesia due to excess dopamine (Sladek et al., 2008), and the anatomical location of the implant influences the origins of host monosynaptic inputs to grafted cells (Adler et al., 2019).

The location of SNpc neurons is likewise relevant for the regulation of areas other than the striatum like the SNpr, which is found adjacent to the SNpc and is a key component of the motor deficits of PD. While there is still active research seeking to correlate activity changes in the basal ganglia with disease progression, a recent study in mice showed that severe pathological activity in the SNpr, developing when dopamine levels drop below 25–35%, serves as a strong predictor of motor symptoms (Willard et al., 2019). Animal studies have indicated that SNpr signaling is regulated in part by somatodendritic dopamine release from neurons in the SNpc (Faynveitz et al., 2019; Rice and Patel, 2015; Zhou et al., 2009). Dendrites from the SNpc are physically close to GABAergic neurons in the SNpr, which express dopamine receptors, and that dopamine may bind to augment their activity (Mallet et al., 2019; Zhou et al., 2009). In cases of dopamine depletion, receptor blockage, or nigral lesions in animal models, GABAergic neurons exhibit reduced firing rates and increased firing irregularity or bursting, whereas increasing dopamine levels or providing agonists depolarizes them (Cáceres-Chávez et al., 2018; Faynveitz et al., 2019; Lobb and Jaeger, 2015; Seeger-Armbruster and von Ameln-Mayerhofer, 2013; Zhou et al., 2009). The dopamine released normally can also act on receptors on axons from the striatum that terminate in the SNpr to inhibit its activity (Rice and Patel, 2015). This research shows the interconnectedness of the components of the motor circuit and the importance of regulation provided by neurons in the SNpc on adjacent regions and by distant neuronal populations on the SNpc. It follows that by not replacing dopaminergic neurons and their innervation in their correct locations, the proper connectivity of the motor circuit would not be restored and dysregulated signaling would likely not be resolved. The anatomical and physiological conclusions of this literature mainly come from animal studies, and thus further studies are needed to confirm if these have an equivalent in the human brain.

2.2. Overview of studies on nigral cell replacement and fiber reconstruction

In the following sections we highlight studies investigating the possibility of encouraging host or grafted neurons in the SNpc to exhibit long-distance axon growth along the nigrostriatal pathway to repair it and to reinnervate the striatum. These studies show how using certain cell types, improving local microenvironments, and providing molecule- or cell-based guidance could be leveraged to reconstruct the pathway *in vivo* from either endogenous or implanted cells (summarized in Fig. 1C, D).

2.2.1. Intranigral grafts alone or combined with other methods

The restoration of the nigrostriatal pathway has been approached in the past mainly by delivering cells into the SNpc and relying on *in situ* axon growth from these grafts. These strategies have ranged from implanting cells in the nigra on their own or combining this with guidance methods to enhance growth and achieve terminal innervation. In cases where the required growth distances were achieved only with cells, it has been hypothesized that this growth was enabled by locally presented guidance cues and pathfinding provided by remaining dopaminergic axons or projections from other areas along the pathway (Thompson et al., 2009). Embryonic mouse neurons implanted in the SNpc of adult mice innervated the striatum, expressed dopaminergic markers, increased striatal dopamine, and significantly improved motor function (Gaillard et al., 2009; Thompson et al., 2009). This was part of growing literature suggesting that the capacity for long-distance axon growth in the adult brain could be seen when grafting immature neuroblasts or young post-mitotic neurons (Thompson et al., 2009). These developments were also possible due to the use of better transplantation techniques, lesion models, and imaging methods (Thompson et al., 2009). Still, innervation was not restricted to the dorsal striatum, the target region for PD, and this "promiscuous" growth may not be desirable. Likewise, long-distance growth has also been seen with human neurons. Cells from human embryos implanted in the rostral midbrain of immunosuppressed rats were able to project axons to the striatum (5−6 mm) and the frontal cortex (10 mm) following the trajectories of the nigrostriatal and mesolimbic pathways and similar innervation patterns (Wictorin et al., 1992). Nevertheless, this study employed a cell source that is not clinically translatable and did not investigate graft-host connectivity or potential motor function benefits. Stem cell-derived neurons represent an ideal source; thus, human ESC-derived ventral midbrain neurons have been used for grafting in the nigra of rats for 6 months and they produced axons along the nigrostriatal and mesolimbic pathways that grew distances totaling over 10 mm (Adler et al., 2019; Cardoso et al., 2018). These axons innervated the dorsolateral striatum, the key target for A9 midbrain dopaminergic neurons, as well as A10 targets such as the nucleus accumbens and prefrontal cortex. The innervation of targets of both A9 and A10 neurons signals a future avenue for optimization, as maximizing the ratio of A9 to A10 neurons in a graft and reinnervation to the dorsolateral striatum is considered to be paramount for motor recovery in PD. Reductions in amphetamine-induced rotations matched the timing and degree of striatal reinnervation (Cardoso et al., 2018). Monosynaptic retrograde rabies tracing and phenotypic analysis also showed host inputs to the graft from the sensorimotor cortex, striatum, and globus pallidus externus. This suggested that cells grafted in the nigra can receive inputs from fibers normally integrated with host neurons in the SN. While these studies showed the remarkable capacity of stem cell-derived neurons to grow long distances *in vivo*, extensive reinnervation of the dorsolateral striatum occurred after 6 months and it is unknown whether the distances needed in the human brain could be achieved. Furthermore, none of these studies characterized the extent of cell loss after implantation, which is a key limitation of current transplant strategies.

In the category of guiding growth from grafts, some have used glial-derived neurotrophic factor (GDNF) overexpression in the striatum to

promote reinnervation given its well-documented role as a chemoattractant for dopaminergic neurons. Nevertheless, unrestricted growth or unnatural innervation densities were observed in the mouse striatum, and retrograde labeling did not offer compelling evidence of striatal connectivity with A9 dopaminergic neurons (Kauhausen et al., 2013; Thompson et al., 2009). In a recent study, lentivirus was injected along the region between the nigra and the striatum in rat brains to overexpress GDNF with either GDNF family receptor α1 (GFRα1) or netrin1 in order to create a guidance pathway for axon growth (Ghosh et al., 2019). Implants of embryonic ventral midbrain neurons exhibited tyrosine hydroxylase (TH+) axon growth towards the striatum following a trajectory adjacent to the guidance pathway, while no growth was seen with lentivirus-green fluorescent protein (GFP) controls. After injecting the retrograde tracer hydroxystilbamidine (FluoroGold) in the striatum, it was confirmed that graft-derived axons did connect with the striatum and that most were of the A9 phenotype. However, only a minority of graft-derived axons reinnervated the striatum, and this growth and motor function were improved only by also overexpressing brain-derived neurotrophic factor (BDNF) in the striatum. Another approach consisted on implanting embryonic mouse dopaminergic neurons in the nigra of parkinsonian mice and injecting chondroitinase ABC (ChABC) into the medial forebrain bundle (MFB) in order to degrade chondroitin sulfate proteoglycans (CSPGs) that inhibit axon growth (Kauhausen et al., 2015). Making the environment less hostile worked, as the number of fibers growing and innervating the striatum was higher compared to no CSPG degradation. Nevertheless, this study did not investigate the effects on dopamine levels or motor function, and there were concerns with ChABC diffusion and a lack of targeted axon growth. In the case of non-human primates, embryonic neurons grafted near the SNpc grew into the striatum that had been injected with adeno-associated virus to overexpress GDNF, but only 1–2% of grafted neurons were labeled with FluoroGold injected in the striatum, and there was no mention of motor recovery (Redmond et al., 2009).

2.2.2. Simultaneous grafts

Another approach for fiber repair has been performing simultaneous cellular implants in the nigral and striatal region. Previous studies with 6-hydroxydopamine (6−OHDA) models showed that simultaneous grafts provide better recovery of rotational behavior and complex sensorimotor deficits when compared to striatal implants alone (Baker et al., 2000; Mukhida et al., 2001). Nevertheless, these studies did not present evidence of long-distance axon growth along the nigrostriatal pathway, rather it focused on reinnervation in the immediate vicinity of the grafts. In the case of humans, a patient was implanted with cell suspensions of fetal ventral midbrain tissue in both the SN and the caudate/putamen (Mendez et al., 2005). This patient improved her motor symptoms and quality of life scores, reduced her L-DOPA dosage, and exhibited increased L-6-[^{18}F] fluoro-3,4-dihydroxyphenylalanine (^{18}F-DOPA) signal and almost total reinnervation in the putamen. However, this study did not draw conclusions about the superiority of co-implants versus intrastriatal-only implants, found less cell survival and expression of A9 markers in the intranigral grafts, and did not look into axon growth from cells implanted in the nigra. In a strategy explicitly trying to achieve long-distance growth, lesioned non-human primates received implants of embryonic nigral tissue adjacent to the nigra and implants of embryonic striatal tissue 2.5, 5.0, or 7.5 mm rostral from the nigral graft in the trajectory towards the striatum to serve as guides for growth (Sladek et al., 2008). While directed outgrowth from the nigral to the striatal grafts could be achieved, there was less directionality and fiber density at the greatest intra-graft distance and long-distance restoration of the nigrostriatal pathway was not observed. Another study also concluded that co-grafts of striatal neurons did not seem to promote outgrowth to the host striatum, rather its effect seemed limited to attracting neurites to their own location (Redmond et al., 2009).

In other cases, simultaneous implants have included cell types different from dopaminergic or striatal neurons. Intranigral grafts have been combined with injections of cells expressing semaphorin 3 (Sema3), a chemotropic agent that promotes axon growth *in vitro* and that is present along the path to the striatum (Díaz-Martínez et al., 2013). Mouse ESC-derived dopaminergic neurons were injected in the SN, and HEK293 cells transfected to express Sema3A or Sema3C were grafted at six locations along the nigrostriatal pathway of rats lesioned with 6−OHDA. These implants resulted in motor recovery similar to intrastriatal-only grafts, evoked dopamine release in the striatum, and TH+ outgrowth from the graft along the pathway and into the striatum as confirmed with histology and retrograde tracing (Díaz-Martínez et al., 2013). Another study centered on a combination of grafting embryonic ventral midbrain neurons and olfactory ensheathing cells (OECs) in rats with the injection of kainic acid, an excitatory amino acid previously used as a guide and bridge for pathway regeneration (Weng et al., 2017; Zhou et al., 1996). For their part, OECs have been suggested to guide axon growth and release neurotrophic factors. There was greater TH+ staining along the nigrostriatal pathway and in the striatum and improvement of rotational behavior with two grafts when compared to neuron implants alone, although the use of kainic acid is controversial since high doses can have neurotoxic effects (Weng et al., 2017).

2.2.3. Genetic-only approaches for endogenous repair

Gene therapy has been employed to drive axon regeneration from cells remaining in the pathway, while disregarding implantation of exogenous neurons. In one case, a constitutively-active form of the Akt kinase was delivered virally into the SN of mice (Padmanabhan and Burke, 2018; Ries et al., 2006). This myristoylated-Akt would enable consistent activation of mTOR signaling, and this pathway has been related to axon growth and the regulation of neurotrophic factors in development (Padmanabhan and Burke, 2018). The same signaling pathway was targeted in another study utilizing a constitutively-active form of Rheb, the GTP-binding protein upstream of mTOR complex 1 (Kim et al., 2012). When the Akt or Rheb intervention occurred after exposure to the 6−OHDA neurotoxin, striatal TH+ staining and axon presence in the MFB were significantly greater compared to controls, and there were significant correlations between striatal TH+ density and behavioral improvement (Kim et al., 2012; Ries et al., 2006). Axon counts in the MFB reached 70–90% in the injured side relative to the contralateral side only with the mutant Akt or Rheb (Kim et al., 2011). Nonetheless, striatal innervation levels were only at ~50% after 4 months, assessment of striatal connectivity with the SNpc was inconclusive, and there was aberrant growth in the striatum and other areas (Kim et al., 2011). An additional problem would be that Akt and Rheb are oncogenes, raising concerns for increased cancer risks as a result of having constitutively active versions of these proteins in the long-term.

2.2.4. Biomaterial- and molecule-based approaches for endogenous repair

Endogenous axon growth has also been explored through the delivery of molecules and non-dopaminergic cells with and without biomaterials. In one study, rats with axotomized nigrostriatal fibers exhibited axon regeneration through the injury and along the pathway and into the striatum only when ChABC was injected to degrade CSPGs impeding growth in the axotomy sites (Moon et al., 2001, 2002). However, this axotomy model bears little resemblance to the pathology of PD. In the case of biomaterials, polycaprolactone scaffolds embedded with OECs have been implanted to connect the SNpc and the striatum, showing axon growth within the scaffold but without striatal reinnervation (Gómez-Pinedo et al., 2019). Various biomaterials have been investigated to deliver GDNF in a targeted manner as a way to reconstruct the nigrostriatal pathway and provide protection to remaining neurons. Poly(lactic-co-glycolic acid) (PLGA) microspheres loaded with GDNF were implanted in the striatum in rats, and increased TH+ fiber sprouting in the striatum and motor improvements were observed when compared to blank microspheres, but the source of the sprouting was unclear (Jollivet et al., 2004). GDNF and vascular endothelial growth

factor (VEGF)-loaded PLGA microspheres implanted in the striatum of lesioned rats led to greater improvements of rotational behavior and neuron/fiber density in the SN relative to empty microspheres (Herrán et al., 2013). Regardless, this study did not look specifically into or show evidence for pathway regeneration and striatal reinnervation originating from the nigra. In a recent study, organotypic cultures were made with membranes having embedded slices taken from the ventral midbrain and the striatum of postnatal mice (Ucar et al., 2021). Collagen hydrogels, polyallylamine, dextran sulfate and tannic acid microspheres, poly(ethylene glycol) diacrylate- and 3-sulfopropyl acrylate-based cryogelated hydrogels, and membranes fabricated by microcontact printing were used to deliver GDNF and study TH+ fiber growth from the midbrain slice towards the striatal slice. Using this system, the study showed more targeted and enhanced TH+ fiber density with GDNF-loaded collagen hydrogels relative to GDNF in the media and empty hydrogels, respectively. Enhanced growth between the slices was also seen with membranes microcontact-printed with GDNF, while GDNF-loaded microspheres contained within a hydrogel did not improve growth over empty hydrogels. Moreover, the study focused on quantifying fiber density in the border between the slices, rather than looking at growth within the striatal sections, and these biomaterials have yet to be assessed *in vivo*.

GDNF has also been infused into the striatum with catheters in both animal studies and clinical trials with the intention of promoting neuroprotection of and/or axonal sprouting from remaining neurons and axon fibers. For example, a report for a single patient indicated that continuous infusion of GDNF in the putamen for 43 months was associated with improvements in motor scores and quality of life, increased ^{18}F-DOPA uptake, and greater TH+ staining (Love et al., 2005). It was unclear if this staining represented axonal sprouting in the striatum and/or upregulation of TH in the remaining fibers. If it was axonal sprouting, it was impossible to know if this growth came from surviving neurons in the SN extending long-distance fibers to the striatum. Moreover, such benefits have not been replicated in randomized, placebo-controlled trials, probably as a result of inefficient and heterogeneous GDNF delivery. Other studies employed the convection-enhanced delivery (CED) technique to ensure a homogeneous presence of GDNF throughout the entire putamen. CED-based infusion of a viral vector encoding for GDNF has been applied in non-human primates with a partial and complete lesion on the left and right hemispheres, respectively (Kells et al., 2010). These animals showed significantly greater improvements of motor deficits, dopaminergic terminal activity with positron emission tomography (PET), and TH+ presence in the putamen and the SN when compared to controls. Nevertheless, there was no increase in the number of TH+ neurons in the SN, and the increased TH+ staining could have been caused by both TH upregulation and sprouting of fibers already present in the putamen. The correlation between GDNF presence and TH+ expression was also weak on the completely lesioned side, suggesting that the intervention was not well suited for restoration of lost neurons and axons. For human patients, a recent randomized, double-blind, placebo-controlled trial used a port in the skull connected to four catheters that reached into the putamen for intermittent CED-based infusions of GDNF every 4 weeks for 40 weeks (Whone et al., 2019). In keeping with past trials, this study failed to show greater improvements in motor function metrics relative to placebo. More work is needed to determine the target disease state for this treatment and to further optimize the delivery method, dosage, and dosing schedule.

2.2.5. Dopaminergic neurogenesis

Some researchers have explored whether dopaminergic neurogenesis occurs in the adult SN and if this could be exploited to restore cells lost in PD (Hermann and Storch, 2008; van den Berge et al., 2013). There is some consensus on the presence of precursor cells in the nigra, so there have been studies attempting to drive them towards a dopaminergic phenotype by the administration of dopamine receptor agonists, various growth factors (*e.g.*, BDNF, transforming growth factor α (TGFα)), and peptide hormones (*e.g.*, exendin-4) (van den Berge et al., 2013). Still, while there have been reports of increased proliferation in the SN as a result, there has not been strong evidence suggesting these new cells differentiate into mature dopaminergic neurons (van den Berge et al., 2013). Thus, this is currently not a feasible avenue to restore dopaminergic neurons through endogenous means nor to promote long-distance axonal growth.

2.3. Challenges for achieving human-scale pathway reconstruction

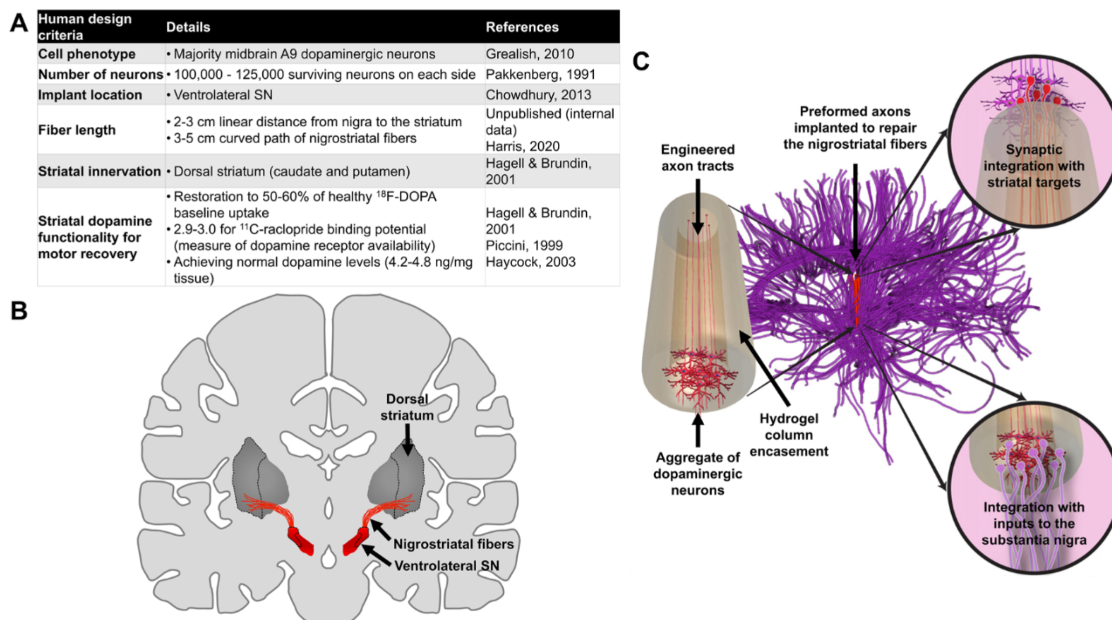
2.3.1. Limitations of existing studies and approaches

Despite the literature surveyed in the previous section, pathway reconstruction for PD has not received the same level of research and attention as the more traditional intrastriatal grafts, perhaps in part due to the pervasiveness of several limitations. Overall, the literature shows that attempting pathway reconstruction is best in combination with neuron implantation in the SN and not by seeking to promote growth from endogenous cells. All of these implants have consisted of cell suspensions or pieces of tissue, without providing the encapsulation or protection with biomaterials needed to improve survival after implantation. Many of the studies surveyed here did not assess motor recovery and/or restoration of dopamine metabolism, which are paramount outcome measures for PD treatment. A vast majority of studies have not specifically examined the presence of dendritic or axonal connectivity between neurons implanted in the nigra and host neurons/fibers from other regions of the motor circuit, which is one of the main benefits of the pathway repair approach. Relying on *in vivo* axon growth can lead to uncontrolled and non-specific growth and/or insufficient striatal innervation and connectivity. It would be relevant to study the temporal progression of *in vivo* axon growth along the nigrostriatal pathway (acute *vs.* chronic) and their branching patterns and innervation targets. Recapitulating native patterns of innervation, particularly the highly dense arborization of nigrostriatal fibers, is important given its relationship with precise temporal and spatial control of dopamine input (Harris et al., 2020). Apart from these missed opportunities in terms of limited study designs and assessment tools, there could also be issues with the translation of some of these methods to humans. Specifically, many of the best growth results were obtained with injections or viral delivery of growth factors and other molecules and with the simultaneous implantation of other cell types, which may complicate clinical studies in terms of safety and off-target effects. Notably, there has not been sufficient evidence to affirm that pathway reconstruction methods attempted to-date outperform traditional intrastriatal grafts in functional recovery. This may be related to the less dense dopaminergic axon growth obtained with implants in the nigra (Adler et al., 2019; Gómez-Pinedo et al., 2019; Winkler et al., 2000). Implants are typically done in the striatum precisely to ensure enough localized dopaminergic innervation in this region and the ensuing therapeutic effect (Adler et al., 2019). Moreover, while intranigral cell implants have grown axons through the pathway trajectory and reached the striatum in rodents and primates, which required distances of at least 5–7.5 mm, it is unclear whether this long-distance axon growth could be obtained to span the greater distance of the nigrostriatal pathway in humans. In fact, axon growth and regeneration after injury in the brain rarely happens in part because of lack of guidance, long distances, and the presence of an inhibitory environment (Harris et al., 2020; Struzyna et al., 2015a). The human brain presents other challenges that have yet to be addressed and are discussed below.

2.3.2. Design criteria for human-scale pathway reconstruction

Any pathway reconstruction method for clinical application should be based on design criteria inspired by the anatomy of the human nigrostriatal pathway and the pathophysiology of PD. The principal design criteria should be: (1) cell phenotype; (2) number of implanted cells; (3) implant location; (4) length and trajectory of reconstructed

nigrostriatal fibers; (5) striatal innervation patterns and locations; and (6) dopamine release capability (Fig. 2A). In the first case, the majority of cells in the graft should be A9 midbrain dopaminergic neurons. These neurons extend axons mainly to the dorsolateral striatum and are selectively lost in PD, while the A10 neurons of the ventral tegmental area project to the ventral striatum and limbic regions and are not as heavily impacted (Grealish et al., 2010; Kauhausen et al., 2013; Kriks et al., 2011; Kuan et al., 2007). The significance of this phenotype has been reflected in studies that demonstrated that A9 dopaminergic neurons intrinsically recognize their targets in the striatum (Adler et al., 2019; Gaillard et al., 2009) and that they are essential for motor function recovery (Grealish et al., 2010). To determine the ideal cell number for implantation we have to look at human neuroanatomy and PD pathology. Each side of the adult human SN has around 250,000 TH+ neurons, and estimates of neuron loss at the onset of symptoms range around 50% (Pakkenberg et al., 1991). Based on these numbers, each side of the human SN requires survival of at least around 125,000 TH+ neurons. Histology of intrastriatal grafts in humans has suggested that significant therapeutic benefits are achieved with having 100,000 surviving TH+ neurons in each side (Hagell and Brundin, 2001). Still, the effective number of cells at implantation may be higher as a safety consideration if more cells are needed to achieve the ideal number of surviving TH+ neurons. The number of implanted cells would vary according to the approach, depending on the ratio of TH+ neurons to other cell types in the graft source, and accounting for the percentage of cells lost during culture and after implantation. A greater number of cells may also be required to achieve sufficient long-distance axon growth and arborization of dopaminergic fibers within the striatum, given that the studies confirming the 100,000 number in humans were performed with striatal grafts and not with implants in the nigra. This safety factor should also be tested to ensure that graft-induced dyskinesia is prevented. These neurons should be precisely placed in the ventrolateral SNpc, the region identified to project fibers to the dorsal striatum using magnetic resonance imaging (MRI) and diffusion tensor imaging (DTI) of patients (Chowdhury et al., 2013; Menke et al., 2010; Zhang et al., 2017) and the region most affected by PD pathology (Dickson et al., 2009). These imaging modalities have been used to identify the trajectory of nigrostriatal fibers in the human brain (Fig. 2B), which emerge from the SN from its dorsal side and follow a path through the medial subthalamic nucleus, then above and lateral to it to reach the putamen (Zhang et al., 2015). Unpublished data from our group suggest a direct distance of 2−3 cm between the nigra and the striatum in humans, but a noteworthy challenge would be recapitulating the curved, tortuous trajectory of nigrostriatal fibers which are expectedly even longer. While from an anatomical perspective it would be ideal to reproduce this geometry, we anticipate that similar results may be obtained just by rebuilding linearly from the nigra to the striatum. It is crucial that these regenerated or repaired fibers reach the putamen in the striatum with sufficient reinnervation volume and density and functionally integrate by forming dopaminergic synapses with MSNs. Using high-performance liquid chromatography of autopsied healthy brains, it has been estimated that the levels of dopamine in the caudate and putamen change with age from around 7.2–8.6 ng/mg wet weight at 30 years to 4.2–4.8 ng/mg wet weight at 69 years (Haycock et al., 2003; Kish et al., 2001). For clinical effect, a pathway reconstruction strategy should increase ^{18}F-DOPA uptake in the putamen to an amount corresponding to at least 50–60% of the healthy baseline value (Hagell and Brundin, 2001). Values of ^{11}C-raclopride binding potential, a measure of the availability of dopamine D2 receptor binding sites and how much is being occupied by released dopamine, should be in the 2.9–3.0 range for full motor recovery (Piccini et al., 1999). While these challenges are substantial, the potential of proper pathway reconstruction in treating PD is immense. One approach to achieve this is the use of tissue engineering to create implantable micro-tissue that addresses the limitations of previous methods and satisfies the design criteria required for pathway reconstruction in the human brain.



Human design criteria	Details	References
Cell phenotype	• Majority midbrain A9 dopaminergic neurons	Grealish, 2010
Number of neurons	• 100,000 - 125,000 surviving neurons on each side	Pakkenberg, 1991
Implant location	• Ventrolateral SN	Chowdhury, 2013
Fiber length	• 2-3 cm linear distance from nigra to the striatum • 3-5 cm curved path of nigrostriatal fibers	Unpublished (internal data) Harris, 2020
Striatal innervation	• Dorsal striatum (caudate and putamen)	Hagell & Brundin, 2001
Striatal dopamine functionality for motor recovery	• Restoration to 50-60% of healthy ^{18}F-DOPA baseline uptake • 2.9-3.0 for ^{11}C-raclopride binding potential (measure of dopamine receptor availability) • Achieving normal dopamine levels (4.2-4.8 ng/mg tissue)	Hagell & Brundin, 2001 Piccini, 1999 Haycock, 2003

Fig. 2. Reconstruction of nigrostriatal fibers in the human brain. (A) Summary of design criteria for strategies seeking to repair the axonal fibers of the nigrostriatal pathway in the human brain. **(B)** Concept figure inspired by MRI and DTI studies presenting the structure of human nigrostriatal fibers and the location of areas in the substantia nigra and striatum that are relevant for the motor symptoms of PD. This image shows the regions where neurons and the axonal cytoarchitecture need to be restored to address some of the causes of PD. **(C)** Tractography recreation of axonal pathways (purple) in the human brain, showing a tissue-engineered nigrostriatal pathway (TE-NSP) replacing and reconstructing the degenerated nigrostriatal pathway (in red). TE-NSPs are constructs fabricated completely *in vitro* for later implantation and that feature a hydrogel encasing an aggregate of dopaminergic neurons that could integrate with native nigral inputs. The aggregate also extends axonal tracts that emulate the structure of nigrostriatal fibers and that may reinnervate the striatum to restore dopamine and thus close the loop of the motor circuit in the brain. Reprinted with permission (Harris et al., 2016).

3. Tissue engineered nigrostriatal pathways (TE-NSPs)

3.1. Overview of preformed neural networks and TE-NSPs

Our research team is developing a unique approach for pathway reconstruction that combines components of tissue engineering, biomaterials, and primary or stem cell sources. Our approach centers on engineering the entire nigrostriatal pathway *in vitro* for subsequent implantation into the host brain to replace/reconstruct the lost neurons and damaged fibers. This platform, termed the tissue-engineered nigrostriatal pathway (TE-NSP), consists of: (1) a hydrogel micro-column with controlled inner and outer diameters and length, (2) a lumen filled with a suitable extracellular matrix (ECM), and (3) an aggregate of dopaminergic neurons seeded on one end that extends long axon tracts along and through the lumen during culture (Fig. 2C) (Struzyna et al., 2018). TE-NSPs mimic the cytoarchitecture of the native nigrostriatal pathway where dopaminergic neurons are restricted to one end (the SNpc) and project axonal tracts (the nigrostriatal fibers) that may grow into the striatum, integrate synaptically with host MSNs, and release dopamine that regulates their activity. The hydrogel encases the engineered dopaminergic tissue, provides necessary physical cues to drive longitudinal axon growth *in vitro*, and protects it during and after implantation, whereas the ECM ensures neurons have the proper environment to survive and grow axons throughout the lumen. Beyond therapeutic implantation, we have also used TE-NSPs to model PD pathophysiology, particularly the role of α-synuclein fibrils in axonopathy (Clark et al., 2020). The TE-NSP technology is built from our previous work with micro-tissue engineered neural networks (micro-TENNs) (Cullen et al., 2012; Harris et al., 2016; Struzyna et al., 2017), a class of engineered micro-tissues featuring preformed axon tracts, which have been explored for corticothalamic reconstruction (Struzyna et al., 2015b), modeling synaptic communication and axon growth (Dhobale et al., 2018; Marinov et al., 2020), and the development of optically-controllable "living electrodes" as a biological brain-machine interface (Adewole et al., 2021; Serruya et al., 2018).

Initial TE-NSPs were fabricated with micro-columns of 1% agarose, an ECM core consisting of polymerized type I collagen, and aggregates of neurons isolated from the ventral midbrain of embryonic rats (Struzyna et al., 2018). Agarose had been used for micro-TENNs due to the generally inert properties of this class of hydrogel and to primarily provide a stiff structural support to control the direction and extent of axon growth (Cullen et al., 2012). Additionally, both impure dopaminergic neurons sourced from embryonic rats (Struzyna et al., 2018) as well as those neurons purified for GFP+ dopaminergic neurons from transgenic rats have been used in TE-NSPs (Struzyna, 2019). These TE-NSPs were made to be "rat-scale", with a 160 μm diameter lumen and axons that grew to be at least 5–6 mm long (Struzyna et al., 2018). The aggregates and axons released dopamine after stimulation and synapsed with an aggregate of striatal neurons. These constructs could also be implanted in rats to span the nigrostriatal pathway, with the aggregate adjacent to the SN and the terminal axon tracts in the striatum. We showed survival of the TE-NSPs *in vivo* and physical integration with the striatum after at least 1 month in a parkinsonian rat model (Struzyna, 2019).

Recently, we have advanced our TE-NSPs with respect to the biomaterial encasement and cell source. For example, we fabricated TE-NSPs from crosslinked hyaluronic acid (HA) hydrogels, and particularly methacrylated HA (MeHA) (Gordián-Vélez et al., 2021). HA hydrogels are comprised of one of the main components of the native ECM in the brain and have been widely used both clinically and in research for a variety of tissue repair applications (Burdick and Prestwich, 2011; Highley et al., 2016; Zhong et al., 2010). When compared to the bioinert agarose hydrogels, HA is cell-responsive through receptor binding and enzymatic degradation. There is ample research into chemical modifications that can be applied to HA to enable fine-tuning of the physical properties of formed hydrogels, the presentation of biochemical cues, and the release of growth factors and drugs (Burdick and Prestwich, 2011; Highley et al., 2016; Zhong et al., 2010). Thus, a wide variety of HA hydrogels could be explored for TE-NSPs depending on the need, making them more biocompatible, versatile, and adaptable to specific design criteria. Indeed, this may contribute to significantly improved outcomes as a result of better graft survival, preservation of the structure of the engineered axons *in vivo*, and integration with brain tissue. Moreover, expanding the functionality of the hydrogel micro-columns beyond structural encasement and protection to now deliver therapeutic molecules may advance TE-NSPs into a more inclusive approach to address other aspects of PD pathophysiology. Using embryonic rat dopaminergic aggregates, we recently confirmed that MeHA can be utilized to create rat-scale TE-NSPs that mimic the nigrostriatal pathway in terms of structure, phenotype, and dopamine release and that can be implanted and survive in the rat brain. Notably, MeHA-encased TE-NSPs featured faster axon growth and elicited a lesser astrocyte and microglia response in the brain after 6 weeks *in vivo* when compared to our traditional agarose encasement (Gordián-Vélez et al., 2021). With eyes towards clinical translation, we are currently testing various human stem cell-derived sources for TE-NSPs. These include neurons differentiated from H9 ESCs and C1.2 iPSCs, midbrain dopaminergic organoids, and commercially-sold fully differentiated neurons. We are focused on characterizing these human TE-NSPs *in vitro* and on evaluating the therapeutic potential of rat-scale human constructs by implanting them in parkinsonian athymic rats and assessing histological, behavioral, and functional outcomes.

3.2. Advantages of preformed neural networks and TE-NSPs

TE-NSPs have been designed to circumvent the limitations of conventional PD interventions and cell replacement and pathway reconstruction strategies. TE-NSPs address the pathology of PD by replacing both SNpc dopaminergic neurons and the axons of the nigrostriatal pathway in their proper anatomical location. In doing so, TE-NSPs should release dopamine in the striatum in a temporally-controlled manner in response to the host brain and with the specificity provided by synaptic integration. This contrasts with treatments that may cause debilitating off-target effects due to non-specific dopaminergic effects or stimulation. Cell-based strategies have involved the injection of suspensions of dopaminergic neurons into the striatum. On the other hand, TE-NSPs are an entire nigrostriatal pathway substitute implanted to match the neuroanatomy and complete the motor circuit. TE-NSPs also have a protective hydrogel that may promote high cell survival during and after implantation and allow greater cell engraftment in the intended anatomical areas, which is in contrast to the low cell survival and the possibility of off-target cell presence observed with traditional cellular injections and implants.

Nigrostriatal fiber repair has focused on injecting neurons in the SN and passively allowing or actively guiding their growth *in vivo*. In contrast, TE-NSPs are created fully *in vitro* to support axon growth prior to implantation, which avoids the need for long-distance growth *in vivo* and the presentation of specific cues in the microenvironment to support it. Additionally, this approach allows us to validate the structure and function of these constructs prior to implantation, specifically in terms of axon growth and density, viability, expression of functional dopaminergic markers, and dopamine release, which improves the reproducibility of our strategy. With TE-NSPs we can also control the phenotype distribution within the aggregate and the kinds of projected axons in order to support the type of reinnervation that maximizes motor improvements. Using a tubular hydrogel for encasing the axons also helps restrict growth to the pathway and reinnervation to the striatum.

As previously suggested, the hydrogel encasement can also expand the capabilities of TE-NSPs to tackle the precise drivers of neurodegeneration. For example, the hydrogel micro-column could be employed to deliver and release neurotrophic factors, such as GDNF,

neurturin, and BDNF, which have neuroprotective effects on midbrain dopaminergic neurons and have shown functional benefits in animals models and clinical trials after injection, viral-induced expression, or delivery with cells (d'Anglemont de Tassigny et al., 2015; Moriarty et al., 2017; Yasuda and Mochizuki, 2010). The hydrogel could also target other components of PD pathology, such as α-synuclein aggregation and mitochondrial dysfunction, as has been investigated through the delivery of heat-shock proteins (Tunesi et al., 2019), vectors to reduce α-synuclein gene expression (Yang et al., 2021; Xhima et al., 2018), and drugs to increase clearance of α-synuclein (Stoker et al., 2018).

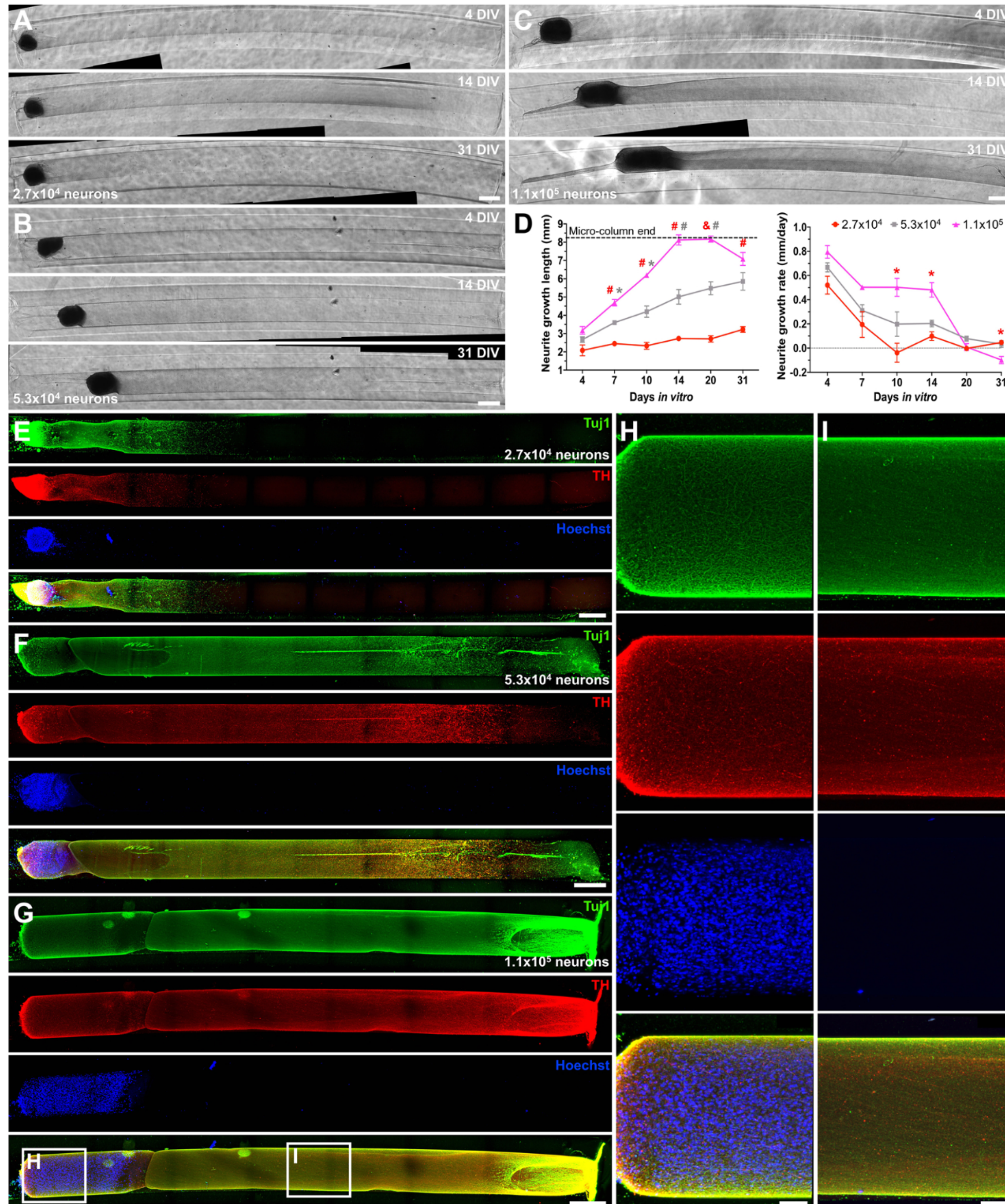


Fig. 3. Growth characterization, optimization, and immunohistochemical assessment of human-scale TE-NSPs. (A–C) Human dopaminergic aggregates having a low (2.7×10^4 neurons; $n = 3$), medium (5.3×10^4 neurons; $n = 4$), and high (1.1×10^5 neurons; $n = 3$) number of neurons were seeded within HA hydrogel (3% MeHA) micro-columns having an inner and outer diameter of 500 and 973 μm, respectively, and a length of ~1 cm. The seeded aggregates can be seen on the left side of the micro-columns in the images. These phase contrast images taken over several days *in vitro* (DIV) are representative of TE-NSPs from each group. **(D)** The neurite growth length and rate were measured over time, with time and cell number having significant effects in both cases ($p < 0.0001$). Data presented as mean ± SEM (*$p < 0.05$, #$p < 0.01$, &$p < 0.0001$; red and grey symbols indicate significant differences between the higher cell number compared to the lower and medium numbers, respectively). (E–G) TE-NSPs from each group were stained at 35-42 DIV to observe neurons/axons (β-tubulin III/Tuj1, green), dopaminergic neurons (TH, red), and nuclei (Hoechst, blue). **(H, I)** Magnification of the aggregate and axon tract regions of the construct in **G** to better observe the cytoarchitecture. Scale bars: (A–C, E–G) 500 μm; (H, I) 100 μm.

3.3. Progress towards TE-NSPs for human-scale fiber restoration

In the past we have transplanted TE-NSPs into the rat brain featuring micro-columns with an inner and outer diameter of 160 and 398 μm, respectively, ~3,000 rat neurons, and axon lengths of 5–6 mm. These dimensions were chosen given the smaller size of rat brains and the length of their nigrostriatal pathway. This inner diameter limited the size of the aggregate that could be fit within the micro-column and thus the number of neurons in a construct. To satisfy the human-scale criteria outlined in Section 2.3.2, particularly the required cell numbers and axon lengths, here we created TE-NSPs using commercially-sold human iPSC-derived dopaminergic neurons (90% TH+) and micro-columns of HA hydrogel (3% MeHA) with inner and outer diameters of 500 and 973 μm, respectively, and lengths >1 cm. The greater diameters and column lengths allow for larger aggregates to be seeded and for more space for axon growth. We seeded 2, 4, or 8 aggregates within one end of the micro-columns, each with 13,269 cells, that fused early on during culture to form a single aggregate with 2.7×10^4, 5.3×10^4, or 1.1×10^5 cells, respectively. In some cases we also seeded an additional aggregate of rat striatal neurons at the opposite end from the human dopaminergic aggregate. These constructs were used to establish their axon growth/density profile, phenotype expression, and dopamine release functionality, as well as their ability to integrate with a striatal target. The methods used to fabricate and characterize the constructs in this section are included in the **Supplementary Information**.

3.3.1. Effect of cell number on axon growth

TE-NSPs with higher initial cell numbers grew neurites for longer distances, at faster rates, and with greater densities than those with lower initial cell numbers. This can be observed in phase contrast images that show more neurites that are positioned farther from the aggregate on the left (noticed by the darker shades in the lumen) at the same time points in constructs with more neurons (Fig. 3A–C). Indeed, cell number had a statistically significant effect ($p < 0.0001$) on both neurite length and growth rate, and constructs with 2.7×10^4, 5.3×10^4, or 1.1×10^5 cells reached average lengths of 3.22 ± 0.16, 5.85 ± 0.48, and 8.17 ± 0.17 mm, respectively, after 1 month of culture (Fig. 3D). Growth rates were highest initially and decreased over time due to neurites slowing/stopping their growth or reaching the end of the micro-column. The higher cell number group also had a more sustained growth rate of around 0.5 mm/day from 7 to 14 days. We expect that TE-NSPs with the highest cell numbers would have grown even more, but growth was restricted as the axons reached the end of the micro-columns and the available space for growth was reduced due to aggregate movement inwards from the end of the lumen. This latter phenomenon could be addressed by further optimizing the hydrogel encasement and fabrication process. Images of immunolabeled TE-NSPs also showed the relationship between cell number and neurite density, and they confirmed the dopaminergic TH+ phenotype of the aggregate and axons (Fig. 3E–G). These constructs were engineered as expected, with neurons restricted to the aggregate and the remaining space of the lumen occupied by a dense, three-dimensional column of axons (Fig. 3H, I). Overall, more cells may directly result in more axons being projected and/or higher cell viability and health, although we do anticipate this effect to reach a plateau due to the mass transfer limitations for larger tissues. We can indeed fabricate TE-NSPs having 100,000 neurons, which is in the range of clinically relevant numbers of dopaminergic neurons. Future studies will assess even greater numbers of cells to determine the maximum number of cells that a single TE-NSP can support before detrimental effects on viability and growth are observed. In the next sections, we will focus on constructs with aggregates having either 5.3×10^4 or 1.1×10^5 cells given their better growth compared to the group with fewer cells.

3.3.2. Effect of cell number on dopamine release

A key feature for any pathway reconstruction strategy is dopamine release. Thus, we assessed human-scale TE-NSPs for electrically-evoked dopamine release in the dopaminergic aggregate and axon tract regions using fast-scan cyclic voltammetry (FSCV) (Fig. 4A). FSCV measures local dopamine concentrations in close proximity to the tip of the working electrode rather than what is being released by all cells. TE-NSPs in all cell number groups released dopamine in the aggregate and axonal tracts after stimulation, as observed by the peaks in the concentration traces, the oxidation peak near 0.6 V in the cyclic voltammogram, and the green region in the colorplot after 8 s (equivalent to ~0.6 V) (Fig. 4B, C). Cell number had a significant effect on dopamine release ($p = 0.0219$), with the mean dopamine release in the aggregate recorded as 579 ± 76 nM for the highest cell number group (1.1×10^5 neurons), which was significantly different from the 5.3×10^4 neurons group (306 ± 56 nM; $p = 0.0090$) (Fig. 4D). There were no significant differences in the axon tract region, with concentrations fluctuating around 365–373 nM. These results reflect that higher cell densities are not detrimental for dopamine release and may even increase it within the aggregate. Dopamine release in the aggregate may point to the existence of somatodendritic release or axodendritic/axosomatic release from connected neurons, while the recordings in the axons show release at presynaptic terminals. This suggests that these constructs could release dopamine from both ends when implanted. It will be crucial to investigate the signaling activity in the basal ganglia after implantation and the associated motor function effects to ensure that the dopamine release along the structure of TE-NSPs is well tuned and regulated according to the needs of the host circuitry.

3.3.3. Integration between TE-NSPs and striatal neurons

We also assessed connectivity between dopaminergic axons and striatal targets, as seen in the native nigrostriatal pathway, with TE-NSPs. We fabricated human-scale TE-NSPs with an aggregate of rat embryonic striatal neurons seeded 5 days after the dopaminergic aggregate within the distal end of the micro-column to examine growth, structure, and dopamine release (Fig. 5A). The presence of the striatal aggregate improved the dopaminergic axon growth length and rate, as displayed in the separation of these outcomes between bidirectional and unidirectional constructs starting around 10 days *in vitro* (Fig. 5B). There was a significant effect of culture type on lengths ($p = 0.0104$) and growth rates ($p = 0.0028$). At 14 days, neurite lengths were 5.01 ± 0.40 and 7.24 ± 0.05 mm (unidirectional vs. bidirectional; $p = 0.0415$), while the growth rates were 0.20 ± 0.03 and 0.56 ± 0.08 mm/day (unidirectional vs. bidirectional; $p = 0.0596$). The case for 1.1×10^5 cells was not included because growth was too similar and fast in both cases to observe a difference before the axons reached the distal end. The data indicate that the striatal target can improve the growth of human dopaminergic axons. This effect is delayed relative to the day in which the striatal target was added, as the effects could be seen starting on day 10 and the cells were seeded on day 5. This could reflect the time required for axons to grow a certain threshold distance from the striatal end or the time for the striatal target to habituate and start to present cues that benefit growth. Future studies will explore what specific growth factors, receptors, and other signaling cues exert this influence in the context of TE-NSPs. Still, it has been reported *in vivo* that dopaminergic axons in their growth phase can follow guidance provided by projections from MSNs expressing dopamine- and cyclic-AMP-regulated phosphoprotein of molecular weight 32,000 (DARPP-32) (Thompson et al., 2009). These MSNs are also the main source of GDNF in the striatum, which can promote the growth of dopaminergic axons that express GDNF receptors (Paratcha and Ledda, 2008; Thompson et al., 2009). Bidirectional TE-NSPs also exhibited the cytoarchitecture of the native nigrostriatal pathway, with the TH+ dopaminergic aggregate and its dense axons filling the lumen and reaching the DARPP-32+ striatal aggregate (Fig. 5C).

We also tested bidirectional TE-NSPs for evoked dopamine release in the dopaminergic aggregate, axon tracts, and axons at the edge of the striatal aggregate (Fig. 6A). We observed dopamine release in all three

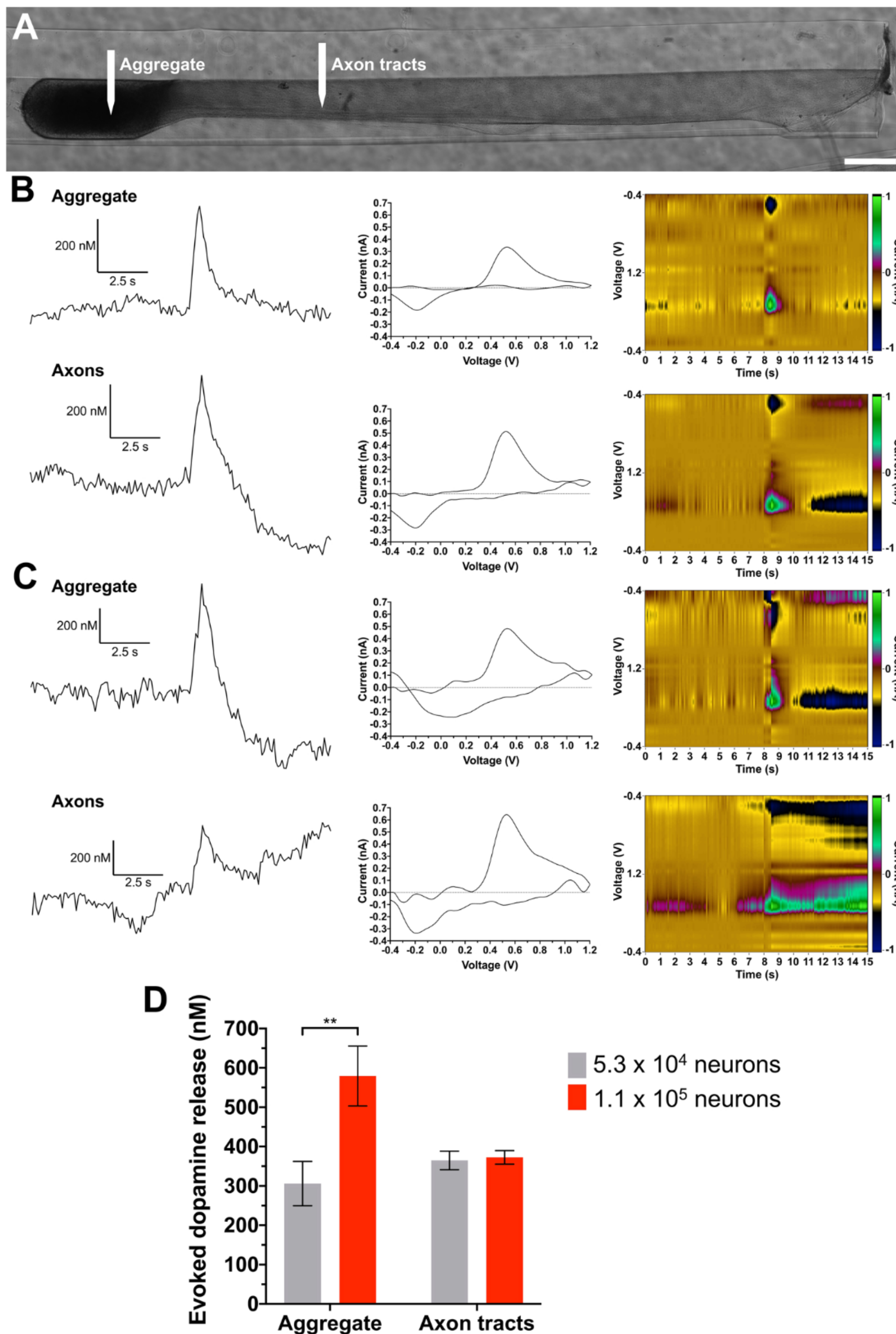


Fig. 4. Functional characterization and optimization of TE-NSPs based on electrically-evoked dopamine release. (A) After 35-42 days, the constructs were analyzed by simultaneously stimulating and recording in the aggregate (left side of the micro-column) and axon tract regions, as shown in this image of a TE-NSP taken before the analysis. **(B, C)** Representative current/concentration traces, cyclic voltammograms at the time of peak release, and colorplots for the analyzed regions in constructs with aggregates having 5.3×10^4 ($n = 3$) and 1.1×10^5 ($n = 3$) cells, respectively. Right after stimulation, the traces show a peak in concentration, and the cyclic voltammograms generally show a peak near 0.6 V, the characteristic oxidation voltage for dopamine. The colorplots show cyclic voltammograms recorded over time, with dopamine oxidation shown as the green region after stimulation at 8 s. **(D)** The quantification of evoked dopamine concentrations shows that there is significantly greater release in the aggregates in constructs with higher cell numbers compared to lower cell numbers, while there are no differences in the axon tract region. Data presented as mean ± SEM (**$p < 0.01$). Scale bar: (A) 500 µm.

regions (Fig. 6B). Of note, the dopaminergic axons at the striatal end could be stimulated to release on average 363 ± 62 nM, which was similar to the concentrations in the inner axon tracts of unidirectional (373 ± 17 nM) and bidirectional (395 ± 125 nM) constructs (Fig. 6C); however, there was no effect for culture type ($p = 0.3810$) and region ($p = 0.1687$). Unlike the case for growth, it seems that the striatal aggregate did not significantly influence dopamine release, although the mean release in the dopaminergic aggregate was higher without a striatal target (579 ± 76 vs. 417 ± 31 nM). Immunolabeled TE-NSPs confirmed the presence of TH+ dopaminergic neurons or axons in all three regions where dopamine was measured and that TH+ axons physically innervated the striatal aggregate and exhibited several patterns of arborization within (Fig. 6D–G). Future studies will determine if these axons are forming axodendritic synapses with the DARPP-32+ MSNs of the striatal aggregate. This would confirm the capacity of these human-scale TE-NSPs to functionally integrate with striatal neurons. Still, the data here showed that bidirectional TE-NSPs built to emulate both ends of the nigrostriatal pathway are functional and that dopaminergic axons connected with striatal neurons can release dopamine.

3.3.4. TE-NSPs with axon tracts with long-distance growth

A key characteristic of any pathway reconstruction method is long-distance axon growth that approaches the length of the native human

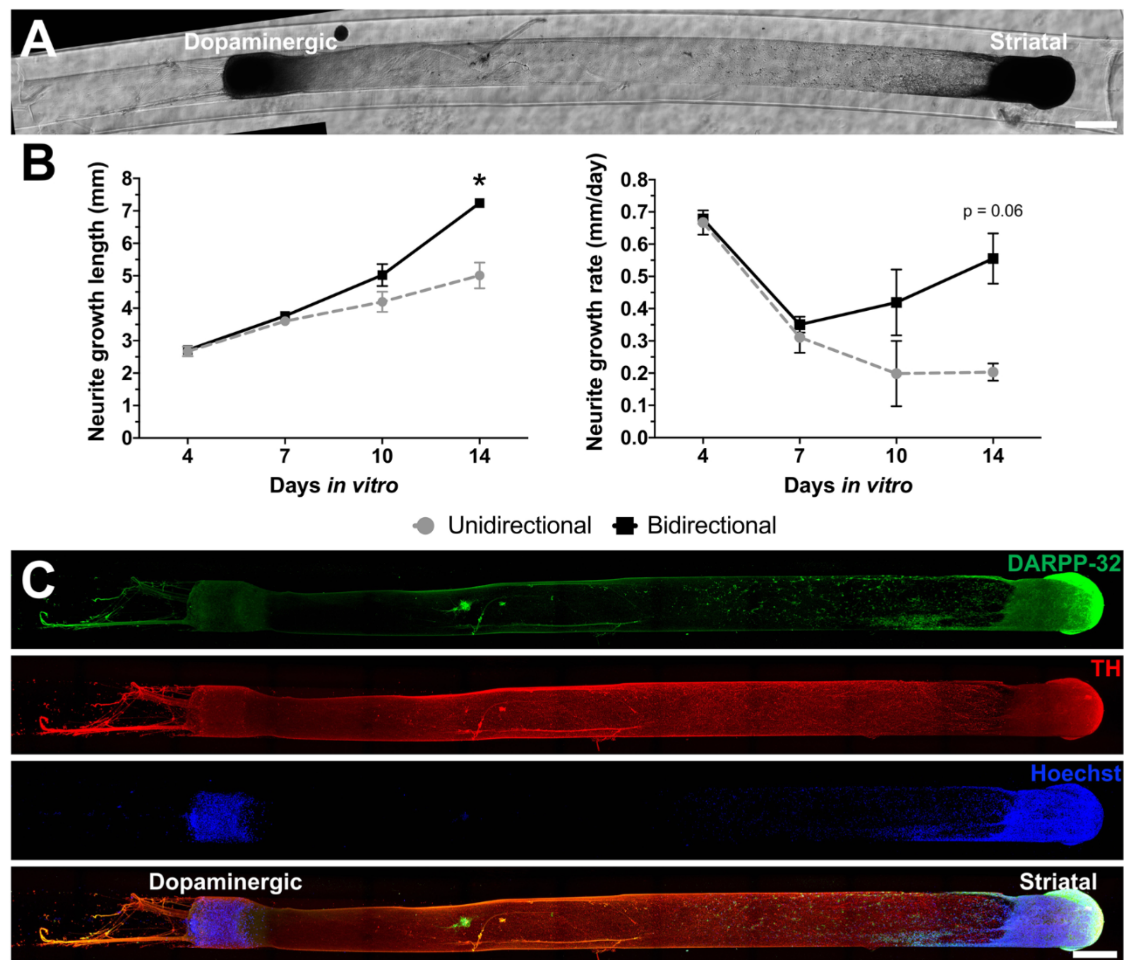


Fig. 5. Recreating and characterizing the growth and structure of the nigrostriatal pathway *in vitro* using TE-NSPs and a striatal aggregate. (A) Representative phase contrast image of a bidirectional TE-NSP at 39 DIV having a rat striatal aggregate (right side; having 2×10^5 neurons) seeded at the end of the lumen opposite to the human dopaminergic aggregate (left side; having 5.3×10^4 neurons). **(B)** The neurite growth lengths and rates were quantified in bidirectional TE-NSPs (500 μm inner diameter, 1 cm length) having a dopaminergic aggregate with 5.3×10^4 neurons. Time and culture type (unidirectional or bidirectional) had a significant effect on neurite length ($p < 0.0001$ and $p < 0.05$, respectively) and growth rate ($p < 0.01$) according to ANOVA. Data presented as mean ± SEM (*$p < 0.05$). **(C)** The construct in **A** was fixed and stained to label striatal neurons (right side; DARPP-32, green), dopaminergic neurons (left side; TH, red), and nuclei (Hoechst, blue) in order to observe the structure of the tissue and the integration of dopaminergic axons with the striatal aggregate. Scale bars: (A, C) 500 μm.

nigrostriatal pathway. Therefore, we created TE-NSPs with micro-columns at or longer than 1.5 cm ($n = 5$) to investigate if the axons could grow fully within these longer hydrogels. Here we show a fully-grown TE-NSP with axon tracts that grew 1.5 cm within a ~1.7 cm long micro-column; the aggregate length and some inward movement occupied the gap between 1.5 and 1.7 cm (Fig. 7A). This construct was kept in culture for 85 days, showing the remarkable resilience of the integrity of the aggregate (Fig. 7B) and axon tracts (Fig. 7C) in TE-NSPs *in vitro*. We anticipate that if these cells had been within an even longer micro-column, we could have achieved longer growths, which we will be testing in ongoing studies.

4. Future directions and challenges for the clinical translation of TE-NSPs

We are working towards transitioning TE-NSPs to a clinical-grade product for use in humans as a regenerative medicine-based treatment for PD. As surveyed previously, we are investigating how to create TE-NSPs with neuronal doses and lengths appropriate for the structural and functional needs of the human nigrostriatal pathway. In parallel, we are planning to optimize TE-NSPs for clinical translation in terms of cell sources, axon growth, manufacturing time, and standardizing their fabrication. In the case of cell sources, similar to other stem cell-based therapies for PD, we are considering human ESCs or iPSCs to obtain differentiated dopaminergic neurons. Each cell type has its own advantages and limitations; for example, ESCs remain the gold standard for stem cell therapies, but there are issues with their availability, ethical concerns, and risks of immune rejection (Jang et al., 2020; Kirkeby et al., 2017). On the other hand, iPSCs offer a way to use patient-derived cells to reduce or eliminate the need for immunosuppression and avoid ethical concerns. Still, the protocols associated with these cells, their differentiation, and their testing are costly, time consuming, and technically complex. These iPSCs also have a higher propensity than ESCs to have variability between cell lines and to exhibit genetic and epigenetic deficits (Das et al., 2020; Jang et al., 2020; Kirkeby et al., 2017). Both ESCs and iPSCs carry risks of tumor formation and of lacking reproducibility in the differentiation efficiency and maturation stage of their products. In any case, TE-NSPs could employ a single cell source, either human ESCs or allogeneic iPSCs, that can be produced and/or differentiated at large scales and cryopreserved to ensure consistent outcomes and to streamline safety and efficacy studies, although this would require immunosuppression. Alternatively, we could consider the creation of individualized TE-NSPs, which would entail the use of iPSCs obtained from each patient or human leukocyte antigen (HLA)-matched cells that are not available on-demand. Any cell source would be extensively tested for pluripotency and differentiation efficacy,

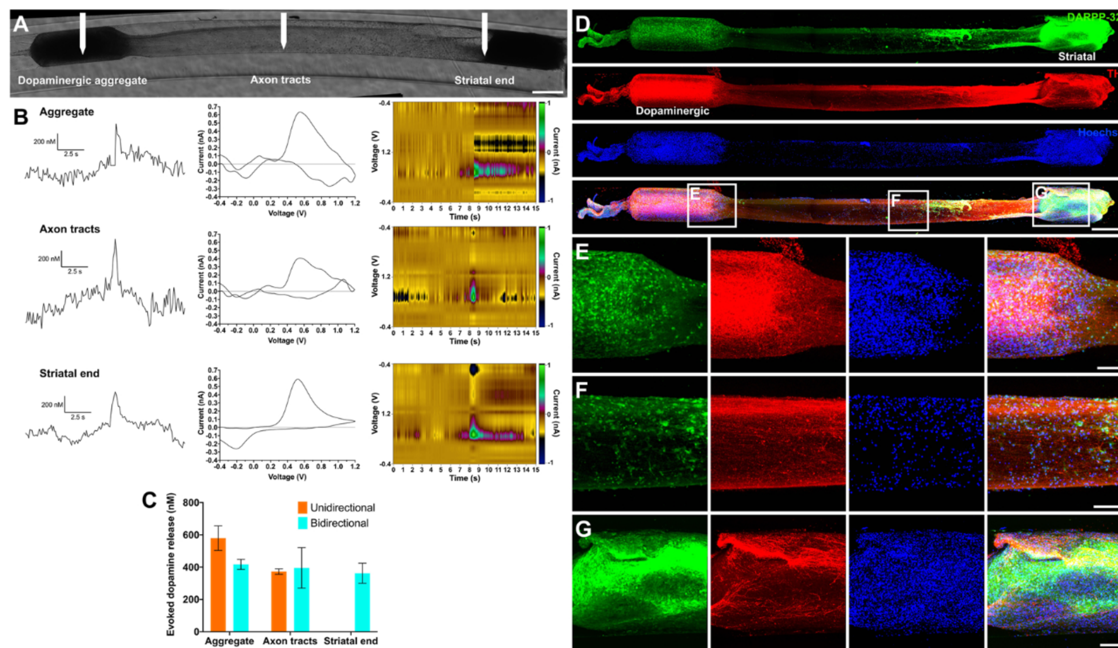


Fig. 6. Assessment of evoked dopamine release in bidirectional TE-NSPs containing human dopaminergic axon tracts and a striatal neuron aggregate end target. (A) The functionality of bidirectional TE-NSPs (having 1.1×10^5 neurons) and their ability to release dopamine in the striatal target was assessed at 33-35 DIV with FSCV by stimulating and recording in the regions shown in the phase contrast image. **(B)** Example of concentration traces, cyclic voltammograms, and colorplots of voltammograms over time in the dopaminergic aggregate, inner axon tracts, and axons at the striatal end. **(C)** Comparison of evoked dopamine release between unidirectional ($n = 3$) and bidirectional ($n = 3$) TE-NSPs shows no significant differences between groups. Data presented as mean ± SEM. **(D)** Confocal image showing the bidirectional TE-NSP in **A**, containing a human dopaminergic aggregate (34 DIV) and a rat striatal aggregate (29 DIV), stained for striatal neurons (DARPP-32, green), dopaminergic neurons (TH, red), and nuclei (Hoechst, blue). **(E–G)** High magnification images of the dopaminergic aggregate, axon tract, and striatal aggregate regions, the regions where dopamine release was tested. The striatal neurons appear to be physically integrated with TH + dopaminergic axons. Scale bars: (A, D) 500 μm; (E-G) 125 μm.

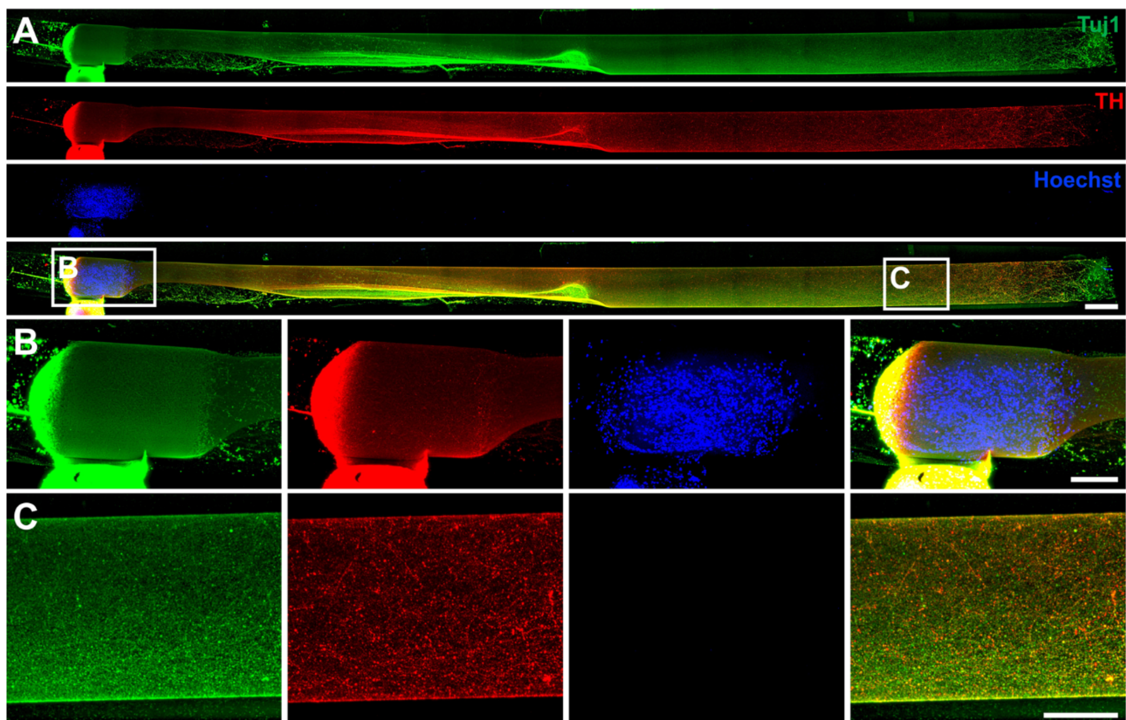


Fig. 7. Long-distance dopaminergic axon tracts in human TE-NSPs. (A) Human TE-NSP having an aggregate with 5.3×10^4 neurons and 1.5 cm long dopaminergic axon tracts, fixed at 85 DIV, and stained for neurons/axons (β-tubulin III/Tuj1, green), dopaminergic neurons (TH, red), and nuclei (Hoechst, blue). **(B-C)** Higher magnification of the dopaminergic aggregate and axon tract regions, respectively. Scale bars: (A) 500 μm; (B, C) 250 μm.

percentage of contaminating cells, presence of tumorigenetic mutations, electrical activity, and dopamine functionality as appropriate for each stage of production. We will need to balance the benefits of each cell type for TE-NSP performance with risks of rejection, effects of immunosuppressant use, and lead time. The selection and optimization of the cell source for TE-NSPs according to these criteria would occur before proceeding to preclinical studies. These processes and protocols also need to be adapted and optimized to follow current Good Manufacturing Practices (cGMPs) and carried out in compliant facilities, which could affect the behavior of the products of stem cell differentiation. In addition, we need to study aggregate size limitations further, in terms of the number of neurons per aggregate that maximizes viability, growth, and functionality. This, in combination with differentiation efficiency, will help establish the effective dose of dopaminergic neurons that an individual TE-NSP can provide. Many of the aspects mentioned here represent potential barriers to the translation of stem cell-derived therapies; nevertheless, there are ongoing clinical trials for PD using these cells. Thus, there are already guidelines and strategies in place to address these complex issues. Moreover, cell therapy is the way forward for the treatment of PD given the immense potential to directly target the pathology of this disease.

In terms of axon length for pathway reconstruction in the human brain, our results thus far indicate that the dopaminergic neuron aggregates are capable of producing 1 cm-long axons after 30 days *in vitro*, while we need to reach lengths of 2 cm for clinical utility. The best-case scenario would be to reach these lengths in 15–30 days consistently throughout different batches and constructs, as culturing TE-NSPs for longer could have adverse effects on viability and differentiation, axon health and integrity, and the reproducibility of fabrication. Thus, one of our most pressing challenges will be scaling up axon growth to achieve longer lengths in shorter time frames. To achieve this, we are pursuing growth acceleration strategies including: ECM alignment (*e.g.*, mechanical, magnetic) (Antman-Passig et al., 2017; Dubey et al., 1999), growth factor supplementation, gradients, or local retention (Yasuda and Mochizuki, 2010), mechanical stretch growth (Chen et al., 2019), presence of glial cells and/or scaffolds (East et al., 2010; Panzer et al., 2020; Purvis et al., 2020), addition of a striatal aggregate (Struzyna et al., 2018), and extrinsic stimulation (*e.g.*, electrical, optical) (Park et al., 2015; Willand et al., 2016). These approaches have been used in the past to improve neuron health, guide and/or augment neurite growth *in vitro*, and promote axon regeneration *in vivo*. In particular, our research group has routinely fabricated up to 5 cm long axon tracts from aggregates of peripheral neurons at controllable rates and in less time using stretch-growth in mechanobioreactors (Katiyar et al., 2021, 2019). Any of these strategies or a combination would introduce additional considerations during preclinical and clinical studies, but these may be justifiable if greater axon tract lengths are achieved at shorter times.

We are also considering automating our fabrication process. Currently, the hydrogel micro-columns are made manually by inserting an acupuncture needle into a capillary tube, filling this with the biomaterial solution, and gelling it inside. The ECM is added to the lumen by pipetting, and the neuronal aggregate is seeded manually with fine forceps. This method produces micro-columns lacking consistency in terms of micro-column dimensions, placement of the lumen, aggregate localization, and radial/longitudinal ECM polymerization. To address these challenges and mass-produce TE-NSPs, we are pursuing 3D-printing to form the hydrogel encasement and ECM core and robotic micro-manipulation to place the neuronal aggregate. This may also pave the way to create the substantially longer hydrogel columns needed for humans without differences in material properties or gaps in ECM. Clinical-grade TE-NSPs would utilize an HA-based hydrogel, and numerous devices of crosslinked HA have been previously approved by the FDA for use for wound dressings, dermal fillers, anti-adhesion films, intra-articular injections, among others (Huerta-Ángeles et al., 2018). Still, we need to conduct studies to assess the safety of this biomaterial after intracranial implantation. In parallel, we will establish stringent characterization and quality control measures, in terms of viability, axon growth/density, expression of relevant dopaminergic markers, presence of progenitor, non-dopaminergic and non-neuronal cells, and evoked dopamine release, to guarantee that representative constructs from each batch of TE-NSPs exhibit optimal properties. Cold-storage and shipping protocols for these constructs will also be validated by optimizing these quality criteria *in vitro* and *in vivo* under these conditions. All of these processes also need to be adapted and validated to comply with cGMPs and standards from the International Organization for Standardization (ISO).

The optimal human-scale TE-NSPs will be used in preclinical studies using lesioned rats or larger animals such as swine or non-human primates. Crucial aspects of TE-NSPs would be verified and characterized in these studies: (1) the acute and chronic survival of cells in the aggregate; (2) the maintenance of axonal tract integrity; (3) the density, volume, growth patterns, targets, and phenotypes of axons reinnervating the striatum; (4) the extent of off-target axon growth innervating other regions; (5) the role of neuronal dose on outcomes; (6) nigrostriatal pathway signaling activity and dopamine release; (7) connectivity with other basal ganglia components; (8) efficacy in terms of histological, dopamine functionality, and motor function outcomes; (9) relationship between therapeutic effects and timing of functional integration; (10) optimal hydrogel encasement properties. Larger animals will allow testing of our constructs in brains similar to humans, particularly to better understand the safety and efficacy of TE-NSPs and resolve any outstanding issues. Importantly, these studies will also serve to probe the more complex surgical methods and anatomical considerations needed for implantation in larger brains, using scaled-up TE-NSPs and possibly delivering more than one construct. For example, we will need to develop a system to safely implant TE-NSPs in larger brains, a process that is fundamentally different from the injection of conventional cell suspensions. We anticipate this could involve scaling up our methodology for rat implantations, where TE-NSPs are loaded into a needle that contains a metal rod adjacent to the construct. The needle is attached to a syringe that has a plunger in direct contact with the metal rod protruding from the needle. After injecting the needle so its distal end is in the SN, the syringe is retracted a distance equal to the length of the TE-NSP while the plunger is maintained in place, thus laying out the construct within the brain. The entire syringe-needle assembly is then pulled out, leaving the TE-NSPs inside. In addition, the preclinical studies will enable us to study other surgical concerns: (1) cell migration to other brain regions; (2) host inflammatory response to the implants; (3) potential graft-derived tumorigenicity; (4) toxicity for non-target organs; (5) effect of administered anti-inflammatory drugs on the survival and integration of the constructs. When possible, key outcomes listed in this section would be characterized in clinical trials as well. We aim to address these challenges, standardize and upscale fabrication, and satisfy safety and efficacy goals in the near future.

For clinical studies we would follow guidelines established for trials under GForce-PD, a global consortium coordinating stem cell studies for PD (Barker et al., 2017, 2015). TE-NSPs would most likely be restricted to patients in the range of 45–70 years with mild to moderate PD who are still responsive to L-DOPA treatment and do not have genetic risk factors, major psychiatric disorders, dementia, or conditions that disqualify them from surgery. TE-NSPs would be implanted using intraoperative MRI-guided neurosurgical methods either in one or both hemispheres, as determined to be the best approach for each patient, and according to the neuroanatomical regions specified in Section 2.3.2 and employing a custom-made device or needle. A crucial consideration will be the number of implanted TE-NSPs per hemisphere, depending on the cell dose in a single TE-NSP and the safety considerations mentioned in Section 2.3.2. Alternatively, trials could be run to test at least two doses of TE-NSPs to optimize the number of implants/cells. These constructs could be manufactured as needed and personalized according to the patient's own cells, specific neuroanatomical features, and/or disease state or they could be taken from a cold-stored stock of

standardized constructs. If TE-NSPs are made with autologous cells, patients would not receive immunosuppression, whereas a regimen would be needed for at least 1 year post-surgery for allogeneic cell sources. After implantation, patients would be evaluated over time using computer tomography (CT) scans, MRI scans, neurological and PD-specific assessments (*e.g.*, MDS-UPDRS, PDQ-39), and ^{18}F-DOPA PET, following guidelines by the FDA and those used in previous trials (Schweitzer et al., 2020). These evaluations would allow us to confirm TE-NSP placement, monitor secondary effects, study dopaminergic activity, and assess motor symptoms and quality of life for patients.

5. Conclusion

When combined with cell replacement, pathway reconstruction as a treatment strategy for PD attempts to directly address two of the main components of the pathophysiology of this disease - dopaminergic neuron loss in the SNpc and the degeneration of their axonal projections that form the nigrostriatal pathway and innervate the striatum. This method of repairing or regenerating these long-distance axon fibers provides a way for dopamine to be released in tune with feedback from the host brain and for the anatomical and functional reintegration of different components of the basal ganglia. Several studies have explored this strategy, but they have mostly relied on fomenting axon growth *in vivo*, which results in growth lacking specificity and being insufficient for restoration at human scales. We have employed the tools of tissue engineering to build TE-NSPs, constructs that feature dopaminergic axons fully grown *in vitro* for subsequent implantation to replace lost neurons, reconstruct nigrostriatal fibers, and integrate with the striatum. We showed recent work to build these constructs with HA-based hydrogels and commercially-available human iPSC-derived dopaminergic neurons, with aggregates of over 100,000 neurons projecting axon tracts that reached lengths of at least 1.5 cm and released dopamine after electrical stimulation throughout its entire somato-axonal structure. Notably, these axons could physically integrate with and release dopamine within an aggregate of striatal neurons. Short- and long-term steps will focus on further optimizing and standardizing the fabrication process and cell sources for TE-NSPs and conducting preclinical studies in rodent and large animal models of PD. We anticipate pathway reconstruction, and TE-NSPs specifically, to be at the forefront of regenerative medicine-based treatments for PD in humans.

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Author contributions

W.G.V. wrote the manuscript, prepared the figures, and designed the experiments, fabricated the TE-NSPs, and carried out imaging, FSCV recordings, histology, and data analysis needed for the original research portion. D.K.C. conceptualized the TE-NSP platform and directly supervised the experiments, analysis, and manuscript preparation. D.C. assisted with image acquisition and manuscript preparation. R.A.E. assisted with the implementation of FSCV and the interpretation, processing, and analysis of acquired data. J.E.D., R.A.E., J.A.B., and H.I.C. contributed to methodology, interpretation of findings, and manuscript preparation with critical feedback, editing, and reviewing. All authors approved the final manuscript.

Data and materials availability

All the data supporting the results and conclusions of this manuscript are available upon request.

Declaration of Competing Interest

D.K.C. is a scientific co-founder of INNERVACE Inc., a University of Pennsylvania spin-out company focused on translation of advanced regenerative therapies to treat central nervous system disorders. Multiple patents relate to the composition, methods, and use of the constructs described in the paper, including U.S. Patent App. 15/032,677 (D.K.C.), PCT International Patent App. PCT/US2014/063720 (D.K.C.), U.S. Patent App. 16/093,036 (D.K.C., H.I.C.), PCT International Patent App. PCT/US2017/027705 (D.K.C., H.I.C.), U.S. Provisional Patent App. 62/758,203 (D.K.C., W.G.V.), PCT International Patent App. PCT/US2019/060585 (D.K.C., W.G.V.), and U.S. Provisional Patent App. 63/190,581 (D.K.C., W.G.V., J.A.B.). No other author has declared a potential conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.brainresbull.2021.07.016.

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