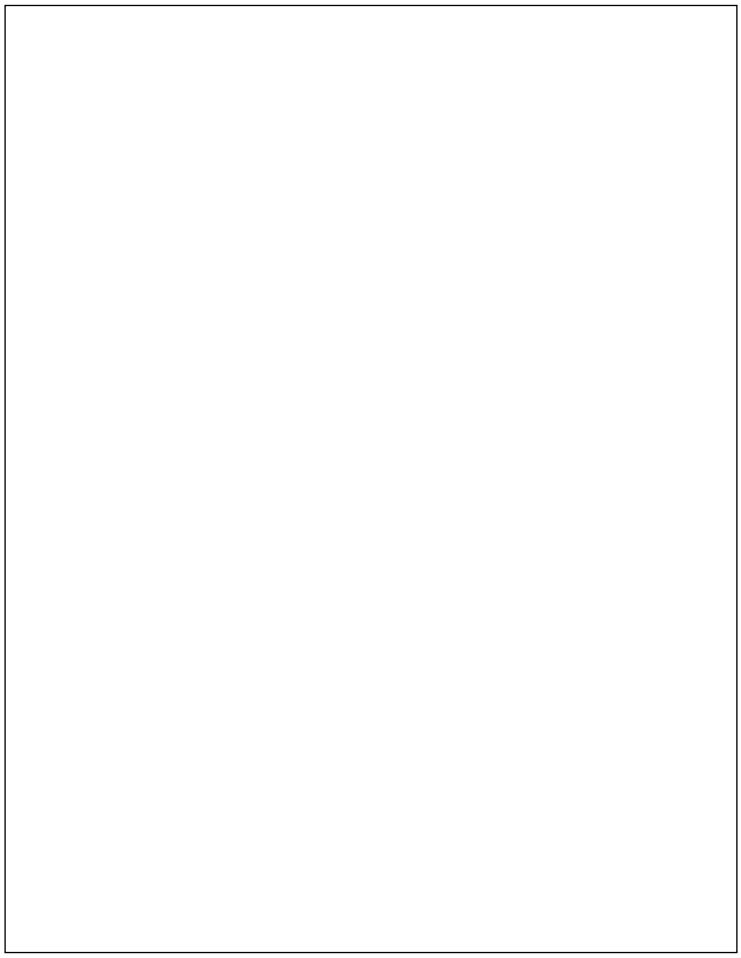

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Current Opinion in Biomedical Engineering

Engineered microtissue as an anatomically inspired model of Parkinson's disease

Elisia M. Clark[1,2], John C. O'Donnell[1,2], Laura A. Struzyna[1,3], H. Isaac Chen[1,2], John E. Duda[2,4] and D. Kacy Cullen[1,2,3]

Abstract

Although traditional small animal and cell culture models of neurodegenerative disease have been valuable in foundational discoveries, their inherent imprecision may have biased our understanding of etiology in humans and hindered translation of therapeutics. Recent breakthroughs to generate human stem cell–derived brain organoids — including from patient-specific cell sources — provide powerful platforms to improve disease modeling accuracy. However, brain organoids do not recapitulate crucial anatomical structures such as long bundled axon tracts — a central systems-level feature of human brains — critical to neurodegenerative disease pathogenesis, including Parkinson's disease. To address this challenge, advances in tissue engineering recapitulate brain pathways with three-dimensional anatomical fidelity to discrete systems-level brain structures, such as the first tissue engineered nigrostriatal pathway. Promulgating anatomically inspired human-derived engineered microtissue may improve translational relevance, empower personalized medicine, and ultimately reduce costs compared to conventional model systems, thus proving useful for targeted development and screening of new therapeutic strategies.

Addresses

[1] Center for Brain Injury & Repair, Department of Neurosurgery, Perelman School of Medicine, University of Pennsylvania, 3400 Spruce St., Philadelphia, PA, 19104, USA

[2] Center for Neurotrauma, Neurodegeneration & Restoration, Michael J. Crescenz Veterans Affairs Medical Center, 3900 Woodland Ave, Philadelphia, PA, 19104, USA

[3] Department of Bioengineering, School of Engineering and Applied Science, University of Pennsylvania, 220 S 33rd St, Philadelphia, PA, 19104, USA

[4] Department of Neurology, Perelman School of Medicine, University of Pennsylvania, 3400 Spruce St. Philadelphia, PA, 19104, USA

Corresponding author: Cullen, D. Kacy (dkacy@pennmedicine.upenn.edu)

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Introduction

For decades, translational research efforts to combat neurodegenerative diseases have relied heavily on rodent models; however, these models are often based on assumptions of causality, gleaned from limited understanding of clinical findings, to project aspects of disease onto a vastly different genetic background. Moreover, rodents often lack relevant human anatomical structures and physiological features (as presented in detail in later sections), in addition to having relatively brief life spans, that collectively may hinder their capacity to emulate the pathological spectrum of many neurodegenerative diseases, including Parkinson's disease (PD). For instance, aging alone has many effects on disease-relevant biological processes, including melanin accumulation, immunosenescence, protein dysregulation, DNA damage, oxidative phosphorylation, and mitochondrial dysfunction [1–4], and are not only often unaccounted for in rodent studies, but may not necessarily manifest even in aged rodents. These factors may limit the translational potential and the relevance of findings to the human condition. Indeed, although the use of rodent models has undoubtedly led to significant scientific discoveries, their limited clinical relevance has likely contributed to the failure of various neurotherapeutics in clinical trials even after demonstrating efficacy in these traditional models.

To compliment studies using animal models, *in vitro* preparations have been developed that have recently seen a vast expansion in complexity and utility owing to developments in stem cell biology and tissue engineering. Initial neural culture preparations used immortalized human cell lines — often derived from tumor cells — or primary cells from rodents, capable of generating planar neuronal cultures with *in vivo*-like synaptic connections and functional properties. However, inherent limitations based on the cell source and the simplicity of planar cultures led to the development of more sophisticated culture systems with improved relevance to the human condition. For instance, critical advancements include the development of protocols to generate induced pluripotent stem cells of human origin, providing a source of differentiated tissue-specific

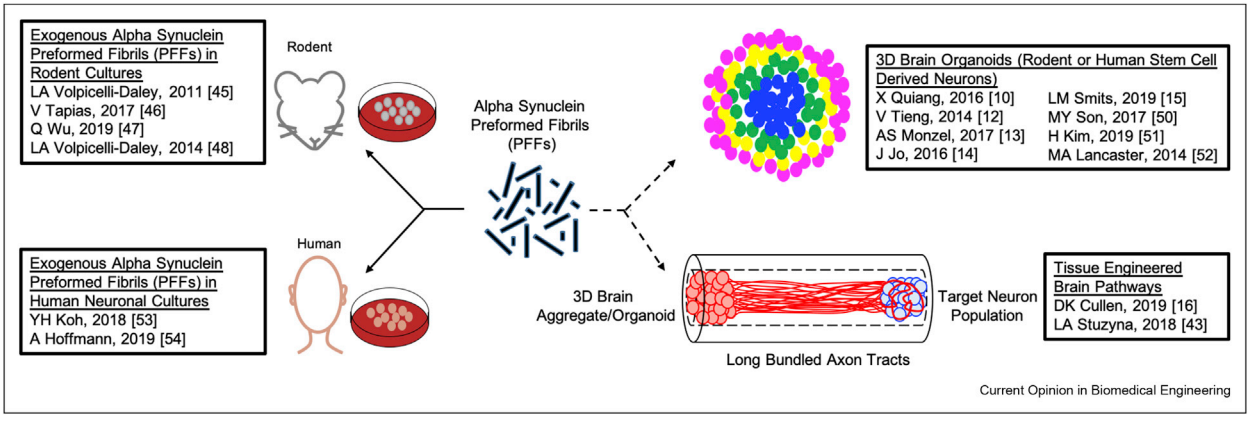
human cells, as well as the ability to growth these as three-dimensional (3D) multicellular organoids that recapitulate tissue-specific architecture.

While there is tremendous promise in generating neural cells from adult stem cell sources and growing them as complex 3D organoids, these platforms alone fail to mimic key neuroanatomical features relevant for modeling neurodevelopment, neurophysiology, and disease responses such as long-projecting axon tracts underlying the systems-level architecture of the brain. Accordingly, this article discusses the state-of-the-art for *in vitro* models of neurodegenerative diseases, with focus on recent advancements in modeling PD. We address the primary features of contemporary model systems, major challenges in the field, and next steps toward the development of more biomimetic *in vitro* models that replicate systems-level axon tract architecture by applying advanced tissue engineering techniques to fabricate 3D brain structures from human cells (Figure 1). While it is unrealistic to expect that even next-generation human stem cell–derived microtissue/organoid models could completely replace *in vivo* studies, current efforts are aimed at developing complimentary model systems that may present unique advantages to improve our understanding of disease sequelae and assess the efficacy of potential treatment strategies.

Parkinson's disease and the nigrostriatal pathway

PD is a progressive neurodegenerative disease with 50,000–60,000 diagnoses annually and over 1 million Americans afflicted in total. PD is characterized by resting tremor, bradykinesia, rigidity, and other symptoms that decrease quality of life, ultimately leading to significant disability via the inability to control motor function [5,6]. PD-associated motor symptoms arise from the selective loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc). As SNpc neurons send long-projecting axons to the striatum, this stereotypical neurodegeneration robs the striatum of crucial dopaminergic inputs and thereby renders an important motor feedback pathway ineffective. Alpha-synuclein protein is known as the pathological hallmark of PD pathology. Its function in the healthy brain is not completely understood; however, it is transported down axons in abundance, is highly enriched in presynaptic terminals, and is believed to be responsible for the transmission and progression of PD pathology across

Figure 1



Toward improved *in vitro* models of Parkinson's disease (PD). Cellular and microtissue testbeds that are anatomically, genetically, and physiologically accurate can help us better understand the pathophysiology of PD. **Center:** One promising strategy to accelerate PD pathology has been to introduce exogenous alpha-synuclein preformed fibrils (PFFs) — a hallmark feature of somatic (i.e. Lewy bodies) and neuritic (i.e. Lewy neurites) inclusions suggested to be a key component of neurodegeneration and pathological spread throughput the brain. To date, this strategy has been applied in cultures featuring dopaminergic neurons from rodents and from human induced pluripotent stem cells (**solid lines with arrows**). In future studies, these techniques may be employed using recently developed 3D brain organoid and/or microtissue preparations (**dashed lines with arrows**). In particular, more sophisticated anatomically inspired microtissue models may be necessary to understand the etiology and sequelae of PD, which features early axonopathy in long-projecting axons that are structurally and metabolically unique. Here, recently developed tissue engineered nigrostriatal pathways (TE-NSPs) may be a useful platform by providing a discrete population of dopaminergic neurons with long-projecting axon tracts that innervate a separate population of striatal neurons, which mimics the anatomy of the nigrostriatal pathway in the brain. The TE-NSP testbed may be utilized to understand relevant systems-level pathophysiological mechanisms underlying PD, such as axonopathy related to alpha-synuclein transport, aggregation and/or transmission. In addition to featuring human cell sources, these various platforms may be fabricated using patient-specific cells, potentially incorporating genetic abnormalities known to predispose and/or accelerate PD pathology. Overall, these platforms and techniques can be employed in combination to improve our understating of pathophysiological mechanisms underlying PD and to establish targeted pharmacologic treatments.

different regions of the nervous system [29]. There is still no cure for PD, and we have an incomplete understanding of the mechanisms that lead to this degeneration.

State-of-the-art: midbrain organoids with human stem cells

Organoids have been one of the most significant scientific advancements toward developing models of later stages of human life when neurodegeneration occurs. As these common diseases afflict older populations, the limited lifespan and simplicity of 2-dimensional (2D) rodent cellular models are simply not sufficient to adequately examine features of human disease pathogenesis and progression. Brain organoids generated from pluripotent stem cell sources, including patient-derived induced pluripotent stem cells, emulate aspects of brain architecture including the tissue-like cell organization and extracellular matrix [7–10]. An elegant example of the brain-like structure that arises in organoids is the formation of rudimentary layers of the cerebral cortex [9,10]. As restorative implants, these factors will likely facilitate improved integration of graft neurons with the brain, especially in highly ordered cerebral regions such as the cortex. In addition, brain organoids derived from human stem cells have significantly advanced the capacity to model and study mechanisms of neurological disorders. To date, various brain regions such as the cerebral cortex, hypothalamus, hippocampus, cerebellum, forebrain, and midbrain organoids have been used to further our understanding of human brain development and network activities as summarized by Gopalakrishnan [11]. While animal models obviously capture these features as well, organoids generated using human stem cells offer a complimentary *in vitro* approach featuring a human genetic background, greater experimental control, and higher throughput of experimentation, making them advantageous for studying specific facets of PD pathology.

Most relevant to modeling PD *in vitro*, midbrain organoids have built upon 2D differentiation protocols to produce 3D structures demonstrating positive tyrosine hydroxylase expression of dopamine neurons by 38 days in culture and specification of A9 dopaminergic neurons of the substantia nigra by 75 days in culture [10]. Over the last few years, protocols for midbrain organoid systems have advanced in sophistication, including the presence of supporting astrocytes and oligodendrocytes [12,13], synapse formation [13,14], functional electrophysiological activity [12–15], and physiological dopamine concentrations [12]. In addition, the fabrication of such 3D organoids using cells isolated from human patients with relevant genotypes and/or of disease-appropriate age may replicate critical underlying factors and, thereby, provide a more accurate representation for disease modeling.

While organoids alone recapitulate some features of gray matter (such as layered organization of somata from different cell types), only recently were these preparations used to emulate the spatial organization of distinct neuronal clusters connected by long axon tracts and interconnected synapses. Indeed, we recently reported *in vitro* fabrication of 3D microtissue consisting of human stem cell–derived, cortical organoids spanned by bundled, centimeter scale axon tracts [16], whereas others have demonstrated long axonal projections from cerebral organoids to spinal cord explants [17]. The necessary next steps will involve development of similar anatomically inspired microtissue featuring long, bundled axon tracts from dissociated human stem cell–derived dopaminergic neurons, which — as detailed in the following sections — we feel is imperative to effectively identify mechanisms underlying axonal pathophysiology and subsequent disease-associated axon degeneration and neuron death in PD.

Axon tracts are crucial for studying axonal pathophysiology

As noted, PD motor symptoms arise from selective loss of dopaminergic neurons in the SNpc, with significant SNpc ventrolateral neuronal degeneration at the time of symptom onset [18]. It remains unknown why dopaminergic neurons are preferentially impacted, but many believe a combination of factors including structure, function, and energetic/metabolic needs make dopaminergic neurons particularly vulnerable [19]. For instance, SNpc dopaminergic neurons feature long-projecting axons (that may be unmyelinated or thinly myelinated) that are highly branched, with this complex axonal arborization calculated to grow up to 15 feet in length within the striatum from each neuron [20]. This need to support such long and distant axonal structures incurs these SNpc neurons with unique — and daunting — transport and metabolic needs. After this stereotypical neurodegeneration, the striatum is robbed of crucial dopaminergic axonal inputs.

Alpha-synuclein is highly enriched in presynaptic terminals, possibly playing a role in regulating synaptic transmission [21–23]. Despite its unclear function, alpha-synuclein has been shown to play a key role in PD pathogenesis. Indeed, many of the genetic alterations associated with PD have been shown to increase the propensity for alpha-synuclein to self-assemble into oligomers, as well as into highly compact amyloid fibrils that are enriched in a beta-sheet structure [24–26]. This fibril structure makes up the primary component of Lewy bodies (LBs) and Lewy neurites (LNs), as well as several other glial and neuronal cytoplasmic inclusions in multiple system atrophy [27]. The contribution of alpha-synuclein to PD pathophysiology has been speculated to involve multiple different mechanisms including interruption of cytoskeletal components and

axonal transport, among others [28]. Normally, alpha-synuclein is produced in the cell soma, transported down the axon, and located primarily at the synaptic terminal. In the neuritic dystrophy hypothesis of neurodegeneration, abnormal aggregation of alpha-synuclein begins as LNs are formed within the axonal compartment of neurons, which enlarge by the sequestration of additional alpha-synuclein, other proteins, and subcellular elements, until they cause an interruption of axonal transport. It also results in supersaturation of the cell soma with alpha-synuclein and other proteins normally distributed by anterograde transport, predisposing the neuron to the formation of somatic alpha-synuclein aggregates (i.e. LBs) [29]. Over time, LB growth continues and alpha-synuclein aggregates form within proximal dendrites as LNs. Ultimately the axonal LNs lead to axonal degeneration with denervation of striatal medium spiny neurons and the possibility of resultant neuronal degeneration [29].

Over the years, much effort has gone into understanding the mechanism by which alpha-synuclein aggregation leads to neurodegeneration and disease pathogenesis. Soon after alpha-synuclein was described as a major component of LBs, alpha-synuclein aggregates were identified within processes [30] as an important early component of Parkinson pathology, including in the striatum [31]. Concurrent pathological and neuroimaging research has suggested that at the time of onset of Parkinsonian features, there is an approximate 30% loss of dopaminergic neurons in the SNpc, but more than 70–80% loss of dopaminergic markers in the striatum, suggesting that axonal terminals of the nigrostriatal pathway are the primary pathology generating Parkinsonian features [32]. This is further supported by severe reduction in staining of axonal terminals in the putamen in PD brains, compared with age-matched controls, supporting the notion that axonal degeneration is an early component of PD pathophysiology [33].

In vitro models for studying axon pathophysiology and alpha-synuclein transmission have provided valuable information to understanding disease pathogenesis. However, limitations to structure (e.g. missing long-projecting axon tracts) and cell source (e.g. non-human) have historically presented major challenges to recapitulating essential features of human disease [34–37]. Animal models are often based on assumptions drawn from an incomplete understanding of disease etiology. These assumptions are then applied against a vastly different genetic background, and then attempts are made to use that model to expand our previous understanding of the clinical condition. For example, pharmacological (i.e. toxin-based) rodent models of PD generally mimic midbrain dopaminergic signaling dysfunction, but this has only led to symptomatic therapies rather than disease-modifying therapies [38–40]. Furthermore, the alpha-synuclein–induced degeneration models demonstrate neuron degeneration without necessarily producing dopamine loss [41–43], making it difficult to correlate the physiological responses to the human condition. Whether this difference in vulnerability is due to interspecies differences in nigrostriatal tract length, density of arborization, or differences in rodent models including shorter life spans, all are open questions for investigation. Although no single model can be expected to recapitulate all features of a disease, the field should strive to embrace multiple modeling approaches to complement their respective shortcomings.

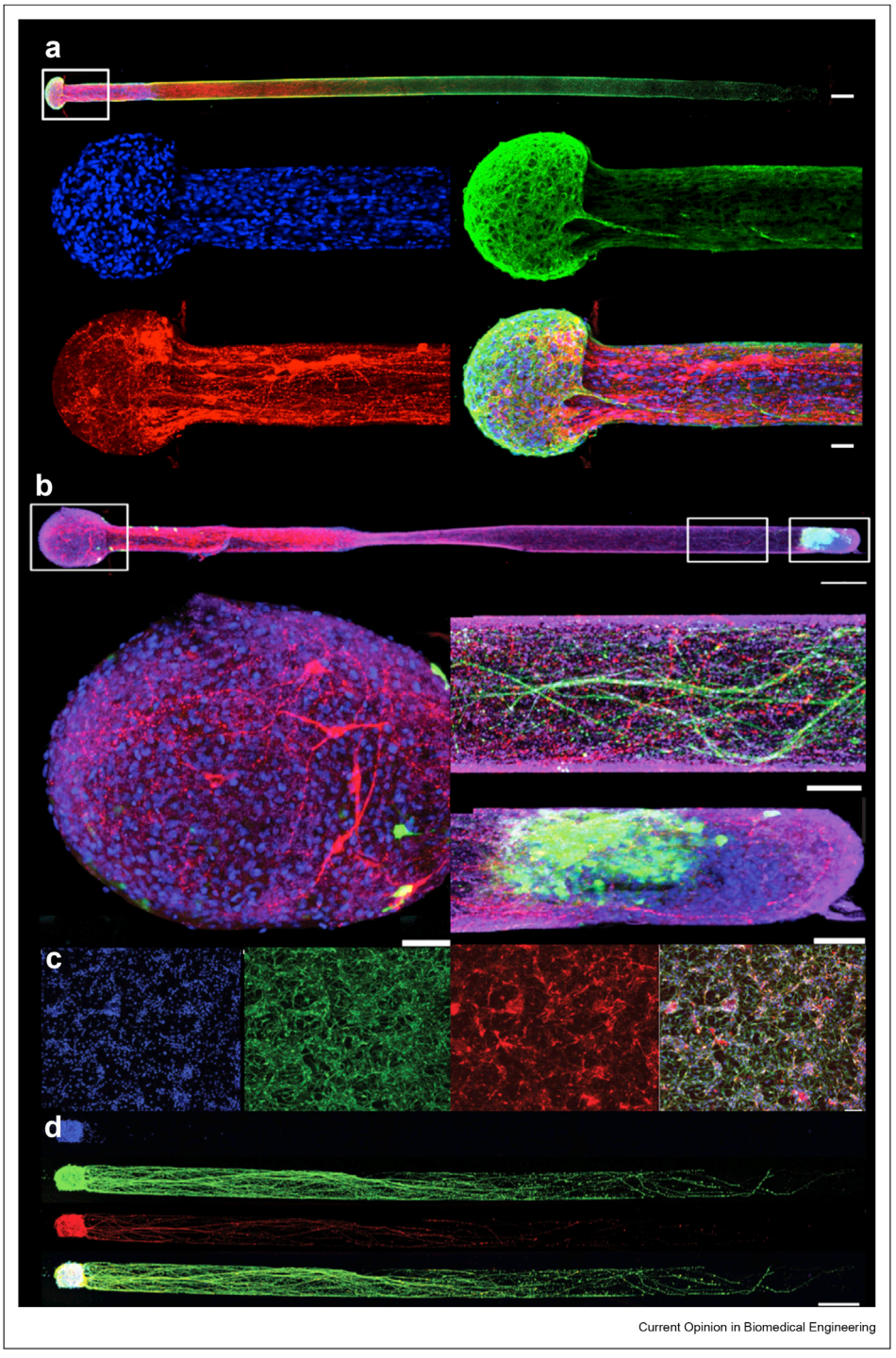
Inclusion of striatal end targets to model neural circuits

PD is a multifaceted disorder involving multiple brain circuits and neuronal populations. Therefore, attention to systems-level responses may be important to appreciate the effects of PD pathophysiology on the overall neurophysiology of neural networks, including intrinsic neuronal properties, axonal function, and synaptic inputs. While organoids are relatively small is size (e.g. millimeter-scale), they generally form small neural networks with localized axonal-dendritic and axonal–somatic connections, making them appropriate to address certain network-level questions. However, expanded engineered microtissue may be necessary to model large-scale networks. For instance, individual engineered microtissue components can be connected as specific ‘nodes’ to systematically build complexity [17]. Currently, there are limited *in vitro* 3D models that mimic the complete nigrostriatal pathway with dopaminergic neurons synaptically integrated with a striatal end target. As detailed in the following section, as we move toward developing a more biofidelic *in vitro* PD model, the presence of a striatal end target may be essential to understand transmission of pathological alpha-synuclein aggregates and degeneration of SNpc dopaminergic neurons.

Applications of exogenous alpha-synuclein fibrils *in vitro*

Recent advances in generating alpha-synuclein aggregates and LBs in cellular models suggest that these pathological entities contribute significantly to decreases in synaptic proteins, impairments in neuronal excitability and connectivity, and neuron death [44–46]. Successful characterization and validated production of alpha-synuclein preformed fibrils (PFFs) by Volpicelli-Daley et al.[47], 2014 recapitulates features of LBs and LNs found in PD brains and other synucleinopathies in both *in vitro* and *in vivo* studies [47,48]. Primary neurons exposed to PFFs led to recruitment and aggregation of endogenous alpha-synuclein into aggregate formation in axons, spread to somatodendritic compartments and induced neuron death as early as 14

Figure 2



Tissue engineered nigrostriatal pathway (TE-NSP) recapitulates features of endogenous neural circuitry and synapse formation. **(a)** TE-NSP with dopaminergic aggregate cultured for 14 days *in vitro* (DIV) labeled by immunocytochemistry to denote cell nuclei (Hoechst; blue); dopaminergic neurons (tyrosine hydroxylase; red), and axons (β-tubulin III; green) (scale = 250 μm). Higher magnification reconstructions show the aggregate rich in dopaminergic neurons (scale = 50 μm). **(b)** TE-NSP with dopaminergic aggregate cocultured with a striatal aggregate target for 14 DIV labeled by immunocytochemistry to denote dopaminergic neurons (tyrosine hydroxylase; red), striatal (medium spiny) neurons (DARPP-32; green), synapses (synapsin; purple), and cell nuclei (Hoechst; blue) (scale = 250 μm). Higher magnification reconstructions depict the dopaminergic neuron aggregate and synapses formed between the dopaminergic axons and striatal end target (scale = 50 μm). **(c,d)** Human stem cell–derived TE-NSPs biofabricated from H9 human embryonic stem cells differentiated into dopaminergic neurons and labeled via immunocytochemistry to denote all neurons/axons (β-tubulin III; green) and dopaminergic neurons/axons (tyrosine hydroxylase; red), with nuclear counterstain (Hoechst; blue). **(c)** Differentiated dopaminergic neurons in 2D culture (scale = 100 μm). **(d)** Differentiated dopaminergic neurons grown as a TE-NSP depicting robust unidirectional axonal extension reaching over 4 mm by 14 days post plating within the microcolumns (scale = 225 μm). Adapted with permission from Struzyna et al., 2018 [49].

days after PFF exposure [47]. These observations support current hypotheses of alpha-synuclein transmission and parallel observations in postmortem brains of PD patients. Currently, PFFs have only been applied to 2D cellular models of rodent and patient-derived neuronal cultures, as well as neuron and astrocyte cocultures, but not 3D models that could provide further insight into how these findings are reproduced within the anatomical context of the human brain cytoarchitecture.

Towards improved *in vitro* models of PD

An important next step toward a more biofidelic *in vitro* PD model involves a system that can be used to study both disease pathogenesis including spread of pathological proteins but also one that has a longer lifespan than typical rodent cultures to examine the downstream effects that lead to progression of disease. Here, the emergence of stem cell techniques and brain organoids created from human cells has shown that relevant human genetic factors can be modeled *in vitro*. As such, a critical next step for the field is to expose midbrain organoids to exogenous alpha-synuclein fibrils. However, as noted, 3D organoids alone do not recreate many of the unique systems-level anatomical features that define neuronal function under healthy and pathological conditions.

To address this challenge, we made a series of advancements in microtissue biofabrication techniques to create the first 3D tissue engineered nigrostriatal pathway (TE-NSP), from both rodent and human cell sources [49]. TE-NSPs may play the dual role of anatomically inspired biofidelic testbeds to model PD, while also potentially providing an implantable microtissue construct to physically and functionally reconstruct the lost nigrostriatal pathway in PD patients [49,50]. Indeed, this work characterized the fabrication of 3D dopaminergic aggregates suitable for transplantation to replace the disease-associated dopaminergic cell loss and has been optimized for axon innervation to a striatal end target to demonstrate complete neural circuitry of the nigrostriatal pathway (Figure 2). TE-NSPs using rat embryonic neurons or human neurons derived from a pluripotent stem cell line serve as proof-of-concept for applying this microtissue platform for studies of pathway development, maturation, function, and disease pathophysiology. Our current efforts are focused on creating next-generation TE-NSPs using adult human stem cell sources to model PD. This will create the opportunity for disease testing from affected patient populations, for instance, based on various single-nucleotide polymorphisms associated with increased risk of PD, or even on an individual basis [51–54].

TE-NSPs provide the ability to manipulate genetic and environmental conditions for controlled mechanistic studies of PD pathogenesis and progression, while providing both structural and functional outcome measures. TE-NSPs fabricated using rat embryonic neurons or human stem cell–derived neurons are each comprised of greater than 50% dopaminergic neurons with projections from the nigral aggregate synapsing with the distal striatal aggregate revealing extensive integration and synapse formation involving the dopaminergic axons and striatal neurons (Figure 2). These dopaminergic axonal tracts span more than 1 cm at average axon growth rates of 500 microns/day through the extracellular matrix lumen and demonstrate functional intrinsic and evoked dopamine release similar to physiological concentrations, measured by fast scan cyclic voltammetry [49].

Moving forward, by applying advanced biofabrication techniques to create 3D brain structures from human (even genotype- and/or patient-specific) cells for use as *in vitro* testbeds, TE-NSPs offer a new approach to studying axon pathophysiology, mechanisms of alpha-synuclein transmission, and neurodegeneration that addresses critical limitations of conventional animal and cellular models. Indeed, the human TE-NSPs may be grown for long time periods — as a surrogate for maturation and/or age — and exposed to human recombinant alpha-synuclein fibrils as a genetically, physiologically, and anatomically relevant *in vitro* model of PD, overcoming the anatomical limitations of 2D cellular models of PD, and the axonal architecture and neural circuit limitations of 3D midbrain organoid models.

Conclusions

Researchers have learned a great deal about PD pathophysiology through conventional cell culture and animal models; yet, findings have had limited translational impact in disease-modifying therapies because of challenges in recapitulating the most disease-relevant attributes of human brain structure and function. Emerging human microtissue models may address these challenges by providing features not possible in rodent systems such as a human genome (which is often necessary to replicate human disease pathways) and human anatomic features (e.g. centimeter scale axon tracts — a necessary feature for replicating the pathophysiology of many axonopathies), while enabling higher-throughput studies owing to inherent attributes of *in vitro* systems. Indeed, models fabricated using human-derived dopaminergic neurons — the human source ensuring a genetic endowment capable of developing and responding to synucleinopathy — featuring long axonal projections to a striatal neuronal source, may be valuable to study the role of axonopathy and metabolic susceptibility in PD pathogenesis. As one example, we developed the first TE-NSP recapitulating key elements of the native pathway [49,50]. Although

human tissue culture models will not be advantageous over rodent models in all cases, it is apparent that such anatomically inspired engineered microtissue holds considerable promise to compliment traditional modeling approaches. Nonetheless, next-generation organoid and/or microtissue models should be adopted with caution as rigorous quality control and experimental validation will be required to assess the relative translatability of these platforms compared with traditional rodent models. However, the measured adoption of more sophisticated tissue culture platforms may accelerate research progress by improving translational relevance and reducing costs (compared with animal models), expanding access to impactful neurodegenerative research, empowering personalized medicine through patient-specific study, and providing a powerful translational paradigm for screening and developing new therapeutics.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

D.K.C. is a co-founder of two University of Pennsylvania spin-out companies concentrating in applications of neuroregenerative medicine: INNERVACE, LLC and Axonova Medical, LLC. There are two patent applications related to the methods, composition, and use of micro-tissue engineered neural networks, including U.S. Patent App. 15/032,677 titled “Neuronal replacement and reestablishment of axonal connections” (D.K.C.) and US Patent App. 16/093,036 titled “Implantable living electrodes and methods for use thereof” (D.K.C. and H.I.C.).

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* of special interest
** of outstanding interest

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