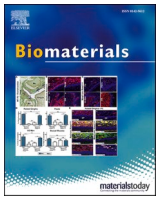
Biomaterials 298 (2023) 122143

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials



Functional bioengineered tissue models of neurodegenerative diseases

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ARTICLE INFO

Keywords:
Bioengineering
Tissue engineering
Neurodegenerative diseases
Human pluripotent stem cells
Organoids
Microfluidics

ABSTRACT

Aging-associated neurodegenerative diseases, such as Alzheimer's and Parkinson's diseases remain poorly understood and no disease-modifying treatments exist despite decades of investigation. Predominant in vitro (e.g., 2D cell culture, organoids) and in vivo (e.g., mouse) models of these diseases are insufficient mimics of human brain tissue structure and function and of human neurodegenerative pathobiology, and have thus contributed to this collective translational failure. This has been a longstanding challenge in the field, and new strategies are required to address both fundamental and translational needs. Bioengineered tissue culture models constitute a class of promising alternatives, as they can overcome the low cell density, poor nutrient exchange, and long term culturability limitations of existing in vitro models. Further, they can reconstruct the structural, mechanical, and biochemical cues of native brain tissue, providing a better mimic of human brain tissues for in vitro pathobiological investigation and drug development. We discuss bioengineering techniques for the generation of these neurodegenerative tissue models, including biomaterials-, organoid-, and microfluidics-based approaches, and design considerations for their construction. To aid the development of the next generation of functional neurodegenerative disease models, we discuss approaches to incorporate greater cellular diversity and simulate aging processes within bioengineered brain tissues.

1. Introduction

The worldwide population is rapidly aging. The proportion of people older than 65 expected to increase from 1 in 11 in 2019 to 1 in 6 by 2050, and the proportion of adult life spent above this age in developed countries has similarly increased over the past six decades [1]. Accordingly, elder care and the treatment of aging-associated disorders will be a major healthcare challenge of the coming decades. Aging-associated neurodegenerative diseases, such as Alzheimer's disease (AD) and Parkinson's disease (PD), are debilitating conditions that remain poorly understood, challenging to diagnose, and lack disease-modifying therapies despite extensive preclinical and clinical investigation. In line with an increasingly aged population, the rates of annual incidences of AD are expected to double by 2050 [2,3], and PD (the fastest-growing neurodegenerative condition) prevalence rates to double by 2040 [4].

AD is the most common cause of dementia, and is symptomatically typified by memory loss and other disruptions in executive functions [2]. PD, the second most common neurodegenerative disorder, is characterized by the loss of dopaminergic neurons in the substantia nigra, resulting in bradykinesia, muscle rigidity, and/or a resting tremor [5]. Pathological protein aggregates are hallmark histological features of both diseases: extracellular amyloid plaques and intracellularly-aggregating neurofibrillary tangles (NFTs) in AD [6,7], and intracellular α-synuclein inclusions in PD, but the degree to which these aggregates are pathogenic remains unclear [5].

These diseases have a complex etiology with protracted prodromal stages, and part of the collective failure in translating disease modifying

Abbreviations: Aβ, amyloid β; AD, Alzheimer's disease; APP, amyloid precursor protein; BBB, blood-brain barrier; BDNF, brain-derived neurotrophic factor; CNS, central nervous system; ECM, extracellular matrix; ESC, embryonic stem cell; fAD, familial Alzheimer's disease; GBA, glucocerebrosidase; GDNF, glial cell line-derived neurotrophic factor; GECI, genetically encoded calcium indicator; GEVI, genetically encoded voltage indicator; GFAP, glial fibrillary acidic protein; HA, hyaluronic acid; HSV-I, herpes simplex virus type I; iPSC, induced pluripotent stem cells; LRRK2, leucine-rich repeat kinase 2; NFT, neurofibrillary tangle; NGF, nerve growth factor; NPC, neural progenitor cell; OPA1, optic atrophy type I; PD, Parkinson's disease; PCL, polycaprolactone; PDL, poly-D-lysine; PLGA, poly(lactic-*co*-glycolic acid); PSEN, presenilin; RA, retinoic acid; ROS, reactive oxygen species; senescence-associated β-galactosidase, (SA-β-gal); sAD, sporadic Alzheimer's disease; SAP, self-assembling peptide; SHH, sonic hedgehog; TNF, tumor necrosis factor.

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https://doi.org/10.1016/j.biomaterials.2023.122143
Received 20 November 2022; Received in revised form 27 April 2023; Accepted 1 May 2023
Available online 2 May 2023

strategies can be attributed to the limitations with current model systems used to study them. Classical 2D culture of primary cells or induced pluripotent stem cells (iPSCs) is limited by lower cell density and higher rigidity of cultureware compared to brain tissue, and the lack of an interstitial compartment prevents pathological extracellular protein aggregation (e.g. amyloid β {Aβ} plaques and NFTs) [8]. Further, monolayer culture limits the portion of cellular membrane exposed to extracellular matrix (ECM), resulting in polarized integrin expression and unnatural cytoskeletal arrangements within the cells [9]. Recent advances in neural organoid culture have enabled high-density, 3D culture environments, but these approaches are currently limited by poor nutrient and oxygen exchange, lack of glial diversity, poor reproducibility and heterogeneity, and a limited culture lifespan [10]. Finally, commonly used murine models of Alzheimer's disease are overwhelmingly reliant on familial early onset AD-associated (fAD) mutations that cause a far more aggressive form of the disease than is observed with the approximately 95% of cases that are sporadic [11,12], and mouse AD models generally cannot recapitulate all disease hallmarks of plaques, NFTs, and neurodegeneration within the same animal [10,13]. Similarly, PD animal models rely on acute toxic insults and hereditary mutations that are not representative of the majority of cases, and show similar limitations in capturing disease hallmarks [14]. These factors, coupled with the species-specific differences in molecular pathways, cell identities, brain networks, lifespan [15–18], and the fact that AD and PD do not naturally occur in non-human animals, limits their relevance for studying these slow-developing human diseases.

While we have learned much about basic pathological molecular and cellular processes from these model systems, they fail to recapitulate essential features of the human brain environment in which neurodegeneration occurs. These collective deficiencies have galvanized the application of bioengineering principles to develop 3D brain tissue models that more faithfully reflect human brain biology and the pathobiology underlying neurodegenerative diseases (Fig. 1).

The goal of this review is to organize the existing literature on functional bioengineered tissue models of neurodegenerative diseases, and to highlight successful neural tissue engineering approaches to guide development of tissue models that more accurately reflect the pathobiology of these diseases in human brains. Our focus is on

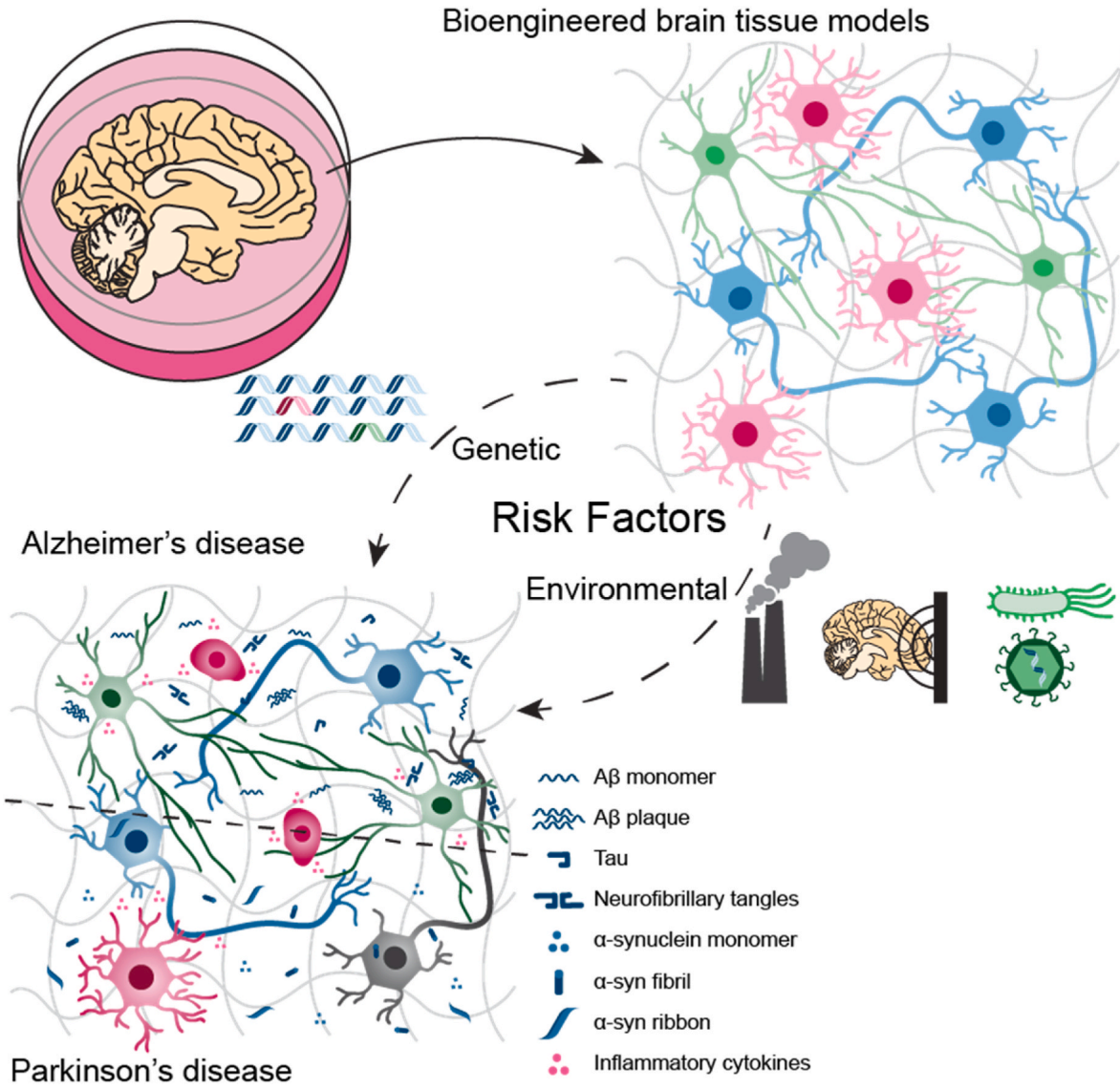

Fig. 1. Functional bioengineered tissue models of neurodegenerative diseases. Bioengineered brain tissues seek to recapitulate the mechanical, cellular, and biomolecular cues of the brain to provide a better mimic of native brain tissue. This enables interrogation of cellular roles and responses in pathological conditions. Such systems could shed light on the contribution of genetic and environmental risk factors to neurodegenerative diseases such as Alzheimer's and Parksinson's disease, and serve as improved platforms for drug development.

functional models of AD and PD, or on functional brain tissue models that are amenable to the study of these diseases. We will discuss biomaterials-based approaches for recreating material and biochemical cues representative of brain cellular niche, and microfluidics-based approaches for controlling and networking cellular microenvironments. We will conclude with perspectives to address two key limitations of existing bioengineered neural tissue models, poor approximation of cellular aging and lack of cellular diversity.

2. Biomaterials: engineering the extracellular matrix

Human brain development takes place in a dynamic and changing environment. Loss of or alteration of the mechanical, biochemical, and/or structural cues can skew stem cell differentiation toward undesired lineages [19,20]. Biomaterials, including naturally-derived and synthetic ECM analogues, can control the presentation of these cues to improve the kinetics and reproducibility of neural tissue development (Fig. 2). Biomaterials need to be cytocompatible, provide adequate mechanical support without being too stiff, and sufficiently porous to allow nutrient and oxygen transport throughout the culture. The biomaterials typically feature intrinsic or chemically-conjugated cell attachment domains. Given the multi-decade pathobiology of sporadic AD and PD, these scaffolds need to be stable under extended culture conditions to capture slow-developing phenotypes. Recapitulating and controlling the presentation of these cues can provide a better mimic of native brain tissue microenvironments, and so improve the reliability of neural tissue synthesis. Finally, these reconstructions of the central nervous system (CNS) ECM need to be chemically defined and synthetically reproducible.

2.1. Hydrogels

Hydrogels, crosslinked networks of hydrophilic polymers that retain their 3D architecture at high water saturation, are one of the most commonly-used biomaterials classes in neural tissue engineering [21]. These intrinsically soft materials are readily modifiable to match brain tissue elastic moduli of <500 Pa, which improves neuronal survival and neurite extension and can promote the selective differentiation of neurons compared astrocytes [19,22]. Recapitulation of these mechanical properties aids neuronal attachment, dendritic organization, and structural rearrangement of F-actin and microtubules during growth cone outgrowth [23–26].

Brain ECM is replete with biochemical cues for developing neurons, accordingly the major constituents of the human brain ECM (glycosaminoglycans {e.g. hyaluronic acid (HA)}, proteoglycans {including neurocan, brevican, versican, and aggrecan}, and glycoproteins {e.g. tenascin}, with small amounts of collagen types I, III, and IV, fibronectin, and vitronectin) [27] are utilized as building blocks for many hydrogel-based 3D culture models (Fig. 2 top). For example, Matrigel, a complex mixture of laminin, collagen type IV, entactin, and other basement membrane components derived from mouse sarcomas [28], is widely-used for neural differentiation and tissue engineering. A notable example is the use of embedded immortalized human neural progenitor cells (hNPCs) overexpressing fAD-associated amyloid precursor protein (APP) and γ secretase subunit presenilin 1 (PSEN1) mutations in Matrigel slabs, which displayed aggregated Aβ phenotypes after 6 weeks and filamentous tau aggregates by 10 weeks [8,29]. Importantly, 3D-differentiated neurons showed increased expression of mature human 4-repeat tau isoforms compared to 2D-differentiated cells, which is critical for reproducing aggregated tau phenotypes in vitro [8]. While Matrigel contains many bioactive molecules necessary for 3D neural tissue culture, its bioengineering applications are intrinsically limited by its poorly defined composition, heterogeneity and batch-batch variability, and the presence of xenogenic contaminants [28]. Further, its complex composition is difficult to modulate for region-specific neural tissue ECM cues [30].

An alternative approach to recapitulate the brain's complex ECM composition has been the use of decellularized brain tissues as cell culture scaffolds. Porcine brain-derived ECM increased average neurite length of N1E-115 murine neuroblastoma cells, which did not occur on spinal cord or urinary bladder ECMs in 2D culture [31]. In contrast to the notion that specifically recreating brain ECM composition enhances neuronal maturation, primary neurons cocultured with astrocytes displayed similar increases in synchronized neural activity and earlier community node formation within a neural network when cultured in decellularized rat pup brain ECM or in MaxGel (non-tissue-specific basement membrane extract) compared to poly-D-lysine (PDL)-only (i.e. no ECM) cultures [27]. However, decellularized tissues are limited by batch-batch variability and mechanical weakness, thus requiring supplementation with other biomaterials to maintain mechanical support [32].

As alternatives to the complex extracts above, a host of naturally-derived and synthetic hydrogels have been generated to provide greater control over composition, molecular and physical cues, and batch-to-batch variability for neural tissue engineering. Examples of naturally-derived materials include HA [33–36], collagen [33], gelatin [33,37], alginate [22,37], and fibrin gels [19], while synthetic hydrogels include polyacrylamide [19,25,26,38,39], polymethylmethacrylate [40], and polylysine [41]. Recently, synthetic self-assembling peptide (SAP) hydrogels have generated interest due to their intrinsic tunability, biocompatibility, and bioactivity due to inclusion of adhesion peptide sequences. The most commonly-used SAP architecture in neural tissue engineering is based on arginine-alanine-aspartate-alanine (RADA) [33,42–45] sequences, although multiple other SAP platforms exist [46–48]. Of relevance to PD, $RADA_{16}$-I hydrogels enhanced the differentiation and maturation of dopaminergic neurons from murine iPSCs and embryonic stem cells (ESCs) [45]. Each of these hydrogel classes contains its own tradeoffs and limitations that need to be navigated. For example, many naturally-derived hydrogels suffer from batch-to-batch heterogeneity and degrade too quickly for long-term culture. Similarly, many SAPs are mechanically fragile, limiting their long-term use. By contrast, synthetic hydrogels are biologically inert and require functionalization with adhesion ligands, and their synthesis often requires environmentally-harmful solvents that must be removed and disposed of post-synthesis.

Coordinating and tuning mechanical and biochemical cues helps to maintain a suitable stem cell niche, which can promote differentiation efficiency and specificity (Fig. 2). Supplementation with retinoic acid (RA) and Sonic Hedgehog (SHH) within collagen type I hydrogels promoted motor neuron differentiation from murine ESCs, while similar supplementation within Matrigel gels promoted dopaminergic neuron differentiation [33]. Advanced applications of these designs have incorporated the release of bioactive molecules or the use of electrical stimulation to improve and directionally guide neuronal differentiation. Hydrogels can control the release of entrapped payloads via degradation of their macroscale networks and individual polymeric units [21], and the gradual release of neurotrophic factors such as nerve growth factor (NGF) [49,50], brain-derived neurotrophic factor (BDNF) [51], glial cell line-derived neurotrophic factor (GDNF) [52], and neurotrophins [53–55] have supported improved neurite or axonal growth. While these applications have been primarily explored in neuroregenerative contexts, controlled morphogen release could provide patterning cues or gradients to enhance neural differentiation within functional models. Electrically conductive components, including metallic nanowires [56], carbon nanotubes [57,58], and conductive polymers (e.g., polypyrole, polythiophene, and polyaniline) [59,60] can be included in composite hydrogels to provide electrical stimulation to developing neurons. Such conductive hydrogels could potentially be used for functionally training neuronal cultures via Hebbian learning. For an excellent review of hydrogels in neural tissue engineering, see Ref. [21].

Hydrogels are essential to the emerging field of bioprinting, an additive manufacturing approach which deposits cellular suspensions

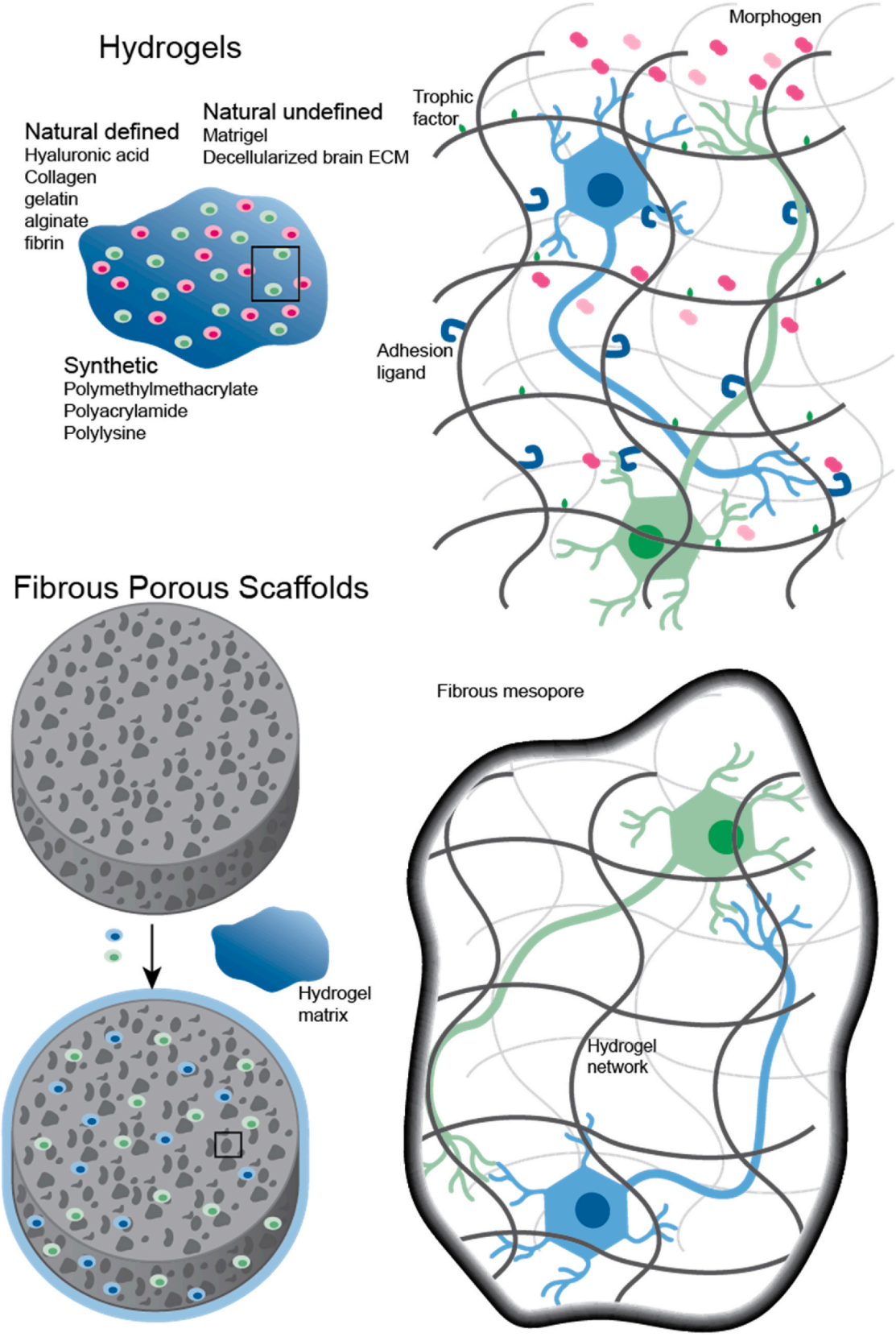

Fig. 2. Biomaterials reproduce brain tissue extracellular matrix. Hydrogels and porous fibrous scaffolds recapitulate the architectural and biomolecular complexity of native brain tissue. This biomimetic environment is a more-favorable stem cell niche, improving neuronal differentiation and viability. Multiple patterning approaches, including supplemented growth factors, immobilized adhesion and trophic factors, and establishment of morphogen gradients, work in concert to enhance differentiation efficiency and specificity. Hydrogels provide a soft substrate to support neurite extension, but can have limited mechanical or chemical stablity. Porous fibrous scaffolds can supplement the mechanical support of hydrogels while maintaining nutrient supply via mesoporous networks.

within hydrogels, termed bioinks, in controlled layer-by-layer patterns. This can provide greater control over the patterning of cellular, structural, and molecular constituents than conventional tissue engineering techniques, allowing production of heterogeneous tissues that mimic physiological organization. Historically, the shear stresses and corresponding low viability associated with bioprinting have limited its utility for sensitive cell types, although recent advances have enabled bioprinting of human neural tissues from iPSC cell suspensions [61], iPSC aggregates [62], and NPCs [63–65]. Bioprinting requires balancing of intrinsic tradeoffs between printing resolution, mechanical stability, cell viability, tissue organization, and cellular diversity [66]. Successful management of these design decisions have allowed construction of complex-tissue architectures like laminated neural sheets [67,68] spatially-controlled hydrogel composition [69], and cell-segregated domains [68].

2.2. Porous/fibrous scaffolds

While hydrogel-based scaffolds can mimic brain mechanical and biomolecular features, they have a limited capacity for long-term culture due to gel contraction-induced heterogeneities [70] and rapid degradation rates [71]. Solid porous or fibrous scaffolds prepared from naturally-occurring or synthetic biomaterials offer enhanced mechanical and chemical stability for long-term culture and long-range porous structures for enhanced nutrient supply and neurite extension (Fig. 2), thus offering attractive alternatives or adjuvants to hydrogel-based 3D culture systems.

One such scaffold system based on porous silk fibroin-collagen hydrogel composite scaffolds has successfully modeled a variety of neurodegenerative conditions. These scaffolds have shown higher neuronal viability and neurite outgrowth than collagen-only systems [72]. They are mechanically stable for months-years timescales, enabling stable culture of iPSC-derived cortical neurons for over two years [73]. In this long-term study, AD-like phenotypes of increased Aβ 42/40 and pTau/tTau ratios and lower spontaneous spike frequency (compared to healthy control cultures) were observed in a subset of cultures derived from an APOE ϵ3/ϵ3 sAD iPSC line, and these phenotypes only occurred in cultures maintained for at least one year [73]. In another study, dopaminergic neurons differentiated from iPSCs harboring the two most prevalent PD-associated mutations, in Leucine-rich repeat kinase 2 (LRRK2, G2019S) or glucocerebrosidase (GBA, N370S), cultured in 3D silk-collagen scaffolds displayed increased α-synuclein expression, altered purine pathway metabolism (dysfunctions of which have been implicated in PD [74]), and increased transcription of dopaminergic neuron genes compared to 2D-cultured neurons [75]. Transcriptomic analysis revealed novel mutation-specific molecular interactions, some of which were unique to 2D or 3D culture, and some of which were shared between 2D and 3D [75]. In one final example of this silk-collagen platform, cortical neural 3D tissues infected with Herpes simplex virus type I (HSV-1) developed Aβ plaques, altered transcription of APP-processing proteins, increased expression of gliosis and neuroinflammatory markers glial fibrillary acidic protein (GFAP) and tumor necrosis factor (TNF), and reduced spontaneous spike frequency compared to uninfected tissues [76]. Excitingly, this viral infection model produced these AD-associated phenotypes within only one week of infection, and this study provided some of the first evidence of HSV-1's direct causality in AD. This viral infection model has since been applied in a neuroprotective compound screen, which identified green tea catechins and resveratrol for displaying anti-plaque and functional neuroprotective benefits with minimal toxicity [77].

Multiple other solid porous scaffold platforms for neural tissue culture exist, often applied in neuroregenerative contexts. However, few of them have been directly applied to studying age-associated neurodegenerative diseases, and accordingly will not be covered in depth. Porous scaffolds based on gaseous salt-leached polystyrene [78], graphene foam [79], and polycaprolactone (PCL) [80] have shown enhanced viability, cytocompatibility, and improved neural induction. Similarly, freeze-drying has been used to synthesize porous scaffolds from a collagen-hyaluronic acid mixture which recapitulates brain tissue porosity and mechanical moduli [81,82], or from collagen modified with adhesive peptides and crosslinked with gluratarаldehyde [83]. Given the long-term mechanical and morphological stability of porous scaffolds, improved nutrient transport, and ability to reproduce native brain tissue mechanical moduli, they are well-suited for studying slow-developing conditions like neurodegenerative diseases. However, the increased labor time required for their preparation reduces their suitability for high-throughput screening. More study is needed to explore their potential advantages as functional neural tissue models in these diseases.

2.3. Scaffolded organoids

Brain organoids generated from iPSCs are fundamentally a self assembly-driven, bottom-up approach to neural tissue engineering. While they are excellent models of early developmental events, organoid approaches are currently limited by batch-to-batch variability, heterogeneity within batches, poor nutrient diffusion beyond the outer 300–400 μm of the organoid [84], and resulting formation of necrotic cores. Bioengineering approaches have been utilized to modify these intrinsic processes to improve the viability and maturation of the cultures.

To overcome organoid diffusional limitations, some groups have mechanically sectioned organoids into thin slices and cultured them using adapted organotypic slice culture techniques (Fig. 4, upper right). Cerebral organoid slices cultured at an air-liquid interface displayed improved neuronal survival and maturation, and reduced the expression of activated astrocyte markers compared to whole organoids [85]. These slice cultures showed more mature axonal outgrowth characteristics, such as anisotropic bundling and axonal tract decussation, than is typically observed in vitro, and these subcortical tracts could innervate mouse spinal cord explants to stimulate coordinated muscle contractions [85]. Another study found that periodically slicing neocortical organoids every 4 weeks improved oxygenation and reduced apoptosis in the core, and enabled the formation of distinct cortical layers (which do not typically segregate in whole organoids) by 120–150 days [84].

Other studies have employed biomaterial scaffold micropatterning to direct organoid development toward a higher aspect ratio to improve nutrient and morphogen supply (Fig. 4, upper left). HiPSCs were seeded onto randomly configured microfilaments, and found the cells adhered well to 10:90 poly (lactic-*co*-glycolic acid) (PLGA) or sea sponge-derived fibers, but not to cellulose fibers [86]. PLGA micropatterned cerebral organoids showed increased forebrain patterning specificity, and a simulated reconstitution of the basement membrane through dissolved Matrigel recapitulated the radial cortical plate organization of the cerebral cortex [86]. Similarly, ventral midbrain organoids patterned with recombinant spider silk microfiber scaffolds functionalized with laminin-111 showed reduced hypoxia and cell death in the inner core, and improved the consistency of dopaminergic neuron marker expression between the outer and inner organoid layers [87]. These trends of enhanced neuroectodermal specification, more homogeneous and functional neuronal maturity, and reduced hypoxia were recently observed for cerebral organoids in a followup study [88]. Another study seeded hESCs suspended in Matrigel onto 3D-printed PCL 12 x 12 × 0.25 mm honeycomb lattices to form flat brain (cerebral) organoids, which demonstrated spontaneous gyrification around the organoid edges within three weeks and showed no interior necrosis [89]. However, these flat brain organoids showed much lower MAP2 transcript expression than spherical organoids, calling into question the maturity of the neurons [89]. Beyond simply improving nutrient diffusion in organoids, these sliced organoid and scaffolded organoid approaches could facilitate drug discovery and mechanistic studies by improving drug distribution within the organoids.

A similar patterning approach has shown promise in 2D neural rosette culture. Controlled neural rosette size was afforded by micropatterned Matrigel-coated surfaces and enabled >70% efficiency in generating singular forebrain and spinal cord rosettes, which could improve the reliability of CNS organoid morphogenesis [90]. A hybrid approach employed 2D laminin-521-micropatterns to constrain model neural tube widths, followed by Matrigel supplementation to trigger in vitro neurulation folding and closing events with 90% efficiency [91]. While the bioengineering community has contributed impressive advancements toward organoid engineering via reproducible, chemically defined matrices with controllable material properties and adequate nutrient supply, an ongoing challenge is the incorporation of morphogen gradients to improve tissue specification [92,93]. As we acquire greater understanding of developmental processes guiding brain tissue formation and differences between cell states and origins in pathology, biomaterials will continue to serve as important tools in next-generation tissue models of neurodegenerative diseases.

3. Microfluidics: controlling microenvironmental culture conditions

While the previous biomaterial scaffold approaches can provide architectural similarity to human brain tissue, they sacrifice microenvironmental control within the culture. An alternative approach applies bioreactor and microfluidic principles to engineer microscale culture chambers with tightly controlled culture conditions, including perfusion rate and establishment of chemotactic or morphogenic gradients. This isolated compartmental approach is well suited for analysis of metabolites, transcripts, or proteins at up to single-cell resolution, or for networking different tissue-on-a-chip systems for inter-tissue pathological studies (so-called microphysiological systems or body-on-a-chip devices) [94]. Many such systems include microsensors for onboard monitoring of culture conditions, e.g. multielectrode arrays for nondestructive repeated measurements of electrical activity, or genetically encoded calcium or voltage indicator (GECI/GEVI)-based optical readouts, and some feature electrodes or LEDs for electrical or optical stimulation of neural networks [95]. Scalability and modularity is a major feature of many microfluidic systems, allowing parallel testing of multiple experimental conditions within standard well plate footprints.

3.1. Compartmentalized models of pathological protein spread and neurodegeneration

Microfluidic systems' fluid flow rate, composition, and compartmental control have proven useful as models for interrogating pathological protein spread in neurodegenerative disease, particularly AD. One commonly-used experimental paradigm with separate soma and axon compartments separated by microchannels [96] established

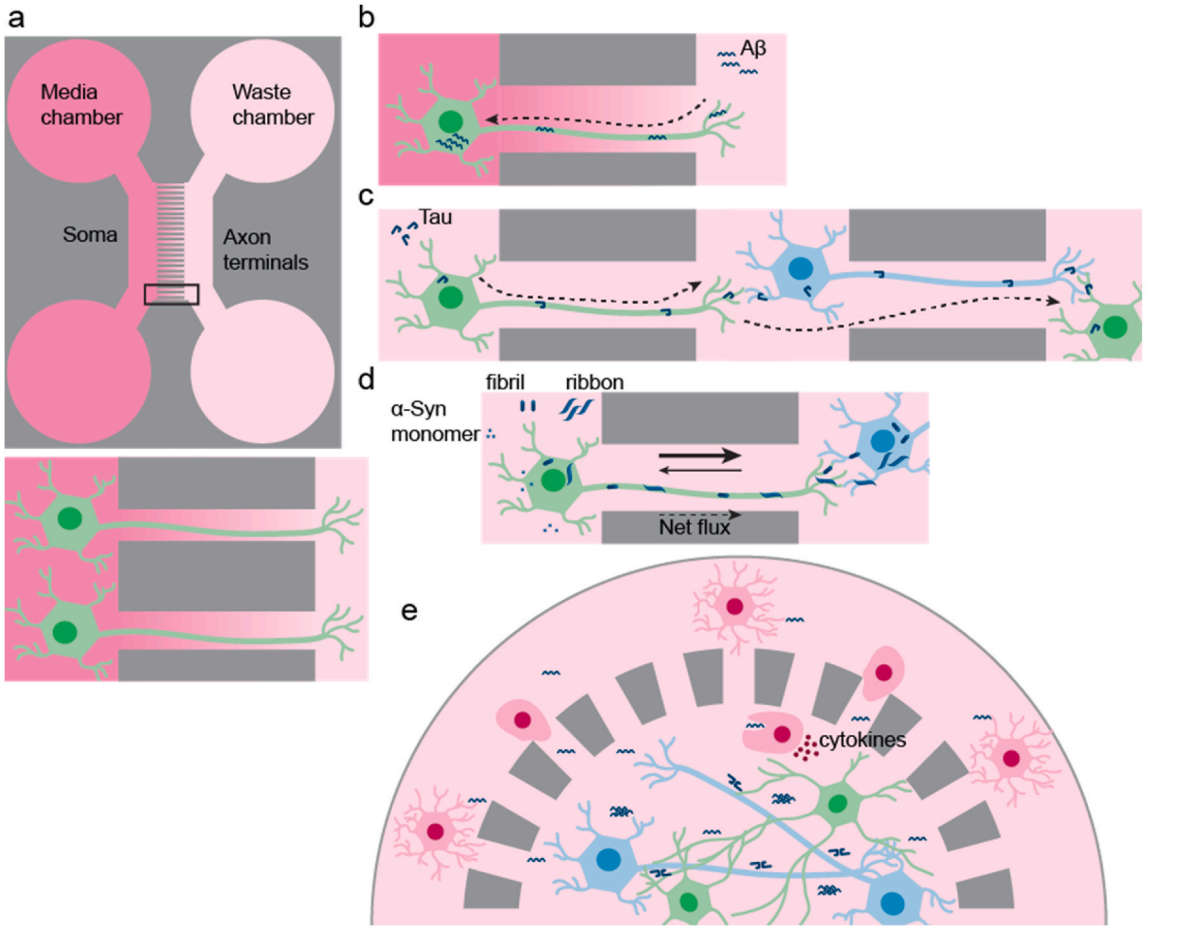

Fig. 3. Microfluidic compartmentalized models of neurodegenerative diseases. a, The most common device architecture, featuring separate neuronal soma and axon compartments that are linked via microchannels. Hydrostic force maintained via perfusion isolates intracellular pathological protein transportfrom diffusive transport. Such devices have established prion-like spread of (b) Aβ via retrograde transport [97] and (c) tau protein via anterograde *trans*-synaptic transport across as many as three different neuron populations [99,100]. d, α-sunclein fibril and ribbon aggregates have demonstrated both anterograde and retrograde transport, with a net anterograde flux. Interestingly, α-synuclein monomer was internalized only transiently and did not traffick to downstream neurons [104]. e, A radial organization of this 2-compartment format modeled microglial chemotaxis, axonal cleavage, and release of neurotoxic factors in response to neuronal Aβ secretion [105].

retrograde monomeric $A\beta_{1-42}$ spread from axonal compartments to soma via plasma membrane-dependent processes within 24 h, resulting in significant cell death by 72 h (Fig. 3b) [97]. This was some of the first direct evidence supporting neuron-neuron prion-like spread of Aβ, explaining the tendency of plaques to spread via neuroanatomically connected regions [98]. Similar compartmentalized models have demonstrated anterograde transsynaptic transport of wild-type human tau protein between primary rat neurons [99] and between up to three different compartments of primary mouse neurons (Fig. 3c) [100]. A higher-throughput iteration on this concept applied high-content imaging for quantification of tau aggregate count and morphological composition, which could be a useful tool for inhibitor screening [101]. Other applications of this compartmental model have identified Aβ oligomer-induced deficiencies in retrograde axonal BDNF transport [102], and that cortical application of Aβ oligomers and fibers induces presynaptic collapse and postsynaptic tau hyperphosphorylation in engineered cortico-hippocampal networks [103]. Similarly to Aβ and tau, compartmentalized microfluidic models have shown that human α-synuclein fibril and ribbon aggregates are trafficked between neurons, and can induce the formation of structures similar to the PD pathognomonic hallmarks Lewy bodies and Lewy neurites (Fig. 3d) [104].

One model recapitulated human nigro-striatal circuits by seeding iPSC-derived dopaminergic and medium spiny neurons into chambers connected via microgrooves to a central synaptic chamber, which showed that dopaminergic neurons derived from patients with a genetic form of PD featuring mutations in dynamin-related GTPase optic atrophy type I (OPA1) had a dramatically reduced mitochondrial count and aberrant mitochondrial morphologies and functions, which were associated with degeneration of dopaminergic synapses [106]. In a two-compartment microfluidic model, the molecular tweezer CLR01 was shown to reduce α-synuclein oligomer aggregation and to interfere with α-synuclein active axonal transport by disrupting its interactions with kinesin [107].

3.2. *Tissue-specific models of cellular pathology*

The precise control of compartments and media flows afforded by microfluidics has also proved a useful tool to investigate sub-tissue and cross-tissue dynamics of neurodegenerative pathobiology. One platform

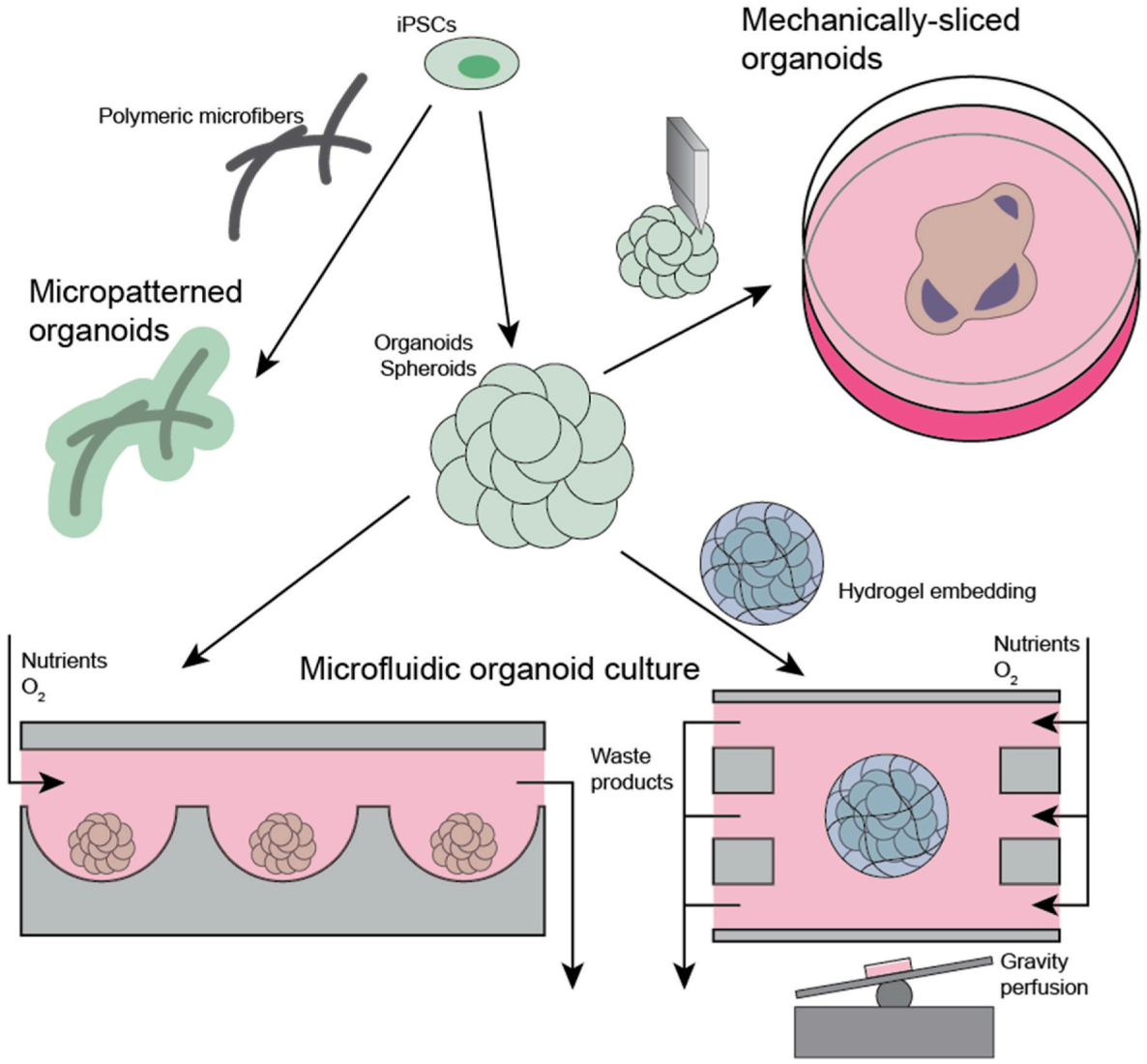

Fig. 4. Bioengineering improves organoid reproducibility and maturity. Upper left, Biomaterial microfibers provide patterning scaffolds for organoid develeopment. The higher aspect ratio of micropatterned organoids improves nutrient and oxygen supply and reduces heterogeneity between organoids. Upper right, Mechanically sectioning organoids into thin slices improves nutrient and oxygen diffusion and enhances neuron maturation. Bottom, Microfluidic bioreactors provide greater control of the organoid culture environment, improving nutrient and oxygen supply, waste removal, and morphogen gradient establishment. This enhances organoid growth, viability, and functional maturation compared to conventional organoid culture.

used a centralized channel with bound Aβ and a gradient of soluble Aβ to investigate microglial chemotaxis from an outer chamber through radial homeostatic microglia-excluding narrow channels, which found that soluble Aβ acts as a recruitment signal and that bound Aβ acts as a targeting signal for developing plaques [108]. Soluble Aβ chemotaxis showed an interesting biphasic response, which revealed that at low Aβ concentrations microglia supplement recruitment via secretion of the cytokine MCP-1 [108]. A later iteration of this radial compartment system seeded neurons and astrocytes overexpressing familial AD mutations in the central chamber as cellular source of soluble Aβ for microglial chemotaxis, which recapitulated microglial axon cleavage and nitric oxide-induced neurotoxicity (Fig. 3e) [105].

Blood-brain barrier (BBB) and vascular dysfunction have been implicated as an important pathogenic component of neurodegenerative diseases [109], but commonly used tissue models lack cellular diversity (e.g. astrocytes and neurons) and the 3D organization typical of the BBB. An innovative microfluidic system used a parallel chamber design, with a 3D NPC chamber and a tubular brain endothelial cell (bEC) chamber separated by an air-gapped chamber during differentiation and maturation [110]. Filling the air-gapped chamber with collagen completed the neurovascular unit, wherein fAD mutation-expressing cultures showed decreased tight junction protein expression, increased BBB permeability, increased reactive oxygen species (ROS) production, and deposition of Aβ on the endothelial wall compared to healthy cultures [110]. Other microfluidic models of the BBB or neurovascular unit have shown that AD patient serum altered brain vascular endothelial cell transcriptional expression towards impaired tight junction function and increased permeability [111], and have demonstrated metabolic coupling between endothelial cells, pericytes, astrocytes, and neurons within the neurovascular unit [112]. Multiple microfluidic NVU cellular compositions have accomplished tight junction formation and BBB permeability benchmarks, including both primary and iPSC-derived endothelial cells, pericytes, astrocytes, and neurons [113–115]. An ongoing challenge is to include all these cellular constituents in the same device to enable analysis of interactions between these cells types in pathological states.

Finally, microfluidic-controlled morphogen gradients have been used to improve the regional specification of developing neural tissues, such as using a glycogen synthase kinase 3 inhibitor to establish WNT-activating gradients, resulting in progressive caudalization of a model neural tube [93]. Similarly, a microfluidic stratified bioreactor using Matrigel-embedded iPSC-derived dopaminergic neurons demonstrated dopaminergic degeneration and mitochondrial dysfunction in cultures with LRRK2-G2019S mutations, but did not show α-synuclein accumulation [116]. Such advances in regional specification could improve differentiation efficiencies to provide a closer mimic of tissues relevant to neurodegenerative diseases.

3.3. Microfluidic-controlled organoid culture

Given concerns over heterogeneous and poorly reproducible organoid differentiation protocols, the microenvironmental control precision afforded by microfluidic bioreactors is an appealing path to enforce constraints on these cultures. One approach deposited NPC spheroids into a series of microwells with connected channels in segregated patterns of healthy and fAD organoids, which were then embedded in Matrigel [117]. Axonal connections formed between spheroids along the channels as the neuronal cultures differentiated and matured, wherein AD-AD connections showed a reduction in axonal bundle density and Aβ plaque aggregation and spread could be followed [117]. In a similar approach, the role of cerebral interstitial flow was investigated via microchannels with concave microwells for rat cortical spheroid culture, and the continuously perfused organoids grew larger and developed more complex neuritic outgrowths than static-cultured organoids (Fig. 4, bottom left) [118]. Interestingly, perfused Aβ monomer caused more neurotoxicity and Aβ aggregation within spheroids than static culture, suggesting an important role for interstitial flow in these pathological processes [118].

Other groups have optimized microfluidic systems toward lower media usage and simpler perfusion apparatuses while maintaining nutrient supply. iPSC-derived cerebral organoids were cultured in a panel of decellularized porcine ECMs derived from brain, intestines, liver, and heart within a microfluidic chamber designed for gravity-based media perfusion, and it was found that brain ECM yielded the largest organoid size and highest neuronal specification (even compared to Matrigel) (Fig. 4, bottom right) [119]. In this landmark study, these bioengineered organoids showed dramatically improved neuroepithelial development, mature and electrically active neurons within 2–2.5 months, and complex brain cell population with a range of neuronal and glial cell identities compared to earlier conventional organoid cultures [95]. Another approach employed a 3D-printed tubular resin-based scaffold with 250 μm mesh pores seeded it with neuro-2a murine neuroblast embryoid bodies, wherein rocking motion provided a steady flow of media through the tubular lumen from connected reservoirs and allowed non-invasive introduction of isogenic iPSC-derived microglia [120]. Tubular forebrain and cerebral organoids improved forebrain neuronal progenitor differentiation efficiency and were less heterogeneous compared to conventional organoids [120].

Microfluidic technology is well-suited for engineering simplified reaction conditions in which to study neurodegenerative pathobiology and constrain neural tissue development. These systems have contributed valuable knowledge concerning the spread of pathogenic proteins in AD and PD, and of critical interactions between CNS cells in diseased states. Their scalability and adaptability to standard well plate formats makes them promising tools for drug discovery and screening. However, long-term cultures remain a challenge for microfluidic systems [121]. Many microfluidic neural tissue models are 2D or 2.5D, and carry the concomitant limitations of limited ECM contact, loss of cell-derived paracrine signals, and limited architectural breadth and complexity [122]. Despite improvements in nutrient supply, waste removal, and morphogen delivery in microfluidic organoid approaches, control of organoid shape, size, and arrangement of cellular constituents remains limited [123]. Finally, microfluidic device synthesis requires microfabrication technical expertise and specialized equipment, which may act as a barrier to entry for neurobiology-oriented laboratories [124]. Addressing these limitations will maintain the important role of microfluidics-based neural in neurodegenerative disease research in the years to come.

4. Current limitations, future directions, and perspectives

4.1. In vitro aging strategies

Despite the promise of iPSC technology to create patient- and disease-specific culture models, recapitulating cellular hallmarks of aging lost during reprogramming is a significant hurdle for modeling age-associated neurodegenerative diseases (Fig. 5a) [125]. This can be partially avoided via direct transdifferentiation [126,127], but the development of such protocols are still in their infancy. Manual aging by designing long-lived culture platforms [73] is one strategy, but carries the downsides of greater cost (simply by using more culture media) and increased risk of contamination compared to shorter-lived cultures. To overcome these limitations, several chemical and biological strategies for inducing aging in cultures have been proposed.

Taking a cue from Hutchinson-Gilford Progeria, an early aging disorder caused by a mutation in nuclear envelope protein lamin A, RNA transfection-induced overexpression of progerin in iPSC-derived neurons produced some aging associated phenotypes, such as neuromelanin accumulation in dopaminergic neurons, nuclear abnormalities, DNA damage, and increased ROS production, along with aging-associated susceptibilities, such as increased apoptosis and neurodegeneration in PD neurons compared to healthy controls (Fig. 5b) [129]. However,

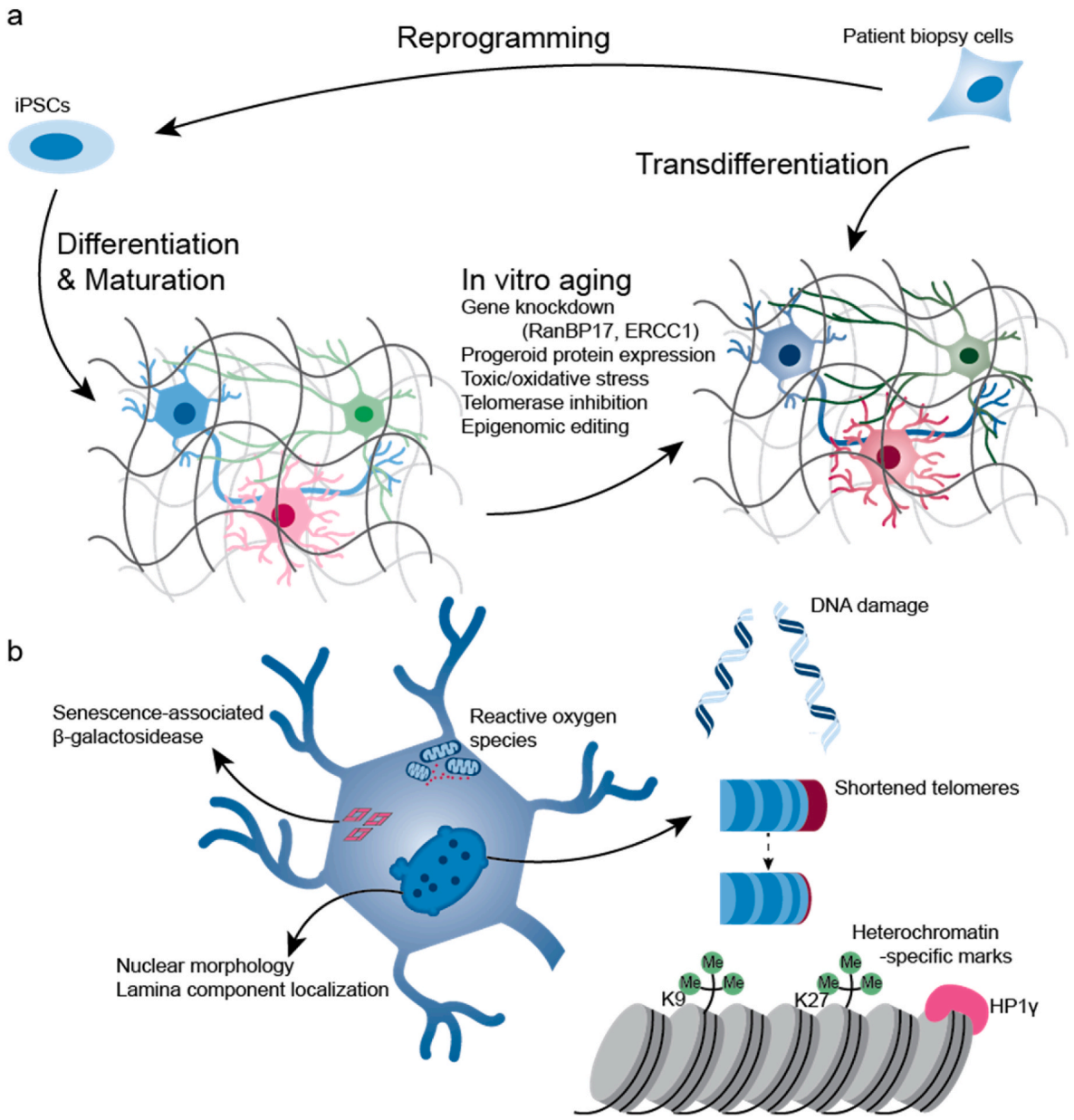

Fig. 5. Recapitulating cellular aging and diversity in bioengineered brain tissues. a, Most signatures of cellular aging are lost upon iPSC reprogramming, some of which can be maintained via transdifferentiation. A complex population of patient-derived cells can be differentiated from iPSCs (depicted are neurons, astroctes, and microglia in blue, green, and magenta, respectively) and seeded into bioengineered tissue platforms to reproduce complex intercell neurotoxic pathways. Aging can be simulated in these bioengineer tissues by knockdown of aging-associated genes, expression of early-aging protein mutants, induction of toxic or oxidative stress, or by altering the epigenomic state. b, The cellular hallmarks of aging include aberrant nuclear morphologies (depicted buds, aggregates, and speckles), altered nuclear lamina component localization, accumulation of DNA damage (particularly double-strand breaks, measured by γH2AX), levels of aging-specific heterochromatin marks (including H3K9me3, H3K27me3, and HP1γ), shortened telomere length, mitochondrial ROS production, and expression of senescence-associated β-galactosidease (SA-β-Gal). For further discussion see Refs. [128,138].[129,138]. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

these artificially aged neurons did not show altered expression of lamina associated-protein LAP2α or heterochromatin markers H2K9me3 and HP1γ, or indications of senescence via senescence-associated β-galactosidase (SA-β-gal) assays, likely a limitation due to CNS expression of microRNAs targeting progerin [129,128]. The relevance of early-aging syndromes as models of normal aging also remains unclear. An alternative approach is to knockdown expression of aging-associated genes, such as the nuclear pore transport protein RanBP17 whose knockdown induces nucleocytoplasmic compartmentalization deficits [127]. The DNA repair protein ERCC1 is another potential knockdown target, mimicking the accumulation of DNA damage in aging [130] and causing synaptic protein and signaling changes, contributing to accelerated neurodegeneration, neuroinflammation, and cognitive decline [131–133]. Toxic stressors such as pesticides and reactive oxygen species have also been used to compress DNA damage accumulation times and induce mitochondrial dysfunction as aging simulants, particularly in PD iPSC models [134–139].

Pharmacological inhibition of telomerase (e.g. using BIBR1532) throughout a dopaminergic neuron differentiation protocol induced a modest shortening of telomeres, along with increased DNA damage and mitochondrial ROS generation, reduced dendrite counts, and reduced production of (dopamine synthesis-associated enzyme) tyrosine

hydroxylase [140]. Finally, recent studies have identified epigenetic DNA methylation signatures for AD [141], PD [142], and chronological aging [143]. These methylation landscapes could hypothetically be induced or restored via epigenomic editing tools such as CRISPRon/off [144]. Although each of these methods contains attractive features, aging remains a complex and poorly understood biological phenomenon. Reduction of this process down to a single element or stimulus may not be able to capture this complexity, so some combinations of these methods may be required. Further, the majority of these studies were performed only in 2D cultures, so it remains to be seen if their conclusions hold true in 3D environments.

4.2. Bioengineered coculture models

Recent studies have demonstrated the contributions of glial-neuron interactions to neurodegeneration and neurotoxicity, including fragmented mitochondria-induced astrocyte activation and neuronal death [145,146] and activated astrocyte release of neuron and oligodendrocyte apoptosis-inducing lipoparticles enriched with apolipoproteins E and J [147]. Further, many AD risk-associated variants identified by recent genome-wide association studies are highly expressed in astrocytes (e.g. APOE, clusterin/CLU, and adenosine triphosphate-binding cassette transporter A7/ABCA7), microglia (e.g. triggering receptor expressed on myeloid cells 2/TREM2, sortilin-related receptor 1/SORL1, complement receptor 1/CR1, CD2-associated protein/CD2AP, phosphatidylinositol-binding clathrin assembly gene/PICALM, and CD33), and oligodendrocytes (e.g. bridging integrator 1/BIN1) [148]. Given these contributions to homeostatic and disease states, glia represent an untapped source for novel therapeutic or diagnostic approaches. For example, microglia in AD could potentially be reprogrammed toward more-homeostatic, plaque-clearing function [149,150], or provide new biomarkers for diagnostics [151].

Despite this evolving appreciation for glial roles in neurodegenerative pathobiology, existing models are overly reliant on neuronal monocultures (or near-monocultures containing low numbers of spontaneously differentiated astrocytes). Few bioengineered brain tissue models include microglia, and fewer include oligodendrocytes. Similarly, most BBB models do not include all cellular components of the neurovascular unit, composed of endothelial cells, astrocytes, pericytes, and neurons, neglecting their roles in BBB maintenance and function and limiting the ability to interpret interactions between these cells in disease states. Future designs of bioengineered tissue culture models of neurodegenerative disease should seek to incorporate a broader swath of this cellular diversity to improve their mimicry of native brain tissue (Fig. 5a). This could shed more light on the specific roles of genetic variants within different cell types in these diseases, or reveal new neurodegenerative or neuroprotective interactive pathways between these cells (Fig. 1). The ultimate hope is that investigating these paths in models that closely resemble the cellular and non-cellular features of human brain tissue will yield novel diagnostic and therapeutic strategies with improved odds of clinical translation relative to historically-used model systems.

4.3. Perspective: How closely does brain ECM need to be recapitulated?

An outstanding question in neural tissue engineering is how closely the brain extracellular environment needs to be emulated to develop functional brain tissue models. Animal models harboring knockouts of or mutations in most brain ECM component knockouts develop aberrant brain morphologies and deficits in neural circuit formation, which would speak to the necessity of the brain ECM microenvironment for healthy development and activity [152,153]. While decellularized brain ECM can clearly reproduce the complexity of native ECM, how well do other ECM substitutes perform in comparison?

A few studies have directly compared decellularized brain ECM to other decellularized ECMs or alternative hydrogels. One study compared decellularized brain ECM, skin basement membrane ECM, and non-ECM PDL coatings [27]. This study found that differences in ECM composition did not affect electrical activity, glial proliferation, or astrocyte phenotypes, but did find that brain ECM shortened the time for electrically active community formation by approximately one week. Both ECM types accelerated network formation compared to the PDL coating controls. Another study from our group compared primary rat neuronal network formation in porous silk scaffolds embedded in Matrigel, Hydromatrix, HyStemC, Puramatrix, collagen I, fetal porcine brain ECM, and adult porcine brain ECM [154]. This study found that the synthetic matrices (Hydromatrix and Puramatrix) degraded too rapidly for network formation, and similarly poor network formation occurred in fibrin and HyStemC matrices. Both Matrigel and collagen I formed networks, but collagen I formed the most-dense networks of the commercially-available gels. Between the decellularized brain ECMs and collagen I, fetal brain ECM produced the most-dense networks and produced electrical activity within one week of culture, whereas adult brain ECM and collagen I were not electrically active at that timepoint.

These comparative studies, combined with ECM deposition and remodeling by neurons, astrocytes, microglia, and other cellular constituents of the brain [155,156], would seem to indicate a minimum engineering requirement to provide adequate mechanical cues and adhesion sites (i.e. not necessarily emulating the precise composition of brain ECM) within a scaffold to allow healthy neural culture survival and development, including network formation and spontaneous electrical activity. Beyond this minimum, incorporating brain-specific ECM components hastens the development and maturation of these cultures, potentially by reducing the reliance on seeded cells for ECM production. However, given our limited understanding of human neurodevelopment, brain regional differences in ECM composition, and precise roles and functions of ECM components in brain health and activity, this issue is far from settled.

4.4. Perspective: How long is long enough for sporadic degenerative disease models?

In vitro models of sporadic AD and PD are of limited utility if they require similar decades-long timescales as in vivo pathogenesis. Ideally, these models can recapitulate as many features of the diseases as possible in as short a time as possible. But a lurking question remains: how long is long enough?

Spontaneous AD phenotypes have been observed in a familial AD cerebral organoid model within 2–3 months [157], while similar phenotypes only presented after one year in spontaneous AD patient iPSC-derived 3D brain tissue models (using porous silk and collagen I gel scaffolds) in our lab [73]. We have observed plaque formation and neurodegeneration in a virally-induced model of sporadic AD within 10 days after infection (totaling 1.5–2 months total culture time in 3D) [76,77], and familial PD 3D brain tissue models in our lab presented characteristic PD phenotypes after 1.5 months [75]. It seems clear that in vitro pathogenesis can occur over much shorter timescales than in vivo, with approximately 1–1.5 years seemingly sufficient from our studies to capture slow-developing phenotypes. However, the evidence base is very limited.

Artificial aging strategies (section 4.1) seem to be a promising approach to shorten this timeline, and telomerase inhibition and oxidative stress are likely to be the most practicable, least pathobiologically-interfering approaches. These approaches are also likely to be useful in glia-containing models, as ROS/RNS production is an essential (and forward propagating) component of neuroinflammation in AD [158]. This remains speculative, as to our knowledge these strategies have not been investigated in the context of sporadic AD or PD. Comprehensive profiling techniques, like RNA sequencing or proteomics, will be essential for validating the relevance of such approaches, such as by directly comparing engineered neural tissues and native brain tissue from healthy and diseased patients. Such

direct comparisons would also be helpful for evaluating the relevance of existing neural tissue models.

5. Conclusions

By recapitulating the complex physical, biochemical, and cellular constituents of human brain tissue, bioengineered brain tissues can provide more physiologically representative in vitro models of human neurodegenerative diseases than existing monolayer, organoid, or murine animal model systems. Despite significant progress in engineering platforms for greater control and reproducibility of neural cultures, much work remains to simulate aging and to integrate a greater diversity of cell types within these cultures with both short- and long-term culture fidelity. Combining these systems with patient-derived cells and modern gene engineering technology offers the tantalizing potential to, after all these decades, finally begin to understand how genetic risk factors interact with environmental factors to produce neurodegenerative diseases. Realizing this potential could aid the long-standing search for reliable, specific diagnostics and disease-modifying therapies.

Credit author statement

ASM and DLK conceived the concept. ASM wrote the initial manuscript. Both authors participated in manuscript revision.

Funding

This research was supported by the NIH (R01AG061838-02, P41EB027062, K12GM133314). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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