



# *In vitro* engineered models of neurodegenerative diseases

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**Key words:** Neurodegenerative diseases, *In vitro* models, Scaffolds, Organoids, Microfluidic devices

**Abstract:** Neurodegeneration is a catastrophic process that develops progressive damage leading to functional and structural loss of the cells of the nervous system and is among the biggest unavoidable problems of our age. Animal models do not reflect the pathophysiology observed in humans due to distinct differences between the neural pathways, gene expression patterns, neuronal plasticity, and other disease-related mechanisms in animals and humans. Classical *in vitro* cell culture models are also not sufficient for pre-clinical drug testing in reflecting the complex pathophysiology of neurodegenerative diseases. Today, modern, engineered techniques are applied to develop multicellular, intricate *in vitro* models and to create the closest microenvironment simulating biological, biochemical, and mechanical characteristics of the *in vivo* degenerating tissue. In THIS review, the capabilities and shortcomings of scaffold-based and scaffold-free techniques, organoids, and microfluidic models that best reflect neurodegeneration *in vitro* in the biomimetic framework are discussed.

## Introduction

More than 600 neurodegenerative diseases (NDs) associated with atrophy affecting the central and/or peripheral zones of the nervous system have been described [1]. NDs are identified as hereditary, idiopathic, and sporadic conditions attributed to progressive nervous system dysfunction. Alzheimer's disease (AD), Parkinson's disease (PD), multiple sclerosis (MS), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS), epilepsy, encephalitis, and brain cancers are some of the known NDs.

Despite significant advances in defining the pathogenesis of NDs, effective treatments are not currently available. This is mainly due to the complexity of human brain physiology, the inability to clarify cause-effect relationships in the pathologies, limited access to human brain tissues, and restrictions of *in vivo* and *in vitro* models that are used in understanding the disease phenotype.

Experimental models of NDs are critical to understanding the pathophysiology better and to evaluate

the potential of new therapeutic approaches. Current experimental models of NDs focus on a particular aspect of a complex pathology, or, more reductively, they are "mechanism models" that provide the closest natural replication of the disease. For this reason, no matter how successful the pre-clinical studies are similar results cannot be obtained in clinical studies [2]. All experimental models, including transgenic rodents, invertebrate animal models, and *in vitro* cell culture models, have distinct characteristics and limitations. Therefore, it is very important to know the targeted pathophysiology in the planned study and to choose the appropriate model.

Animal models of NDs cannot precisely recapitulate the pathophysiology of the disease in humans since NDs rarely occur spontaneously in animals, and the pathological mechanisms in animals poorly reflect those in humans [3]. *Ex vivo* disease models have become important tools to complement *in vivo* models to closely examine damage mechanisms. While organotypic slice cultures provide advantages in terms of examining cell dynamics and disease mechanisms, the chance of modifying and engineering them as much as *in vitro* models is low and is limited to applications allowed by tissue integrity [4]. *In vitro* models allow the use of human cells, unraveling the doubts due to species differences. The common *in vitro* models are in two

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Received: 24 August 2023; Accepted: 21 November 2023;

Published: 30 January 2024



different culture systems: two-dimensional (2D) and three-dimensional (3D). Classical 2D *in vitro* models are the most basic culture systems studied, and essential information about cellular interactions is obtained from these models. 2D models simply comprise the induction of the relevant cell type (or types) *in vitro* and the application of a neurotoxin to damage the cell type of interest (Fig. 1). However, these systems fall short of modeling the complex cellular, biochemical, and physical microenvironment of human tissues. Therefore, it is necessary to engineer functional 3D *in vitro* models that mimic the microenvironment of tissues and cell-to-cell interactions that affect pathophysiology to be examined much more realistically. These engineered models allow the co-culture of cells, enabling the study of the interaction of different cell types, as well as the extracellular elements involved in disease pathogenesis [5].

Engineered *in vitro* disease models can be divided into three: scaffold-free systems, scaffold-based systems, and microfluidic platforms.

Scaffold-free tissue-engineered models produce matrix from cells based on cell-to-cell interactions without requiring exogenous materials. Organoid technology, one of the leading scaffold-free techniques, has great potential in disease modeling and allows researchers with the chance to produce more complex human disease models that can precisely describe clinical pathophysiology. Organoid technology has been developed from cultures of embryoid bodies, which are 3D aggregates of self-organizing stem cells to develop different tissues *in vitro* [6,7]. These embryoid bodies can spontaneously differentiate to form a variety of organized tissues or can be induced by factors to differentiate into specific lineages [8]. They physically resemble the organ's *in vivo* cellular organization, architecture, and composition, in whole or in part, and may also mimic sectional or complete functions of the organ

[9,10]. It is thought that human-derived brain organoids may help bridge the gap between *in vitro* model systems and therapeutic trials with a patient-specific, personalized medicine approach [11,12].

The most important limitation in the use of scaffold-free techniques is the lack of vascularization. Although existing brain organoid systems simulate the successful formation of the brain in the embryonic period, they are far from mimicking the mature brain structure [13]. Vascularization is notably required for oxygen penetration, nutrient supply, and effective neural differentiation [14,15]. The failure of oxygen delivery causes the formation of necrotic areas in the organoid center, affecting its natural development and potential neuronal migration pathways [16]. Constructing a vascular structure that provides adequate oxygen and nutrient delivery within the organoid is necessary to overcome the limited size of the organoids [17]. From the translational medicine and commercialization point of view, another limiting factor of scaffold-free models is the high number of cells required to produce the 3D tissue [18].

To overcome the disadvantages of the scaffold-free system, scaffold-based tissue-engineered ND modeling strategies have been developed. Scaffold-based models prevent premature cell death by providing nutrients and oxygen to the cells in the core, thanks to the porous structure they form. Scaffold-based, 3D *in vitro* models also remain viable for a longer period due to the mechanical stability of the scaffolds [19].

Tissue engineering scaffolds can be from natural or synthetic polymers and can be produced to have very different physical and microstructural properties such as a sponge, fiber, and hydrogel [20,21]. Commercially available Matrigel, which consists of four extracellular matrix (ECM) proteins, collagen IV, laminin, entactin or heparin sulfate proteoglycan perlecan [22], is often preferred in the creation of 3D *in vitro* disease models in hydrogel form. However,

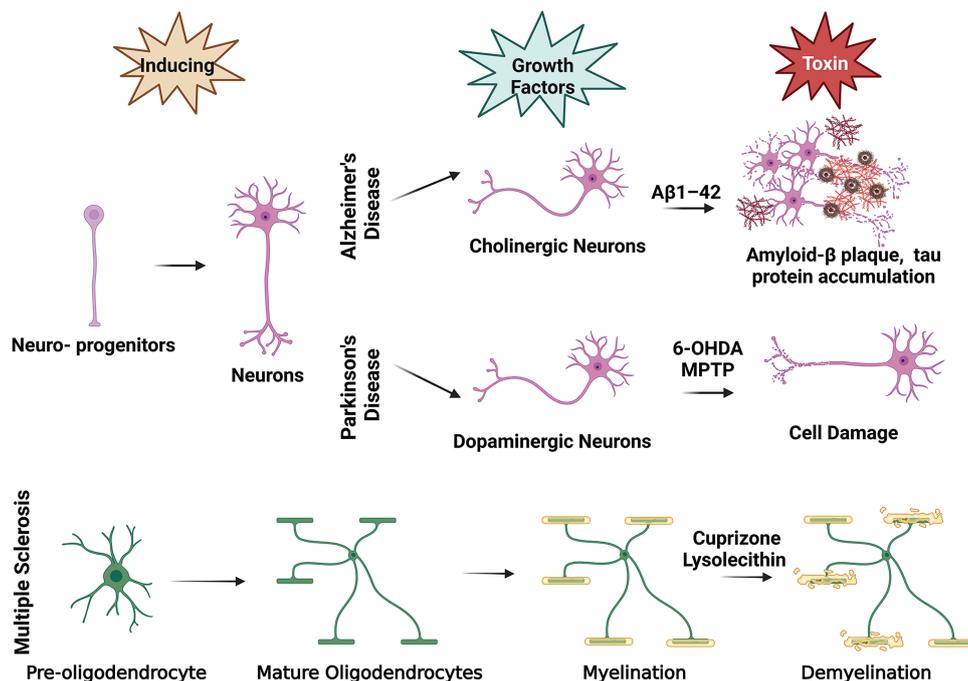


FIGURE 1. *In vitro* ND modeling basic illustration. This figure is covered by the Biorender license.

Matrigel poses a limitation in constructing a reliable model because it varies from batch to batch. This causes heterogeneity in the models created and affects the efficacy. Synthetic hydrogels, as well as natural and synthetic polymeric scaffolds in different microstructural forms, have been used to overcome this limitation [19].

Microfluidic platforms that provide controllable microenvironments offer the possibility to incorporate more cell types, as well as a dynamic perspective to disease models. Microfluidic platforms that apply mechanical forces over the cells (flow rate, pressure, shear stress) and more adjustable ambient conditions (temperature, pH) compared to scaffold-free and scaffold-based systems have the potential to increase the reliability of *in vitro* ND disease models [23].

This review compiles and summarizes the current examples in different 3D *in vitro* models of the most common NDs and discusses the advantages and shortcomings of different approaches.

### Alzheimer's Disease

AD accounts for 75% of dementia cases worldwide and affects an estimated 35 million people. By 2050, it is predicted that 152 million people will be affected by AD and other dementias [24]. Both hereditary and environmental factors trigger the development of the disease. Current therapies for AD are only at the level of slowing the progression of the symptoms and increasing the patient's quality of life. A reversible and curative therapeutic approach has not yet been found.

Early-onset AD is genetically inherited in an autosomal dominant manner and accounts for less than 5% of all cases. AD reflects the presence of one of 200 mutations discovered in the amyloid precursor protein (*APP*), presenilin 1 (*PSEN1*), and presenilin 2 (*PSEN2*) genes [25]. On the other hand, late-onset is sporadic and cannot be genetically determined [26].

The physiopathology of AD is based on extracellular amyloid- $\beta$  ( $A\beta$ ) plaque accumulation, hyperphosphorylated tau (p-tau) protein accumulation, intracellular neurofibrillary tangle formation, loss of synapses, neuroinflammation, oxidative stress, and neuronal cell death.

Experimental models in AD have been animal models consisting of transgenic mice expressing human genes that cause the development of  $A\beta$  plaques and neurofibrillary tangles. The development of the first transgenic AD mouse model with  $A\beta$  units in 1995 paved the way for over 200 transgenic rodent lines. However, approximately 80% of the pre-clinical tests completed and deemed successful with these transgenic animal models failed in clinical trials [27]. These results showed that animal models simulated pathological events like  $A\beta$  accumulation, only mimicking early onset AD, but they did not completely carry the salient physiological features, such as neuronal loss and neurofibrillary tangle development [28].

Toxins like scopolamine, streptozotocin, colchicine, and heavy metals have been used in *in vivo* dementia models. However, these toxin-induced models do not reflect human pathophysiology as they cause local brain damage, lack disease progression, and induce high mortality.

Nevertheless, toxin-induced models have been frequently used as they are the most realistic models so far, reflecting cognitive dysfunctions and dementia [29]. But *in vitro* modeling of dementia is much more difficult. The necessity of reflecting the cellular pathologies (inflammation, gliosis, autoimmunity, complement factors) occurring in AD has always been a limiting factor, apart from inducing AD. The most common toxins used to reflect reactive oxygen species (ROS), NADPH oxidase (NOX), inducible nitric oxide synthase (NOS), and neurotoxins induced by  $A\beta$  aggregates *in vitro* are  $A\beta$  monomers and oligomers [30–33].

### Scaffold-free techniques

Models created by neurons derived from immortalized cell lines (e.g., SHSY-5Y; human-derived neuroblastoma cell line) do not provide reliable results, as they show a quite different amyloidogenesis phenotype from degenerated neurons in AD [34]. 1–5  $\mu$ M  $A\beta$  concentrations are required to cause cell death *in vitro*, while it is known that this concentration is 50 nM in natural brain tissue [35]. In studies with PC-12 cells,  $A\beta$ 1–42 treatment increased the phosphorylation of tau at 10  $\mu$ M, but not at lower concentrations [36,37]. In another study, 25  $\mu$ M  $A\beta$ 1–42 administration on PC-12 cells increased cell apoptosis [38].

3D *in vitro* models can mimic the physiology more realistically, as the 3D configuration of neurons affects cytoskeletal dynamics [5]. Similar to AD pathophysiology, there was glutamatergic rather than GABAergic toxicity in human embryonic stem cell (hESC)-derived neurons exposed to oligomeric pre-fibrillar  $A\beta$  forms. In this study, time-dependent  $A\beta$  accumulations and subsequent cell death were reported in Matrigel-coated ultra-low attachment plates [34]. Induced pluripotent stem cell (iPSC) based models enabled the discovery of some basic differences between healthy control groups and individuals with AD. Researchers have characterized several iPSC lines from donor cells from familial AD and sporadic AD patients. Studies on these cells have revealed differences such as increased  $A\beta$ , specifically amyloid  $\beta$ 42 ( $A\beta$ 42) and tau hyperphosphorylation compared to those in iPSCs derived from healthy controls. iPSC lines also reflected additional AD-related pathologies such as increased glycogen synthase kinase-3 beta (GSK3 $\beta$ ) activation, increased large endosome number, and accumulation of intraneuronal  $A\beta$  oligomers [39].

PSEN1 and PSEN2 mutations are genetic factors that cause early-onset familial AD. In a study conducted by differentiating iPSCs produced from AD patient-derived fibroblasts with these mutations into neurons on gelatin-coated plates, the ratio of  $A\beta$ 40 and  $A\beta$ 42 synthesized by these neurons was higher than healthy controls in embryoid bodies [40]. This shows that the model reflects the familial AD pathophysiology.

Since it is impossible to obtain primary human neuronal cells from healthy (or diseased) donors, attention has been drawn to stem-cell-induced neurons for more realistic models. In a model with iPSCs obtained from blood cells of AD patients, 3D neuronal spheroids were created in 100 mm ultra-low-attachment plastic plates by inducing iPSC colonies with ROCK inhibitor and growth factors

(epidermal growth factor (EGF), fibroblast growth factor-2 (FGF2), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT3)). Researchers compared 3D spheroids with 2D culture and found that while there were differences in A $\beta$  clearance between individual iPSCs in 2D, these differences were less in 3D [5]. Another research group using iPSCs derived from AD patients also generated brain organoids showing AD-like pathophysiologies such as hyperphosphorylated tau protein, A $\beta$  aggregation, and endosome aberration. These pathologies were observed in a time-dependent manner harboring APP duplication or PSEN1 mutation [41]. This was a result revealing the similarity of the model to *in vivo*. In a similar study with the differentiation of iPSCs into neurons, it was reported that p21-activated kinase-mediated sensing of A $\beta$  oligomers was only in the 3D model created in Puramatrix and F-actin-related proteins were clearly associated with disease processes in 3D [42].

Another *in vitro* AD model is produced by differentiating iPSCs derived from AD patients with the PSEN1  $\Delta$ E9 mutation into astrocytes. They demonstrated the pathological features of AD, such as increased A $\beta$  production, altered cytokine release, and impaired Ca<sup>2+</sup> homeostasis. These pathologies caused oxidative stress in astrocytes and suppressed neuronal support by reducing lactate production. This study also shows that PSEN1 mutant astrocytes display a serious disease phenotype and may contribute to AD progression [43].

#### Scaffold-based techniques

Scaffolds that allow for greater physiological interactions in three dimensions between neurons and glia have also been used in AD models. Microenvironments that mimic the ECM, the presence of biochemical factors, and functional and structural molecules play a direct role in *in vitro* disease models.

In their study, Ranjan et al. formed a porous poly (lactic-co-glycolic acid) scaffold using a wet electrospinning technique and cultured familial AD-derived iPSCs on these scaffolds. Increased neuronal differentiation and spontaneous enhanced pathogenic A $\beta$ 42 and p-tau levels were observed in neurons carrying familial AD mutations, compared to age-matched healthy controls on scaffolds within seven days [44].

Cui et al. investigated the effect of self-assembled peptide hydrogel containing the YIGSR (Tyr-Ile-Gly-Ser-Arg) functional domain on the survival and neuronal differentiation of neural stem cells (NSCs) in *in vitro* and *in vivo* AD models. To examine the neuroprotective effect, 40  $\mu$ M A $\beta$ 1–40 was applied to the cells simultaneously with the peptides. The researchers found that self-assembled peptides not only increased cell survival, but also reduced the number of cells undergoing apoptosis. In addition, neuronal differentiation was increased on these peptides when compared to self-assembled peptides without YIGSR. In rat AD models, NSCs with self-assembled peptide containing YIGSR increased cognitive rescue and decreased the number of cells proceeding to apoptosis and the A $\beta$  production [45].

The development of cells in an organoid can also be achieved by using scaffolds. In the study of Choie et al., a

3D organoid was developed with human neural stem cells (hNSC) in Matrigel scaffolds, and extracellular A $\beta$  aggregation and accumulation of tau in the dystrophic neurites and cell soma was observed *in vitro* for the first-time [46]. AD virus models were also used to understand disease pathophysiology. A 3D AD model was created in a study with Herpes simplex virus type I (HSV-1), which has recently been shown to play a role in AD development pathology. Silk sponge scaffolds with 500 to 600  $\mu$ m pores were prepared as 6 mm discs, and 2 mm holes were drilled in the center to form annular scaffolds. After the scaffolds were coated with laminin (0.5 mg/mL), iPSC-derived hNSCs were seeded onto the scaffolds, and the next day, type I rat tail collagen gel was added. Organoids cultured for 4 weeks were infected by HSV-1, and A $\beta$  plaque-like formations, gliosis, and neuroinflammation were reported [47].

#### Microfluidic systems

Basic pathologies of AD were provided in a model tri-culture of neurons, astrocytes, and microglia on a 3D co-centric two-chamber microfluidic platform. High levels of A $\beta$ -producing human neural progenitor cells (hNPCs) with FAD mutations and astrocyte-differentiated cells were cultivated in the central chamber, while microglia were cultivated in the outer angular chamber. The central and angular chambers were connected by migration channels. Using this platform, migration of microglial cells has been observed. A $\beta$  aggregation, p-tau deposition, axonal cleavage, and neuroinflammatory activity such as NO, CCL2, IL8, TNF- $\alpha$ , and IFN- $\gamma$  release were demonstrated [48]. This model, which is useful in elucidating neuro-glia interactions, can be used not only for AD but also for understanding other disease pathologies such as PD and MS.

Due to the experimental challenges of neurospheroid cultures and the fact that they are affected by variability, including size differences, variable cell culture procedures, including cell density and media composition, the researchers further developed uniformly sized neurospheroids. These neurospheroids were derived from NSCs with FAD mutations and human-derived iPSCs (hiPSCs) with a PDMS microarray platform produced by photolithography for high-throughput drug screening [49]. In the developed platform, dendrite formations were observed in neurospheroids containing mature neurons. After 8 weeks of culturing, these neurospheroids were reported to possess A $\beta$  plaques and p-tau deposits [49].

The disruption of blood-brain barrier (BBB) in AD has been shown to precede disease pathology. In this regard, to investigate the role of BBB in AD, a three-channel microfluidic system was developed. Researchers used NSCs with FAD mutations in AD modeling and hCEMC/D3 cells (brain endothelial cell line) in BBB modeling. To modulate the differentiation periods of these two cells separately, sequential seeding was performed on the developed chip, and the intermediate channel was left filled with air until co-culture was initiated. Human NSCs were cultivated in Matrigel, and hCEMC/D3 cells were cultivated in collagen-filled channels. After the endothelial barrier was formed, the intermediate channel was filled with collagen type 1, and the interaction between differentiated NSCs, and the endothelial

layer was ensured by diffusion of soluble factors. It was stated that this model showed pathologies such as increased BBB permeability, decreased endothelial junction markers (claudin-1, claudin-5, zonula occludens-1, occludin, VE-cadherin), increased expression of matrix-metalloproteinase-2 and ROS, and accumulation of A $\beta$  peptides in the vascular endothelium [50].

The researchers investigated the effects of A $\beta$  on accumulations of microglial cells in a microfluidic chemotaxis platform by subjecting the cells to different A $\beta$ 42 gradients and surface bound A $\beta$  concentrations. Microglia chemotaxis for A $\beta$  monomers and oligomers was determined to be most effective at two different A $\beta$ 42 concentrations (23 pg/mL and 23 ng/mL), and at lower concentrations of A $\beta$ 42, microglial migration was further enhanced by monocyte chemoattractant protein-1 (MCP-1) secreted by the microglia [51]. This study created a model to elucidate the microglial pathology in AD.

### Parkinson's Disease

PD is the second most common ND after AD. The disease is characterized by the destruction of dopaminergic (DA) neurons in the substantia nigra (SN), particularly affecting the ventral component of the pars compacta [52].

In 1817, British physician James Parkinson reported his observations of six people as “shaking palsy.” Later, the studies on the understanding of PD with its modern name were made by Jean-Martin Charcot. In 1912, German neurologist Friedrich Lewy defined the cardinal neuropathological lesion that later bears his name: Lewy body (LB) [53].

LBs are cytoplasmic inclusions comprising several neurofilament proteins, together with the proteolytic proteins found in the remaining DA neurons. LBs are filamentous in nature and contain predominantly alpha-synuclein ( $\alpha$ -Syn) and ubiquitin proteins. Although LBs are also found in other diseases, they are thought to be the pathological hallmark of PD [54]. Age is the primary risk factor for PD. The incidence of PD is 1%–2% between the ages of 50–65 and around 3.5% between the ages of 85–89 [55].

Although genetic mutations causing PD have not been identified, it is known that there are defects in  $\alpha$ -Syn, parkin (PARK2), and ubiquitin C-terminal hydrolase-L1 (UCHL-1) genes, which play a role in the formation of LB protein aggregates characterized in neuropathology.

#### Scaffold-free techniques

Various *in vitro* culture procedures have been developed to model the pathogenesis of PH. The most commonly used cell lines in PD studies are the PC12 cell line derived from rat pheochromocytoma and human-derived SH-SY5Y neuroblastoma cells with ion channels and neurotransmitter receptors that secrete catecholamines such as dopamine and can exhibit DA neuron-like properties [56,57]. Since these cells can be obtained more easily than primary cells and can maintain their structural stability, ensuing genetic manipulations, they provide great advantages in the production of toxicity-induced PD models and the use of these models in pharmaceutical trials.

Neurodegeneration, oxidative stress, loss of function of mitochondria, and formation of protein aggregates are among the known causes of PD. Models created by inducing PD by triggering the conversion of ROS by neurotoxins such as 6-hydroxydopamine (6-OHDA), 1-methyl 4-phenyl 1,2,3,6-tetrahydro-pyridine (MPTP), paraquat and rotenone have led to important discoveries in understanding the molecular pathways that occur during the degeneration and death of DA neurons [58]. One of the catecholaminergic neurotoxins, 6-OHDA, and its noradrenergic analog have been used for more than 30 years in the creation of *in vitro* and *in vivo* experimental models for PD studies. Since 6-OHDA has a chemical structure similar to dopamine and norepinephrine, it is recognized with high affinity by the dopamine transporters (DATs) and norepinephrine transporters (NETs) in the plasma membrane. Thus, it causes oxidative stress on the catecholaminergic pathways of the peripheral nervous system (PNS) and central nervous system (CNS) through ROS and hydroxyl radicals and causes degeneration of DA and noradrenergic neurons [59].

A significant change in PC-12 morphology and DNA and chromosome breaks in cells was observed with the apoptosis-triggering effect of 6-OHDA [60]. Similarly, 6-OHDA has been tested with neuroblastoma, cerebellar granule, and primary mesencephalic DA cell lines, which are frequently used as model cell lines in the literature, and the data obtained support that 6-OHDA activates oxidative stress and apoptotic pathways in 2D culture [61,62].

MPTP, unlike 6-OHDA, can quickly pass through the BBB and be metabolized to its active form, 1-methyl-4-phenylpyridinium (MPP<sup>+</sup>), which is stored in neurons via the DAT, and as a result, it plays a role in the pathophysiology of mitochondrial dysfunction by affecting the mitochondrial electron transport chain [63].

Many researchers have generated iPSCs derived from PD patients to obtain cells with susceptibility alleles in PD-related genes such as leucine-rich repeat kinase 2 (LRRK2), glucocerebrosidase (GBA), PARKIN,  $\alpha$ -Syn, synaptophysin, and phosphatase and tensin homolog-(PTEN)-induced gene (PINK1) [48].

Developing an idiopathic PD model for the first time with organoids generated from iPSCs, the researchers were able to demonstrate a complex network of DA neurons. They observed statistical differences in expression levels of the *LIM homeobox transcription factor alpha* and *tyrosine hydroxylase* genes between organoids developed from healthy and PD cells [64]. In a study with iPSCs developed from a patient with a mutation in the *LRRK2* gene, 3D neuro-ectodermal and intestinal organoids were created to examine the brain-gut relationship dependent on *LRRK2*. As a result of the differences seen in the gene expression profiles of these organoids, the researchers stated that PD may affect intestinal functions [65].

In a study aiming to standardize reproducible organoid production, human neuronal progenitor cells (NPCs) were cultured for 8 days in 96-well ultra-low adhesion plates until they formed colonies. On day 8, the colonies were embedded in geltrex (a gel containing ECM proteins such as laminin, fibronectin, collagen, entactin, and heparin sulfate proteoglycans) droplets, and maturation was initiated by

differentiation media in 24-well ultra-low adhesion plates under shaking media for 30 days. This protocol developed standardized organoids that retained the basic aspects of midbrain development, such as DA neuron and astrocyte differentiation and compartmentalization, and can be utilized in PD models [66].

#### *Scaffold-based techniques*

In a PD model developed using 3D bioprinting, DA neurons were cultured in ultra-short self-assembled tetrapeptide Ac-Ile-Val-Phe-Lys-NH<sub>2</sub> (IVFK) and Ac-Ile-Val-Cha-Lys-NH<sub>2</sub> (IVZK) scaffolds. DA neurons encapsulated in tetrapeptide gels responded to 6-OHDA. They recorded spontaneous electrophysiological activity of encapsulated DA neurons by multi-electrode arrays. The model supported vascularization (by human umbilical vein endothelial cells (HUVECs)), and neuronal culture was continued for more than one month [67].

In another study, PD was modeled by culturing SH-SY5Y and U-87MG glioblastoma cell lines on polyacrylonitrile (PAN) and Jefamine<sup>®</sup> doped polyacrylonitrile (PJ) electrospun scaffolds. PAN and PJ nanofibrous scaffolds promoted proliferation, differentiation, and 3D interaction of DA neuron and astrocyte-like cells and increased neuronal survival against PD mimetic toxin treatments, including rotenone, MPTP, MG132 proteasome inhibitor and buthionine sulphoximine (BSO) [68].

Researchers developed a different approach using iPSC, genetic, and scaffold technologies together in a model. Human iPSC-induced DA neurons derived from healthy people and PD patients harboring LRRK2/GBA mutations were cultured on 3D silk scaffolds produced by the melt-molding technique [69]. Sodium chloride particles with a diameter of 415–500 µm were added to the silk solution and removed by washing, creating pores on the silk scaffolds. The 3D platform supported DA neuronal network formation, whereas α-Syn and purine metabolite profiles showed changes in PD-derived cells, offering a realistic approach to PD pathophysiology [69].

#### *Microfluidic systems*

There are several microfluidic models for PD. One of them is the model in which primary cortical neurons were cultivated on a microfluidic platform in the presence of recombinant α-Syn fibrils. They reported that neurons were able to internalize the α-Syn fibrils and transported them along axons resembling the characteristic spreading pattern of LBs [70].

Differentiating neuroepithelial cells derived from iPSCs into DA neurons within phase-guided microtiter plate format bioreactors brought a distinct perspective to PD modeling. Cells differentiated to functional DA neurons in a Matrigel-loaded microfluidic bioreactor with differentiation media in 30 days, and 19% showed high tyrosine hydroxylase levels [71].

In another study using the same type of microfluidic reactor, patient-specific PD-derived iPSCs carrying the LRRK2-G2019S mutation were differentiated into DA neurons. The DA differentiation, neurite length, and branching complexity in LRRK2-G2019S neurons decreased,

and cell death increased due to changing mitochondrial morphology. DA phenotypes were found to be improved with the administration of an LRRK2 inhibitor [72].

The differentiation of PD-derived human neuroepithelial stem cells into DA neurons was performed on an automated cell culture platform integrating the microtiter plates with a robotic system for long-term monitoring and maintenance of the culture system. This system, called Pelican, has the potential to automate patient-specific disease modeling, enabling a versatile set of *in vitro* analyses [73].

#### **Multiple Sclerosis**

MS is an inflammatory ND of the CNS with myelin destruction and is one of the most important causes of disability in young adults. It affects the quality of life of approximately 2.1 million individuals worldwide. The first clinical and pathological description of the disease was made by Charcot in 1868. Its prevalence varies between 2–200 per 100,000, depending on geographical features. It is more common in the 18–50 age range and in women. There are three phenotypes: clinically isolated syndrome, which is the first neurological picture associated with MS, a relapsing-remitting form with attacks and remissions, and a progressive form with gradually increasing disability [74,75]. MS is diagnosed by clinical findings meeting the McDonald criteria based on the clinical lesions and the presence of demyelinating plaques in brain magnetic resonance imaging (MRI) in regions typical for MS (periventricular, juxtacortical/cortical, infratentorial, and spinal) [76].

MS pathology is characterized by demyelinating areas called plaques or lesions that indicate the destruction of myelin sheaths and loss of myelin-forming cells (oligodendrocytes) in the white and gray matter of the CNS [77]. Although axons and neurons are mostly spared in early MS, neuroaxonal loss occurs as the disease progresses. The result is CNS atrophy and the production of multiple sclerotic glial scars in the lesions. Demyelinating plaques in white matter can be partially remyelinated [77–79].

Contrary to the accepted views that the CNS has a limited capacity for regeneration, a partial regenerative response (remyelination) is seen with the formation of new myelin sheaths following the damage to oligodendrocytes [80]. However, for many patients, remyelination eventually fails and the metabolic support that myelin sheaths normally provide to axons is lost, accompanied with axonal degeneration, and progressive degeneration that characterizes the later stage of MS progresses.

Experimental autoimmune encephalomyelitis (EAE) is generally considered a disease model for MS. However, this is insufficient—although EAE mimics an acute MS lesion, acute and chronic inflammation are lacking for modeling the neurodegeneration that characterizes progressive MS [81]. Disease models that reflect MS pathology require other mechanism patterns that are usually created using toxins that damage oligodendrocytes (thus leading to primary demyelination) or other cell types to varying degrees.

Animal studies have tended to the understanding of MS; however, they partially model the underlying pathogenic mechanisms [81]. Most of the commonly used CNS *in vitro*

culture systems are of animal origin. At this point, *in vitro* models using human cells are more realistic since there are fundamental differences between the rodent and the human brains [82].

One of the biggest obstacles to *in vitro* myelination studies is understanding how two different cell types communicate effectively to perform a specific task. Myelination occurs as a result of neuro-glial interaction and provides efficient and rapid conduction of action potentials throughout the CNS. During CNS development, inductive and/or inhibitory signals originate and propagate with neuro-glial interaction that induces myelination. Mitogenic and trophic factors produced by neurons also modulate the proliferation and migration of oligodendrocyte precursor cells (OPCs). This multifactorial interaction resulting in myelination is difficult to study using *in vitro* methods [83,84].

#### *Scaffold-free techniques*

Different toxins or immune-mediated inducers are frequently used in demyelination studies. One of the toxin-based demyelination models is the use of lyssolecithin (LPC), which disrupts cell membranes, including myelin, by acting on lipid bilayers to form micelles. It has been shown to damage directly the myelin layer *in vitro* [85–88].

Lipopolysaccharide (LPS), which induces innate immune cells, is also used in modeling MS pathology [89–92]. Researchers investigating the effect of dimethyl fumarate (DMF) used in the treatment of relapsing-remitting MS *in vitro* used the oli-neu (primary mouse oligodendrocyte) cell line. By applying LPS-mediated inflammation to cells, researchers demonstrated that DMF induces differentiation of oli-neu cells into mature and potentially myelinating cells, and it has anti-inflammatory and antioxidant effects [93].

The cuprizone demyelination model is of particular interest among toxic models [94]. Cuprizone [bis(cyclohexanone) oxaldihydrazone] is a copper chelator. After cuprizone administration, cytochrome oxidase and monoamine oxidase are inhibited in mature oligodendrocytes, resulting in apoptosis due to mitochondrial damage. Cerebral microglia activation is observed *in vivo* as a result of the demyelination of axons and the production of tumor necrosis factor- $\alpha$  (TNF $\alpha$ ) and interferon- $\gamma$  (IFN $\gamma$ ) by oligodendrocytes is increased [95–97].

*In vitro* systems that culture OPCs with CNS or PNS neurons are relatively simple, inexpensive, high-throughput models [98]. Oligodendrocytes express genes encoding cell-specific myelin structural proteins [99–101]. Isolated rat and mouse primary oligodendrocytes were shown to express most of the proteins and lipids associated with myelin membranes, but primary cultures of human oligodendrocytes are difficult to study due to the difficulty of reaching live human brain tissue. For this reason, immortalized human oligodendrocyte cell lines are preferred [82]. The human oligodendrocyte cell line (HOG) is a cell line created from a surgically removed oligodendroglioma and has been shown to express myelin basic protein (MBP) and some oligodendrocyte-specific proteins such as A2B5 [102]. It is frequently used in myelination and remyelination studies [103–105].

Myelinated brain organoids have recently turned into a new and important model system for myelination. Human cortical organoids contain GABAergic interneurons, a small number of oligodendrocytes, and OPCs located in the ventral forebrain, mimicking the dorsal forebrain composition [106]. A single-cell sequencing study reported that oligodendrocytes in ventral organoids go through developmental stages similar to those observed in the developing human brain [107]. During ventral organoid differentiation, with the addition of growth factors such as platelet-derived growth factor (PDGF-AA), Insulin-like growth factor 1 (IGF1), and NT3, the number of OPCs increases for about 2 months [108].

Neurons derived from iPSCs can be co-cultivated with iPSC-derived or primary OPCs and oligodendrocytes. Mixed culture approaches like this could enable the investigation of interactions between oligodendroglia and neurons. A group working with hiPSCs studied oligodendrocyte developmental stages by developing human oligodendrocyte spheroids in microwells. The researchers reported that cell morphologies change as they mature and begin to myelinate neurons by 100 days. They also showed that LPC causes oligodendrocyte loss, a process that can be monitored with live imaging [107].

Another study on hiPSCs reproducibly obtained oligodendrocytes and myelin in ‘oligocortical spheroids’ using MSC-derived extracellular vesicles (EVs). Myelin-related protein expressions were reached at week 20, and myelin structures were observed at week 30 [109].

Chesnut et al. developed another iPSC-derived brain organoid that can be used to study myelin damage. They examined the damage by applying multiple toxins to examine the myelination disruption on the brain organoid containing highly myelinated axons they developed in eight weeks in non-coated plates under constant shaking at 88 rpm. Researchers who tried cuprizone, bisphenol A, tris (1,3-dichloro-2-propyl) phosphate, and ibuprofen determined that myelination was affected only in cuprizone application [110]. All spheroid studies revealed that co-culture conditions and interactions of oligodendrocytes with neurons and/or astrocytes are important for mature myelination in *in vitro* scaffold-free culture conditions.

#### *Scaffold-based techniques*

Studies to investigate the need for axonal signals to initiate oligodendrocyte myelination have shown that myelination does not depend solely on co-culture with neurons, but oligodendrocytes can also form myelin on surfaces such as paraformaldehyde-fixed axons [111].

The development of scaffolds that mimic axons has prompted the investigation of their effects on oligodendrocytes. With this approach, OPCs or oligodendrocytes are studied by culturing on nanofibers or microcapillary arrays. Such synthetic approaches are important not only to screen drugs for multiple sclerosis therapeutics but also to screen for oligodendrocyte association of all NDs.

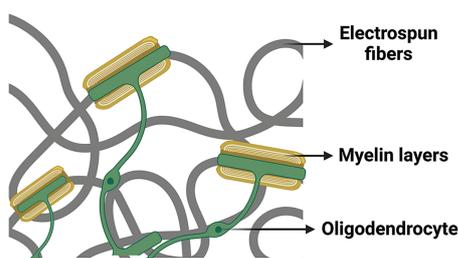
Appropriate biomaterials that mimic the structure of axons and the three-dimensional microenvironment in the brain with their fibrous microarchitecture can also support

cell migration, attachment, and survival, and increase the success of ND models as well as therapeutic studies. Electrospinning is an advantageous method for the reproducible and modifiable production of the physical properties of the extracellular matrix (e.g., pore and fiber size), chemical properties (e.g., cell adhesion proteins), and mechanical properties (e.g., Elasticity). In the literature, beneficial effects of electrospun fiber scaffolds on neural cell proliferation, viability, differentiation, and neurite formation have been demonstrated [112–114]. Furthermore, the functionalization of electrospun scaffolds with ECM components can control cell attachment and neurite outgrowth.

Lee et al. reported that the use of nanofibers offers occasions to study myelination mechanisms and is a complementary approach to induce myelination without requiring the presence of co-cultured neurons. Nanofiber scaffolds facilitate the investigation of oligodendrocyte dynamics and enable the testing of therapeutic agents without confounding neuronal effects. As demonstrated in the study, OPCs and oligodendrocytes are sensitive to the minimum fiber diameter threshold of 0.4  $\mu\text{m}$  to initiate myelination-related processes (Fig. 2) [83].

A research group has also developed synthetic mimics of biological axons that represent key geometric and mechanical characteristics by 3D printing of polydimethylsiloxane (PDMS), poly (2-hydroxyethyl methacrylate) (poly-HEMA) and polyhexanediol diacrylate-co-star-polyethylene glycol (poly (HDDA-co-star PEG)) by projection micro-stereolithography. The fiber surfaces were coated with laminin and poly-D-lysine. These artificial axons reflected the mechanical cues presented by neurons *in vivo* with similar stiffness to axons, enabling the study of myelination. Researchers reported that laminin-coated pillars were more ensheathed by oligodendrocytes compared to poly-D-lysine ones. For  $\sim 16\ \mu\text{m}$  diameter laminin-coated pillars, the artificial axon with a stiffness of 140 kPa showed approximately three times more oligodendrocyte interaction compared to those with a stiffness of 0.4 kPa. Also, more oligodendrocyte interactions were observed in 10  $\mu\text{m}$  axons compared to 20  $\mu\text{m}$  diameter axons [115].

In addition to fibrous microstructures, myelination, and demyelination modeling studies are also carried out in hydrogels that allow cells to move in 3D. It was reported that myelination increased in Schwann cells co-cultivated with dorsal root ganglion (DRG) cells in gelatin methacryloyl (GelMA) hydrogels with Young's modulus of  $32.6 \pm 1.9$  kPa and mean pore size of 10.3  $\mu\text{m}$ . Schwann cells had a



**FIGURE 2.** Myelin wrapping of oligodendrocytes around electrospun fibers. This figure is covered by the Biorender license.

higher rate of myelination on GelMA compared to poly-D-lysine and collagen-coated surfaces [116].

Co-culture systems using rat DRG/Schwann cells are widely used to fundamentally study myelination *in vitro*. Researchers developing co-culture using polyvinylidene fluoride-trifluoro ethylene (PVDF-TrFE) scaffolds showed that aligned fibers-oriented Schwann cells and neurites and supported longer neurite extension of the DRG compared to cultures without scaffolds and monoculture. In addition, they reported that myelin structures formed around neurites in scaffolds and emphasized the importance of oriented fibers in myelination studies [117].

In another study, OPCs cultured on collagen microspheres differentiated into oligodendrocytes and formed myelin sheath in co-culture with DRG neurons [118]. This study shows that microspheres produced from biocompatible materials can also be used in myelination and neurite modeling studies and provide a supportive environment for cell growth.

Scaffold-based myelination models provide substantial evidence of the importance of mechanical as well as biochemical regulation of myelination.

#### Microfluidic systems

Microfluidic devices are advantageous platforms for co-culturing neurons and oligodendrocytes in *in vitro* myelination models. Spontaneous formation and random orientation of myelin sheaths have made the analysis of myelination difficult in systems reported to be used in scaffold-based and scaffold-free myelination models. In organ-on-chip models, myelin formations can be examined by channeling the chip. In a study performed on the 3D-printed microchip, oligodendrocytes co-cultivated with DRG neurons interacted with neurites extending into an independent channel from the somas. Myelination capacity was demonstrated by overlapping MBP- and  $\beta$ III-tubulin-positive staining and MBP segments were reported to be located along neurites. The myelin segment distribution followed the neurite network distribution, and the differences in myelin appearance were evident when compared with the classical culture [119].

Sharma et al. developed a microphysiological biomimetic chip that demonstrated neurite outgrowth (0.4–0.55  $\mu\text{m}$ ), myelination of Schwann cells (4.7%), and assessment of nerve conduction velocity (0.13–0.28 m/s) [120]. They created hydrogel scaffolds using micro-photolithography. Cell adhesion restrictive surfaces were created using 30% w/v polyethylene glycol dimethacrylate 1000 and 1.1 mM lithium phenyl-2,4,6-trimethylbenzoylphosphinate solution. After the formed channels were filled with Matrigel, motor neurons and Schwann cell co-cultured spheroids were transferred into the chip. For 4 weeks, the elongation of neurites along the canal and myelin formation of Schwann cells were monitored and evaluated [120].

Researchers who developed another 3D neuron culture platform on a microfluidic chip (Neuro-IMPACT) modeled neuronal network, BBB, and myelination processes in this high-throughput scanning platform [121]. The developed chip enabled 3D neuron culture for a long time and enabled the examination of neuro-endothelial and neuro-glial

interactions on the same platform. The functionality of the model was demonstrated by BBB permeability testing, imaging of myelin sheaths, and observing neuronal functionality with intracellular calcium changes.

Table 1 gives a summary of the selected studied *in vitro* models of ND.

### Other Neurodegenerative Diseases

Adult-onset HD, which mostly affects striatal and cortical neurons in the brain, is characterized by motor abnormalities, cognitive decline, and psychiatric symptoms, with an incidence of 7–10 per 100,000 [122]. HD is an ND

TABLE 1

#### *In vitro* studies on the various NDs

|                            | Cell  | Surface & scaffold  | Outcome   | Ref.      |
|----------------------------|---|---|---|-----------|
| <b>Alzheimer's disease</b> | PC-12                                       | Polystyrene   | Increased phosphorylation of tau, cell apoptosis  | [36–38]   |
|                            | hESC-derived neurons                        | Matrigel-coated plates  | Time dependent A $\beta$ accumulations and subsequent cell death  | [34]      |
|                            | iPSCs                                       | Matrigel  | Increased A $\beta$ production, altered cytokine release, and impaired Ca <sup>2+</sup> homeostasis       | [43]      |
|                            | iPSCs                                       | Poly (lactic-co-glycolic) acid                                      | Increased cell proliferation and differentiation  | [44]      |
|                            | NSCs  | Matrigel  | The first-time <i>in vitro</i> culture that extracellular A $\beta$ aggregation and accumulation of p-tau | [46]      |
|                            | NSCs  | Silk sponge   | Amyloid plaque-like formations, gliosis, and neuroinflammation  | [47]      |
|                            | Human neuronal progenitor cells (hNPCs)     | Matrigel-filled microfluidic system                                 | A $\beta$ aggregation, phosphorylated tau deposition, and neuroinflammatory activity                      | [48]      |
|                            | hiPSCs                                      | Matrigel  | A $\beta$ plaques and p-tau deposits  | [49]      |
| <b>Parkinson's disease</b> | hNPCs hCEMC/D3                              | Matrigel<br>Collagen filled channel                                 | Accumulation of A $\beta$ peptides in the vascular endothelium  | [50]      |
|                            | PC-12, SH-SY5Y                              | Polystyrene   | Oxidative stress, apoptosis   | [56,57]   |
|                            | NPCs  | Geltrex droplets  | DA neuron proliferation and astrocyte differentiation   | [66]      |
|                            | SH-SY5Y, U-87MG                             | Polyacrylonitrile and Jefamine <sup>®</sup> doped polyacrylonitrile | Proliferation, differentiation, and 3D interaction of dopaminergic neuron and astrocyte-like cells        | [68]      |
|                            | iPSCs                                       | Silk  | Proliferation, differentiation, and 3D interaction of dopaminergic neuron and astrocyte-like cells        | [69]      |
|                            | DA Neurons                                  | Tetrapeptide  | Supported vascularization and spontaneous activation of dopaminergic neurons                              | [67]      |
|                            | Primary mouse neurons                       | Poly-D-lysine and laminin-coated microfluidic channels              | Internalization and transportation of fibrillar $\alpha$ -syn through neurons                             | [70]      |
|                            | PD-derived iPSCs                            | Matrigel-coated OrganoPlates  | Improved dopaminergic phenotype   | [72]      |
| <b>Multiple sclerosis</b>  | PD-derived human neuroepithelial stem cells | Matrigel-coated OrganoPlates  | Increased dopaminergic neuron differentiation   | [73]      |
|                            | Oli-neu                                     | Polystyrene   | Induced maturation and myelination  | [88]      |
|                            | HOG   | Polystyrene   | Express myelin basic protein form and some oligodendrocyte-specific proteins                              | [103–105] |
|                            | hiPSCs                                      | Matrigel coating  | Myelin-related protein expressions  | [109]     |
|                            | DRG   | Gelatin methacryloyl  | Higher rate of myelination compared to poly-D lysine and collagen-coated surfaces                         | [116]     |

(Continued)

Table 1 (continued)

| Cell          | Surface & scaffold   | Outcome   | Ref.  |
|---------------|--|---|-------|
| DRG           | Polyvinylidene fluoride-trifluoroethylene  | Aligned fibers-oriented Schwann cells and neurites, and supported longer neurite extension                      | [117] |
| OPCs          | Collagen microspheres  | Provide a supportive environment for cell growth  | [118] |
| DRG           | 3D-printed PDMS microchip  | Myelin segment distribution followed the neurite network distribution, and the differences in myelin appearance | [119] |
| Schwann cells | Polyethylene glycol dimethacrylate 1000 and lithium phenyl-2,4,6-trimethylbenzoylphosphinate hydrogel loaded | Neurite elongation and myelin formation   | [120] |
|               | Neuro-IMPACT culture platform  | Neuro-endothelial and neuro-glial interactions  | [121] |

characterized by the increase of cytosine-adenine-guanine (CAG) trinucleotide repeats in the gene encoding the Huntington protein (HTT) and forming a polyglutamine (PolyQ) extension. HTT is a protein involved in the removal of protein misfolding and clearance of abnormal gene expressions, and directly affects neuronal apoptosis and cytoskeletal elements. The role of HTT in the pathogenesis of HD has also been used as a tool for *in vitro* experiments, and HTT accumulation was induced *in vitro* as a result of transfection of cell lines (SH SY5Y, PC12, Neuro2a) with vectors containing poly (CAG) such as HTT exon-1 protein [123–125].

As in other NDs, many iPSC-based HD models have also been studied [126–128]. In the first study to report iPSCs derived from HD patients, cells reflecting the underlying disease pathologies, such as increased lysosomal activity, CAG stability, and increased lysosomal activity were obtained [129].

On-organ-chip approaches have also been developed for modeling HD. Researchers modeling presynaptic, synaptic, and postsynaptic areas with HD-mutant mouse cells in 3 separate regions of a chip examined intracellular events. Having developed a platform that models the pathophysiology of HD, researchers have observed cellular and network dysfunctions [130]. Presynaptic and postsynaptic pathways were examined in another HD on-chip study, which was developed to examine drug interaction with the pathogenic mechanism, and pridopidine was found to maintain synaptic transmission and synapse homeostasis [131].

Tissue engineering scaffold usage is still limited in developing HD models, but they are used to develop therapeutics in HD [132,133].

Prion Disease is similar to AD and PD in terms of its pathology related to misfolding of another protein, cellular prion-related protein (PrPC). Although 2D *in vitro* models have failed to represent the disease mechanism, 3D spheroid models based on murine NSCs (Collins & Haigh, 2017) [134] and hiPSCs [135] showed more promising results for

simulating the accumulation of prions. Also, the advantage of iPSCs was the presentation of the genetic mutations related to the disease, as well as the simulation of astrogliosis, which is a hallmark of several NDs.

Different than other NDs, ALS affects mainly motor neurons and progresses faster. The majority of the *in vitro* models used for ALS are based on the genetic mechanisms of the disease, although only a minority (5%–10%) of ALS cases are familial [136]. Motor neurons derived from iPSC of ALS patients were used to model the disease [137]. These models showed increased misfolded superoxide dismutase 1 (SOD1), accepted as a distinct marker of ALS [138]. However, SOD1 expression is much higher in mouse iPSC models compared to human iPSCs, raising concern about the validity of human models [139]. The fused-in sarcoma (*FUS*) gene, Tar DNA binding protein (*TDP-43*), and chromosome 9 open reading frame 72 (*C9ORF72*) are also other common markers used in iPSC derived ALS models [137].

Neuromuscular junctions (NMJs) are also affected in several neurodegenerative and neuroimmunological disorders like ALS and Myasthenia gravis. *In vitro* models of NMJs have been recently established using microfluidic platforms and bioreactors [140,141]. Two chambered microfluidic platforms were used to culture motor neurons that can extend their axons through microchannels to reach and innervate muscle cells [142,143]. It is also possible to incorporate electrical stimulation to these compartmentalized systems [144].

Microfabrication techniques allow the functionalization of surfaces to achieve better attachment of neurons and myocytes. A PDMS platform that utilized micro-cantilevers was used for the co-culture of motor neurons and myocytes and allowed to measure muscle contraction force due to stimulation. This platform is accepted as the first tissue-engineered model of the NMJ [145]. In a spheroid model of NMJ, skeletal muscle cells were co-cultured with iPSC-derived motor neurons [146]. In an on-chip system using optogenetic technology, iPSC cells were differentiated into

motor neurons, and a motor unit model was created by co-culture of skeletal myocytes to facilitate the examination of ALS pathogenesis [147]. The same group enhanced their microfluidic platform by also incorporating the BBB into the same device [148].

Since neuroinflammation is a major hallmark of NDs, *in vitro* systems that study microglia are also of crucial interest. There are various murine and human microglia cell lines, as well as *in vitro* models for culturing primary microglia or differentiating microglia from stem cells; however, 2D cultures have failed to mimic the necessary microenvironment for the presentation of the correct phenotypes [149,150]. Successful integration of iPSC-derived microglia in brain organoids [151], electrospun polycaprolactone nanofibers [152], collagen [153] and RADA-16 peptide hydrogels [154] were shown to possess more success in representing neuroinflammation in 3D. The existence of ECM proteins in the scaffolds provide a more *in vivo*-like microglial morphology [153]. Functionalization of PCL fibers with graphene also contributed to a similar, ramified, non-phagocytic morphology closer to the *in vivo* resting state of the glia [155].

There is increasing evidence that gut microbiota plays an important role in NDs pathophysiologies (microbiota-gut-brain axis (MGBA) [156]. Microfluidic chips would provide an excellent platform for *in vitro* simulation of MGBA, since they furnish the possibility to incorporate different interconnected channels that can be designed to culture various microbiota, as well as the different cell types of the CNS simultaneously on a single device, and to study their one-to-one and/or collective interactions. A recent European Research Council (ERC) funded project (MINERVA, Grant no. 724734) aims to engineer platforms to study the effects of MGBA on AD. Studies have reported intestinal inflammation in chip models [157]. Although challenging, MGBA-on-chip models would provide a significant impact on our understanding of the complex relations between our gut microbiota, our immune system, and the NDs.

Although two-dimensional *in vitro* models reflect some pathophysiological conditions, they are insufficient because they cannot fully reflect the complex brain physiology. To fill this gap between conventional 2D *in vitro* models and clinical applications, 3D-engineered *in vitro* models form a bridge (Table 2).

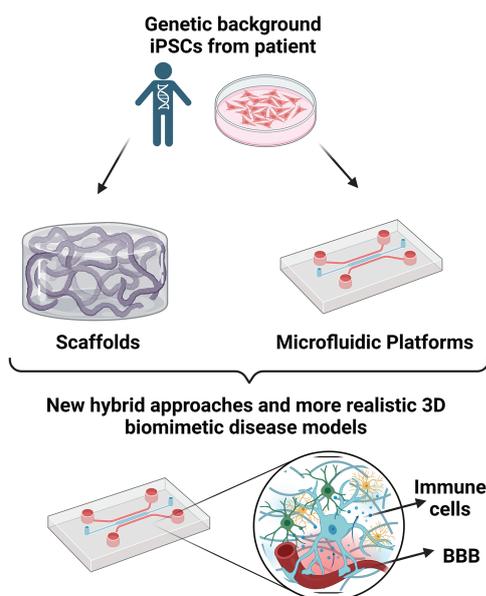
TABLE 2

## Advantages and disadvantages of culture systems

| Culture system              | Advantages  | Disadvantages   | Ref.          |
|-----------------------------|---|---|---------------|
| <b>Standard 2D cultures</b> | Easy circulation of nutrients and oxygen to the cells<br>Easy removal of wastes from the cells,<br>Fast results,<br>Cost efficient,<br>Easy to analyze and characterize   | Single surface adhesion,<br>Unnatural flat and elongated cell morphology,<br>Low cell differentiation efficiency,<br>One-way contact stress,<br>Lack of natural extracellular matrix,<br>Variable gene/protein expression compared to <i>in vivo</i>  | [158,159]     |
| <b>Spheroid models</b>      | Cell-based 3D physiological environment,<br>Natural cell morphology,<br>High cell differentiation efficiency,<br>Multiple-way contact stress,<br>Reproducible—few experimental variations,<br>Easy to analyze and characterize,<br>Similar gene/protein expression compared to <i>in vivo</i> | Limited circulation of nutrients and oxygen to the cells,<br>Restrained removal of wastes from the cells,<br>Lack of vascularization and subsequent development of internal necrotic layers,<br>Variable spheroid diameter and size   | [23,160]      |
| <b>Organoid models</b>      | Cell-based 3D physiological environment,<br>Natural cell morphology,<br>High cell differentiation efficiency,<br>Multiple-way contact stress,<br>Mixed population,<br>Similar gene/protein expression compared to <i>in vivo</i>  | Limited circulation of nutrients and oxygen to the cells,<br>Restrained removal of wastes from the cells,<br>Complex to analyze and characterize,<br>High experimental variations,<br>Variable diameter and size,<br>Lack of vascularization and subsequent development of internal necrotic layers,<br>High cost | [159,161–163] |

(Continued)

| Table 2 (continued)          |   |   |                  |
|------------------------------|---|---|------------------|
| Culture system               | Advantages  | Disadvantages   | Ref.             |
| <b>Scaffold-based models</b> | Material-based 3D physiological environment,<br>Natural cell morphology,<br>High cell differentiation efficiency,<br>Multiple-way contact stress,<br>Cell-cell and cell-material interactions,<br>Modifiable and controlled biodegradability, chemical composition, mechanical properties,<br>Easy to functionalize,<br>Reproducible depending on scaffold material source,<br>Similar gene/protein expression compared to <i>in vivo</i> | Challenging circulation of nutrients and oxygen to the cells and removal of wastes from the cells,<br>Complex to analyze and characterize,<br>High cost | [158,164,165]    |
| <b>Microfluidic systems</b>  | Cell-material-based 3D physiological environment,<br>Natural cell morphology,<br>High cell differentiation efficiency,<br>Multiple-way contact stress,<br>Can be functionalized by adding a scaffold,<br>Dynamic culture can be applied,<br>Usable as a micro bioreactor by applying mechanical forces,<br>Similar gene/protein expression compared to <i>in vivo</i>   | Require specialized equipment and expertise,<br>Complex to analyze and characterize,<br>High cost   | [23,166,162,167] |



**FIGURE 3.** The development of hybrid approaches can provide more realistic disease models. This figure is covered by the Biorender license.

### Conclusion and Future Aspects

NDs are a serious health problem characterized by progressive loss of cells in the brain. The mechanisms that cause NDs are still being identified. *In vitro* ND models are vital to understanding the mechanisms of these diseases, developing early detection methods, and finding effective treatments.

With the 3D-engineered models discussed in this review, it is possible to characterize the mechanisms of occurrence of NDs and enable the discovery of new treatment strategies. Particularly, ND-on-chip models offer the opportunity for high-throughput screening of potential therapeutic compounds. Researchers can assess the efficacy and safety of a large number of drug candidates, leading to faster and more efficient drug discovery processes. However, it should be noted that these models are still incomplete.

The development of better 3D *in vitro* models based on cells from the patient will be able to reflect the genetic and epigenetic background that contributes to the pathology of NDs. In addition, the development of precision medicine treatments will accelerate with these personalized models. A

major drawback of iPSCs in modeling NDs is the intrinsic rejuvenation of somatic cells during the reprogramming stage and subsequent failure in replicating the aging effects closely associated with most NDs.

One of the biggest challenges in the progress and testing of drugs targeting the CNS is the BBB. Therefore, complex *in vitro* models should include fluidic systems capable of mimicking the BBB. Recent studies include vascularization, considering this obstacle. In addition, it is necessary to develop 3D *in vitro* models in which immune system cells are included to examine the role of neuro-immune relations on neurodegeneration in detail.

Continued advancements in bioengineering techniques, such as microfluidics, bioprinting, and organ-on-a-chip technologies, are enhancing the capabilities of 3D-engineered ND models. Employment of these technologies in an orchestrated, hybrid approach enables the creation of more complex and interconnected multicellular networks, better control of cellular microenvironments, inclusion of the biochemical and mechanical extracellular matrix cues, and the integration of vascular and immune components, further enhancing the model's physiological relevance (Fig. 3).

While 3D-engineered *in vitro* ND models hold immense promise, several challenges need to be addressed. These include improving the reproducibility and standardization of model generation, enhancing the complexity and maturation of neuronal networks, and integrating multiple cell types and functions into the models. Future research should focus on addressing these challenges to maximize the potential of 3D-engineered models.

In conclusion, *in vitro* ND models are one of the remarkable areas open to development. By establishing more accurate and realistic *in vitro* disease models, faster and more effective drug discovery will be achieved, and personalized patient care will be improved in NDs.

**Acknowledgement:** None.

**Funding Statement:** The authors received no specific funding for this study. Zehra Gül Morçimen is supported by the TUBITAK-BIDEB 2211-A National Scholarship Program for Ph.D. Students and the YOK 100/2000 Scholarship in the field of smart and innovative materials.

**Author Contributions:** The authors confirm contribution to the paper as follows: literature review and discussion: Morçimen ZG, Taşdemir Ş, Şendemir A; draft manuscript preparation: Morçimen ZG, Taşdemir Ş, Şendemir A. All authors reviewed and approved the final version of the manuscript.

**Availability of Data and Materials:** Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

**Ethics Approval:** Not applicable.

**Conflicts of Interest:** The authors declare that they have no conflicts of interest to report regarding the present study.

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