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Review

Modelling neurodegenerative diseases *in vitro*: Recent advances in 3D iPSC technologies

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Abstract: The discovery of induced pluripotent stem cells (iPSC) 12 years ago has fostered the development of innovative patient-derived *in vitro* models for better understanding of disease mechanisms. This is particularly relevant to neurodegenerative diseases, where availability of live human brain tissue for research is limited and post-mortem interval changes influence readouts from autopsy-derived human tissue. Hundreds of iPSC lines have now been prepared and banked, thanks to several large scale initiatives and cell banks. Patient- or engineered iPSC-derived neural models are now being used to recapitulate cellular and molecular aspects of a variety of neurodegenerative diseases, including early and pre-clinical disease stages. The broad relevance of these models derives from the availability of a variety of differentiation protocols to generate disease-specific cell types and the manipulation to either introduce or correct disease-relevant genetic modifications. Moreover, the use of chemical and physical three-dimensional (3D) matrices improves control over the extracellular environment and cellular organization of the models. These iPSC-derived neural models can be utilised to identify target proteins and, importantly, provide high-throughput screening for drug discovery. Choosing Alzheimer's disease (AD) as an example, this review describes 3D iPSC-derived neural models and their advantages and limitations. There is now a requirement to fully characterise and validate these 3D iPSC-derived neural models as a viable research tool that is capable of complementing animal models of neurodegeneration and live human brain tissue. With further optimization of differentiation, maturation and aging protocols, as well as the 3D cellular organisation and extracellular matrix to recapitulate more closely, the molecular

extracellular-environment of the human brain, 3D iPSC-derived models have the potential to deliver new knowledge, enable discovery of novel disease mechanisms and identify new therapeutic targets for neurodegenerative diseases.

Keywords: induced pluripotent stem cells; neural stem cells; 3D; culture model; microenvironment; matrix; neural differentiation; neurodegenerative diseases; Alzheimer

Abbreviations: AD: Alzheimer's disease; ALS: Amyotrophic Lateral Sclerosis; AMPA: Aminomethylphosphonic Acid; APP: Amyloid precursor Protein; ECM: Extracellular matrix; EMT: epithelial-mesenchymal transition; EPSC: excitatory postsynaptic potential; ESCs: embryonic stem cells; fAD: familial Alzheimer Disease; FTD: Frontotemporal dementia; GABA: Gamma-Aminobutyric Acid; GSK: glycogen synthase kinase; HGPS: Hutchinson-Gilford progeria syndrome; iN: induced neurons; iPSC: induced pluripotent stem cells; LIF3: Leukaemia Inhibitory Factor 3; MEAs: microelectrode arrays; NMDA: N-methyl-D-aspartate; NSCs: neural stem cells; PS1: presenilin1; RAN: Repeat-associated non-ATG; TGFβ: transcription growth factor beta; UPR: unfolded protein response; 3D: three-dimensional; 2D: two-dimensional

1. Introduction

In 2006 Yamanaka and Takahashi published their seminal work on induced pluripotent stem cells (iPSC) technology, by forcing expression of key transcription factors, Klf4, Sox2, c-myc and Oct3/4 to reprograme somatic cells back to an undifferentiated state capable of unlimited proliferation. This provided laboratories with a tool for continuous supply of human pluripotent cells [1]. Since then, the use of iPSCs in research has expanded exponentially. Over a decade later, researchers can now use patient-specific iPSCs to create many neural cell types, including those that are involved in neurodegeneration. It is now possible to study patient-derived mature neurons and glia in two-dimensional (2D) monolayer culture and, more recently, in three-dimensional (3D) culture thereby investigating disease pathogenesis in a more translational context. There is currently intensive research into the feasibility of 3D neural cultures for disease modelling and drug screening; however, recapitulating maturation and aging in a model of age-related disease is still a challenge.

Can 3D cultures deliver what they promise or will they just be another research tool to complement traditional disease models?

2. iPSCs as a tool for *in vitro* research relevant to neurological diseases

One major advantage of iPSCs is that they negate the ethical concerns regarding the use of embryonic or foetally-derived cells, and they can now be produced using virus-free methods [2–5]. The use of adult somatic cells for iPSC production is widely ethically approved and provides autologous non-immunogenic cells that can be used for cell transplant and personalised medicine. In particular, these cells can generate autologous dopaminergic neurons for the treatment of Parkinson's disease to replace the use of implanting neural progenitor/stem cells from foetal/embryonic sources which can cause tumour formation and illicit a deleterious immune response [6,7]. One other

advantage of iPSCs is their isogenicity to a patient, recapitulating the exact genetic information leading to the disease. For example, iPSC-derived from familial AD (fAD) patients harbouring mutations in amyloid precursor protein (APP), presenilin-1 (PS1) or -2 genes can be used to recapitulate *in vitro* the cellular events that induce early stages of disease pathology. This enables scientists to unravel the underlying mechanism(s) underpinning the disease [8–10]; it also overcomes the issue of using model species that do not develop these human diseases *de novo*.

IPSCs can now be easily inducted along the neural lineage using dual smad inhibition, Noggin and SB431542 [11,12]. This method has also been refined by modifying the original protocol and relies on three small molecules to inhibit the following signalling pathways: glycogen synthase kinase (GSK)-3 beta with CHIR99021, transcription growth factor beta (TGFβ) with SB431542, and Notch with compound E, along with human Leukaemia Inhibitory Factor 3 (LIF3) [13]. This new small molecule-based neural differentiation protocol increased neural differentiation kinetics and allowed the derivation of truly multipotent neural stem cells (NSCs) that respond to regional patterning cues specifying forebrain, midbrain, and hindbrain neural and glial subtypes (see Table 1).

In several neurodegenerative diseases, age is a major risk factor. This suggests a long incubation time for the pathology to develop, taking years for clinical symptoms to appear. This period has been defined as the biochemical and cellular phases occurring before the clinical phase, in a recent review on Alzheimer's disease (AD) [14]. The biochemical phase is described as the phase of abnormal aggregation of Abeta and processing of APP, tau aggregation and hyperphosphorylation, up to the generation of amyloid plaques and neuronal tangles. The cellular phase is described as a phase with defective clearance mechanisms of Abeta and tau, happening both within the cell, occurring possibly as a result of impaired autophagic and proteosomal clearance, and outside the neurons within the neurovascular unit. Both these phases are essential in the appearance of clinical symptoms and are the focus of research into the causes and mechanisms of the disease. Using a model that recapitulates these phases in human cells is a promising avenue. Cell culture models from patient iPSCs recapitulate this long incubation period corresponding to the biochemical and cellular phases, with disease-causing mutation present during development, maturation and aging of the cells. In these models, observations are possible at different time points to study the development of the pathological features. The use of isogenic iPSCs allows the observation of early disease phases in physiologically relevant conditions, without the need to transduce or overexpress specific components of the disease pathology.

Neurodegenerative disease pathophysiology involves degeneration of neurons, with possible involvement of astrocytes, endothelial cells, microglia and oligodendrocytes. The use of iPSC models allows for the generation of isogenic cell types and design of culture with these cells in isolation or combination to study their individual role in the disease pathophysiology. This might allow the identification of specific cells to target in the design of new therapeutic strategies. Indeed, key roles of glial cells in schizophrenia [15], Amyotrophic Lateral Sclerosis (ALS) [16–18], Frontotemporal dementia (FTD) [19] and AD [20] have for example been shown using iPSCs.

In the field of toxicology and drug testing, high-throughput experiments are usually necessary to screen large numbers of candidates. Using an iPSC-derived model allows the generation of an almost unlimited source of functional lineage-specific cells and is thus perfectly compatible with the high-throughput experimental design required. Moreover, toxicity testing on diseased and healthy cells can be done in parallel on isogenic differentiated cell types and 3D reconstructed tissue *in vitro*, an area in which 3D cultures could promise cost effective toxicity testing. For example,

iPSCs-derived neurons have already been used in high-throughput screening and resulted in the identification of several compounds with some activity to reverse Fragile X syndrome [21].

One of the main issues with the use of iPSC-derived models is the variability within cell lines and derivation techniques. To overcome this to some extent, established iPSC lines (following standard protocols and characterization) are now expanded and banked in open access or commercial cell banks (Table 2). This provides uniformity in cells used across laboratories and will hopefully lead to reproducible results across research groups.

Overall, iPSC-derived models provide a promising tool for neurodegenerative disease research, and due to their increased accessibility to the large scientific community through cell banks, they are already leading to new discoveries in this area.

Table 1. Differentiation methods of human iPSC-derived neural progenitors.

Cell types	Protocols	Results	References
Astrocytes	cAMP (1 μM)	S100ß+ (6 weeks), GFAP+ (12 weeks)	[22]
	BDNF & GDNF (20 ng/ml)	GFAP+ (19% at 8 weeks)	[104]
	CNTF, FBS (10%)		[110]
	BDNF & NT3 (20 ng/ml)	GFAP+	[102]
	BDNF (10 ng/ml), SHH (200 ng/ml), GDNF (10 ng/ml), IGF (10 ng/ml), CNTF (10 ng/ml), FBS (1%), FGF1/2 (50 ng/ml)	GLT1+ GLAST+	[111]
	CNTF (5 ng/ml), BMP2 (10 ng/ml), heregulin (10 ng/ml)	~70% GFAP+ (5 weeks)	[112]
Oligodendrocytes	T3, PDGF-AA, IGF1, NT3	O4+ (16 weeks)	[22]
	BDNF& GDNF (20 ng/ml)	OLIG2+ , CNPase+, NogoA+ , BMP+ (42%) and myelinating-like processes (8 weeks)	[104]
	PDGF-AA (5 ng/ml), IGF-1 (5 ng/ml), NT3 (5 ng/ml), BDNF (10 ng/ml)	20–40% CD140a+, 5–10% O4+ (21 weeks)	[113]
	all-trans RA (100 nM) followed by SHH agonist SAG (1 μM)	44–70% O4+ (11 weeks)	[114]

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Cell types	Protocols	Results	References
Neurons	Retinoic acid(100 nM), SHH (100 ng/ml), cAMP, (1 μM) cultured on laminin substrate	TUJ1+, HOXC8+ (6 weeks)	[22]
	BDNF & GDNF (20 ng/ml)	TUJ1+, VGlut+, SYN+ (8 weeks)	[104]
	Growth factor removal, immunopanning using NCAM.	MAP2+ (90%), Voltage gated Na+ & K+ currents and TTX sensitivity (8 weeks)	[115]
	FGF8, PMA and AA on matrigel, followed by BDNF, GDNF & TGF-ß3 (10 ng/ml), AA (200 μM), & cAMP (500 μM).	TUJ1+, evoked APs & TTX sensitivity (4 weeks)	[92]
	FBS (10%) on a gelatin substrate	TUJ1+, SYN+ (2 weeks)	[91]
Cortical neurons	Growth factor removal on laminin substrate	MAP2+, PSD95+. SYN+, VGlut1+, Tbr1+, Ctip2+ Evoked APs	[12]
	BDNF & GDNF (10 ng/ml) on laminin substrate	MAP2+, SYN+, Tbr1+, Brn2+, Ctip2+ (4 weeks)	[110]
	BDNF & NT3 (20 ng/ml)	NeuN+, MAP2+	[102]
Motor neurons	Laminin substrate, BDNF, GDNF, IGF1 (10 ng/ml)	ChAT+, BTX+, HB9+ (10–23%), ISL1/2+, spontaneous post synaptic currents detected (>8 weeks)	[116]
	Purmorphamine(1 μM) & CHIR99021(3 μM) followed by BDNF(10 ng/ml)	ISL1 & HB9(d42), evoked Aps (d30)	[117]
Mid-brain neurons	FGF8a, SHH, AA	TH+, En-1+, FOX2A+, DAT+. VMAT2+, AADC+	[118]
	FGF8a, SHH, RA	FOX2A+, TH+, TUJ1+	[119]
	FGF8, PMA and AA on matrigel, followed by BDNF, GDNF & TGF-ß3 (10 ng/ml), AA (200 μM), & cAMP (500 μM).	TH+, FOXA2+ (50%)	[92]

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Cell types	Protocols	Results	References
Dopaminergic neurons	Laminin substrate, matrigel, BDNF & GDNF added at day 70	TH+, En-1+, FOX2A+, DAT+. VMAT2+, AADC+, spontaneous APs, EPSC, voltage gated Na+& K+ currents and NMDA and GABA gated currents	[118]
	BDNF& GDNF (20 ng/ml)	TH+ (8 weeks)	[104]
Glutamatergic neurons	FGF2 & Cyclopamine (400 ng/ml) followed by BDNF (10 ng/ml)	Glutamine+, VGluT1+ (6 weeks)	[120]
	Cyclopamine(1 μM) followed by BDNF(10 ng/ml)	VGlut1+ TBR1+ (d35) & Glutamine+ (d55)	[117]
	Ngn2 induction followed by Laminin (1 μg/ml) BDNF (10 ng/ml) & NT3 (10 ng/ml)	NeuN+, MAP2+ , TUJ1+, spontaneous post synaptic currents blocked by CNQX (d28)	[121]
GABAergic neurons	FGF2 followed by BDNF (10 ng/ml)	GABA+ (6 weeks)	[122]
	FGF8, PMA and AA on Matrigel, followed by BDNF, GDNF & TGF-ß3 (10 ng/ml), AA(200 μM), & cAMP (500 μM)	GABA+(45%)	[92]
Cholinergic neurons	FGF8, PMA and AA on Matrigel, followed by BDNF, GDNF & TGF-ß3 (10 ng/ml), AA(200 μM), & cAMP (500 μM)	ChAT+ (3%)	[92]
Serotonergic neurons	SHH & FGF4 followed by BDNF, GDNF, IGF1(all 10 ng/ml) & TGFβ3 (1 ng/ml)	Serotonin+, TPH2+, serotonin release in response to tramadol (6 weeks)	[123]

Table 2. Some of the major iPSC banks with notably cells from neurological patients.

Cell bank/ Company	Location	Disease types	No. of lines developed
StemBANCC	EU	Neurological diseases Diabetes Drug Testing	500
EBiSC- IMI	EU	Age-related macular degeneration Frontotemporal dementia Bardet-Biedl syndrome Amyotrophic lateral sclerosis Parkinson's disease Diabetes	367
Cellular Dynamics International - Fujifilm Company	USA	Viral Hepatitis and non-alcoholic Steatohepatitis Autism Spectrum disorders Cardiovascular diseases Idiopathic Pulmonary disease Alzheimer's disease Neurodevelopmental disabilities Blinding Eye disease	1,424
HipSci (Sanger institute)	UK	Bardet-Biedl syndrome Monogenic diabetes Usher syndrome Congenital eye defects	700 control lines and 100 disease lines
CIRM – Coriell Institute for Medical Research (California Stem Cell Agency)	USA	Cardiomyopathies Blinding Eye Diseases Lung Diseases Neurodevelopmental Disorders Alzheimer's Disease Liver Diseases	1,429

3. Methods of iPSC differentiation and maturation for modelling aspects of neurodegeneration

A variety of cells can now be generated from iPSC-derived neural epithelial cells using timed exposure to different growth factors and small molecules, to encourage the differentiation of neural progenitors along specific lineages (Table 1). For models of neurodegeneration, different protocols are required to obtain the specific neural cell types involved in different diseases e.g. dopaminergic neurons for Parkinson models, Schwann cells for multiple sclerosis whereas cholinergic cortical neurons are of interest for AD models (specific protocols for different phenotypes are listed in Table 1).

The iPSC-derived cells can be patterned in the same manner as neural stem cells, however there is often large variability and reduced efficiency in producing neurons from iPSCs compared to embryonic stem cells (ESCs) [22] along with an apparent delay in maturation. Mature

electrophysiological responses in ESC-derived neurons occur after 3 weeks of differentiation [23,24], but appears to be delayed to around 8 weeks in iPSCs-derived neurons [22,25], although both neuronal populations show the same functional properties [26]. It has been suggested that using late rather than early passage iPSCs, increases the capacity of neuronal differentiation and maturation in these cells [27].

Evidence of functionality is one of the most important feature to be observed in any 3D iPSC model through neurotransmitter release and firing of action potentials. To date there is a lack of electrophysiological characterisation of 3D cultures, particularly from patient-derived neurons. Choi *et al*. conducted electrophysiology in their premature and mature control ReN cells to show they contained voltage-gated sodium and potassium currents, the former being blocked with TTX [28]. However, more patch clamp, biochemical and immunohistochemical studies are needed in this model to determine the ability of differentiated neurons to elicit sustained action potentials, form functional synapses expressing key structural proteins and receptors such as N-methyl-D-aspartate (NMDA) receptors, required for induction of long-term potentiation and memory and other AD-relevant proteins (e.g. the sodium calcium exchanger 3) [29].

Producing different neuronal subtypes from iPSCs is now becoming routine (Table 1) and there are efforts to standardise protocols between laboratories. However, neuronal maturation to produce terminally differentiated and functional neurons to study disease mechanisms is still a challenge. In the developing brain there is sequential expression of gamma-aminobutyric acid (GABA) a, NMDA and aminomethylphosphonic acid (AMPA) receptors [30] along with receptor subunit switching to initiate synaptogenesis and maturation. Immature cells of the mammalian dentate gyrus are highly excitable and exhibit reduced GABAergic inhibition; this allows initial communication, enabling formation of synapses, but the excitability then decreases. One may therefore predict nascent neuronal networks, formed *in vitro*, to exhibit high levels of excitatory postsynaptic potential (EPSC) and action potentials that reduce in frequency over time. So far only a gradual increase in excitability has been recorded using patch clamp or multielectrode array (MEA) methods [22,31]. Investigations into levels of GABAergic interneurons and receptor subunit expression in 3D neural cultures may indicate how representative these models are of the *in vivo* situation.

Sensory deprivation during olfactory bulb development is known to reduce survival and density of excitatory neurons [32,33]. The environment in which neuronal cultures are maintained does not recapitulate this variation of stimulation: incubators have constant oxygen tensions and cells are in highly buffered media void of exogenous factors, except for those that are provided (Table 1). To increase external stimuli, neurons can be cultured under electronic stimulus using conductive scaffolds and MEAs, Pires *et al.* found these conditions increased neuronal differentiation and neurite outgrowth [34]. In addition, a new culture media 'BrainPhys' has exhibited improved excitability *in vitro*, by supporting neuronal survival and activity, and, with long-term exposure, by enhancing neuronal synaptic function [35].

As many neurodegenerative diseases are associated with aging, and iPSC-derived neurons usually age only for a few weeks, their utility as a model for age-related neurodegenerative diseases has been questioned. Some studies have attempted to partly address these issues: for example, by using cells from patients with early onset neurodegenerative disease patients as in fAD or by forcing the iPSC-derived neurons to age prematurely. For the latter, there has been interest in expressing progerin, a protein involved in severe premature aging. This protein is implicated in Hutchinson-Gilford progeria syndrome (HGPS), a rare genetic disorder in which a truncated form of lamin A,

progerin, is expressed, leading to defects in DNA repair mechanisms, premature senescence and a very shortened lifespan [36]. IPSCs-derived neurons expressing progerin induce multiple aging-related markers and characteristics such as a decrease of dendrite branching and an increase of neuronal aging process transcripts) [37]. Moreover, progerin-expressing neural stem cells have a reduced proliferation rate similar to aged neural stem cells as well as some accumulation of damaged proteins [38].

Another route to obtain 'aged' neurons is through direct conversion of fibroblast to neurons by reprogramming with a set of transcription factors without reversion to pluripotent state [39–42]. These induced neurons (iN) provide a new approach for patient-specific disease modelling as they preserve donor age-dependent transcriptomic signatures which are erased in iPSCs as they are reverted to a undifferentiated state [43]. A recent study showed generation of induced dopaminergic neurons exhibiting functional dopaminergic neurotransmission by direct differentiation method from human fibroblasts bypassing the lengthy *in vitro* differentiation time. These cells relieve locomotor symptoms when transplanted in Parkinson's disease animal model [44]. Although the iN technology has been available for over 5 years, it is not widely used, possibly due to the low efficiency along with limited proliferative capacity, restricted lineage and difficulties in scaling up the process.

With increasing interest in producing neural subtypes from iPSCs, many labs are developing methods to produce specific cell types (summarized in Table 1) as well as new methods for maturation and aging, but there is a need for homogenisation and validation of protocols if these neural models are to be compared and utilised for drug discovery.

4. 3D iPSC neural models

4.1. 2D versus 3D cell cultures

2D iPSC cultures are suited to individual cell populations or co-cultures, allowing easy access to cells and enabling electrophysiological recordings to study synaptic function [45–47], but 2D cultures do not recapitulate *in vivo* environment as well as 3D cultures. Monolayer cultures have less paracrine cell communication and cell-cell contacts. These cell communications provided by oligodendrocytes and astrocytes are crucial for neuronal behaviour and plasticity. For example, Burkhardt *et al.* observed electrical activity in iPSC-derived neurons at 2 weeks when cultured on astrocytes compared to 3 months when cultured without [8,48]. Pre *et al.* observed improved resting membrane potentials and a greater number of cells producing action potentials in the iPSCs-derived neurons co-cultured with glia. Moreover, 3D cultures show several advantages over 2D including increased viability, neurite outgrowth and synaptic density [49–52].

4.2. Current 3D culture models

Several different types of 3D neural models now exist, each with their own distinct properties; one unifying advantage of 3D culture design is the structural components in their extracellular milieu. By providing the 3D space for cells to secrete extracellular matrix (ECM) proteins that can sequester secreted proteins, as well as retain growth factors and signalling molecules that would otherwise be washed away in 2D cultures, 3D cultures will more accurately model the *in vivo* environment. For instance, chemokines secreted by microglia were found to increased NSC polarisation and migration

in 3D graphene foams but not on 2D graphene films [53], demonstrating that neural cells grown in 3D culture are more responsive to chemokine cues, possibly due to greater retention of these compounds in the 3D environment.

Cerebral organoids developed from iPSCs are ideally suited to study early brain developmental processes, as they spontaneously recapitulate a multi-layered structure, similar to the mammalian cortex [54]. Cerebral organoids have been used to study different diseases such as the microcephaly caused by the ZIKA virus. Indeed, they have been used to decipher the virus mode of action [55–58], showing that the virus targets neural progenitors to kill them. Cerebral organoids were also used to screen libraries to find anti-ZIKA virus compounds targeting neural progenitor cell death as well as viral replication [59–61]. Cerebral organoids have thus been essential in these steps forward to clinical trial for ZIKA infection treatment.

Organoids can grow much larger than 3D cultures but their size is limited to around 400 μm in diameter by the nutrient availability and waste removal in the organoid core, which can become necrotic. They also exhibit high variability between experiments and are generally low throughput. They are limited in differentiation and maturation as their maturation is usually equivalent to human mid to end of gestation brain maturation stage.

By contrast, human organotypic slices from adult brain tissue are suited to study disease/normal state in adults, with most examples coming from epilepsy or brain tumour tissue [62–65]. Only a few studies have been carried out with "healthy" brain tissue [66,67], including one using Abeta oligomers [68]. These *ex vivo* cultures are still the closest we can achieve to a human model, the innate structure is maintained and they include microglia, blood vessels and parts of the blood-brain interface. However, availability of tissue from neurodegenerative patients is very low, healthy brain tissue from patients is rare and mostly comes from tumour de-bulking surgeries or cortex removed to access deep brain regions. Long-term culture remains a challenge and genetic manipulations to test disease hypotheses are not yet possible.

An alternative is 'Hi-spots' [69], dis-aggregated tissue from post-natal brain shown to re-assemble functional networks *in vitro* providing a human model in which patient iPSCs could be incorporated. However, this system remain highly variable and with limited scalability. In 2015, Schwartz *et al.* built a multi layered neural construct, containing endothelial cells and microglia, and used it for successful predictive toxicology using RNAseq and machine-learning technologies [70]. Although these 3D composite neural constructs are in the early stages, they do provide hope of the possibility to engineer tissue that fully mimics human brain in the future.

4.3. Scaffolds

Structural scaffolds to support cell growth in a 3D environment have long been a subject of interest for regenerative medicine, in particular for *de* novo generation of bone [71] and cardiac tissue [72]. Several companies now produce structural biomaterials with the aim of mimicking the mechanical and biophysical properties of human tissue to promote the 3D culture of cells into tissue-like structures for research and drug discovery (Table 3). 3D scaffolds for human cultures ideally should not use animal-derived products and they should allow cell adhesion; for technical applications, desirable qualities include transparency and exclude autofluorescence, to allow imaging. Porous scaffolds, e.g. Alvetex, Mimetix and graphene foams, are produced from animal free, inert materials which are cheap to produce and suitable for large scale, high-throughput applications. They

can be cut to size and are easy to use in the lab but often require coating in ECM proteins for cells to adhere to the scaffolds [71]. Electrospun scaffolds show increased neurite outgrowth [73,74]. In addition, graphene foams show increased conductivity compared to carbon based polymers [75], a property that could advance neuronal maturation in 3D cultures [76].

It has long been known that the stiffness of bio-scaffolds affects the neural differentiation. Ma *et al.* found that a soft graphene foam maintained stemness in NSCs whereas stiffer foams enhanced astrocyte differentiation [74]. As the brain parenchyma lacks rigid structural proteins, many groups have utilised hydrogels to create 3D models more representative of the *in vivo* environment (Table 3). Hydrogels, as the name suggests, are comprised of water and one or more ECM component e.g. hyaluronic acid. They can be tailored to contain any proteins desired and therefore a truly representative extracellular environment containing proteoglycans e.g. brevicans and tenascins could be achieved. Not only can the chemical properties be adjusted but also physical properties including viscosity and stiffness, which has been shown to encourage colony formation [77] and neurite extension [78].

Table 3. Examples of structural matrices used in 3D cultures and their application for neural cultures.

3D Structural matrices	Composition	Neural culture evidence
Alvetex	Polystyrene	Formation of functional glutamatergic synapses [124]
Mimetix	Electrospun PLLA microfibres	Allows differentiation and neurite outgrowth of NSC (ReNeuron) http://www.electrospinning.co.uk/case-studies/stem-cell-differentiation/
Hydrogel containing hyaluronan and cellulose	hyaluronan and methylcellulose in artificial cerebrospinal fluid	Improved survival and integration of neural stem cells [125]
Hydrogels	Various ECM components and H_2O	PEG hydrogel for neurite outgrowth [126] Hydrogel stiffness affects NSC behaviour [127–129]
Pyrolysed carbon	Carbon rods	Increased synapse formation and neurite outgrowth compared to 2D [76]
Graphene foams	Graphite	Biocompatable scaffold for NSC proliferation and differentiation [75] Increasing rigidity enhances differentiation of NSCs [74]
Graphene hybrids	Graphene oxide and polycaprolactone nanofibres	Promotes oligodendrocyte lineage in NSCs [130]

4.4. Biochemical matrices and brain ECM relevant to neurodegenerative models

Matrigel, an ECM protein mix comprising mainly Laminin111 from mouse sarcoma, has traditionally been used as scaffold protein in models for epithelial-mesenchymal transition (EMT), to investigate mechanisms of cell invasion. It has recently been used by several groups to generate 3D neural cultures, showing that 3D Matrigel cultures promoted survival, neurite outgrowth, synapse density and electrophysiological properties compared to 2D Matrigel cultures [51,79] or other 3D substrates [80]. However, its composition is variable and poorly defined, and there are questions about the inclusion of animal-derived products (substrates, serum and growth factors) in models designed to develop and test new human therapies. There are also questions about the suitability of laminin111 as a physiologically relevant structural protein in *in vitro* neural cultures as it is only present in the foetus with expression decreasing during development. Human recombinant laminin 511 (expressed in basement membrane/blood vessel walls of adult human brain) has been suggested as a suitable alternative [81].

To avoid the issues of non-representative ECM proteins, the ideal scaffold for 3D neural models of degeneration would be decellularised human tissue from adult brain. As this is not available, characterisation of the brain ECM would permit synthesis of scaffolds to truly mimic that of the *in vivo* tissue.

Most scaffolds are designed for generic culture rather than to specifically mimic individual tissue types. Brain ECM is less rigid than most other tissues comprising mainly hyaluronic acid, tenascins, brevicans and sulphated proteoglycans [82]. Changes in brain stiffness with age [83] and disease [84] would need to be considered in neurodegenerative 3D models where aging is a factor. Therefore a more tailored scaffold is required to mimic brain ECM, the rigidity of hydrogels are easily manipulated and they can be synthesized to contain any combination of ECM proteins desired making them the best candidate for high-throughput and drug screening assays. Hydrogel degradation also affects stemness and differentiation of NSCs [85].

4.5. Limitations

The main limitation of current 3D models is the lack of blood and cerebral spinal fluid, to supply nutrients and remove waste, and the absence of the immune system. This is particularly important in diseases where inflammation is implicated in neurodegeneration. Advances in perfusion systems may go some way to resolve this. Microfluidic chips are commercially available and can, in part substitute for the lack of continuous nutrient provision and waste removal. As commercial interests in highly reproducible and robust 3D human neural cultures lie in the high-throughput drug screening market, microfluidic chips have been useful in this area as they have been used to assess the cytotoxic effects of anti-cancer agents on tumour cells [86]. However, this principle cannot automatically be applied to systemic therapies targeted towards CNS disorders. The lack of blood-brain interface and therefore the inability to assess fully drug and toxicology outcomes in these models may slow progress through to clinical trials.

In conclusion, the current evidence highlights the potential advantages of 3D cultures to better recapitulate the *in vivo* environment over planar cultures. A range of 3D matrices are currently undergoing investigation, where further work is required to identify matrices that allow neural cultures to faithfully recapitulate the environment of the mature or aged adult brain.

5. iPSC models of AD

5.1. Notable discoveries using 2D iPSC models in AD research

Neurodegenerative studies using 2D iPSC-derived culture systems have for over a decade consistently mimicked biochemical observations in transgenic AD mice of the same genotype. Notably, both models displayed increased production of both Abeta40 and Abeta42 or an increased ratio of Abeta42/Abeta40 [87]. 2D culture models have provided evidence of how iPSCs could be used to test the efficacy of prospective therapies [88]. There is also proof of concept of high-throughput models of tau aggregation with potential for drug screening [89], which have since been replicated in 3D cultures [90].

The use of 2D models has also uncovered mechanisms of neurodegeneration, including disrupted calcium homeostasis, neurotoxicity, accumulation of insoluble and cleaved tau and aberrant phosphorylation at disease-relevant epitopes, tau-mediated mitochondrial dysfunction, oxidative stress [91] and interactions of tau with other proteins, such as GTPase and the cytoskeleton [92]. Many models exhibit physiological as well as pathological tau changes [93–95]; but they do not fully recapitulate the AD biochemical phenotypes as described by Choi *et al.* [87] where Abeta42 levels produced in fAD iPSCs were consistently significantly lower than those observed in AD brain. These 2D models crucially do not form amyloid deposits or hyper-phosphorylated tau aggregate and there is no conclusive evidence as to the synaptic function in 2D iPSC-derived neural cultures. Only two studies [96,97] have so far reported increased activity of tau kinase GSK3 and resultant tau phosphorylation in APP mutant neurons but without changes in synaptic function, therefore questioning the use of this model for uncovering the mechanisms of memory loss in early AD.

5.2. The advent of 3D iPSC models

3D cultures have the potential to be used as more physiological human-derived high-throughput systems for drug screening. Thus, they have the potential to reduce the use of animal models, save time and costs, and reduce inter-assay variability. However, this requires that the conditions and characteristics of the cells (e.g. genotype, source, patient-specificity) be standardised for compound effect readouts. These cells should arguably also be mature enough to express key disease-relevant proteins and exhibit fully functional synapses.

In 2014, Choi *et al.* published their seminal work, which, for the first time, showed evidence of extracellular amyloid deposition and tau hyperphosphorylation and aggregation into filamentous structures in a 3D matrix [28]. They used human neural progenitor cells (ReN cells) transfected with mutant APP (K670N/M617L with V717I) or APPV717I with PS1 E9 and then selected for high overexpression of PS1, APP and APP C-terminal fragments. Although not a physiologically relevant model due to greatly increased protein expression, it opened a new avenue for dementia research. After 3 weeks of culture, the authors reported that the cells were differentiated in neurons (expressing VGlut1) and glia and secreted 1000 fold higher Abeta than physiological levels. After 6 weeks, they noted a 17 fold increased Abeta40 and Abeta42 levels in APP/PS1 culture media, with a 5-fold increased Abeta 42:40 ratio in PS1 cells. This effect was attenuated by beta/gamma-secretase inhibitors, as was the presence of extracellular amyloid deposits. By 7 weeks, both 3R and 4R tau were present at a 1:1 ratio, which is comparable to human brain, and at 10 weeks, phosphorylation of

tau was increased at AT8 and PHF1 epitopes along with presence of tau aggregates in the somatodendritic compartment. These findings were in line with animal studies (e.g. [98]). The authors determined that none of the ReN cells were expressing the high AD risk ApoE e4 isoform; all had an e3/e3 genotype, controlling for any effects of this risk factor in the analyses. The authors explained that the above pathological alterations were evident in a 3D and not 2D environment due to the ability of 3D networks to sequester proteins in the extracellular matrix, which would otherwise be washed away in 2D. There is also the propensity for more complex neural connections to form than on planar cultures, which is key for synaptic maturation and in turn pathogenic mechanisms related to synaptic function and activity. Indeed, neurons exhibited extensive processes after 2–6 weeks of differentiation and this is likely to have facilitated the increased formation of mature tau isoforms compared to 2D—which is integral for modelling tauopathy.

Since Choi *et al.* 2014 paper, the interest in 3D models has vastly expanded to cover a variety of models of neurodegenerative diseases. In 2016, Raja *et al.* used 3D brain organoids comprising iPSCs-derived cells from fAD patients expressing either mutant PS1 or duplicated APP [99]. The fAD brain organoids consistently showed age-dependent pathological changes (including Abeta aggregation, tau hyperphosphorylation and endosome abnormalities) in several different mutant cell lines compared to controls. Treatment of organoids with beta/gamma-secretase inhibitors attenuated accumulation of disease-associated proteins, with the authors optimistic about the translatability and tractability of this 3D AD model for drug development. These cultures were subjected to biochemical assays at 60 and 90 days post-plating. At this time however, the centre of growing organoids began to show necrosis, as indicated by caspase-3 activation. In order to control for this, the authors had to establish a limit of 250 μm away from the surface where cells could be lysed for measurements and not confounded by the necrosis. The authors showed increased soluble, intracellular and aggregated Abeta 37–42 after 90 days and Western blotting revealed the presence of oligomeric peptides. Tau phosphorylation at the Ser394/Thr181 residue was observed by immunostaining and phospho-tau level was increased after 90 days in culture, as opposed to amyloid accumulation after 60 days. This may imply that phosphorylation of tau is an Abeta aggregation-induced effect. Alternatively, it might take longer for levels of phospho-tau, compared to Abeta aggregation, to increase past detection threshold. This model might support the amyloid hypothesis and evidence in AD brain [100]. FAD organoids also exhibited increased sizes of both small and large endosomes compared to controls, in line with findings in mice and both sporadic AD and fAD 2D iPSCs.

IPSCs derived from patients with sporadic AD are now available and can be cultured in 3D. These may provide a crucial parallel between the mechanisms underlying two forms of AD and this may provide important insight into therapeutic strategies for the most common sporadic condition, the causes and early mechanisms of which are yet to be understood [97,101]. Lee *et al.* produced 3D neuro-spheroids using iPSCs from the blood of five advanced sporadic AD patients with no familial mutations [102]. These spheroids showed increased amyloidogenesis, in line with fAD models; interestingly, beta and gamma secretase blockers showed a lower potency for reducing amyloid load under 3D conditions than under 2D conditions, and only 4/5 patient-derived spheroids showed any efficacy of these inhibitors. Proteomic analysis revealed reductions of several proteins that the authors suggest might have contributed to the lower secretase potency in reducing amyloid load. MAP2, PAX6 and the loss of stem cell markers were used to show differentiation. While 2D neurons were treated after 6–8 weeks post-differentiation, 3D neurons were treated after 9 weeks, for a 48-hour period. Abeta 40/42 was measured in conditioned media. There was reduced APP levels in

the 4 patient lines that showed secretase inhibitor efficacy, compared to the line where these inhibitors showed no effect. This finding was associated with reduced levels of clathrin heavy and light chains (involved in endocytosis). This study is the first 3D model of sporadic AD; however, non-demented control patients were not included in the experimental design, which prevents further insight into the amyloid production seen in sporadic AD spheroids. If Abeta levels were indeed measured and observed, it would have confirmed the Abeta hypothesis of sporadic AD and that aggregation of Abeta seen in fAD iPSC studies is not a mere result of mutations or mutant protein overexpression.

Zhang *et al.* modelled mechanotransduction in sporadic AD using a pharmacological approach on differentiated neurons from iPSCs [103]. The 3D cultures displayed elevated levels of phospho-PAK and cytoskeletal proteins such as Debrin, and a decreased level of Cofilin, compared to 2D iPSCs, as observed in late stage AD brains. Treatment of 3D cultures with Abeta oligomers redistributed activated phospho-PAK from nuclear and cytosolic locations to submembraneous regions resembling the AD brain, an effect not observed in 2D iPSCs. There was a high degree of co-localisation of F-actin and Debrin observed in neuronal growth cones of AD cultures. As changes in PAK are shown in late stage AD brains, the above study provides proof-of-concept that 3D cultures can be used to model disease mechanisms.

Reports of neuronal myelination observed in 3D mixed neural cultures [104] along with reduced variability in organoid production makes these highly variable models more amenable for drug screening. Experimentally, both 2D and 3D iPSC-derived cultures can be studied using a multidisciplinary approach as they offer a tractable *in vitro* environment for readouts into AD-relevant mechanisms, namely: electrophysiological recordings of synaptic function and plasticity (thought to be disrupted in early AD and lead to memory loss [105]), live imaging of calcium dynamics (to model early calcium changes in AD [106]) or axonal transport (which is impaired as a result of tau dysfunction in AD models [107,108]) and an array of molecular and biochemical analyses to identify the transcriptional or post-translational protein changes that are induced in AD and play a role in development of pathology and cell loss (such as modifications in tau protein [109]).

6. Conclusion

Modelling pathological processes by expressing disease-associated proteins or studying cells from patients with sporadic forms of disease in iPSCs is not without challenges. However, iPSCs offer several advantages over other cellular or tissue models: (i) most importantly, they are isogenic to patients enabling the study of physiologically relevant mechanistic aberrations; (ii) they allow for the creation of models capable of displaying the early stages of diseases and the long pre-clinical phases; (iii) like ESCs, they are multipotent, and thanks to the recent rapid expansion in our understanding of directing cell fate towards functionally-defined and region-specific neuronal subtypes, 3D models can now be created to mimic specific brain regions associated with disease aetiologies. Moreover, iPSCs do not gather the ethical concerns associated with ESCs. Other concerns raised over the use of iPSCs are being overcome by stem cell banks through protocol standardization, higher availability of patient-derived cells and development of maturation protocols to generate fully functional cells.

The use of patient-specific or disease-modelled 2D iPSC cultures has provided evidence of disease-associated events, yet these models lack certain key features associated with disease

aetiology. 3D cultures incorporate a heterogeneous mix of cell types and produce a more complex ECM capable of retaining molecules in the intercellular space to assist in paracrine signalling. These factors taken together improve cell maturation and functionality to provide a model that more closely mimics the *in vivo* environment, and so far have shown promising results in term of matrices and environment recapitulation. 3D models are amenable to high-throughput experiments, where functional outputs of neuronal activity can be measured through calcium signalling and electrophysiology. These models are certainly applicable to the study of neurodevelopment. However, for neurodegeneration, the crucial factor for use as an *in vitro* model is the presence of fully matured, functioning neurons in which changes in synaptic events can be observed. This remains a challenge which, with all the vested interest, will soon be overcome. With the advent of ever more specific protocols to produce subtypes of neurons, there is a need to standardise these protocols across research and industry. To become viable pre-clinical disease models, 3D cultures must first be validated against current animal models and *post mortem* tissue [131]. This validation is currently ongoing in both academia and industry.

Models of neurodegeneration should be 'fit for purpose' and their design will ultimately depend on the research questions being asked of them. It is also prudent to design models that are easy to image and analyse, especially if investigating synaptic events. For high-throughput drug screening, reproducibility and automation are the key and drug exposure can be aided by the development of sophisticated microfluidic systems. For the 3D iPSC set up to be used routinely in drug screening, challenges such as high-throughput provision or standardized methods still need to be addressed but proof of principle that these models are well-suited have started to accumulate in the literature.

Conflict of interest

All authors declare no conflicts of interest in this paper.

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