3D human brain cell models: New frontiers in disease understanding and drug discovery for neurodegenerative diseases.

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27 **Highlights:**

28 • New approaches to understanding neural cell function are urgently needed.
29 • There have been rapid developments in human stem cell technologies and
30 microfluidic platforms, leading to development of new cell models.
31 • 3D brain cell models and 'brain on a chip' approaches have the potential to
32 produce improved translational outcomes for therapeutics to treat brain
33 disorders.
34
35 **Abbreviations:**

36 APP: amyloid precursor protein
37 BBB: blood brain barrier
38 ECM: extracellular matrix
39 HTP/HTS: high throughput/ high throughput screen
40 iPSC: induced pluripotent stem cell
41 MPTP: 1-methyl-2-phenyl-1,2,3,6-tetrahydropyridine
42 NSC: neuronal stem cell
43 PS1: presenilin 1
44 SOD1: superoxidise dismutase 1
45 TEER: transepithelial/transendothelial electrical resistance
46 Aβ: beta-amyloid

47 **Abstract:**
48 Neurodegenerative disorders have an enormous impact on society and healthcare
49 budgets. There has been a high degree of failure in many recent clinical trials for
50 disease-modifying therapeutics. A major factor in this failure is the difficulty of
51 translating findings from animal-based cell models to human patients. The majority of
52 non-animal neurodegenerative disease research has been conducted in 2 dimensional
53 models of rodent neonatal neurons and glia. While these systems have provided
54 valuable insights into neural cell function and dysfunction, they have largely reached
55 the end of their useful life, as human stem cell technologies combined with major
56 advances in microfluidic technologies have opened the door to development of patient-
57 derived 3D brain cell models. These have major advantages in providing a micro-
58 physiological system more closely reflecting the in vivo brain environment, and
59 promote the interaction between different patient-derived brain cell-types. However,
60 major challenges remain before these model systems will replace the 2D rodent models
61 as the workhorse for neurodegenerative disease studies. Despite these challenges, we
62 are likely to experience a rapid transition of research from old models to new patient
63 derived 3D brain cell systems, which will likely improve translational outcomes for
64 disease therapeutics.
65

66 **Poor translational outcomes for neurodegenerative disease drug development:**
67 Neurodegenerative diseases include the more common disorders such as dementia
68 (characterized by Alzheimer's disease, AD), and Parkinson's disease (PD), together
69 with a large number of rarer disorders such as motor neuron disease, prion diseases,
70 Huntington's disease and many others (reviewed in (Poon et al., 2017)). Despite
71 differences in etiopathology and brain regions affected, neurodegenerative diseases
72 have in common the progressive dysfunction and breakdown of neuronal networks,
73 often together with neuronal cell death, neuroinflammation and elevated oxidative
74 damage in the brain. As the world population ages, neurodegenerative disorders claim
75 an increasing portion of the healthcare budget with an estimated current cost in excess
76 of US$600 billion worldwide. Despite several decades of intense research, there are
77 limited outcomes for therapeutic treatments for any of these disorders, with only a few
78 approved pharmaceutical compounds offering short-term symptomatic relief.
79
80 This deficit of therapeutic options has not come from lack of trying. For dementia
81 treatment alone, there have been more than one hundred new drugs that have reached
82 phase II clinical trials since 2003, the last year when a significant new pharmaceutical
83 (memantine) was approved for treatment of AD (Hung and Fu, 2017). There are a
84 number of likely reasons for this including treatment too late in the disease process,
85 failure to understand the true genotype/phenotype relationship to disease (resulting in
86 patients with different disorders being grouped together), patient variability, potentially
87 incorrect molecular targets, etc. However, one of the key issues, which will be dealt
88 with in this review, is the contribution of poor translational outcomes from
89 neurodegenerative disease models used as a basis for developing treatments.
90
91 **Translational failure of animal models of neurodegeneration:**
92 Understandably, one of the key components of the drug development chain is the testing
93 of potential therapeutic compounds in animal models of disease. This also applies to
94 neurodegenerative disorders where animal (mostly mouse)-based models are used for
95 pre-clinical drug testing. These models are largely based on over-expression of mutant
96 human genes (e.g. APP/PS1 model of AD and G93ASOD1 model of ALS), or induction
97 of disease with chemicals (e.g. MPTP treated mice as model of PD) (Moujalled and
98 White, 2016; Koprich et al., 2017; Sasaguri et al., 2017). While such models have
99 provided valuable insights into disease pathways and identify potential drug targets, as

100 described above, these models have produced very little success in translational
101 outcomes for drugs entering human clinical trials.
102
103 This may be due to 6 reasons: (1) differences between human and animal biology,
104 especially in complex brain structure and function, and overall different metabolism of
105 drugs, (2) the fact that even though the animal phenotypes may be partially comparable
106 to human, the signaling cascades and pathogenic mechanisms in animals may be
107 completely different. This is highlighted by the fact that none of the animal models
108 show Aβ plaque, neurofibrillary tangles and frank neuronal loss at the same time, all of
109 which are important hallmarks of the human disease. In addition, the genetic inbreeding
110 of the animals as well as the housing in germ-free environment limits their
111 comparability to humans; (3) the fact that most models are based on key protein over-
112 expression and often combination of multiple mutations which is not the case in the
113 human disease, (4) simplification of the disease down to a single component i.e.
114 neuronal death in PD models, expression of beta-amyloid (Aβ) in AD models, (5) the
115 difficulty in assessing disease-specific phenotypes in animal models where there is a
116 major limitation on how animals can respond to illness, compared to the complexity of
117 human responses, and (6) possible failures in interpretation of the models (Langui et
118 al. 2007; Jucker, 2010).
119
120 New animal models are very costly and time consuming (although these are somewhat
121 negated by CRISPR/Cas-9 gene editing) (Pamies et al., 2014; Yan et al., 2018), and
122 limitations on how complex human brain disease processes can be modeled in rodents.
123 One advancement in modeling neurodegenerative diseases in rodents are the attempts
124 on creating humanized mouse models in which mouse glia are replaced by
125 transplantation of human iPSC-derived glial progenitors in immunodeficient mice
126 (Windrem MS et al., 2017, Benraiss A et al, 2016). These models allow the
127 investigation of human glial functions in living animals. However, these mice still do
128 not combine multiple cell types even if this is the direction of future development. The
129 ability to combine the action of multiple cell types is especially important when human
130 growth factors substantially differ from those of rodent counterparts.
131

132 At present there are few options to move beyond the use of rodent models. There are
133 major ethical and cost issues associated with using large animal models for drug testing.
134 It is more likely at present that the ability to develop patient-specific complex human
135 cell culture models combining several cell types that can be used to high throughput
136 drug screening will drive advanced drug development using non-animal model systems.
137 They will likely never completely replace animal models: animals will likely always be
138 used to test for drug toxicity due to the need for examination of the entire physiological
139 system with peripheral tissues and pharmacokinetics and pharmacodynamics
140 assessment. We are still a long way from developing a cell-based system that can model
141 the entire human body, but the ability to greatly improve in vitro drug testing is on the
142 horizon and this has the potential to significantly advance translational impact.
143
144 **Translational failure of current cell culture models of neurodegeneration:**
145 As with translational failure from animal models of neurodegenerative diseases, so too
146 have our cell culture models failed in providing accurate interpretation of disease
147 processes and drug action. Cell culture is essential to allow high throughput, ease of
148 manipulation, and cost-effectiveness in models to understand the basic disease
149 pathways and processes, and identify new drug targets. Due to the complexity, time
150 frame of animal breeding, and costs, such basic studies are very difficult to perform in
151 animal models.
152
153 Neuronal and glial cell models have certainly provided much valuable information on
154 many brain disorders over the past few decades, especially on the mechanical
155 background of the diseases (Whitesides, 2006; Horvath et al., 2016), but serious
156 limitations are leading to the need to develop greatly improved models. The major
157 limitations of our current models include (1) they are based largely on rodent brain cells
158 with the same issues pertaining to cellular and molecular differences between animal
159 and humans as described above for animal models, (2) the fact that most of these models
160 have been based on use of neonatal (embryonic or post-natal) brain cells as these have
161 been the easiest to grow, but fail to represent the ageing cells in the brain common to
162 most neurodegenerative diseases, (3) they are mostly used in a 2 dimensional state, but
163 cells growing as a monolayer have very different phenotypes including altered gene
164 expression, organization of cell surface receptors, morphology, and cell-cell
165 connections (Fig. 1 and Table 1), (4) they are mostly used as pure or near pure neurons,

166 astrocytes or microglia rather than mixed cultures, but the ratio of different cell-types
167 is critical to cell responses, (5) human brain cells have traditionally been studied by
168 using transformed (cancerous), neuroblastoma lines of adrenal gland origin that
169 perpetually divide unlike healthy neurons in the brain and behave very differently to
170 normal neurons, and (6) generation of disease models has been largely based on highly
171 artificial single component analysis such as treatment with synthetic amyloid peptide
172 for AD modeling, or treatment with toxins to model PD (Pamies et al., 2014; Horvath
173 et al., 2016). Although rodent cells and secondary cell lines do not represent fully the
174 complexity of human disease, they still provide information on disease mechanisms
175 and are valuable to studies of specific pathways involved in brain diseases. Yet, as with
176 animal models, only modeling one aspect of a disease and ignoring many other changes
177 that occur can lead to a gross mis-understanding of the physiological changes that occur
178 *in vivo* and this should be taken into consideration especially in drug discovery studies.
179
180 In combination with the limitation of animal models above, one can begin to understand
181 why it has been so difficult to generate translational success in drug development for
182 neurodegeneration. However, as discussed below, the confluence of patient stem cell-
183 derived brain cell technology with new microfluidic 3D platforms (Fig. 2) is providing
184 an exciting environment for innovative and complex human-based neurodegenerative
185 disease models to study for example specific interactions between different cell types
186 (neurons-astrocytes-microglia), effect of vasculature and involvement of extracellular
187 matrix to the cell signaling (Majumdar et al., 2011; Pamies et al., 2014; Yi et al., 2015;
188 Murphy et al., 2017; Phan et al., 2017).
189
190 **Human stem cell-based cell culture models for neurodegenerative diseases:**
191 Recent advances in stem cell methodology have opened up a new frontier for human
192 brain cell cultures and models of neurodegeneration. Induced pluripotent stem cells
193 (iPSCs) and neural stem cell cultures (NSCs) allow the generation of human neurons,
194 astrocytes, oligodendrocytes, and more recently microglia, from human, and where
195 possible, patient cells (Sanses et al., 2016; Chandrasekaran et al., 2016; Muffat et al.,
196 2016; Abud et al., 2017). iPSCs, in particular, can be generated from skin fibroblasts
197 and through induction of pluripotency can then be used for subsequent generation of
198 neurons of different sub-types e.g. motor neurons, cholinergic neurons etc., or specific
199 forms of glial cells. Cells can be compared between patients and healthy controls. In

200 patients where a specific gene mutation is the underlying cause e.g. presenilin 1
201 mutation in AD or superoxide dismutase 1 (SOD1) mutation in an ALS patient, the
202 mutation can be 'corrected' by CRISPR editing to produce the ideal isogenic control
203 cell line, with the only difference between the two lines being a mutated gene
204 (Mahairaki et al., 2014; Sproul et al., 2014; Yi et al., 2015; Park et al., 2016; Zhang et
205 al., 2016b; Bhinge et al., 2017; Ortiz-Virumbrales et al., 2017; Wang et al., 2017b).
206 This has the advantage of reducing the number of patients that need to be examined to
207 determine a disease phenotype.
208
209 Unfortunately, as many neurodegenerative diseases e.g. late onset AD, still have
210 unknown, non-genetic causes, many studies still require larger patient cohorts. Despite
211 this limitation, iPSC-derived neural cells have already revealed a number of key
212 differences between patient and control cells for some neurodegenerative diseases
213 (Schwab et al., 2017; Ochalec et al. 2017; Oksanen, et al., 2017; Birnbaum et al., 2018;
214 García-León et al., 2018). AD is one of the most extensively studied disease using
215 iPSC-models. As examples, astrocytes derived from AD patients carrying a deletion in
216 the exon 9 of the presenilin 1 (PSEN1) gene, show increased Aβ production, oxidative
217 stress, altered Ca^{2+} homeostasis and cytokine production and have decreased ability
218 for neuronal support (Oksanen et al., 2017). AD patient derived neurons, on the other,
219 have been shown to exhibit changes in mitochondrial protein expression (Birnbaum et
220 al. 2018) and increased sensitivity to oxidative stress (Ochalec et al. 2017). iPSC-
221 derived neurons from PD patients carrying mutation in their leucine-rich repeat kinase
222 2 (LRRK2) show altered mitochondrial distribution and trafficking corresponding to
223 decreased sirtuin deacetylase transferase activity and leading to bioenergetic defects in
224 dopaminergic neurons (Schwab et al., 2017). iPSC-derived model for tauopathies
225 showed increased electrophysiological activity, and upregulation of stress and
226 neurodegenerative pathways (García-León et al., 2018). The most exciting potential for
227 iPSC-based models is the ability to understand patient-specific characteristics of brain
228 cell models. This, together with high throughput handling processes may lead to the
229 realization of personalized medicine for neurodegenerative disorders. One of the key
230 limitations of iPSCs is that the reprogramming of cells to induce pluripotency may lead
231 to a loss of epigenetic markers that are likely to have a key role in non-germ line
232 mutation-based disease (i.e. late onset Alzheimer's disease) (Imm et al., 2017).

233 However, surprisingly, studies are finding that this is not always the case (Matsumoto
234 et al., 2016).
235
236 Moreover, new approaches are on the horizon in which cells could be induced directly
237 from one cell type e.g. skin fibroblast, into another e.g. neuron by addition of a specific
238 set of growth factors selected by algorithms (Rackham et al., 2016). Despite the exciting
239 future for human and patient-derived iPSCs and inducible neurons/glia as a basis for
240 neurodegenerative disease models, this does not overcome all of the key problems with
241 current cell culture. As described above, the growth of pure populations of neurons or
242 glia in 2D monolayers also produces a large volume of artifactual information that
243 greatly impedes translational discovery. New developments in 3D neural cell culture
244 systems are enabling iPSC-derived neurons and glia to more accurately model the
245 neurodegenerative disease brain.
246
247 **3D human neural cell models to understand neurodegenerative diseases:**
248 ***Organoids:*** The terms 'brain on a dish' and 'brain on a chip' have become synonymous
249 with new advances in cell culture-based brain modeling. But what they mean is still
250 quite a fluid concept. To some, they are referring to the advances in extra-body organ
251 development known as 'organoids'. These are primitive versions of human organs that
252 can be grown in culture. This technology has been applied to the brain and iPSCs have
253 been used to generate human brain organoids (Di Lullo and Kriegstein, 2017). These
254 are small structures that develop the correct layering of some neural cell types and are
255 extremely valuable for studies on development and structure of the brain as well as
256 some neurological diseases including autism (Mariani et al. 2015), schizophrenia (Ye
257 et al., 2017) and Rett's syndrome (Mellios et al. 2018). However, there remain several
258 key limitations to the application of brain organoids for neurodegenerative disease
259 studies and associated drug development. The organoids are currently difficult to grow
260 (although this is likely to change, (Tomaskovic-Crook and Crook, 2017)) and very time
261 and labor intensive, which is not ideal for a straightforward model to understand disease
262 pathways and drug targets. Together with the complexity of the organoid structure,
263 these models are perhaps more akin to a simplified animal model, than a complex cell
264 model. There remains little direct control over the growth and it is complicated by
265 internal necrosis due to lack of vascularization (Yin et al., 2016). The problem with
266 using organoids in studying neurodegenerative diseases may also be the lack of ageing

267 of the cells. The cells in organoids have shown to be closer to fetal tissue than the real
268 age of the patient (Camp et al., 2015). However, Raja et al. (2016) showed that brain
269 organoids derived from AD patient cells had increased Aβ accumulation and tau
270 hyperphosphorylation as well as defects in endosomal activities, proving that the brain
271 organoids may be used also in the research of neurodegeneration. Development of
272 midbrain organoids (Monzel et al., 2017) will also pave the way for studies of PD using
273 organoids. Thus, despite the current limitations with organoids, they are likely to be
274 overcome in the future and organoids will certainly have a key role in
275 neurodegenerative disease modeling. However, due to fact that organoids are time
276 consuming and difficult to grow, the more simple 2D monolayer cultures are likely to
277 remain the mainstay of neuroscience research.
278
279 ***3D cultures of iPSC-derived neural cells:*** An alternative form for 3D modelling is
280 achieved through combinations of brain cells combined to produce a simplistic 3D
281 model comprising neurons, glia, and if possible, a blood brain barrier (BBB, discussed
282 in more detail later) (Pamies et al., 2014; Yi et al., 2015; Choi et al., 2016; Murphy et
283 al., 2017). Such models can obviously be formed using animal cells as well as human,
284 but as mentioned above, the development of human iPSC-derived neurons and glia
285 from patients and controls is driving the use of these highly valuable cell-types in 3D
286 modeling. The development of more complex 3D models that combine several cell
287 types has been boosted by major advances in microfluidic engineering, producing
288 platforms that allow the integration of small numbers of different cell types together
289 with media perfusion, and high-throughput technology (Hogberg et al., 2013; Alépée
290 et al., 2014; Pamies et al., 2014; Lee et al., 2015; van Duinen et al., 2015; Nierode et
291 al., 2016) (Fig. 2). Some of the systems also provide unique platforms that allow
292 integration of neurons, glia and a BBB without separating membranes, thus providing
293 a more accurate model of in vivo neural cell growth, interactions between different cell
294 types, connection of the cells with ECM and the formation of functional neuronal
295 network with synaptic connections between the cells (Phan et al., 2017; Wang et al.,
296 2017b). The advantages of microfluidics will be further discussed below.
297
298 ***The importance of a 3D environment:*** Why is it important to grow neural cells and
299 investigate neurodegenerative disease pathways in 3D? While it is obvious that brain
300 cells do not grow as 2D monolayers in the human brain, what advantages are gained by

301 going to the trouble of generating more complex 3D cultures? To some extent this need
302 is driven by the fact that we are coming to a limit of useful data that can be generated
303 from 2D cultures, as our understanding of (3D) brain complexity rapidly accelerates.
304 We need model systems that can be used to interrogate in vitro the concepts developed
305 from a greater understanding of brain function in vivo (Yi et al., 2015; Phan et al.,
306 2017). Recent studies have shown that neural cells grown in 3D do in fact more
307 accurately mirror in vivo cell growth and function in many ways (Fig. 1 and Table 1).
308 These include differences in morphology (cells in 2D often form unnatural shapes),
309 gene and protein expression and localization, synapse formation, neuroinflammatory
310 responses and neuronal-glial interactions, faster cell differentiation, and cell
311 polarization (Edmondson et al., 2014; Lee et al., 2015; Yi et al., 2015; Murphy et al.,
312 2017; Phan et al., 2017). For example, Zhang et al. (2016b) showed that neuronal
313 maturation was accelerated in 3D culture and they were able to receive
314 electrophysiologically active neurons in only 3 weeks compared to 4 weeks in 2D
315 culture. They also used 3D culture in a migration assay thus avoiding traditional
316 membrane-based assays and giving more reliable and *in vivo*-like results (Zhang et al.
317 2016b). Another study showed that levels of phosphorylated active p21 activated kinase
318 (pPAK) were significantly higher in neurons cultured in 3D environment than in 2D
319 cultures. This kinase regulating F-actin cytoskeleton and dendritic spine dynamics has
320 been shown to be decreased in AD patient brains. In 3D neurons treated with Aβ, pPAK
321 was significantly depleted and its localization was different than in untreated cells,
322 which is comparable to what is seen in AD patients and in rodent models of AD. In 2D
323 cultures, these differences were not seen (Zhang et al., 2016a). In addition, recent study
324 used 3D environment formed by starPEG-heparin-based hydrogel promoting the
325 proliferation of neural stem cells (NSC) and showed that externally administered Aβ-
326 42 decreased the plasticity and regenerative capacity of NSCs that could be restored by
327 anti-inflammatory cytokine interleukin 4 (Papadimitriou et al., 2018). The importance
328 of 3D-modeling is further highlighted by a study showing that proteomic profile of 3D
329 cultured iPSC-derived neuro-spheroids is similar to that of AD patient brain including
330 alterations in proteins involved in axon growth, mitochondrial function and antioxidant
331 defense (https://www.ncbi.nlm.nih.gov/pubmed/29709615). Although 3D models have
332 not yet been extensively used for initial drug screening, Medda et al. demonstrated that
333 a 3D culture model consisting of iPSC-derived cortical neurons can be applied for high
334 throughput screening against tau aggregation (Medda et al., 2016).

335

336 ***The 3D environment and the extracellular matrix:*** One of the key reasons for why a
337 3D environment promotes neural cell growth and function more closely resembling that
338 seen in vivo is due to a much richer extracellular matrix (ECM). The brain ECM is a
339 'hydrogel' of hyaluronic acid with laminins, fibronectin, collagens, vitronectin,
340 tenascins, and proteoglycans (Murphy et al., 2017). These structures provide key cell
341 binding domains, and bioactive motifs that coordinate cell-cell interactions, and
342 signaling from growth factors (Pamies et al., 2014; Murphy et al., 2017). Altered
343 signaling patterns leads to changes in gene and thus protein expression and
344 subsequently cell behavior (Fig. 1 and Table 1). The ECM is also critical for interaction
345 between glial cells and neurons and this process is dependent not only on astrocyte-
346 neuron interaction but also microglia-neuron interaction and microglia-astrocyte-
347 neuron interactions (Pöttler et al., 2006; van Duinen et al., 2015; Murphy et al., 2017).
348 In vivo, this occurs in a 3D manner with cells interacting with many other cells, but this
349 is impaired in 2D cultures. A 3D matrix also controls movement of oxygen, nutrient
350 transfer and removal of wastes in a manner that occurs similar to the brain rather than
351 the batch media of 2D cultures that bath cells in high concentrations of compounds and
352 allow rapid diffusion of waste (Lee et al., 2015; Watson et al., 2017) (Fig. 1 and Table
353 1). Formation of the microenvironmental conditions optimal for neural cell function
354 depends on a 3D matrix and its interacting proteins and other factors (Lin and Bissell,
355 1993). Matrices differ from their composition and there are different ways how the cells
356 are plated to form the wanted 3D structures. They may be synthetic, such as
357 polyethylene glycol, or natural, such as collagen or Matrigel^{TM}. These 3D matrices can
358 be used together with different cell culture systems, such as microfluidic systems and
359 cell-containing modules that increase the possibilities to form gradient of different
360 substrates and metabolites and the lifespan of the cells (Lee et al., 2015).

361

362 ***The need for 3D co-culture models of neurodegeneration:*** While human neural cells
363 grown in 3D cultures can greatly improve modeling of neurodegeneration, and several
364 key examples have already been reported (Yi et al., 2015), ensuring that cultures
365 accurately represent the correct combination of cell types is also important, albeit this
366 is still a major challenge. Combining neuronal cell cultures with astrocytes,
367 oligodendrocytes and microglia is crucial if we are to accurately model brain function.
368 Non-neuronal cells have key regulatory roles on synaptic formation and function,

369 cytokine and chemokine levels, glutamate, glutathione, lactate levels and drug
370 metabolism (Alépée et al., 2014; Phan et al., 2017; Watson et al., 2017). Critically,
371 modeling the correct cell ratios is also important as this directly influences all of the
372 above parameters. Correct modeling of neurodegeneration also depends on a
373 combination of neural cell types. AD is a complex multifactorial disorder and involves
374 synaptic and neuronal degeneration, tau accumulation in specific neurons,
375 inflammatory responses from microglia, accumulation of extracellular amyloid of
376 uncertain cellular origin, oxidative and energy perturbations to astrocytes, changes to
377 the BBB permeability, etc (Kunze et al., 2011; Choi et al., 2013; Choi et al., 2016).
378
379 To move past single component disease studies, cultures need to be generated to model
380 all of these factors together, yet still remain amenable to rapid and simple, cost-effective
381 experimental protocols for standard research and drug screening purposes (Astashkina
382 et al., 2012). However, while this may sound straightforward, and with the current iPSC
383 technology, certainly achievable, there remain major challenges to overcome. Most of
384 the current assay techniques used on 2D cultures cannot easily be adapted for 3D use.
385 For example transepithelial/transendothelial electrical resistance (TEER) is very
386 difficult to apply in many 3D chip-based cultures (Phan et al., 2017). It also remains a
387 challenge to correctly identify cells of a specific lineage and phenotype in combined
388 co-cultures (Pamies et al., 2014; Lee et al., 2015). Many cell markers are not exclusive
389 for a particular cell-type or stage of development, and for ongoing live cell analysis a
390 multitude of cell dyes or constitutive fluorescent markers may be needed. Some of these
391 culture systems limit the ability to recover cells for subsequent analysis e.g. by PCR,
392 limiting the studies to mostly imaging-based (although this too can be hampered by the
393 3D nature of the culture) (Pamies et al., 2014). Another key issue is ensuring that cells
394 express the correct phenotype, markers and functional state when assaying the culture.
395 This is hard to address when neurons may take several weeks to mature while glia might
396 take several months to reach maturity. Microglia may move through phases of different
397 phenotypes (Bohlen et al., 2017). Trying to time all these different growth states
398 correctly is likely to be highly problematic especially in terms of modeling a particular
399 stage of disease. Likewise, co-culture systems often mean a compromise on the type of
400 culture medium used as each cell type generally has it's own optimized conditions
401 (Table 2). In vivo, obviously this is not the case, yet the microenvironment surrounding
402 each cell is conditioned to its own needs. Growing cells in 3D matrices like hydrogels

403 does help to overcome this to some extent as the matrix reduces movement of nutrients,
404 cytokines, and other molecules allowing cells to better condition their localize
405 environment (Fig. 1). On the other hand, different cells may secrete growth factors
406 supporting each other. This may be the case with microglia, which are supported by the
407 CSF-1 secreted by neurons and astrocytes. In comparison, batch liquid culture, which
408 is routinely replaced, interferes with the micro-conditioning process. Despite the
409 challenges, advances are being made to bring different brain cell types together for
410 modeling neurodegeneration (Choi, 2016; Pamies, 2017}.
411
412 **Microfluidic 3D culture systems for modeling neurodegeneration:**
413 The growing need for new 3D model platforms has coincided with recent exciting
414 developments in microfluidic systems and nanofabrication. Microfluidic technology
415 has driven miniaturization of 3D culture systems allowing very small quantities of cells
416 and media to be grown in mono- or co-culture systems, and often integrated into HTS
417 platforms (Joshi and Lee, 2015; van Duinen et al., 2015; Choi et al., 2016; Wang et al.,
418 2017b) (Fig. 2). The microfluidic platforms allow the generation of controlled micro-
419 physiological systems that better model in vivo brain function leading to faster and
420 improved differentiation and survival of the cells. The rapid evolvement of 3D
421 microfluidic modelling is now driving neurodegeneration research from pathological
422 investigation through to drug screening and personalized medicine. Major advantages
423 are gained through microfluidic platforms including spatially controlled co-culture,
424 membrane-free separation of cell types, perfusion flow of media imitating blood flow
425 and vascularization of the tissue, and gradient control for nutrients, oxygen to model
426 for example hypoxia, metabolites and drugs (Funamoto et al., 2012; van Duinen et al.,
427 2015; Choi et al., 2016; Wang et al., 2017b). The platforms can also promote shear
428 stress and improved metabolite and oxygen gradients, which are necessary for ideal
429 BBB models (van Duinen et al., 2015; Choi et al., 2016; Wang et al., 2017b). For these
430 reasons, microfluidistic platforms are ideally suitable especially in the research of
431 vascular aspects of neurodegeneration and in BBB studies, where the artificial flow of
432 fluid is taken advantage of. This type of platform was utilized by Herland and co-
433 workers, who investigated the astrocyte-pericyte crosstalk in neuroinflammation using
434 a human 3D blood-brain barrier on a chip -model (Herland et al., 2011). These systems
435 can be used for evaluation of BBB permeability and transendothelial electrical

436 resistance (Griep et al.) 3D-microfluidic technology on vascular biology research is
437 very nicely reviewed by Cochrane et al.
438
439 3D microfluidic platforms are often ideal to incorporate new synthetic scaffold
440 materials rather than biological hydrogels. The former has the advantage of being
441 highly reproducible and allow controlled structure, but there have been issues with bio-
442 compatibility (Murphy et al., 2017). In addition, many microfluidic systems remain
443 highly complex with proprietary materials, and pump systems to control fluid flow, and
444 there are difficulties recovering cells for post-experiment assays. While some of these
445 systems have clearly already demonstrated important physiologically relevant findings
446 in central nervous system studies, such as axonal growth and guidance (Morel et al.,
447 2012; Dupin et al., 2013; Park et al., 2014), localized protein synthesis and degradation
448 (Deglincerti et al., 2015), synaptic function and formation of connections with other
449 neurons (Coquinco et al., 2014), and in neurodegenerative disease studies, such as
450 mitochondrial transport in PD (Lu et al., 2012), neuro-vascular coupling in motor
451 neuron disease (Osaki et al., 2018), neuronal defects in cortico-striatal circuit in
452 Huntington's disease (Virlogeux et al., 2018) and increased Aβ and
453 hyperphosphorylated tau accumulation in AD model (Park et al. 2018), they remain out
454 of reach for most biologists and clinicians who need rapid, simple to use systems, based
455 on conventional techniques and lab-based assays (Lee et al., 2015). There are some
456 commercial microfluidic systems that offer many advantages to a non-bioengineer user
457 (Moreno et al., 2015; van Duinen et al., 2015; Murphy et al., 2017) and it is likely that
458 the number of commercially available systems will continue to grow in this competitive
459 space.
460
461 **Examples of 3D human cell culture systems for modeling neurodegeneration:**
462 As reviewed elsewhere (Watson et al., 2017), 3D brain models have been extensively
463 generated using animal (mostly rodent) cells. However, due to the relatively recent
464 advent of human NSC and iPSC-derived brain cells, 3D modeling using such cells is
465 still in its infancy. Despite this, several key models have been established and have
466 shown promising outcomes to support the use of this approach for modeling human
467 neurodegenerative disease. The most prominent of these was the human 3D model of
468 AD reported in 2014 by Choi et al., (Choi et al., 2014; Choi et al., 2016). Using an
469 immortalized human neural stem cell line (ReN VM cells), the researchers transfected

470 the cells to over-express human constructs for APP and PS1 with disease causing
471 mutations. The subsequent cell lines were grown in 3D hydrogel for several months.
472 The researchers reported the formation of amyloid-plaque like deposits in the cell
473 cultures, probably due to a combination of high level of amyloid peptide secretion
474 together with long culture periods and an extracellular hydrogel matrix that prevented
475 diffusion of the peptide, leading to localized aggregation (Choi et al., 2016)
476 Importantly, this was the first (and currently remains the only) report of amyloid-plaque
477 like structures naturally forming in cell culture, and is therefore a major step forward
478 in modeling AD. The authors also demonstrated the formation of human AD-like tau
479 protein hyperphosphorylation and paired helical filament formation in the same
480 cultures, providing two of the major AD hallmarks in a cell culture model for the first
481 time.
482
483 The obvious next step from the 'Choi et al' 3D AD model is whether similar cultures
484 can be generated using human patient-derived cells rather than artificially-transfected
485 cells? At time of publishing this has not been reported, and likely reflects the difficultly
486 of growing iPSC or NSC-derived cells that naturally secrete enough amyloid peptide to
487 generate plaque like structures within a reasonable culture time frame. However, one
488 anticipates that such models may soon be available by modifying culture conditions to
489 increase amyloid generation or reduce diffusion/loss of secreted peptide. In the
490 meantime, additional exciting 3D models of AD have been reported. Raja et al.,
491 reported the establishment of organoids from AD patients with mutant APP, or PS1,
492 and demonstrated that these models formed amyloid aggregates (although short of
493 actual plaques), tau phosphorylation and additional abnormalities including endosomal
494 changes (Raja et al., 2016). Similar outcomes were reported by Lee et al., (Lee et al.,
495 2017) who generated organoids from late onset AD patients (non-mutation).
496
497 Although perhaps not strictly models of neurodegeneration, additional 3D human
498 neural cell platforms have been used to identify the toxicity of compounds to human
499 brain cells. These models are critical to advance our understanding of drug toxicity and
500 efficacy and the adaptation of these systems to HTS programs could greatly improve
501 drug analysis (Cheng et al., 2008; Neto et al., 2016; Terrasso et al., 2017). Schwartz et
502 al., have grown human embryonic stem cell-derived neural progenitors, microglia and
503 additional cell types in 3D hydrogel. Using 60 chemicals that were known as toxic or

504 non-toxic the study showed that the model could be used in conjunction with machine
505 learning to predict whether a chemical has neural toxicity based on gene expression
506 patterns (Schwartz et al., 2015). This model provides a significant advance over current
507 systems where a single assay of neurotoxicity is performed. In a similar study, Nierode
508 et al., described the use of a 3D micro-platform to compare neurotoxicity in
509 differentiated and undifferentiated NSCs. (Nierode et al., 2016). A 3D model has also
510 been generated involving human iPSC-derived neurons in 3D hydrogel by Zhang et al.,
511 (Zhang, 2016b), and this was used to examine the role of methyl-CpG-binding protein-
512 2, a protein involved in the neurodevelopmental disorder, Rett Syndrome. The study
513 showed that iPSC-derived neurons from Rett Syndrome patients had reduced migration
514 and differentiation (Zhang et al., 2016b). 3D human neural cell models have
515 additionally been developed to model the BBB. These have been reviewed in detail by
516 Phan et al., (Phan et al., 2017) and while none have yet been directly applied to
517 neurodegenerative disease studies, the ability of the model to incorporate patient cells
518 holds great potential for modeling BBB dysfunction and/or drug delivery in
519 neurodegenerative disease studies (Brown et al., 2016; Geraili et al., 2017).
520

521 **The future:**

522 Due to major advances in human iPSC and NSC methodology and 3D microplate and
523 microfluidic technology, we are on the verge of a new frontier in neural brain cell
524 culture modeling. The next decade will likely see the rapid expansion of patient-derived
525 brain cell models in neurodegenerative disease research, combined with increased ease
526 of use in commercially available 3D microfluidic systems and organoid culture
527 methodology. These models will provide major advantages to neural cell research
528 including the co-culture of major brain cell types, inclusion of BBBs, modeling of blood
529 flow, 3D-dependent cell structure, function and interaction, better integration of cells
530 with ECM, etc. These factors will greatly help us understand the pathological processes
531 underlying neurodegenerative diseases and allow improved translational outcomes for
532 drug targets and compound development (Horvath et al., 2016).
533

534 However, there also remain a number of challenges ahead (Table 2). The processes for
535 developing human microglia are just becoming widely available, and these cells need
536 to be combined with neurons, astrocytes and oligodendrocytes to more correctly model
537 brain processes, particularly neuroinflammation and synapse modeling. There still

538 remain difficulties of combining multiple cell types into a single model but this may
539 eventually be overcome by development of carefully defined media and scaffold
540 matrices that support growth of multiple neural cell types. In addition, new suites of
541 assay systems and technologies will need to be developed to allow assessment of
542 distinct cell types and functions in combined 3D model systems, as well as allow
543 compatibly with HTS systems. The combination of 3D co-culture modeling with
544 patient-derived brain cells and single cell sequencing and Nanostring technology will
545 provide rapid advances in our understanding of cell-cell interactions in
546 neurodegenerative disease.
547
548 Modeling of BBB needs to overcome problems associated with incorrect size and shape
549 of supporting structures and the fact that vasculature in vivo is highly branched. For
550 studies on specific illnesses such as MND, models will need to generate long axons to
551 model the spinal cord. Matrices that mimic natural hydrogels need to be developed that
552 are highly reproducible and available to the science community. On top of all these
553 requirements, one must remember that the most frequent users will not be bioengineers
554 but biologists and clinicians who want easy to operate models to answer key questions.
555 However, with the confluence of biology and engineering approaches, we can
556 anticipate that 3D human brain models are likely to make a major impact on our
557 understanding and treatment of neurodegenerative diseases in the near future.
558
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563
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565 **Table 1:** 2D culture limitations that are partially overcome by using 3D mixed neural
566 cell cultures.
567

	Limitation	Rectification by 3D culture
1	Contact with plastic surface	Cell morphology and growth characteristics are more aligned with normal brain when cells are grown in 3D rather than on plastic surfaces.
2	Limited ECM formation	Complex ECM in 3D culture improves cell signaling that underlies neural cell differentiation and function
3	Cells bathed in culture medium	Many 3D systems allow media perfusion as occurs in vivo, improving exchange of oxygen, nutrients and metabolites, as well as creating a more in vivo-like microenvironment of chemokines, cytokines, growth factors etc
4	Limited interaction between cell types	Studies show that improved interaction in 3D between neurons and glia drives more in vivo-like differentiation and functional outcomes for all cell types
5	Poor representation of blood-brain barrier (BBB) interface	Cells grown in 3D form more in vivo-like cell-cell interactions involving endothelial cells, astrocytes and pericytes, improving modeling of BBB.

568
569

570 **Table 2:** Challenges of 3D microfluidic brain on a chip models

571

1	Incompatibility of certain scaffolds with cell interactions
2	High cost of complex microfluidic systems
3	Difficulty of using novel and complex 3D systems
4	Incompatibility of 3D systems with current post-treatment assays
5	Balancing media requirements for multiple cell types
6	Balancing the timing of maturation of each cell type
7	Difficulty of achieving optimal drug diffusion in 3D systems
8	Reproducibility issues with organoids
9	Modeling capillary-like branched vasculature in BBB models

572

573 **Figure legends:**

574

575 **Figure 1:** Comparison between 2 dimensional (2D) and 3D neural cell culture
576 systems. In 2D cultures, the cells are grown on artificial plastic surface without a
577 touch to ECM that is essential in the actual cellular functions. All the cells are freely
578 in touch in nutritionally rich cell culture medium which does not occur *in vivo*. There
579 is also limited contact and interaction between different cell types. In 3D culture the
580 cells are grown inside hydrogels or ECM they have fabricated by themselves (as in
581 the case of organoid cultures). This limits the contact within the cell culture medium
582 and thus the availability of nutrients and oxygen resembling more the *in vivo*
583 environment. Several cell types may be cultured together allowing complex
584 interaction between the various cell types. ECM = extracellular matrix.

585

586 **Figure 2:** Convergence of technologies driving the growth in 3D ‘brain on a chip’
587 platforms that enable the growth of human brain-like structures of the patient’s own
588 cells, HTP genetic analysis of the patient and HTP drug screening to clear out what
589 treatment suits the best for each patient leading to more personalized medicine. iPSC
590 = induced pluripotent stem cells; HTP = high throughput.

591

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