Alzheimer's Disease & Treatment

Chapter 2

Preclinical Modeling of Alzheimer's disease - Success and Limitations

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Abstract

Preclinical neurodegenerative disease models have been the cornerstone of neurodegenerative research in the past century. Although these models are inherently flawed, it is undeniable that they have provided rare access to the complexities of the nervous system and linkages between mechanistic and behavioral changes in the study of neuropathology. In this chapter, we discussed the development of models used in Alzheimer's disease (AD) research. We have also looked at the insights obtained about AD pathology and the possible limitations of using these models.

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1. The Development of Alzheimer's disease

Before the first pathological report of Alzheimer's disease (AD) by Alois Alzheimer in 1906 [1], the medical and scientific understanding of dementia was shaped by the observation of mental decline with age [2], which was associated with neurosyphilis or cerebral damage [3,4]. The description of illnesses was heavily influenced by individual perceptions and the cultural backdrop of society [5]. Due to a lack of understanding of the underlying cause, medical conditions associated with the loss of memory and cognitive impairment were generally recognised as a form of dementia. Interestingly, dementia was also thought of as a disease of demon possession and occult behavior, with the domination of theocracy in 5th century Roman [6]. It took a further 80 years from Alzheimer's reports, for molecular biology advancements to provide an objective understanding of AD (**Figure 1**).

For most of the late 20th and early 21st century, research strategies and the management of clinical symptoms of AD were heavily influenced by relatively premature conclusions about AD pathology. Due to the infancy of AD research and a total lack of experimental models of AD, dementia research at this stage was conducted solely on post-mortem human brain tissues. Neurotransmitter content was first examined in the early 1980s in an attempt to further understand dementia pathology in the absence of resources for an in-depth biochemical examination of AD. A marked decrease in choline acetyltransferase was observed in various regions of the AD brain [7,8]. This premise has since served as the basis for AD treatment, albeit with limited efficacy [9]. It was later found that an amyloid-bearing neuritic plaque in the AD cortex likely contained degenerating neurites of varying neurotransmitter identities [10–12]. Each of the neurotransmitter systems in the central nervous system (CNS) can be equally affected. Recent studies using modern imaging modalities and advanced biochemical methods presented controversies in the role and overall relationship of the cholinergic pathways in AD pathology [13,14].

As the main pathological hallmarks of AD, it is natural that extracellular senile plaques and intraneuronal protein tangles garnered much attention in the early stages of AD research. Electron microscopy and basic biochemical methods revealed that neurofibrillary tangles (NFT) are composed of human tau protein assembled into distinct paired helical filaments structures (PHF) [15–18]. As NFTs were identified in several etiologically distinct neurological disorders [19,20], it was initially thought that tau aggregation was a non-specific outcome of neuronal damage. On the other hand, amyloid-beta (Aβ), the primary component of the plaques found in AD brains, was first identified from plaque samples isolated from meningeal blood vessels of AD and Down's syndrome patients [21]. It was later confirmed that this same Aβ protein was present in senile plaque cores isolated from the AD cortex [22,23]. There is also a relative abundance of extracellular plaques in AD and trisomy 21, compared to normal brain aging and other age-related degenerative brain diseases, suggesting its importance in

dementia and AD.

1.1. Understanding the development of AD pathology

The search for detailed mechanistic descriptions of processes leading to AD pathophysiology led researchers to look for a more amenable model system than autopsy samples from deceased human patients. Three main types of culture systems have since been widely adopted for AD studies - organotypic tissue culture, 2D cell and primary culture, and neuron-like neuroblastoma cell culture.

The technologies for living cell culture were first developed in 1910. Beginning with the initial hanging drop method of tissue or cell culture with semi-coagulated serum or lymph [24], cell culture methods have been modified throughout the years to allow for aseptic and precise spatial and temporal control of nutrient availability in culture. A comprehensive review of the development of cell culture methods was presented by Millet and Gillette [25] and Yao and Asayama [26]. A neuroblastoma is an embryonic malignancy of the sympathetic nervous system, which shares features of plasticity with developing neural crest stem cells. Depending on the line used [27], chemical differentiation of neuroblastoma cells into neuron-like cells with dopaminergic and cholinergic properties is possible. Furthermore, due to their potent renewal capabilities and the relative ease of transfection, neuroblastoma cell lines are often used in situations where rodent brain tissue is not readily available or when genetic manipulation of the cells is essential for a study.

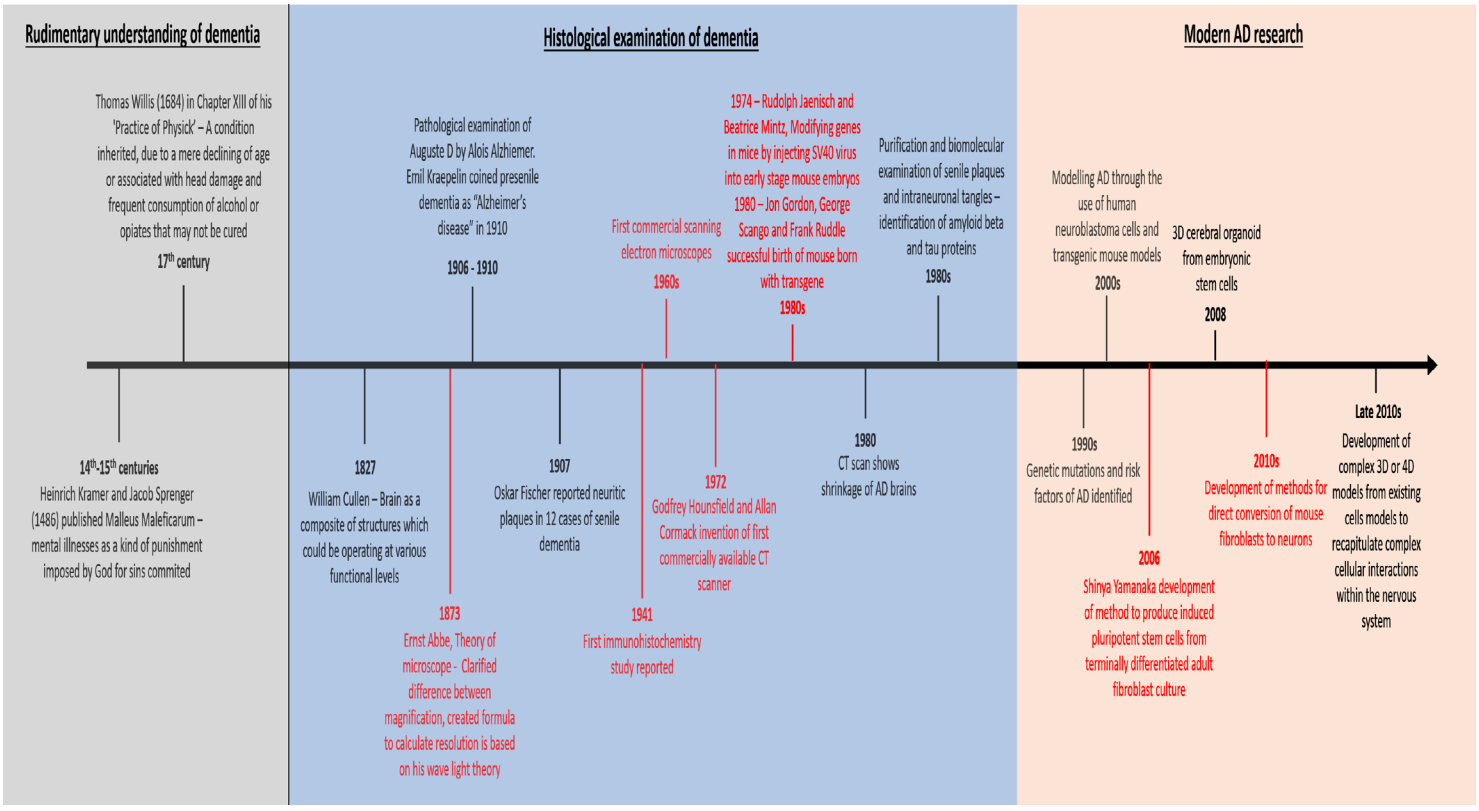

Figure 1: Timeline of AD research. Key milestones from the dependence of diagnosis on the subjective clinical description and cultural experiences of dementia to the current evidence-based delineation of AD pathology. Recent advances in imaging and biomolecular methods (red) have provided new avenues and accelerated AD research.

Initial studies using cell culture with immunostaining methods have established that amyloid precursor protein (APP) and presenilin-1 (PS1) are widely expressed throughout the rodent brain [28–30]. APP likely performs an as yet unknown cell signalling function within neurons as it is preferentially expressed on the neuronal surface and possesses characteristics of glycosylated cell-surface receptors [28,31]. The presence of AD-associated APP mutations have resulted in an enhanced Aβ1-42 peptide generation in cell culture models [32], reflecting a similar observation in familial AD patients carrying PS1 mutations [33]. When present in the extracellular environment, Aβ exerts toxic effects on primary neurons through induction of oxidative stress [34], reactive astrocytosis [35], and direct damage [36]. The relative contribution of different Aβ species to neuronal toxicity is currently under debate [37].

A more comprehensive understanding of tau in the diseased state has been obtained from studies of human tissues. Tau is preferentially hyperphosphorylated in AD brains and the extra phosphorylation prevents its interaction with microtubules [38,39] and promotes its dissociation from the cytoskeleton [40]. Protein kinases and phosphatase such as mitogen activated protein (MAP), protein phosphatase-2B (PP-2B), and glycogen synthase kinase-3 (GSK-3) are capable of changing the relative electrophoretic mobility of AD patient's brain tissue-isolated tau on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [41–43], suggesting their involvement in abnormal tau hyperphosphorylation. Further examination of PHF in vitro shows that an increase in PHF formation is linked to the reduced turnover of tau protein [44]. Furthermore, advanced glycation end products (AGE)-modified tau proteins observed in sporadic AD samples can induce oxidative stress in SH-SY5Y neuroblastoma cells [45], pointing to the potential role of modified tau proteins in promoting neuronal dysfunction. However, cultured cells and neurons expressing genetic mutations associated with Down's syndrome and AD are hypersensitive to neuronal peptide or growth factors-mediated cell survival and DNA damage [46–50]; this suggests that a genetically encoded predisposition towards neuronal death is the main cause of AD development in familial AD patients.

1.2. Modelling AD development

Even though research on APP, PS1, and tau proteins has provided a good understanding of AD pathology, the majority of AD patients suffer from late-onset AD (LOAD) with unknown causes. The presence of classic AD hallmarks, Lewy bodies, synaptic dystrophy, and loss of neurons without neurological problems are frequent observations in aged brains [51]. A reduced body defense system [52,53] and cellular compensatory mechanism [54,55] likely amplified the effect of accumulated cellular damage and long-term changes triggered by the long-term environment, driving neuronal demise. The complete impact of various environmental and man-made contaminants on neurological function has been reviewed by Dr Halden's and Dr Moklas' group [56,57]. To mimic the environmental exposure of neurotoxins and stressors of the nervous system and their impact on neurological function, cultured neurons and neuron-like

cells or rodent brains are directly treated with neuronal modulating or damaging conditions, and biochemical and functional outcomes are examined.

Drugs and chemicals which induce cellular defects observed in AD are often used directly to model AD pathology. Scopolamine is a natural alkaloid and a selective competitive muscarinic receptor inhibitor previously used to treat motion sickness and postoperative nausea [58]. The scopolamine model was used to study the clinical correlates associated with cholinergic deficiency [59] following a study showing that the administration of the drug into healthy young volunteers caused a decline in memory profile similar to that observed in the aged [60]. However, due to controversial cognitive and physiological changes that do not necessarily mimic actual AD alterations, scopolamine was never used as a standard model for the assessment of AD drug efficacy. On the other hand, colchicine is more preferentially used in drug screening. Colchicine is an anti-inflammatory drug used to treat gout that was shown to be neurotoxic towards cholinergic neurons [61]. It binds to soluble tubulin to form a stable colchicine-tubulin complex and prevent microtubule elongation [62]. Furthermore, colchicine triggers cox-2-dependent neuroinflammation thought to be a prominent pathological phenotype and contributes to neuronal vulnerability in AD [63,64]. In the same vein, lipopolysaccharide (LPS), and sodium azide or okadaic acid, known to induce neuroinflammation and oxidative stress are administered directly onto cultured cells or into the brain of animals to investigate the role of inflammation and cellular dysregulation in AD development.

Alterations of cerebral blood vessels resulting in brain infarcts and the impaired the ability to deliver the metabolic substrates required for the basic neuronal functioning are linked to the development of vascular dementia [64,65]. Hypoxia can be induced by chemicals, surgery or brain injury. Chemicals frequently used to trigger a hypoxic-like state include carbon dioxide, carbon monoxide and sodium nitrite [66–69], while localised and global cerebral ischemia can be induced by radio-lesioning of the medullary lamina of the thalamus or a bilateral carotid artery occlusion followed by prolonged reperfusion, respectively [70,71]. The hypoxic condition has been shown to reduce lipoprotein receptor-related protein-1 (LRP-1) expression in an immortalized mouse cerebral microvessel endothelial cell line, resulting in the reduced clearance of Aβ [72,73]. Furthermore, there is an increase in tau seeding and accumulation, resulting in memory deficits [67], and thus suggesting that hypoxia accelerates both Aβ and tau accumulation and initiates the development of AD pathology.

Personal dietary habits have been linked to various cognitive states. Obesity or alcoholism-associated cognitive impairment is a rising area of research due to its negative impact on public health. Chronic ethanol exposure followed by subsequent withdrawal [74] and high fat diet treatment are common models adopted to mimic alcoholism and obesity states. Genetic risk factors such as the dopamine D2 receptor gene (DRD2) and leptin deficient ob/ob or leptin receptor defective state (db/db) have also been investigated. High alcohol consumption

leads to the formation of alcohol-related dementia (ARD) and accelerated cognitive decline with age [75–77]. Moderate to heavy alcohol consumption results in a 57% increased risk of developing dementia, whilst studies have suggested that AD patients who frequently drink are diagnosed with dementia at an earlier age than those who do not consume alcohol [78,79]. N-methyl-D-aspartate receptor (NMDAR)-dependent excitotoxicity and compromises in thiamine metabolism are key theories for the development of ARD. Epidemiological studies have revealed that middle age obesity increases the risk of dementia and AD [80–82]. Cardiovascular diseases and diabetes, common comorbidities of obesity further compound the risk of dementia development [83,84]. Neuroinflammation and vascular damage are the most common mechanisms implicated in the development of dementia and AD in ARD patients and obese individuals.

Heavy metals are recognized environmental pollutants that persist in the environment, and are known to induce nervous system toxicity. They have been increasingly released into the environment with the advent of the industrial age [85,86]. Heavy metals entering the human body have been found to accumulate in the brain-periphery barriers [87], leading to vascular damage and cerebral hemorrhage [88–90]. Upon entry into the CNS, methylmercury likely suppresses the tropomyosin receptor kinase A (TrkA) pathway and induces apoptosis as seen in differentiating PC12 cells [91]. Lead (Pb), on the other hand, inhibits heme synthesis, compromises energy metabolism [68,92] and interferes with neurotransmitter release [93] and calcium (Ca)-dependent neuronal function [94,95]. Dysregulation of metals vital to biological processes can also exacerbate age-dependent neurological disorders. Copper (Cu) is obligatory for enzymes involved in aerobic metabolism such as cytochrome c oxidase and a cofactor of superoxide dismutase, which protects cells from free radical damage. An increase in the concentration of serum copper has been observed in some AD patients [96] and the 2495 A>G ATP7B polymorphism is present at higher frequency in mild AD patients [97]. ATP7B encodes for a copper transporting ATPase 2 responsible for the sequestering of free inorganic Cu^{2+} and regulating free Cu^{2+} uptake and transport out of the brain [98]. By itself, free Cu^{2+} accelerates Aβ deposition and aggregation [99] and the presence of low concentrations of Cu^{2+} (0.12 ppm) in the drinking water of a rabbit model of AD enhanced AD pathology and accelerated memory loss [100], although presence of a similar concentration in chow caused no toxicity [101]. Similarly, a study has shown that humans consuming supplements that contain Cu^{2+} displayed cognitive deterioration at six times the rate of controls [102]. Therefore, the actual significance of Cu^{2+} dysregulation in the development of cognitive defects and AD requires further interrogation.

1.3. Modelling AD using transgenic animal models

The development of methods for the specific introduction of transgenes into animals [103], has led to the generation of many transgenic animal models for a myriad of physiological

diseases. Transgenic modelling of AD is often performed using mice as their use is relatively inexpensive, they have a comparatively short life span, and they are closer phylogenetically to humans. Hence, experimental outcomes can be achieved at a relatively lower cost and shorter time scale, which is more translatable to the pathology observed in human patients. A comprehensive list of the common transgenic AD rodent models, their original source, and their disease phenotypes has been elegantly presented by the Alzforum [104]. The switch from simpler 2D culture models to 3D animal models is essential to investigate the synergistic effects of various neurological cell types and different physiological systems in the development of AD. It reveals the dynamic nature of pathology development that is impossible to capture in a reduced 2D model system.

The process of neuroinflammation is an essential contributor to brain atrophy and subsequent cognitive decline characteristic of AD, yet this aspect is often overlooked in 2D modelling. A great number of genetic risk factors implicated in sporadic AD are directly associated with neuroimmune processes and microglial activity [70,105,106]. Microglia cells are brain-resident immune cells and one of their roles is to prune synapses via the immune complement pathway, in order to clear apoptotic neurons [107]. However, their overactivation and subsequent release of pro-inflammatory cytokines have detrimental effects on neurons by modulating astrocyte activation and inflammatory status [108]. In AD their overactivation leads to excessive engulfment of neuronal synapses, resulting in reductions in neurite lengths and density and consequent neuronal hyperexcitability [109]. Inhibition of complement pathway components, as well as the depletion of microglia, have both been reported to exert neuroprotective effects on neurons and reduce pathology in AD mouse models [110–112]. Inhibition of complement pathway components was also shown to reduce tau pathology [106,110], suggesting that microglial activation not only aggravates neuronal dysfunction directly via synaptic engulfment, but also indirectly via the amplification of tau pathology. Microglial activity has also been associated with Aβ pathology; microglia have protective functions in clearing pathological Aβ, mediated by Trem2 and ApoE proteins [111–114]. However, variations in TREM2 and APOE genes have been strongly linked with the presentation of sporadic AD [115–117], and have thus been proposed to lead to microglia malfunction and increased Aβ plaque accumulation [118,119]. The consequence here is a spiralling of pathological effects; Aβ induces further microglial overactivation, pro-inflammatory cytokine release and complement pathway alterations, which exacerbate Aβ plaque deposition [120,121]. Abnormal neuronal excitability has been observed in the key transgenic human amyloid precursor protein (hAPP), APP/PS1, and 3xTG familial AD models [122–125]. The mechanism here is likely an initial neuronal dysfunction [126] that promotes a compensatory mechanism, leading ultimately to reduced long-term potentiation and increased long-term depression of synapses which correlates with cognitive deterioration in later stages of the disease [127].

2. Failure in Preclinical to Clinical Translation of Developed Treatments

Despite the potential from drug development in non-human AD models, translation of preclinical research outcomes into effective treatments for human patients has been largely unsuccessful. There has been a consistent failure of numerous high-profile clinical trials to reduce Aβ and tau pathology and improve behavioral outcomes. Many promising Aβ-targeting monoclonal antibodies have failed their Phase III trials. Although aducanumab was recently approved by the FDA for AD treatment, there have been high levels of scrutiny surrounding its potential clinical effect. Similarly, alternative tau therapies involving the use of small-molecule drugs to inhibit tau modifications, and aggregation, have had limited success. These failures place doubts on the accuracy of the amyloid and tau theories of AD development. In the preclinical context, the role of normal physiological levels of soluble and insoluble Aβ is, at best, contradictory. While exposure to low Aβ dosage promotes the release of neuroprotective BDNF, larger doses of Aβ induce presynaptic and postsynaptic defects and neuronal dysfunction. In the hope of finding a novel drug target for AD, research into alternative AD mechanisms is in progress. There has been an increasing focus on metabolic and physiological processes such as mitochondrial dysfunction, insulin resistance, neuroimmunomodulation, and cerebral hypoperfusion. Nonetheless, there remains no satisfactory explanation that can fully describe the initiation and development of AD.

Rodents do not spontaneously develop Aβ or tau lesions without genetic manipulation [128], suggesting that the internal mechanisms which drive the pathological expression of these proteins may not even be present in these species [129]. Even in transgenic mouse models, reproducing tau pathology has proven tricky; models that express mutations associated with the development of Aβ, such as in APP or PS1, do not develop tau tangles despite their presentation being heavily linked with Aβ activity [129,130]. Models such as the 3xTg model which also contain a tau transgene associated with frontotemporal dementia, must therefore be harnessed to ensure Aβ and tau pathologies are accurately reflected as much as possible, particularly as they appear essential components of the pathophysiology of the disease. However, care must still be taken as transgenes in such models become randomly incorporated into the host genome, meaning expression patterns may not be biologically accurate. For example, transgenic mice containing the APPswe mutation (such as APP/PS1 and 3xTg models) have reported to result in Aβ pathology, but also overproduction of other APP fragments, which can be seen as overexpression-related artifacts. Therefore, pathological changes that occur in these strains cannot be attributed entirely to the overproduction of Aβ [131,132].

There is a fundamental mismatch in the physical and functional characteristics of rodent and human neurons and glia that affects AD development. Due to the importance of dendrites in the processing of input signals propagating towards the cell soma [133,134], the difference in size of dendritic arbours of human pyramidal neurons compared to those in rodents [135,136]

can lead to differences in neuronal outcome. For example, there is an increase in electrical compartmentalisation and changes in input-output properties, as a result of decreased channel densities within human neurons [137]. There is also an absence of h-channels in mouse supragranular layers, although these are abundant in human subgranular layers [138], further pointing to differences in signal processing in the brains of the two species. Furthermore, human astrocytes are more susceptible to oxidative stress likely due to their higher basal mitochondrial respiration rate and lower expression of enzymes required for the detoxification of peroxisomal reactive oxygen species [139]. Furthermore, in response to amyloid, human and mouse microglia have been shown to take diverging approaches [140]. In the human AD brain, microglia increase the expression of homeostatic genes and AD risk genes which are likely controlled by the transcription factor IRF8, reminiscent of the IRF-8-deriven response observed in spinal cord microglia following peripheral nerve injury [141]. On the other hand, mouse microglia will increase expression of their disease-associated microglia genes, which are related to positive disease control [142].

3. The Advent of *in vitro* Human Models in AD Research

In order to overcome animal model limitations, researchers have looked to utilize human induced pluripotent stem cell (iPSC) technologies. Yamanaka and Takahashi initially showed that the addition of the transcription factors Oct3/4, Sox2, Klf4 and c-Myc could maintain pluripotency of mouse embryonic cell cultures, and convert adult fibroblast cell back to pluripotent status [143]. They subsequently attempted to implement the same techniques on adult human fibroblasts, and were able to successfully reprogramme fibroblast cells into pluripotent stem cells [144,145]. This was important, as the reprogramming of human somatic cells into pluripotent cells enables the derivation and study of stem cells from somatic cells of AD patients, combating the limited access and ethical concerns using brain samples from patient post-mortem. The use of human cells further overcome rodent model limitations. Additionally, the gene expression and epigenetic status of human pluripotent cells induced from fibroblasts was found to be similar to that of human embryonic stem cells. Therefore, these techniques enable the study of not only AD pathology, but also further examination of potential genetic and epigenetic modifications which may contribute to disease pathology.

3.1. AD induced pluripotent stem cell-derived neurons in drug testing and mechanistic studies

Once pluripotent cells are obtained from human fibroblasts, they can be converted to mature neurons with direct or indirect cellular reprogramming. In indirect lineage reprogramming, pluripotent stem cells are sequentially converted to mature neurons, first using the small molecule inhibitors Noggin and SB431542 to inhibit the SMAD signalling for neural fate induction [146]. The resulting neural progenitor cells can be further converted into

neurons, astrocytes and oligodendrocytes in the presence of FGF-2 [80] or small molecules which impact FGF, ERK, Notch and Wnt pathways [148], mimicking the natural development of the human nervous system. Alternatively, the ectopic expression of transcription factors can convert differentiated non-neuronal cell types or stem cells directly to neurons [149–151]. Reduction in steps of neural induction and avoidance of transcriptome reprogramming accelerates neuron production and preserves the age and experience-dependent epigenetic profile of source cells [152–154]. The development of microglial cells in 2D systems was initially more of a challenge since microglia are derived not from neural progenitor cells but a macrophagic lineage. However, microglial cell differentiation has also been achieved following iPSC addition to a microglial differentiation medium containing colony-stimulating factor 1 (CSF-1) and IL-34 [155].

As reviewed by Arber et al., [156] 2D iPSC models have advanced understanding of the normal function and processing of APP and PS1 proteins, as well as downstream signalling events which lead to their toxicity in AD. For example, the application of Aβ to iPSC cultures has been shown to reduce vesicle clusters in neuronal axons and impair AMPA receptor function [157], which may contribute towards excitatory/inhibitory imbalances and deficits in long-term potentiation of synapses. iPSC models have also enabled the study of genes associated with the formation of sporadic forms of the disease. For example, variations of the SORL1 gene, which encodes the neuronal ApoE receptor, have been shown to increase risk of sporadic AD, due to an increase in Aβ expression as a result of alterations in brain-derived neurotrophic factor (BDNF) signalling [158]. Finally, 2D models have enhanced drug-screening possibilities; the inhibition of β-secretase and γ-secretase has been shown to differentially reduce Aβ pathology [159], whilst the Aβ42 inhibitor compound W, as well as the non-steroidal anti-inflammatory drug sulindac sulfide, have been shown to reduce the Aβ42:Aβ40 ratio in iPSC AD models [159,160].

Despite these advancements, spontaneous presentation of plaques and tangles in human neurons has not been observed [129], which is a considerable limitation of these models. Furthermore, iPSC-derived neurons and CNS cell types are inherently heterogeneous in nature [161], and making use of techniques which reduce this experimental variability is essential [162]. To ensure reproducibility of phenotypes observed in iPSC studies, researchers are often requested to repeat their experiments in multiple independent iPSC lines, whilst using isogenic iPSC-derived models of AD is an alternative way of addressing the problem of variability. Additionally, similarly to rodent models, 2D iPSC models are limited in their ability to incorporate the effects of aging and environmental factors to disease pathology. Neuroimmune processes and dysfunction in such interactions are thought to be a major contributor towards disease pathophysiology, demonstrating the need for co-culturing of multiple cell types. The development of 3D cerebral organoids looks promising to address these downfalls, enabling

the study of both familial and sporadic genetic risk factors and interactions between multiple cell types, whilst maintaining relevance to pathological mechanisms occurring in human AD patients.

3.2. Importance of 3D cerebral organoid models in AD research

The development of cerebral organoids has been a significant advancement in the modelling of AD, and is a promising step in attempts to increase translational validity of research to the clinic. The development of organoids initially follows that of 2D models as described above, namely; neural induction (SMAD inhibitors), patterning (FGF-2 or small molecules impacting FGF, ERK, Notch, Wnt pathways) and terminal differentiation into mature neurons. Organoids are then produced by allowing cell autonomous signals and spatiotemporal signalling events to determine cell migration and the self-organised generation of cellular subtypes [156]. During this time, cells are suspended in Matrigel containing laminin, entactin and collagen, which resembles the extracellular matrix. These proteins provide a scaffold for the adhesion, strengthening and structuring of cells. Supplementation with neurotrophic factors such as BDNF, growth-derived neurotrophic factor (GDNF), ascorbic acid and dp-cAMP further enhance neurogenesis, synaptogenesis, and cell differentiation, development and survival [163].

AD research harnessing 3D organoids may enhance understanding of mechanisms of the disease which have been difficult to emulate in both animal models and 2D models, as it allows the addition of AD-risk-associated genes to mixed cultures, and subsequent experimentation to establish how these contribute to pathophysiological dysfunction. For example, the role of the APOE4 allele has been studied via the expression of the gene in organoids using CRISPR-Cas9 technology [164]. Alternatively, iPSCs can be obtained directly from APOE4 carriers and subsequently cultured and studied; upon harnessing this technique, Zhao et al., found that carriers of the APOE4 allele had exacerbated neuronal death and synaptic loss compared to APOE3 carriers [165]. Importantly, organoids may allow the study of risk-factor gene interactions. Various risk genes such as the APOE4, TREM2, BACE1 and numerous neuroimmune-related genes have been implicated in sporadic AD, but are not fully causative alone. Organoids will enable the study of gene interactions and also cell-type specific interactions, if mutations are introduced into just one cell type in an otherwise functioning mixed-cell culture [166]. The ability to investigate the contribution of genetic risk-factors, combined with successful recreation of amyloid and tau pathology reflective of that seen in human AD, and how these factors interact, will be an essential component of future AD research using organoids.

The development of cerebral organoids that contain specific neuronal subtypes would be beneficial in elucidating the mechanisms of synaptic dysfunction, and this seems attainable; dopaminergic-specific organoids have been successfully developed [163]. Cholinergic

dysfunction and imbalances in glutamatergic/GABAergic function in AD are likely to be important contributors to synaptic hyperexcitability and subsequent cognitive decline. Therefore, integrating multiple neuronal subtypes into a model containing microglial cells should be prioritised going forward, to allow the coordinated circuit properties which contribute towards these effects to be more effectively and representatively modelled. AD organoids also display hopeful signs that they may be useful as drug-screening and mutagenesis platforms; for example, it has been shown recently that treatment of APP- and PSEN-1- mutated organoids with β- and γ-secretase inhibitors attenuates both Aβ and tau pathology [167]. Park et al., have proposed a strategy by which drug-screening in organoids may be developed via the integration of mathematical modelling, in order to enable the testing of drugs in large quantities whilst limiting variation between models as much as possible [164]. Because many cerebral organoids can be grown simultaneously, drug-screening using these methods can occur on a large scale, leading to the acceleration of efficacious drug identification [129].

An important future addition to the use of organoids in drug-screening processes is the incorporation of neuroimmune components, such as microglial cells. This has so far been difficult to achieve due to the fact that microglia are derived from macrophages in the yolk sac, as opposed to neural progenitor cells [168]. Encouragingly, a 3D human triculture system modelling AD pathology has been developed that contained not only neurons and astrocytes but also microglia, via the use of a microfluidic platform [169]. Neuronal-microglial interactions similar to those seen in AD animal and 2D models were successfully observed, such as the retraction of neurites and reduced surface area of astrocytes and neurons upon co-localisation with microglia. Additionally, as mentioned previously, Muffat et al., derived a model containing microglial-like cells from iPSCs in a culture medium designed to support their growth [155]. These studies indicate that the incorporation of microglial cells into cerebral organoid models of AD is possible. Since the neuroimmune response is an essential component of AD pathology, it is essential that 3D models designed to investigate underlying pathophysiological changes, or act as drug-screening platforms, successfully integrate the microglial response. Organoids have the potential to act not only as such drug-screening platforms but also regenerative and personalised medication tools, which have so far been discussed in iPSC models [170,171]. These will be particularly useful in the study of sporadic cases, in which numerous interactions between genes and environmental-related factors, which vary amongst cases, have been supposed to contribute to disease onset.

As disease modelling using cerebral organoids is a relatively novel approach, the optimisation of current limitations is an important step in ensuring accurate AD pathology is reflected as much as possible. For example, as with 2D and animal models, organoids are unable to recreate the impacts of aging on neurodegeneration. However, this could potentially be addressed via the overexpression of pro-aging proteins/reduced expression of anti-aging

proteins using CRISPR-Cas9 technology. Additionally, DNA and mitochondrial damage, or upregulation of the production of reactive oxygen species, all of which occur naturally during aging, may be implemented via the application of toxins [172,173]. Another important advancement would be the introduction of vasculature, which would not only result in a more representative model of human AD, but may also improve the health of 3D cultures by facilitating the delivery of oxygen and nutrients to cells. The introduction of vasculature to human brain organoids has been accomplished via the ectopic expression of the ETS variant-2 protein [174], which plays an essential role in the development of vascular endothelial cells. Interestingly, the development of vasculature has also been achieved by implanting organoids into mouse brains and obtaining grafts [175]. The maintenance of age- and environmental-related factors and intact vasculature are consequently two ways in which AD modelling using organoids can be optimised, and studies should look to harness techniques which allow these to be achieved, in order to reflect true AD pathology more completely. Finally, an essential reduction in variation and consequent increase in homogeneity between organoid models via the introduction of miniature spinning bioreactors [176], neurospheres [177] or quality control steps [164] have all been shown. It is important to utilize these methods to limit variability amongst organoids both within and between studies, enabling the collection of more reproducible, representative results. As with 2D models, if variation is not restricted as much as possible, it could be a substantial limitation of organoid models.

3.3. New perspectives of AD with innovation of human models of AD

Neuronal hyperexcitability, particularly in frontal and temporal areas [178] has been reported in mouse models, human induced pluripotent stem cell (iPSC) models of AD, and human AD patients [131,179–182], and has been attributed to disruptions in a number of processes. Several of these have been discussed by Ghatak et al., who conducted whole-cell patch-clamp recordings on iPSC neuronal cultures and cerebral organoids which contained PSEN-1 and hAPP mutations associated with the formation of pathological Aβ [182]. Observed hyperexcitability in the form of enhanced spontaneous action potentials and increased frequency and amplitude of excitatory postsynaptic currents (EPSCs) was partly owing to a rise in sodium current density, due to increased expression of sodium channels. Previous studies have shown that γ-secretase and β-secretase (encoded by the BACE1 gene, overexpression of which has been linked to the development of sporadic AD), which cleave APP to produce Aβ, regulate the surface expression of voltage-gated sodium channels [183,184], which is enhanced in AD [182,185]. Deletion of the BACE1 gene and the blocking of sodium channels with antiepileptic drugs have both been shown to individually attenuate hyperexcitable activity and Aβ plaque accumulation [11,12].

Aside from atypical sodium channel characteristics, abnormal neuronal morphology has also been strongly linked with the presentation of neuronal hyperexcitability in AD. A

reduction in neurite length in AD cerebral organoids compared to controls has been reported [182], which corroborates with several studies in AD patients and mouse models [13–16]. The amplification of synaptic output and integration of postsynaptic currents are neuronal structure-dependent [109,182], and disruption to neuronal morphology therefore leads to deficits in these processes, and consequent hyperexcitability. Additionally, a reduction in dendritic density leads to synaptic loss, which has been directly linked with cognitive dysfunction present in AD [191,192]. Synaptic loss contributes to neuronal excitatory/inhibitory imbalances, further exacerbating hyperexcitability. These imbalances may occur due to atypical regulation of glutamatergic and GABAergic transporters; Ghatak reported an increased number of glutamatergic transporters (vGluT) in parallel with reduced levels of GABAergic transporters (vGAT) in AD cerebral organoids compared to controls [182], which mirrors studies in AD patients and mouse models [193–196]. Increased glutamatergic spill-over due to reduced synaptic uptake [197], an enhancement of presynaptic glutamatergic release [198], altered glutamatergic and GABAergic receptor expression [199,200] and GABAergic signalling deficits [201,202], have all additionally been associated with excitatory/inhibitory imbalances in AD, as they collectively lead to increased glutamatergic tone and reduced GABAergic inhibition, increased probability of release at excitatory synapses and consequent hyperexcitability [127].

Abnormal hyperexcitability of neurons in Alzheimer's can therefore be attributed to a combination of neuronal irregularities, including increased sodium channel density, changes to neuronal morphology, and imbalances in excitatory/inhibitory activity. Aspects of all of these mechanisms have been strongly associated with the presentation of pathological Aβ plaques and tau neurofibrillary tangles (NFTs) [182–185,188–190,193–195,197–199,201,203], so elucidating the ways in which these proteins interact with neurons is of high importance. One mechanism that has been repeatedly proposed is the interaction of Aβ plaques with the protein kinase, glycogen synthase kinase-3 (GSK-3), which has been studied in AD cerebral organoids [167], 2D models [204] and animal models [205,206]. The GSK-3 enzyme plays a role in the regulation of glycogen metabolism [207], and modulates the function of a number of proteins via the Wnt signalling pathway, including microtubule associated proteins (MAPs) such as tau [208]. In Alzheimer's disease, GSK-3 has been linked to Aβ-induced tauopathy [167] and neuronal cell death and hyperexcitability [208,209]. Selectively inhibiting the actions of the enzyme can consequently be seen as a promising therapeutic target in the reduction of Aβ-induced pathology and associated neuronal hyperexcitability and cognitive decline [205,210–212]. Additionally, diverse interactions amongst numerous signalling pathways such as the Wnt, MAPK and PI3K-AKT pathways have been implicated in AD pathology via effects on amyloid and tau pathology [164]. Further proposed mechanisms by which pathological Aβ and tau may lead to neuronal dysfunction and hyperexcitability include the triggering of mitochondrial dysfunction and subsequent production of toxic levels of reactive oxygen species

[203,213–215], and the induction of cholinergic dysfunction [212,216–218]; a mechanism which the majority of, currently limited, Alzheimer's treatments (acetylcholinesterase inhibitors donepezil, galantamine and rivastigmine) aim to target. Pathological Aβ has also been shown to play a role in the remodelling and exacerbation of calcium activity, further contributing towards hyperexcitability. The enhancement of Ca2+ levels has been reported to promote long-term depression of synapses and subsequent cognitive dysfunction [219]. Calcium hyperactivation has been observed in cerebral organoids containing high levels of Aβ aggregation [164].

4. Future Development of Neurodegenerative Disease Research

Cerebral organoids have the potential to further our understanding of AD pathology. Organoids already hold several advantages over 2D and animal models and are favorable for investigating disease pathology. Optimization of current techniques enabling the introduction of neuroimmune components, vasculature, age-related factors, and neuronal subtypes, and increased homogeneity amongst models will allow research to become more representative of human AD. They may also have the capacity to act as drug screening and personalized medication platforms with high clinical translatability, promoting the advancement of currently limited treatments.

Preclinical AD research has gained traction in recent years due to failures to stall disease progression after severe neuronal loss and disease diagnosis in patients. Prodromal changes in tau and Aβ levels, white matter distribution, and retinal nerve fiber loss have been found to precede the key amyloidosis and hippocampal damage driving the classical diagnosis of dementia. Attempts to understand the preclinical AD changes may provide insights to the processes responsible for the widespread protein accumulation and neuronal death observed in late-stage AD.

Beyond the pathology, it is surprising that the physiological functions of the APP protein family and its resulting amyloid protein are only beginning to be understood. Products of APP gene isoforms and homologs likely perform overlapping functions and possess differences in tendencies to produce Aβ. Hence, single-gene knockout models of APP do not present full AD pathology. In relation to this, there has been much debate on the contribution of Aβ to prodromal AD. How does the presence of Aβ in the preclinical AD brain reconcile with the toxicity of Aβ observed in various AD models? If Aβ is the key causative factor of AD, why are there individuals who suffered from extensive amyloid accumulation in the brain but never exhibit cognitive deficits prior to death? Further interrogation of the role of the APP family of proteins and interaction partners throughout different stages of brain development is essential to reconcile the problems in translation of in vitro observations into in vivo functions.

As much as experimental models have been essential for mechanistic studies of

neurological disorders and AD, there has been limited success of rational target selection for the development of treatment modalities for CNS conditions. With the exceptions of rare familial genetic events, many patients suffer from an unique phenotypic outcome resulting from a combination of low abundance genetic variations. Even as rare, low impact genetic risk factors are researched, there is a current lack of insight to reconcile the expected physiological variations with such variability of genetic combinations. Attempts to subtype AD through the use of single cell methods and molecular biomarkers, have provided insights to the development of personalised medicine for AD patients [220].

It is also a challenge to recapture the effects of aging on pathology using models of AD; yet aging is the single largest risk factor for developing AD [221]. Rodents simply do not age in the same way as humans [222]. Even though human 3D cerebral organoids recapitulate the cell types present in actual cerebral cortex [223], culture time restriction resulting from difficulty to maintain long term healthy organoid cultures have restricted the organoid's developmental endpoint to that of a fetal neocortex [224]. The trade-off between relevance to human physiology and the ease of experimentation will always be a limitation in human disease modelling. If the in vitro human organoid model developmental time frame is reminiscent of actual human developmental profile, with current time points at which the organoids are terminated, a mature phenotype will not be possible, least to say the aging condition.

The ability to account accurately for the complex interactions between ion channel function, neuronal activity, and network activation in a single behavioral outcome remains a major problem of neuroscience research. Bio-realistic models have been used to supplement biochemical studies to answer questions that cannot be effectively resolved using reduced experimental models [225]. Alternatively, some have proceeded to understand the possible dynamics of a single neuronal subtype or compartments of a single neuron from commonly used parameters or proxies of neuronal activity and probe the possible computation outcome from a similar input [226]. Some aspects of cognition such as the loss of consciousness observed in late-stage AD patients may never be fully understood in brain simulations, biochemical and physiological studies

Preclinical neurodegenerative disease models are essential for neurodegenerative research as they allow the examination of mechanistic changes in the native, complex environment of the CNS. Furthermore, biomolecular alterations can be correlated with behaviour changes, allowing a better comprehension of the role of genetic mutations in neuropathology and neurophysiology. As we dive deeper to understand nervous system function and pathology, an increase in integrative and system-based research using multiple research models is essential. Existing models will remain relevant in areas they are first designed for, complementing studies using alternate models.

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