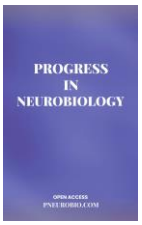

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Review Article

Promising Prospects for Human Cerebral Organoids to Advance Alzheimer's Disease Research

Patricia Mateos-Martínez[1,2,3], Rosa Gonzalez-Sastre[1,2,3], Raquel Coronel[1,4], Andreea Rosca[1], Sabela Martín-Benito[1,2,3], Victoria López[2*] and Isabel Liste[1*]

[1]Unidad de Regeneración Neural, Unidad Funcional de Investigación de Enfermedades Crónicas (UFIEC), Instituto de Salud Carlos III (ISCIII), Majadahonda, Madrid, Spain

[2]Unidad de Biología Computacional, Unidad Funcional de Investigación de Enfermedades Crónicas (UFIEC), Instituto de Salud Carlos III (ISCIII), Majadahonda, Madrid, Spain

[3]Ph.D. Researcher of Universidad Nacional de Educación a Distancia (UNED), Madrid, Spain

[4]Departamento de Biología de Sistemas Facultad de Medicinay Ciencias de la Salud, Universidad de Alcalá, Alcalá de Henares, Madrid, Spain

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ABSTRACT

The causes of the alterations found in the brains of patients with alzheimer's disease (AD) begin before the first signs of memory loss appear, and are still unclear. Adequate research models are essential to understand the mechanisms that cause the onset of these alterations, as well as to advance in the diagnosis, development and testing of treatments for the AD. Animal research models fail to recreate the great diversity and complexity inherent to the human brain, so *in vitro* systems based on human pluripotent stem cells (hPSCs) present themselves as an important alternative. Differentiation of hPSCs into two-dimensional (2D) cell culture models allows recreation of various brain functional processes and the three-dimensional (3D) cell culture models or human brain organoids (hCOs) recapitulate the cellular diversity and structure of the human brain. hCOs from human induced pluripotent stem cells (hiPSCs) from patients with familial (*APP*, *PSEN1* and *PSEN2* mutations) or sporadic AD allow identifying and studying changes due to this pathology. This review presents an overview of the research models used to study the AD, and recapitulates the advantages and discusses the challenges of the hCOs as an innovative and promising technology that will aid in the understanding of AD.

1. Introduction

Alzheimer's disease (AD) is the principal cause of dementia in the elderly population and one of the leading causes of death worldwide [1-4]. The AD incidence is expected to increase dramatically by 2050 mainly due to the rise in life expectancy of the world population [5, 6]. Moreover, AD is a disabling disorder, and the maintenance of the patients reaches a high cost for families and society, which makes AD a problem for public health [6, 7]. In the last decades, great advances have been made in the field of AD, but the main causes that trigger AD and its etiology are still unclear.

The neurodegenerative process of AD appears in adults and older people, beginning with some progressive memory impairments that involve alteration of cognitive processes, self-awareness and memory loss. In later stages, AD patients also manifest aphasia, amnesia, agnosia and apraxia [8-11]. The neurological changes are accompanied of two main histopathological features observed in the post-mortem brains of AD patients: the abnormal intracellular hyperphosphorylation of Tau protein (p-Tau) and the presence of extracellular amyloid plaques formed by accumulation of the long variants of β-amyloid (Aβ) peptide. On the one hand, p-Tau protein would lead to the formation of aggregates or neurofibrillary tangles (NFTs) inside the cells that would cause failures in the normal functioning of the neurons. By the other hand, Aβ peptide first would appear extracellularly as monomers that would associate with each other, forming oligomers and finally their aggregation would give rise to senile plaques (Figure 1) [8, 11-13]. Recent studies indicate an interrelation between both alterations found in AD [14, 15].

Correspondence to: Isabel Liste, Unidad de Regeneración Neural, Unidad Funcional de Investigación de Enfermedades Crónicas (UFIEC), Instituto de Salud Carlos III (ISCIII), Majadahonda 28220, Madrid, Spain; E-mail: iliste@isciii.es

Victoria López, Unidad de Biología Computacional, Unidad Funcional de Investigación de Enfermedades Crónicas (UFIEC), Instituto de Salud Carlos III (ISCIII), Majadahonda 28220, Madrid, Spain; E-mail: victorialopez@isciii.es

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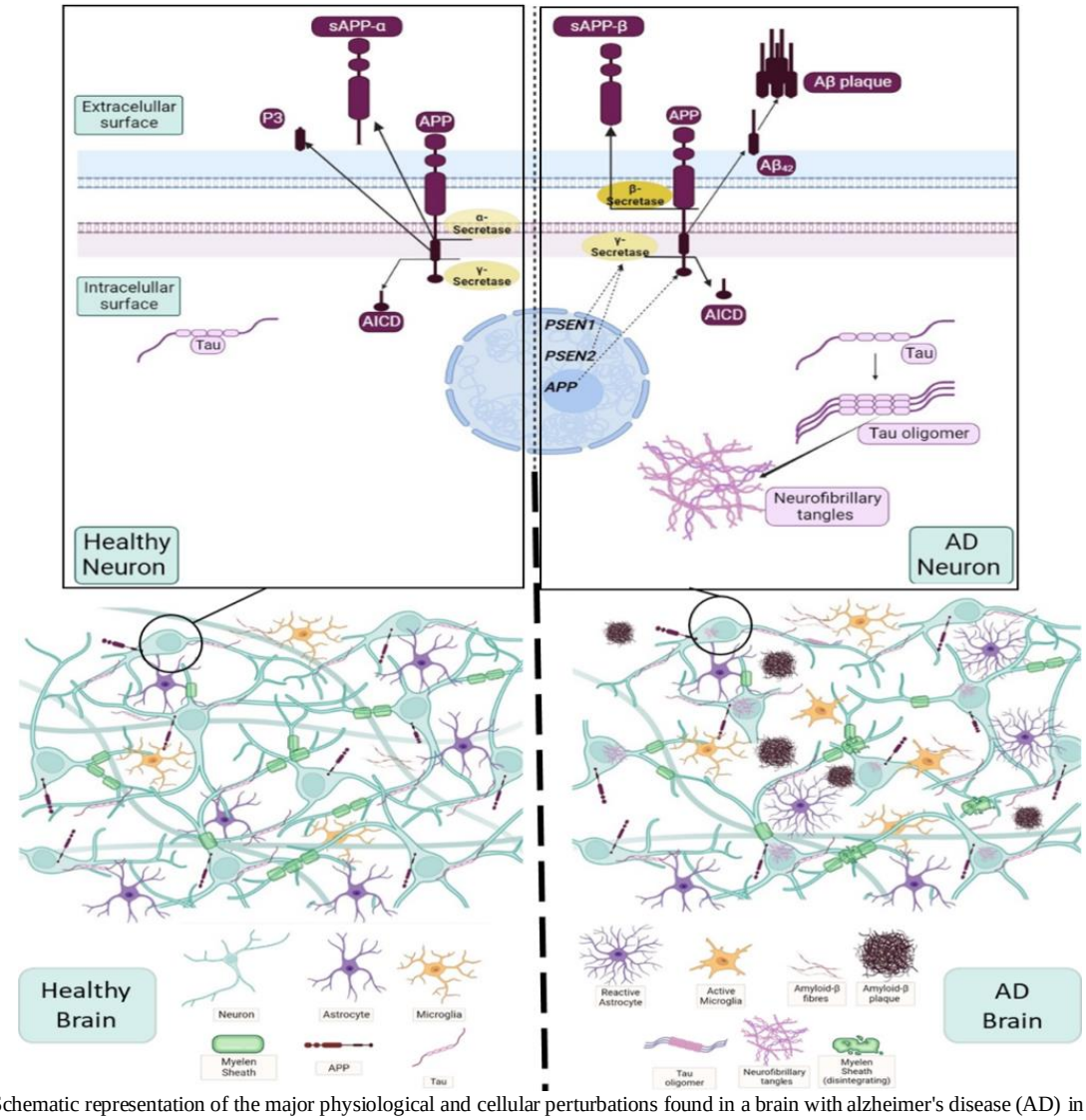

FIGURE 1: Schematic representation of the major physiological and cellular perturbations found in a brain with alzheimer's disease (AD) in comparison to a healthy brain.

In the brain with AD (right) in comparison to a normal brain (left) the main physiological changes found are: presence of Aβ fibers and plaques, NFTs of p-Tau, damage of the myelin sheath, disintegration and reduction of dendrites in neurons, activation of microglia and presence of reactive astrocytes. The presence of Aβ plaques and NFTs in the neuron with AD is shown in the upper right of the figure together with the schema of amyloidogenic pathway of amyloid precursor protein (APP) (β-secretase pathway) and the genes that encode the catalytic subunit of γ-secretase, *PSEN1* (*presenilin-1*) and *PSEN2* (*presenilin-2*) implicated in the development of familial AD.

Both events (NFTs of p-Tau and senile plaques of Aβ peptide) appear in patients with sporadic AD (sAD) and familial AD (fAD). At least 90% of all cases of AD are sporadic and occurs in patients with more than 65 years. It is associated with aging, the allelic composition of apolipoprotein E (APOE), failures in the correct functioning of microglia and brain metabolism [16-19]. The prevalence of fAD is less than 10% of the AD cases and appears in patients with ages younger than 65 years. It is due to autosomal dominant mutations on the gen of the amyloid precursor protein (APP, located on chromosome 21) and mutations on genes of *Presenilins 1* and *2* (*PSEN1* and *PSEN2*, located on chromosome 14 and chromosome 1, respectively). It should be noted that APP is the precursor protein of Aβ peptides (Aβ40 and Aβ42) and *Presenilins 1* and *2* are part of the γ-secretase catalytic complex, one of the enzymes that cleaves APP during its proteolytic processing. It is important to highlight that most of the mutations described for *APP*, *PSEN1* and *PSEN2* genes favor production and accumulation of Aβ peptide in the brain parenchyma of AD patients [11, 20].

Probably because the pathogenic mechanisms that cause AD have not yet been determined currently, there is no effective cure for this disease. In recent decades, only a few drugs have been approved for the palliative treatment of AD, such as inhibitors of acetylcholinesterase (donepezil, galantamine or rivastigmine) or antagonists of N-methyl-D-aspartate receptor (memantine) [21-23]. More recently, the FDA has approved a controversial new treatment, aducanumab (Aduhelm™), an anti-Aβ peptide monoclonal antibody, hoping to reduce their accumulation in

brains of AD patients [24, 25]. However, none of these pharmacological treatments is capable of stopping the neurodegenerative process in AD.

Because the causes of this disease are still not well understood, many efforts are being carried out to know the origin, development and evolution of AD. One of the reasons why the causes of the disease are still not well known is the absence of good study models that allow replicating this disease. Animal models with human mutations (in *APP*, *PSEN1* or *PSEN2* genes) have been used to approach the study of AD. Nevertheless, there are many differences regarding the development of the disease in humans [26-29]. Secondly, the technology of human stem cells has allowed progress in the knowledge of AD, especially the use of induced pluripotent stem cells (iPSCs) derived from AD patients [10, 30-32]. However, the two-dimensional (2D) models have limitations, such as the lack of three-dimensional (3D) organization and cell diversity that make up the human brain [33, 34]. For this reason, the development and use of human cerebral organoids (hCOs) can be a good alternative to deepen and advance in the pathogenic mechanisms of AD.

In this review, we present the milestones reached in relation to the study of AD with animal models and *in vitro* models. Finally, we will focus on the use of hCOs as a promising technology to study and model of both sAD and fAD.

2. Animal Models of Alzheimer's Disease (AD)

Several animal species (*Drosophila melanogaster*, *Caenorhabditis elegans*, *Danio rerio*), but mainly mice, have been used during last decades to understand the pathology of AD [29]. Most of the studies have focused on *APP* mutations [27-29], although mutations in other genes have also been analyzed, like *PSEN1*, *APOE* or *MAPT* [29]. For studies at behavioral, phenotypical and morphological level, these models have been very useful; however, they have shown limitations in modelling AD. In fact, the failure rate with these models in drug discovery has been of 99% [26, 35]. In addition, they are not realistic enough because neurodegeneration, like neuronal loss or the development of NFTs, cannot be observed in their brains.

Furthermore, there are important anatomical differences between brain morphology [29, 36-38] and physiology of the mouse and human brain, as the organization of ventricular zones. Animal models have a bias in the information they provide, since the genes that are modified only represent a minority of cases (fAD) and do not represent the totality of the pathology [22].

3. *In vitro* Models of Alzheimer Disease (AD)

3.1. Stem Cells and Adherent Models (2D)

Recent advances in the field of stem cells and new knowledge related to cell reprogramming have opened up a new world of opportunities and the creation of more reliable models for a better understanding of the etiological and molecular process of AD. During the last decades, the study and characteristics of stem cells has been deepened, as well as their possible therapeutic applications or as an alternative method to *in vivo* procedures [39, 40].

Stem cells are defined as cells with a high renewal potential and the capacity to differentiate into more specialized cell types [41]. There are four types of stem cells depending on how many different types of cells can be obtained from them: totipotent, pluripotent, multipotent and unipotent [42]. The most used in research are the multipotent (MSCs) and pluripotent stem cells (PSCs) [23, 37, 41]. MSCs can be derived from foetal, neonatal or adult tissues, but also from PSCs [42]. PSCs can be obtained from embryonic stem cells (ESCs) or by reprogramming somatic cells to induced pluripotent stem cells (iPSCs) [41].

The technique for creating iPSCs from fibroblasts was developed by Takahashi and Yamanaka, through a retroviral infection, that allowed the introduction of the factors Oct4, Sox2, Klf4 and c-Myc [40]. The same year, the group of Yu *et al.* also reported the reprogramming of human somatic cells to PSCs by the use of the Oct4, Nanog, Sox2 and Lin28 factors [43]. In recent years, much progress has been made in reprogramming techniques, to the point of obtaining safer and more efficient non-integrative methods [44-46].

In 2011 were designed and used in research the first iPSCs derived from patients with fAD [47, 48], and a year later from patients with sAD [30]. In these studies the presence of *PSEN1* mutations found in fAD patients were associated with accumulation of Aβ peptides due to alterations in the ubiquitin kinase system [49], γ-secretase activity [50], Tau proteostasis [51] and up-regulation of calcium-controlling receptors in the endoplasmic reticulum (ER) [52]. In the early stages of AD were also observed diverse alterations in astrocytes [53-55].

Studies with iPSCs from fAD patients with *APP* mutations found that some iPSCs are more prone than others are in accumulation and aggregation of Aβ peptides [56]. APP alterations were studied in relation to p-Tau levels [57], cholesterol receptors involved in endocytosis and clearance of Aβ [58], and mitochondrial dysregulation [59]. Because of these studies, new treatments have been proposed to reduce Aβ peptides such as the use of statins [60], or various chemicals to increase Tau autophagy [61]. Despite these studies, many of the functions of APP involved in the disease are still unknown [62]. Another approach to study AD is to use iPSCs from individuals with down syndrome (DS), who present in most cases with early-onset dementia similar to patients with fAD due to the triplication of chromosome 21, where APP is encoded [63-65]. Deletion in these models of the extra copy of APP reverses and normalizes the Aβ40/Aβ42 ratio [66].

iPSCs from sAD patients has allowed the study of the *APOE* gene increasing the ratio of Aβ42 similar to what was observed in post-mortem brains [67, 68]. Not all *APOE* gene isoforms are aggressive; the *APOE2* is neuroprotective, whereas *APOE4* is the most toxic [69]. Several studies have found *APOE4* to be associated with proinflammatory profiles and increased TREM2 [70], and hPSC-derived astrocytes with *APOE* mutations are generated for drug screening [71]. Recently a link between the *APOE4* and the SARS-CoV-2 infection has been established with increased neurodegeneration and synapse loss [72].

Other 14 genes with direct involvement in fAD have been described with these models [73]. In addition to the alterations described above, increased oxidative damage [74] leading to alterations in membrane

permeability [75] has been found. Microglia is also affected, due to failures in TREM2 [76], with an increase of microglial cells involved in the clearance of Aβ and Tau around Aβ accumulations in AD patients [77, 78]. For phagocytosis to be successful, support from mitochondria is required to mobilise all the proteins involved [79], although these also end up being affected by increased reciprocal Aβ42 and Tau deposition [80]. The degradation of Aβ, likewise, is mediated by insulin [81], so its accumulation induces insulin resistance, along with toxic and apoptotic reactions, increasing AD symptoms [82]. Oher studies focus in the regulation of miRNAs that could protect against AD symptoms, such as the miRNA124 [83].

2D stem cell models have been a great advance in the study of neurodegenerative diseases. However, in relation to AD, there is a need to recreate the different stages in time and space, as well as some key events such as the aggregation of extracellular peptides. The appearance of the 3D models, which are now possible due to advances in stem cell technology and knowledge of iPSCs, are closer to simulate that occur *in vivo* and open the research for understanding AD [33, 34].

3.2. 3D Models: Human Cerebral Organoids

In addition to 2D cultures, the ability to develop hCOs revolutionized the field and reduced the use of other techniques. Lancaster and collaborators [84] made the first hCO protocol, with the main goal of understand the development of the human brain. This first hCO protocol from human PSCs could recreate different brain regions capable of influencing each other. The protocols in which organoids generate various brain regions spontaneously are called unguided protocols [84-86]. The authors defined organoids as 3D cultures with two or more cell types in an order and function similar to those that would be found in the organ [85]. Another simple definition can be a primitive organ that is obtained from a tissue sample using PSCs [87].

Currently, other type of protocols for the generation of hCOs exists. Guided protocols employ patterning factors that give rise to organoids of a specific brain region [88-95]. For example, cortical organoids have the possibility of combining them to achieve a better understanding of cell, synapse, and neuron interaction and astrocyte maturation in these complex structures. In addition, this type of protocols can be used without the need of support [89, 90]. Table 1 lists the main publications with hCOs to study AD. Moreover, the identity of hCOs with the human foetal cerebral cortex has been confirmed [96]. Other studies indicate that 45-day-old organoids have a large number of pathways and functions typical of neural tissue: metabolism, cell-cell adhesion, development of the cerebral cortex, organisation of the cytoskeleton, and others like the human brain at 16 weeks gestational age [32].

TABLE 1: Summary of the main publications with hCOs as research models to study AD, classified attending to the protocol employed to generate the hCOs (non-guided or guided) with mutations corresponding to familial AD (fAD), sporadic AD (sAD), or other type.

Protocol	[a]Origin human cell line	[b]Mutations	[c]AD Pathology	[d]Cell types	Studies	Refs
fAD non-guided hCOs						
Lancaster, 2013	AD patient & DS Fibroblasts iPSCs	*PSEN1* (1)	Aβ42/40 ratio Aβ Agg p-Tau Tau Agg	Neurons Astrocytes	In hCOs found elevated levels of Aβ, p-Tau and cell death similar to those observed in AD and DS brains	[111]
Lancaster, 2013 (STEMdiff ™)	AD patient Dermal Biopsy iPSCs	*APP* (2) *PSEN1* (3, 4)	Aβ42/40 ratio	Neurons	Neuronal hyperactivity due to increased VGLUT1 and decreased VGAT expression Nytrosynapain reduces neuronal hyperactivity	[105, 108]
Lancaster, 2013 (STEMdiff ™)	AD patient Fibroblasts iPSCs	*PSEN2* (5)	Aβ42/40 ratio	Neurons	Smaller organoid size and calcium dysregulation in AD hCOs versus control hCOs	[103]
Lancaster, 2013	AD patient Fibroblasts iPSCs	*APP* (6) *PSEN1* (7, 8, 9, 10, 11)	Aβ38, Aβ40 Aβ42, Aβ43	Neurons	PSEN1 and APP mutations alter the neurogenesis and Aβ secretome, and favour aging and neurodegeneration	[104, 106]
fAD guided hCOs						
Kadoshima, 2013 (modified)	AD patient Fibroblasts iPSCs	*APP* dup *PSEN1* (1, 7)	Aβ42/40 ratio Aβ Agg p-Tau, Tau Alt Endosomes	Neurons	hCOs recapitulate the AD pathology and the use of β- and γ-secretase inhibitors significantly reduces this pathology	[97]
Qian, 2016	AD patient Fibroblasts iPSCs	*APP* (6) *PSEN1* (8,9 ,10)	Aβ Agg p-Tau	Neurons	A risk factor of fAD is the failure of the genetic regulator 5-hydroxymethylcytosine	[110]
Qian, 2016 (modified)	AD patient Fibroblasts iPSCs	*PSEN1* (1) *PSEN2* (5)	Aβ Agg Tau Agg	Neurons Astrocytes	Using an adenovirus to introduce Tau into fAD hCOs enhances aggregation and phosphorylation of the Tau protein	[102]

Park, 2023	AD patient Fibroblasts iPSCs	*PSEN1*	Aβ Agg Alt astrocytes Alt dendrites	Neurons Astrocytes	Spheroid protocol to use hCOs in pharmacological assays	[99]
Yan, 2018	AD patient Fibroblasts iPSCs	*PSEN1* (4)	Aβ42 p-Tau Neuroinflammation	Neurons	hCOs to study of the microenvironment in AD brains	[100]
Paşca, 2015 (modified)	AD patient Fibroblasts	*APP* (6) *PSEN1* (11)	Aβ42/40 ratio p-Tau	Neurons Astrocytes	Hippocampal hCOs for the study of AD. NeuroD1 to help in AD gene therapy	[109]
Sloan, 2018	AD patient Fibroblasts	*PSEN1* (12)	Aβ Agg p-Tau	Neurons	Nanoparticles, as STB-MP, help to reduce the pathology of the AD	[101]
Amin, 2018 Birey, 2017	AD patient Fibroblasts iPSCs	*PSEN1* (13, 14, 15)	Aβ42/40 ratio p-Tau	Neurons	Mutations in *PSEN1* increase the Notch pathway, promoting generation of neuronal precursors	[107]
sAD non-guided hCOs						
Lancaster, 2013	AD patient Skin Biopsy iPSCs	*PITRM1* KO	Aβ42/40 ratio p-Tau	Neurons	Absence of *PITRM1* causes a pathology similar to AD due to mitochondria dysregulation	[126]
Lancaster, 2013 (STEMdiff ™)	AD patient Skin Biopsy iPSCs	*APOE ε3/ε3* *APOE ε4/ε4* *APOE* KO	Aβ Agg p-Tau	Neurons Astrocytes	*APOE4* increases p-Tau as well as cell apoptosis. APOE-/- hCOs to study pathological mechanisms in AD	[19, 123]
Lancaster, 2013 (STEMdiff ™)	AD patient PBMCs iPSCs	*APOE ε3/ε3* ε4/ε4	Aβ42/40 ratio p-Tau, Tau	Neurons Astrocytes	Development a platform to evaluated new drug to treat AD	[118]
Lancaster, 2013 (STEMdiff ™)	Healthy Fibroblasts iPSCs	*APOE ε3/ε3* *APOE* ε4/ε4	Aβ secretion p-Tau	Neurons Astrocytes	*APOE4* neurons exhibited an increase of the synapsis	[121]
Lancaster, 2013 (STEMdiff ™)	AD patient Fibroblasts iPSCs	*APOE ε3/ε3* *APOE* ε4/ε4	Aβ40 p-Tau	Neurons Astrocytes	*APOE4* produces neuronal dysregulation due to the reduction of translocation to the nucleus of the repressor silencing transcription factor (REST)	[114]
Lancaster, 2013 (STEMdiff ™)	Healthy Fibroblasts iPSCs	*APOE ε3/ε3* *APOE* ε4/ε4	Aβ Agg p-Tau Alt lipids	Neurons Astrocytes	Role of *APOE4* expression in neurons and astrocytes related to AD pathology	[115]
Lancaster, 2013 (modified)	H9 hESCs	ND	Aβ Agg Neuroinflammation	Neurons Astrocytes	Herpes Simplex Virus 1HSV-1 induces AD like pathology	[128]
Lancaster, 2013 (STEMdiff ™)	Control ASE-9109 iPSCs	*BIN1* KO	*APP*	Neurons Astrocytes Oligodendrocytes	*BIN1* KO hCOs present smaller primary endosomes, and early AD pathology	[124]
sAD guided hCOs						
Raja, 2016	AD patient Fibroblasts iPSCs	*APOE ε3/ε3* *APOE* ε4/ε4	Aβ Agg p-Tau	Neurons Astrocytes Microglia	Transform of *APOE4* hCOs to *APOE3* hCOs reduces AD symptomatology	[113]
Paşca, 2015	AD patient PBMCs iPSCs	NS	Aβ Agg Tau Agg	Neurons Astrocytes	First neuro-spheroid model derived from AD patients' blood	[116]
Paşca, 2015	AD patient PBMCs iPSCs	NS	Neuroinflammation	Neurons	Found of deregulated proteins in AD related with axon development, platelet aggregation, RNA translation and inflammation	[117]

Lin & Chen, 2008 Seki, 2012 Paşca, 2015	AD patient Fibroblasts iPSCs	NS	Aβ Agg p-Tau	Neurons	CKD-504 (HDAC6 selective inhibitor) reduces levels of p-Tau	[120]
Other non-guided hCOs						
Lancaster, 2013	DS Fibroblasts iPSCs	*APP* dup	Aβ Agg	Neurons Astrocytes	*BACE2* as a dose-sensitive AD-suppressor gene	[139]
Lancaster, 2013 Sheridan, 2012	Fibroblasts iPSCs (CRL-2522)	ND	Aβ Agg	Neurons Astrocytes	Treatment with Aftin-5 (β-42 inducer) modulates the posttranslational pathways of *APP*	[132]
Pavoni 2018	Fibroblasts iPSCs (CRL-2522)	Tau (16)	p-Tau	Neurons Astrocytes	Overexpression of Tau to study neurodegenerative disease	[137]
Lancaster, 2013	Fibroblasts iPSCs	ND	Aβ Agg Tau Agg	Neurons	Treatment with serum obtained from post-mortem AD patients increases Aβ and Tau in hCOs	[135]
Lancaster, 2013 (modified)	PBMCs iPSCs	ND	Aβ Agg p-Tau Alt Endosomes	Neurons	hCOs co-stimulated with Aβ. Estrogens reduce AD symptomatology	[133]
Lancaster, 2013 (STEMdiff ™)	hESCs (UE02302)	*APP* (2) *BACE2* (17)	Aβ42 Neural apoptosis	Neurons Astrocytes	The loss-function of *BACE2* induce AD-like pathology	[140]
Other guided hCOs						
Raja, 2016	Patient FTD iPSCs	Tau (18)	Tau Agg	Neurons	Inhibition of p25 reduces p-Tau	[136]
Camp, 2015 Trujillo, 2019 Yao, 2020	DS iPSCs (DS1-iPS4)	ND	Aβ Agg Tau Agg	Neurons	DS hCOs show accumulation of Aβ42 and Tau, as observed in AD	[112]
Zhang, 2023	iXCells Biotechnologies iPSCs	ND	Aβ42 Alt Mitophagy	NS	hCOs treated with Aβ42. Galangin reduce Aβ42 mitophagy and produce an increment of PTEN-induced kinase 1 (*PINK1*)	[134]

[a]iPSCs: induced Pluripotent Stem Cells; hESCs: human Embrionic Stem Cells; DS: Down Syndrome; PBMCs: Peripheral Blood Mononuclear Cells; FTD: Frontotemporal Dementia.

[b]1: A264E; 2: Swedish; 3: ΔE9; 4: M146V; 5: N141I; 6: London; 7: M146I; 8: int4del; 9: Y155H; 10: M139V; 11: R278K; 12: 14q24; 13: L435F; 14: M146L; 15: D385A; 16: P301S; 17: G446R; 18: P301L; KO: Knock Out; dup: duplication; ND: Not Disease mutation.

[c]Aβ42/40: Aβ42/ Aβ 40; p-Tau: hyperphosphorylation of Tau protein; Agg: Aggregates; Alt: Alterated.

[d]NS: Not Specified.

3.2.1. Familial Alzheimer Disease (fAD)

One of the first AD studies performed using COs was that of the Raja group, using different hiPSCs from patients with fAD (*APP* duplication or *PSEN1* mutations) and the protocol described by the Kadoshima group in 2013. In this work, AD-associated pathology was observed in these organoids, such as amyloid aggregation, p-Tau protein and endosome abnormalities compared to controls. They also observed that the use of β-secretase and γ-secretase inhibitors resulted in a reduction of Aβ and p-Tau [97].

Many authors have focused on studying diverse *PSEN1* mutations that appears in most of the fAD cases [98]. Using guided hCOs models, researchers have observed AD pathology events as increased levels of Aβ42, decreased neural dendrite size [99], elevated gene expression of proinflammatory cytokines (IL-6 and TNF-α), upregulated syndecan-3, altered expression of matrix proteins and prominent levels of p-Tau [100]. In addition, some research models of hCOs of neurons and astrocytes from AD patients have also been proposed to test drug efficacy reducing Aβ aggregation [99]. hCOs has been used to study the microenvironment and test how to decrease the pro-inflammatory profile found in AD [100], and also as screening platform for novel AD treatment assessments as the effect of nanoparticles [101].

PSEN2 mutation has hardly been considered in hCOs, probably due to its lower incidence [98]. The few studies using *PSEN2* mutations have observed an increase in Aβ42/Aβ40 ratio, increased p-Tau, asynchronous calcium transients, enhanced neuronal activity, and smaller hCOs size [102, 103]. An increase in caspase-3 in *PSEN2* hCOs has also been detected associated with a high level of apoptosis [103].

The increase in the levels of p-Tau with adeno-associated virus was used in these *PSEN2* hCOs as a research model to study tauopathies [102].

In addition to analysing the effect produced by mutations in *PSEN1*, it is interesting to compare them with the effects found due to mutations or duplication of *APP*, also present in fAD. hCOs with mutations in *PSEN1* or in *APP*, generated with unguided protocol, show an increase in the Aβ42/ Aβ40 ratio, although present differences in the length of the Aβ fragments [104], and in the ratios [105]. hCOs with some mutations in *PSEN1* produce premature neuronal differentiation, possibly due to a reduction in the notch pathway [106]. However, other *PSEN1* mutations show upregulation of the notch pathway leading to an increase in neural progenitors as well as the reduction of post-mitotic neurons [107]. These *PSEN1* mutations causing alterations in the notch pathway suggest that neural stem cell biology is affected in aging and disease. Regardless of the mutation, all fAD hCOs exhibit synaptic dysfunction, showing increased expression of VGLUT1 and decreased VGAT. In addition, further studies have observed that NitroSynapsin could be a good candidate to palliate these alterations [105, 108].

Other authors have opted to compare *PSEN1* and *APP* mutations using hCOs from guided protocols. They observe Aβ aggregation, increase in the Aβ42/Aβ40 ratio, alterations in synaptic proteins or increase in p-Tau [109, 110], as seen using hCOs from non-guided protocols [104-106, 108]. Differences in the alterations are found also depending of the mutation [104, 109]. Alterations at the epigenetic level have been found using these models with a decrease in 5-hydroxymethylcytosine [110] and an increase in miRNA125b. The alterations at the mitochondrial level appear to be able to be regulated using NeuroD1 gene-based therapy [109].

Apart from using iPSCs from AD patients, it is also possible to use lines derived from down Syndrome (DS), since the extra copy of *APP*, producing as *PSEN1* hCOs, presence of Aβ plaques, NTFs [111], or p-Tau [112], regardless of the protocol used to generate hCOs.

3.2.2. Sporadic Alzheimer's Disease (sAD)

Most studies have focused in obtaining models of organoids based on fAD mutations rather than sAD, in part, because studies conducted on sAD hCOs have observed that they take longer to present associated histopathology [113]. Even with this handicap of time, this type of model can be useful when recapitulating the AD phenotype, such as acceleration of maturation and synapses, increase in the Aβ42/Aβ40 ratio, increase in p-Tau [19, 114, 115], as well as the increase in lipid droplet [115] allowing studying the alterations that trigger AD without being subject to mutations of fAD.

These hCOs sAD models have also laid the foundations for the generation of platforms that allow us to detect new useful drugs against AD. This is because apart from collecting the AD phenotype, they also allow studying drug penetration, as well as studying alterations in numerous pathways: axon development, platelet aggregation, RNA translation, inflammatory ions [116, 117] when comparing the spheroids with post-mortem brains from AD patients. Platforms that are more recent have used organoids with *APOE3* and *APOE4* identity together with RNA-seq, calcium, and protein quantification analysis against controls to establish a mathematical platform for more realistic drug testing [118].

The investigations carried out on hCOs originating from lines of sAD patients, in contrast to the studies carried out with fAD organoids and, as already mentioned, with their use to generate drug testing platforms, have been considered from the perspective of obtaining possible drugs to alleviate symptomatology. An example would be its use to learn more about tauopathies. Based on the 2D culture background where a relationship between Tau and acetylated histone 6 (HDAC6) was observed [119], the use of the compound CKD-504, a selective and dose-dependent anti-HDAC6, has been studied on these hCOs, obtaining a reduction in p-Tau levels [120].

This use of hCOs to search for alternatives to alleviate the disease has also occurred in organoids obtained from lines with the main risk factor in sAD, the *APOE* gene. Mainly, lines with *APOEε4/ε4* have been used for this purpose, as they are the most aggressive and the only isoform where p-Tau and lipid droplet in hCOS can be seen [19, 114, 115, 118], as well as *APOEε3/ε3*. Some factors, such as the repressor element silencing transcription factor (REST) 1, have been identified as delaying the onset of symptoms in hCOs *APOE4* [114]. A reduction in symptoms has also been observed when *APOE4* lines are converted to *APOE3* using CRISPR/CAS9 techniques [19, 113], although there are authors who consider that these differences are due more to variations in the culture of hCOS than in the isophorm used for its generation [121].

Despite the lack of depth on how *APOE* affects to trigger AD [122], its relationship in the loss of typical synapses in AD is known, by acting on the synaptic protein α-synuclein [123] as well as its importance in lipid control, mainly at the neuronal level [115, 123]. However, *APOE* is not the only gene of interest in sAD. Recent studies are beginning to focus on other genes that might predispose to AD. This is the case of bridging integrator (BIN), which influences the size of the primary endosomes, depending on the isoform present, with isoform 1 (BIN1) being highly associated with early alterations in AD [124]. Likewise, another gene whose alteration is known to affect the correct degradation of Aβ in mitochondria and alters the potential of the mitochondrial membrane is *PITRM1* [125]. Studies on hCOs *PITRM1* knockout showed AD-like pathology: protein aggregates, tau pathology, and cell death. Mitochondrial alteration was also appreciated, as well as deregulation of astrocytes [126].

Apart from these, various risk factors for sAD, certain studies have considered focusing on the risks of developing sAD due to viral infections. It has been observed that we start from healthy lines infected with HSV-1 [127, 128], or from lines of AD patients infected with the zika virus [129], organoids develop with an increased AD phenotype. Another virus that could have implications for AD is SARS-CoV-2, as it has been associated with destruction of microglia [130], as well as infection of cells with the *APOE4* phenotype [72]. Even though it can be considered a way to model AD, using antivirals results in a decrease in AD-like [127, 128], so they end up being a realistic model of the pathology. In addition to viruses, also bacterial infections could influence the onset of AD. It has been observed that in these infections there is an increase in the Aβ level, since it would have a protective role against antimicrobials [131].

3.2.3. Other Approaches to the Study of AD

Another way to model AD without using fAD or sAD patient lines is by inducing the disease from healthy lines. A factor that allows this modulation is aftin-5, which when used on healthy hCOs, it is observed that produces an increase in Aβ42, which is also modulable [132]. Another alternative is based on inducing the pathology by stimulating them with Aβ [133, 134] or with serum from AD patients, which makes it possible to observe, in addition to Aβ and Tau aggregates, neuronal loss and other alterations [135].

Apart from inducing the pathology with various factors, we can submit healthy lines to CRISPR/Cas9 genomic editing or through the use of vectors, so that they express AD. Following these methods, hCOs have been developed in a way that allows us to study the tauopathies [136, 137]. Tau is observed to appear early in human brain development and the concentration of its mRNA is greatly increased in mature neurons [138], for this reason, it is sought how to counteract its effects. For example, p25, a proteolytic fragment of the p35 regulatory subunit, has been shown to induce aberrant cyclin-dependent kinase 5 (Cdk5) activity, which is associated with neurodegenerative disorders such as AD. In organoids, in which Tau effects were induced, p25 blockade was shown to reduce p-Tau levels and increase synaptophysin expression [136]. This type of model allows us to focus on specific causes of AD for a preliminary study where we can simplify the disease.

Although the genes or histopathologies associated with AD are usually studied, some groups have preferred to focus on learning more about genes or factors that are protective against the pathology. One of these genes is *BACE2*, located on chromosome 21. Using organoids from patients with DS (with an extra copy of *APP*) and AD (APOE3 phenotype), the effects of *BACE2* have been studied. This gene was found to produce high levels of amyloid-free peptides and Aβ degradation products [139]. However, in addition, if we start from a line with mutations in *BACE2*, the organoids that are generated will present a pathology similar to AD [140]. Similarly, *ADAM10*, the gene encoding α-secretase, is interesting, since this secretase can cleave APP instead of β-secretase without giving rise to Aβ peptides. In organoids that have been induced for AD by Aβ peptides, it is observed that activation of *ADAM10* reduces the pathology. The levels of these peptides can be reduced by adding oestrogens to the medium, since these are involved in ADAM10 activity [133]. Continuing with this type of stimulation to obtain AD in organoids, a decrease in mitophagy has also been observed, as well as one of its associated genes, *PINK1*. It was observed that this reduction could be alleviated with the addition of a flavonoid called galangin [134].

4. Future Perspectives

Recent advances in the field of cell cultures have opened up a wide range of opportunities in the study of human diseases, making it possible to overcome the disadvantages of previous models. Despite the advantages of animal models, such as their easy genetic manipulation, physiological differences with the human brain have led to the emergence of *in vitro* 2D models [141]. These 2D cultures allow obtaining highly pure, homogeneous and reproducible cell cultures, but they cannot recreate some neurodevelopmental events, such as cell-scaffold interaction or the influence of some cells on other cell types [34]. For this reason, hCOs have caused a great revolution [142]. Even so, animal models and 2D cultures remain important for preliminary steps in research [104, 127], as they allow the results to be verified at different levels. Also, when it comes to neurodegenerative diseases and age-related disorders, it should be noted that, hESCs and other stem cells have a fetal or embryonic cell age, which is a limitation for these studies [105, 127].

As explained above, *in vitro* 3D models have compensated for the shortcomings of previous models, providing a more reliable view of cell interaction, drug response, and spatiotemporal development. Other advantages are that it can allow us to obtain organoids from patients for more personalized treatment [82] whole organoid image analyses can be performed for a global understanding [143]. It also allows us to reproduce and study the extracellular environment [100], how to use them for the creation of virtual platforms, allowing us to test new strategies without long protocols or ethical conflicts [118, 144, 145], or delving into stem cell therapy [146]. However, obtaining these organoids requires long-term experiments due to slow growth and the times required for cell maturation, aging and senescence [147].

One of the disadvantages of hCOs is ageing, which is due to hypoxic areas in deep regions due to poor perfusion of nutrients and other substances, as they lack vascularisation [148]. This limits in-depth study of neurodegenerative phenotypes and chemical detection. Several strategies have been proposed to address this. A first option would be to use support materials, such as matrigel, porous structures, hydrogels or polymeric materials [149-151]. Even so, these structures are difficult to use, so improvements are still being sought [152], or not relying on supports for their cultivation [153]. Another option that has been considered is to vascularize hCOs. To this end, it has been proposed to transplant organoids into mice [154, 155], but also to directly generate this vasculature [156-158]. Another option for vascularisation would be through co-culture with cells [159-161]. Studying vascularisation would allow us to learn more about the blood-brain barrier (BBB), whose permeability increases in AD, allowing us to improve drug screening [162]. The last strategy that has been proposed is to reduce senescence [163, 164].

The heterogeneity of organoids is another of its limitations. The use of embryoid bodies is a bottleneck for organoid formation, decreasing efficiency and homogeneity. Other factors that increase their heterogeneity are the culture conditions themselves [121], the AD mutation to be studied [129], the material of the plates where they are cultivated or in which at the time of the protocol, matrigel is used [165].

Another drawback is that, so far, most protocols have failed to recapitulate all of the cell types that make up the brain. Even so, one group had recently created a 2D triculture of neurons, microglia, and astrocytes [166, 167]. This opens up an opportunity to create three-culture organoids for more complex models. For example, it is already possible to obtain spheroids that also include oligodendrocytes [168]. The same as obtaining mixed organoids of neurons and astrocytes by fusing them after culturing each cell type independently [115].

5. Conclusion

As seen throughout this review, 3D culture models, with an emphasis on hCOs, appear to be one of the best models for advancing AD research today. Compared to previous models (*in vivo* animals and 2D *in vitro* cultures), hCOs have been found not only to be more efficient, but also to recapitulate some of the most striking features of AD pathology. This has allowed enormous progress in the field in recent years.

Human hCOs, by recapitulating a large part of the complexity of the human brain and due to the innovation of their technology, are quickly positioning themselves in a very interesting model for the study of the molecular and cellular pathology characteristic of AD at the brain level. These hCOs can be generated from control iPS cells or from AD patients, both fAD and sAD, allowing mutations or genes of interest to be introduced or deleted by gene editing. Thus, new therapeutic targets or possible treatments could be determined more quickly.

Another advantage of hCOs is that it is a very suitable technology to further investigate the interactions of the different phenotypes of brain cells (neuron-astrocytes-oligodendrocytes-microglia), under not only physiological conditions, but also pathological ones. Although, there are still challenges to improve and deepen the use of these models, even with the current limitations, great results have already been achieved, allowing for a better understanding of AD, as well as advances in potential therapies.

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Conflicts of Interest

None.

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