New insights in AD research as a result of 3D cell cultures

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Abstract

Despite Alzheimer's disease (AD) being the most prevalent form of dementia, very few therapies are currently in use and none of these has a curative potential in AD. Due to its complex pathology and the fact there are no animal disease orthologues, many proposed therapies fail to show an effect in clinical trials. Human cerebral organoids provide a model system to study AD in human cells, providing a more accurate model system to study pathology and test therapeutic compounds. The goal of this narrative literature review is to describe recent findings that are a direct result of the use of cerebral organoids and to theorize possible implications of these results.

Introduction

Alzheimer's disease (AD) is the most prevalent form of dementia in the world.[1] AD has an intricate pathology that is not fully understood to date, however, the most important aspects of AD pathology that have been identified are extracellular aggregation of amyloid-beta (Aβ)[2] and intracellular tangles formed by phosphorylated Tau (p-Tau) protein.[3] In the past few decades, studies have focused on understanding the pathology of AD and finding therapeutics. These studies have largely been performed in mouse models[4] and therefore lack many characteristics of AD. Many of these murine models are transgenic mice with genes associated with familial AD (fAD) while only around 5% of total AD cases is fAD[5] the remaining 95% is classified as sporadic AD (sAD). In order to study the pathology in this majority of cases, a new type of model has been generated: cerebral organoids.

Organoids are small, organ like structures grown from pluripotent stem cells. Using cellular reprogramming, induced pluripotent stem cells (iPSCs) can be generated from somatic cells. These iPSCs are then cultured and form small clumps of stem cells called embryoid bodies. Neural differentiation is promoted in the embryoid bodies to induce the formation of neurons and astrocytes.[6] Using this method, cerebral structures can be generated consisting of multiple cell types.

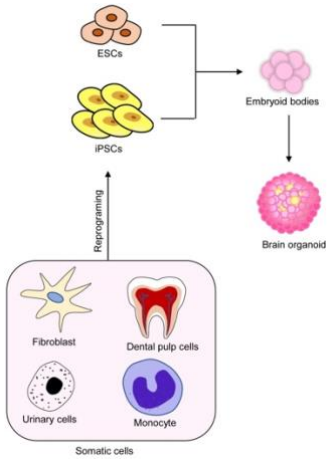

Figure 1: a schematic representation of organoid generation. Image adapted from Shou et al. (2020)

These organoids are typically derived from human cells and therefore have a greater capacity to recapitulate the intricate pathology in human tissue. Furthermore, these organoids can form complex tissues consisting of multiple cell types. Compared to cell lines, which consist of only a single cell type, these organoids can be used to study the interactions between different cell types within a disease. As this type of model system is increasingly used in AD research, this study will try to give a small insight into the different ways this technique can be used to study AD in ways that were not possible using the old models. This review serves as a comprehensive summary of recent studies using organoids. Papers were selected from the past three years (2020-2022) in which researchers used human cell-derived organoids to gather new insights into AD pathology.

Organoids are used to study blood-brain barrier breakdown.

The development of AD has been associated with leakage of blood serum into the brain, through the Blood-Brain Barrier (BBB).[7] This has been suggested in a study by Wu et al. (2021)[8] where BBB leakage was induced in an fAD mouse model by mild traumatic brain injury (mTBI). However, this study had two main pitfalls. Firstly, it was not proven whether the AD pathology was a result of the BBB leakage or more directly caused by the mTBI. Secondly, since there are no murine models to study sAD,[9] this research is only relevant to 5% of AD cases. Therefore, Chen et al. (2021)[10] focused on studying BBB breakdown in an sAD organoid model.

This model showed increased Aβ and p-Tau levels, the primary hallmarks of AD. Furthermore, they showed that elevated Aβ and p-Tau can independently be induced by serum exposure. In the study by Chen et al., human brain organoids were generated following a protocol published by the same authors.[11] The resulting brain organoids were shown to mimic the cortical regions of the brain as they contained a $TBR2^+$ subventricular zone-like layer and neurons that were both $SATB^+$ and $CTIP2^+$. Furthermore, these organoids were shown to contain $GFAP^+$ cells, indicating the presence of astrocytes. Organoids were then exposed to human serum for about 12 days and analyzed. The serum treated organoids showed significantly higher p-Tau and Aβ expression. The insoluble fraction of Aβ in the organoids was also analyzed and showed an increase after serum treatment. This means that the elevated levels of Aβ can also result in the plaques that are representative of AD. The expression of β-Secretase 1 (BACE), an enzyme involved in the cleavage of the Amyloid precursor protein, was also increased compared to control, indicating this may be the mode of action via which serum exposure induces high Aβ levels. To confirm the role of BACE in the increase in Aβ, organoids were treated with BACE inhibitor IV during serum exposure. Treatment with this compound resulted in a substantial decrease in Aβ levels.

In fAD models, BACE inhibitor IV has been shown to reduce p-Tau levels as well as Aβ. In this study p-Tau levels remained high after treatment with the inhibitor, leading to the understanding that serum exposure can increase p-Tau levels, irrespective of Aβ. Similarly, organoids treated with CHIR, a small molecule inhibitor of GSK3α/β that has been shown to reduce p-Tau levels in murine models,[12] resulted in a reduction in p-Tau levels, yet had no effect on Aβ levels. This means that both p-Tau and Aβ can be increased as a result of serum exposure irrespective of one another. Besides p-Tau and Aβ levels, RNA expressions were also analyzed. Using single cell RNA sequencing and subjecting the differentially expressed genes to gene set enrichment analysis against the gene ontology database, several cellular functions that were up- or downregulated were identified. In the serum exposed organoids, genes associated with mitochondrial function were significantly enriched, which is in accordance with relevant literature.[13] [14] Genes involved in synaptic function, however, were significantly downregulated after serum exposure, indicating that serum exposure can lead to reduced synaptic functioning. These results persisted after combined treatment with BACE inhibitor IV and CHIR. From this it can be concluded that synaptic function is decreased by serum exposure, irrespective of Aβ and p-Tau levels.

In this study, human blood serum is used to mimic the effects of BBB breakdown in AD. However, it has been shown that BBB breakdown not only results in serum exposure but also infiltration of blood cells and proinflammatory cytokines and aberrant clearance of extracellular molecules,[15] [16] which can decrease cerebral blood flow,[17] consequently leading to more neuronal damage. Since only blood serum was used in this study, it is not fully representative of BBB breakdown. Therefore, a follow-up

study should be performed using whole blood, in order to provide a more extensive model for studying the effects of BBB breakdown in AD. As shown in this study, this model would be suitable to test prospective therapeutic compounds in a human based model system that is representative of sAD development. In this study it was also shown that serum exposure can result in reduced synaptic functioning, independent of Aβ and p-Tau levels. Following these results, therapies that focus on reducing Aβ and p-Tau will not suffice in the treatment of AD, as the serum exposure can still result in reduced synaptic functioning. Therefore, research should be directed to finding a therapy that targets the BBB breakdown and its subsequent serum exposure in the cortex.

Viral infections can accelerate AD pathology

Many risk factors for AD have been established in the past, one of which is infections with certain viruses. Zika virus (ZIKV) is among the viruses associated with an increased chance of developing AD later in life.[18] However, little is known about the molecular mechanisms involved. Therefore, Lee et al. (2022)[19] investigated the molecular effects of ZIKV infection in neural organoids and studied the pathways involved in this pathology. Several known AD pathologies were studied in organoids generated from both wild-type and AD patient-derived hiPSCs. These AD pathologies were compared between ZIKV, vaccinia virus (VACV) and non-infected organoids. ZIKV infection showed an accelerated AD pathology, including elevated p-Tau and Aβ levels. These pathologies were shown to be caused by upregulated BACE and GSK3α/β, respectively.

For this study, Lee et al. used two organoid models, generated from WT iPSCs and AD patient-derived iPSCs. This means that for this study they mainly focused on the effects of ZIKV infection in the development of fAD. After generation of the organoids, following a protocol described by Lancaster et al,[6] the AD organoids were exposed to ZIKV, VACV or mock conditions for a period of 24 hours, after which the growth medium was changed for all organoids. Using RT-PCR, researchers showed successful productive infections for both viruses. Using immunostaining, Aβ aggregates were visualized in the organoids. For both ZIKV and VACV infected organoids, aggregate formation was continuous for the first 6 days post infection (dpi) and slightly decreased after 14 dpi.

At 14 days dpi the organoids were lysed and analyzed. At this timepoint, aggregate count and size of Aβ were shown to be increased in ZIKV infected organoids compared to WT, mock treated and VACV infected organoids, indicating that ZIKV had an effect on the formation of Aβ aggregates. To investigate the molecular basis for this increased Aβ aggregate count, BACE expression levels were studied using western blotting. BACE levels were increased in ZIKV infected organoids compared to WT and mock treated, BACE levels in VACV infected organoids were not studied. As discussed previously, elevated BACE expression is commonly seen as cause of increased Aβ levels.

Using AT8 antibodies, hyperphosphorylated Tau was also shown to be increased in ZIKV infected AD organoids compared to mock and VACV infected organoids. GSK3α/β levels were not increased in ZIKV compared to mock-treated organoids. However, p-GSK3α/β levels were significantly increased in ZIKV organoids, suggesting that the increased levels of p-Tau could be caused by this increase in activated GSK3α/β. Furthermore, activated-double stranded RNA-dependent protein kinase-like ER-resident (PERK), a marker for endoplasmic reticulum stress, was upregulated in AD organoids and more strongly in ZIKV infected AD organoids. Increased PERK has been associated with an increase in

BACE activity[20] and p-Tau induction.[21] Therefore, Lee et al. propose that increased PERK activity as result of ZIKV infection could induce the AD pathology in human brain organoids. To demonstrate this link, AD organoids infected with ZIKV and mock treated organoids were treated with a GSK2656157, a PERK inhibitor (PERKi). PERKi treatment reduced the level of Aβ in both mock and ZIKV infected AD organoids. It also negated the effect of ZIKV infection on p-Tau levels as both ZIKV infected and mock treated AD organoids had similar p-Tau levels after PERKi treatment compared to mock treated organoids that received no PERKi.

Using hiPSC-derived brain organoids, Lee et al. show that ZIKV infections can accelerate AD pathology and clarify the molecular basis of this induction. Increased PERK activity is shown to be related to increased Aβ and p-Tau levels and inhibition of PERK was found to negate this effect. The model system used in this study relies on patient-derived iPSCs and is therefore only representative of fAD pathology. To investigate the effects of ZIKV infections and increased PERK activity in more prevalent sAD, it would be advised to repeat parts of the study using a different model system that is able to recapitulate the pathologies in sAD, such as the model described by Chen et al.[10]. Using this other model, compounds that inhibit the effects of PERK could be tested for their efficacy against sAD development.

APOE4 is shown to exacerbate AD pathology

Studies have identified the apolipoprotein E (APOE) ε4 allele to be one of the major risk factors for late onset AD.[22] [23] However, little is known about the extent to which APOE4 increases the risk of AD development. Previous studies into the effects of APOE4 have mostly been conducted in murine models for fAD,[24] limiting the scope of the research and its translatability to human pathology. In order to provide more insight in how APOE4 increases the risk of developing AD and to what extent APOE4 is responsible for this increased risk, Zhao et al. (2020)[25] set out to use hiPSC-derived brain organoids to study the effects of the APOE4 allele on the development of AD pathology.

New iPSC lines were generated from volunteers that were divided into 4 groups: AD patient APOE3, AD patient APOE4, cognitively unimpaired APOE3 and cognitively unimpaired APOE4. APOE3 in this context being homozygous for the APOE ε3 allele and APOE4 homozygous for APOE ε4. During the study no significant difference in size was observed between the different sets of organoids. For quantification of AD pathology, researchers focused on three of the most established pathologies in AD, namely Aβ levels, p-Tau levels and neurodegeneration. To show neurodegeneration, the apoptotic protein Caspase-3 (CASP3) and the activated cleaved Caspase-3 (cCASP3) were measured. Measurements were taken from the edge of the organoids as not to be affected by the center of the organoids where a lack of oxygen had resulted in necrosis in all organoids.

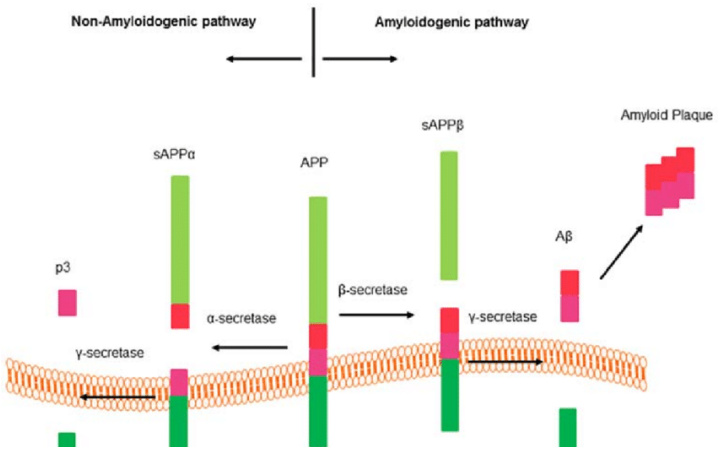

Figure 2: a schematic representation of the Aβ pathway. Image derived from Zhao et al. (2016)

Aβ levels were measured using ELISA and western blot. An increase was seen in both Aβ-40 and Aβ-42 in AD organoids when compared to the organoids derived from healthy individuals. This effect seemed to be slightly, but not significantly, exacerbated by

APOE4. Data also suggested that the Aβ-42 over Aβ-40 was slightly elevated in APOE4 organoids, however, this was again not significant. There was no significant difference observed in the levels of the other derivatives of the amyloid precursor protein (sAPPα, sAPPβ and CTF-β), suggesting that elevated Aβ levels were a result of reduced clearance rather than increased production. This is contrary to the results from the previously discussed studies where increased Aβ was accompanied by an increase in BACE. BACE levels were not measured in this study, however, the lack of increased sAPPβ, a byproduct of Aβ production, is a good indicator that generation of Aβ is not altered in the organoids used in this study. The insoluble fraction of Aβ was below the measurable threshold in all organoids, therefore no conclusions can be drawn about the ability of the Aβ to form aggregates in the organoids.

Immunostaining with AT-8 was used to quantify p-Tau levels in the organoids. Total Tau was measured using western blot. Both APOE4 and AD were shown to increase p-Tau in cerebral organoids and a positive correlation was shown between the p-Tau/Tau ratio and both APOE4 and AD. These results indicate that APOE4 can exacerbate tauopathy in AD and can induce it independently of AD status. This means that APOE4 is not the only risk factor in AD to be responsible for increased p-Tau. However, it can exacerbate the pathology significantly.

Lastly, cCASP3 was measured as quantification of neurodegeneration. The absolute levels of cCASP3 were only significantly increased in AD-APOE4 organoids. However, the ratio of cCASP3/CASP3 was shown to be increased by both AD and APOE4 with APOE4 having a stronger correlation. These data suggest APOE4 could be one of the major factors in the neurodegenerative pathology of AD. As a control, a parental AD APOE4 line was compared to an isogenic AD APOE3 line. Conversion to APOE3 showed an attenuation in both the tauopathy and cCASP3 levels.

In conclusion, the research by Zhao et al. shows APOE4 to be responsible for an exacerbation in both tauopathy and neurodegeneration. No insoluble Aβ fraction was observed as this is one of the later stage AD pathologies. Therefore, in a new study it might be advisable to study the organoids during a longer period of time. As the center of the organoids were already becoming necrotic due to a lack of oxygen, this longer study would benefit from using a protocol for vascularized cerebral organoids, such as the one described by Pham et al.[26] Overall, this study showed the importance of APOE4 in the development of AD and that by conversion of the APOE4 allele to APOE3 several of the important AD pathologies could be attenuated, suggesting APOE4 as possible therapeutic target in future research.

Effects of APOE4 differ with cell type

Another approach to study the importance of APOE4 has been taken by Huang et al. (2022).[27] In a recent study, they compared the effects of the APOE4 allele in astrocytes and neurons. To this end, Huang et al. generated a new type of organoid, described as a chimeric human cerebral organoid, which consisted of neurons and astrocytes with different APOE genotype. The study showed that APOE4 in neurons was responsible for the pathological increase in Aβ levels, whereas APOE4 in astrocytes resulted in increased lipid droplet formation and cholesterol aggregation. Only in the organoids in which both cell types were positive for the APOE4 allele, tauopathy was observed.

Following a novel protocol, Huang et al. generated organoids consisting of two distinct genotypes. A parental APOE3 cell line was used, as well as a genetically altered line where, using CRISPR/CAS9, the APOE3 gene was replaced by an APOE4 gene. Astrocyte production was induced by NFIB and SOX9 in

a number of cells from each of these cell lines. Organoids were then generated from a combination of a transfected cells and non-transfected cells. This resulted in four groups of organoids, namely APOE3 organoids with APOE3 astrocytes, APOE3 organoids with APOE4 astrocytes, APOE4 organoids with APOE3 astrocytes and APOE4 organoids with APOE4 astrocytes. Besides these chimeric organoids, control organoids were generated from a separate hiPSC line.

The presence and activity of astrocytes in the chimeric organoids was confirmed by a glutamate uptake assay. Astrocytes isolated from the organoids were able to take up glutamate and this activity was reduced by a competitive inhibitor of the excitatory amino acid transporters. Neurogenesis was compared between the control organoids and the chimeric organoids, as they consisted of a larger number of astrocytes, which have been found to promote neurogenesis.[28] By labeling the neural progenitor cells and later co-staining the organoids with a neuronal marker, newborn neurons were identified. The presence of newborn neurons was shown to be increased in the chimeric organoids compared to the control organoids. Consistent with these findings, the chimeric organoids showed an increase in $MAP2^+$ neurons and pre- and postsynaptic proteins synapsin I (SYN) and PSD95. Furthermore, higher levels of Aβ, p-Tau and lipid droplets were observed in chimeric organoids compared to the control organoids, signifying an exacerbation of the observed AD pathologies.

Several pathologies of AD, such as increased Aβ, have been extensively studied throughout the years. However, a pathology that has not seen much research is the altered lipid metabolism in AD.[29] When comparing between the different chimeric organoids, an increase in neuronal cholesterol levels was observed as a result of APOE4 in either neurons or astrocytes. Neuronal cholesterol was highest in the organoids with APOE4 in both neurons and astrocytes. These results show that APOE4 is responsible for altered lipid metabolism in both neurons and astrocytes and that they, independently of one another, contribute to elevated neuronal cholesterol.

The Aβ pathology was shown to only be increased by neuronal APOE4. Astrocytic APOE4 had no significant effect on both Aβ40 and Aβ42, whereas neuronal APOE4 increased the levels of both molecules. When comparing the ratios of Aβ42/Aβ40, neuronal APOE4 seemed to increase the ratio slightly, in contrast to astrocytic APOE4, which seemed to lower the ratio slightly, however, neither of these trends reached statistical significance. Tauopathy was only significantly affected by the combination of neuronal and astrocytic APOE4 as only this group saw an increase in p-Tau and in the ratio of p-Tau/Tau.

As it had been shown that astrocytic and neuronal APOE4 increased neuronal cholesterol and previous studies had shown that neuronal cholesterol is involved in elevated p-Tau and Aβ,[30] Huang et al. also investigated the effects of two cholesterol inhibitors (simvastatin and atorvastatin) on the observed AD pathologies. P-Tau was significantly reduced by both therapeutics, however, simvastatin reduced p-Tau levels irrespective of APOE4, leading to below-normal levels of p-Tau in all groups except neuronal APOE4 with astrocytic APOE4. In contrast, atorvastatin only reduced p-Tau levels significantly in the APOE4/APOE4 group, suggesting this therapy could be more suited for clinical use. The effects of simvastatin and atorvastatin on Aβ pathology was not investigated.

To conclude, the use of chimeric human cerebral organoids is an interesting new development as it broadens the possibilities for future research. In this study, Huang et al. showed that APOE4 in neurons is responsible for the APOE4-mediated increase in Aβ in AD. Furthermore, they showed that neuronal and astrocytic APOE4 are both essential for the increase in p-Tau. These results together suggest neuronal APOE4 expression to be an important therapeutic target. For this research, a singular AD cell line was used. Therefore, it cannot be ruled out that other genetic drivers of AD may influence the results that were observed. However, the data from this study suggest a possible

therapeutic use of statins for the treatment and prevention of AD. These data are in line with the results from recent studies into the effects of statins on AD risk and progression.[31] [32]

Discussion

In this study, I have shown that the emergence of cerebral organoids in AD research has led to new discoveries in different aspects of the disease. The use of a human based model system made it possible to study sAD in more detail. Furthermore, these models more closely resemble the complex pathologies observed in patients and, therefore, are better suited for testing therapeutics. This is needed as several drug candidates that have been established in mice have not shown a therapeutic effect in patients.[33] [34] The newly developed, human based model systems discussed in this study have already identified new therapeutic targets, such as the BBB breakdown and its consequent leakage of serum into the brain.[10] Other studies have used organoids to show therapeutic effects of drugs that are currently prescribed for other diseases.[27] As these drugs are already FDA approved, these drugs would provide a good addition to AD therapy.

However, the use of cerebral organoids also has limitations, especially in the field of age-related diseases, as most organoid models start showing signs of necrosis after several weeks due to a lack of oxygenation of the center of the organoid[25] and, therefore, the pathology of the disease cannot occur spontaneously and needs to be induced by external factors. This can be attenuated to some extent using vascularized organoids,[26] however, this would markedly decrease the simplicity of the system and increase the variability between the organoids. Secondly, numerous studies have reported “epigenetic memory” in iPSC lines.[35] These iPSCs retain the epigenetic markers of the somatic cells they were derived from. Therefore, the iPSCs and consequently the organoids would not be fully representative of the cerebral cells they portray. Lastly, although organoids consist of multiple cell types and can form complex structures, they are not fully representative of the human brain, they lack certain cell types, including immune cells, and many of the structures found in the human brain, such as ventricles. Therefore, organoids can be used to study AD pathology in a more accurate model compared to mouse models, however, they still need improvement and, as of yet, are only usable in combination with other models.

In the first article discussed in this study, cerebral organoids were subjected to human blood serum exposure as to mimic the BBB leakage that has been established in AD.[10] It was shown that serum exposure in organoids induced both Aβ and Tau pathologies independently. These results propose the use of BBB breakdown as a key therapeutic target in AD. Since BBB breakdown was only represented by serum exposure in the brain, the full extent of this pathology could not be reproduced. Therefore, future research should induce BBB breakdown in vascularized cerebral organoids[26] in order to study the full extent to which this pathology can contribute to the development of AD.

Besides studies into the possible therapeutic targets in AD, organoids have also been used to gain further insight into the pathology. An example of this is the study by Lee et al.[19] where zika virus infection was shown to accelerate the development of AD pathologies. These results suggest ZIKV infection as indication for therapies that can delay the onset or reduce the risk of developing AD.

In contrast, the research by Zhao et al.[25] further establish the importance of therapies targeting APOE4. It was shown that conversion of APOE4 to APOE3 could attenuate AD pathology in AD

patient-derived cerebral organoids. Since it is not possible or ethical to alter the genotype of the human brain, either in the embryo or later in life,[36] new research should focus on ways to inhibit the pathological activity of APOE4 without disrupting the normal function of the APOE gene.

Lastly, Huang et al.[27] discussed the different effects APOE4 has in neurons and astrocytes. Their findings showed neuronal APOE4 to be mainly responsible for Aβ pathology whereas both neuronal and astrocytic APOE4 were needed to show tauopathy in chimeric human cerebral organoids. These results, combined with the data from Zhao et al. establish neuronal APOE4 as one of the major targets for therapy. Since their research also showed an increase in neuronal cholesterol, Huan et al. studied the effects of statins on AD pathology. These clinically approved drugs showed a reduction in tauopathy, implying their possible use as AD therapeutics. Lipophilic statins, such as atorvastatin and simvastatin, have been shown to be able to cross the BBB[37] and studies have even shown statins to attenuate BBB damage.[38] These results, combined with the data from Chen et al. suggest lipophilic statins as a multipronged approach to targeting AD pathology.

References

Figures

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