APOE4 exacerbates synapse loss and neurodegeneration in Alzheimer’s disease patient iPSC-derived cerebral organoids

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APOE4 is the strongest genetic risk factor associated with late-onset Alzheimer’s disease (AD). To address the underlying mechanism, we develop cerebral organoid models using induced pluripotent stem cells (iPSCs) with APOE ε3/ε3 or ε4/ε4 genotype from individuals with either normal cognition or AD dementia. Cerebral organoids from AD patients carrying APOE ε4/ε4 show greater apoptosis and decreased synaptic integrity. While AD patient-derived cerebral organoids have increased levels of Aβ and phosphorylated tau compared to healthy subject-derived cerebral organoids, APOE4 exacerbates tau pathology in both healthy subject-derived and AD patient-derived organoids. Transcriptomics analysis by RNA-sequencing reveals that cerebral organoids from AD patients are associated with an enhancement of stress granules and disrupted RNA metabolism. Importantly, isogenic conversion of APOE4 to APOE3 attenuates the APOE4-related phenotypes in cerebral organoids from AD patients. Together, our study using human iPSC-organoids recapitulates APOE4-related phenotypes and suggests APOE4-related degenerative pathways contributing to AD pathogenesis.
Alzheimer’s disease (AD) characterized by progressive neurodegeneration is the most common form of dementia. While formations of senile plaques and neurofibrillary tangles (NFTs) are two major neuropathological hallmarks in AD, these pathologies composed of amyloid-β peptides (Aβ) and phosphorylated tau often precede the onset of symptomatic dementia by decades. However, the relevance of the observations APOE4 and pathological changes in age-related disorders. APOE4 risk allele is the strongest genetic risk factor for AD among the three polymorphic alleles (APOE2, APOE3, and APOE4). Apolipoprotein E (apoE) is produced primarily by astrocytes in the central nervous system as a carrier of cholesterol and other lipids to support membrane homeostasis, synaptic integrity, and injury repair. Increasing evidences from animal models and postmortem human brains have shown that APOE4 is associated with multiple aspects of AD pathogenesis. In particular, mouse models carrying human APOE4 have accelerated Aβ seeding and suppressed Aβ clearance, as well as disturbed synaptic plasticity and blood–brain barrier integrity; however, whether these findings are translatable to humans is not clear. In addition, studies with human postmortem brains represent only the characteristics of end-stage disease. Thus, there is an urgent need to establish human-relevant models to define APOE4-related pathogenic pathways in AD.

The induced pluripotent stem cells (iPSCs) derived from human somatic cells with AD-linked mutations or risk alleles are promising in vitro models by recapitulating the earliest molecular and pathological changes in age-related disorders. Elevated levels of Aβ and phosphorylated tau, as well as increased cellular stress markers have been reported in iPSC-derived neurons from AD patients. For example, Aβ oligomers accumulate in iPSC-derived neurons and astrocytes in cells from patients with a familial amyloid precursor protein (APP) p.E693A mutation and sporadic AD, leading to endoplasmic reticulum (ER) and oxidative stress. In addition to altered APP processing, an increase in levels of total and phosphorylated tau is observed in iPSC-derived neurons from AD patients carrying the APP p.V717I mutation or APOE4 risk allele. However, the relevance of the observations from two-dimensional (2-D) cell cultures to AD is questionable as AD pathology is intricate and involves diverse cell types, tissue structures, and cellular pathways. Thus, in this study, we utilize a large number of human iPSC lines from healthy subjects and AD patients carrying APOE ε3/ε3 or ε4/ε4 genotype, and investigate AD-related phenotypes using the iPSC-derived three-dimensional (3-D) cerebral organoid model system, which is highly reminiscent of human brain structure with diverse cell types. Here, we show that APOE4 aggravates neurodegeneration in iPSC-derived cerebral organoids from AD patients; however, its effects on tauopathy are significant in both healthy subject-derived and AD patient-derived cerebral organoids. Interestingly, the levels of Aβ are increased in AD organoids independent of APOE genotype. Importantly, isogenic conversion of APOE4 to APOE3 attenuates AD-related phenotypes in iPSC-derived cerebral organoids. These findings reveal APOE4-related pathways in 3-D models that are directly relevant to humans.

**Results**

**Characterization of human iPSC-derived cerebral organoids.** To study the effects of APOE genotype on AD-related pathways in a physiologically relevant environment, we generated 3-D cerebral organoid models using human iPSC lines from cognitively unimpaired individuals carrying APOE ε3/ε3 (Con-E3; N = 5), cognitively unimpaired individuals carrying APOE ε4/ε4 (Con-E4; N = 5), AD patients carrying APOE ε3/ε3 (AD-E3; N = 5) and AD patients carrying APOE ε4/ε4 (AD-E4; N = 5), collected from multiple sources (Supplementary Table 1).

Among them, 5 iPSCs lines were generated de novo from fibroblasts by transfecting with three episomal vectors encoding five transcription factors (OCT3/4, Sox2, L-MYC, KLF4, and LIN28) and p53 shRNA. Expression of pluripotency stem cell-specific markers was confirmed by immunostainings for SSEA4, Nanog, and TRA-1-60 (Supplementary Fig. 1A). The pluripotency of the iPSC lines was also validated by their ability to differentiate into endodermal, mesodermal, and ectodermal origin cells upon immunostaining of Brachyury (Mesoderm marker), Sox17 (endoderm marker), and Nestin/Sox2 (ectoderm marker) (Supplementary Fig. 1B). The iPSC lines maintained a normal karyotype after reprogramming (Supplementary Fig. 1C). All other iPSC lines utilized in published studies or from California Institute for Regenerative Medicine (CIRM) (FUJIEILM Cellular Dynamics, Inc.) have been fully validated and characterized previously.

To generate cerebral organoids from iPSCs with efficiency and reproducibility, we followed an optimized protocol developed by Stemcell Technologies. Cerebral organoid formation was initiated through an intermediate embryonic body (EB) formation step followed by expansion of neuroepithelia in a matrigel scaffold. On day 12, the iPSC-derived organoids were transferred to an orbital shaker in neuronal differentiation medium and maintained under rotary conditions for maturation (Fig. 1a). On week 4, cerebral organoids showed a predominantly dorsal forebrain region specification, containing fluid-filled ventricle-like structures aligned with Sox2-positive neural progenitors in a ventricular/subventricular-like zone (VZ/SVZ) and beta-tubulin III (TuJ1)–positive neuroblasts in an outer layer (Fig. 1b). A deep cortical layer marker Ctip2–positive neurons were detected as early as week 4 (Fig. 1c), whereas a superficial cortical layer marker Satb2–positive neurons emerged in the later stage at week 12 (Fig. 1d).

These observations revealed the sequential emergence of different neuronal layers along with the differentiation, which is consistent with previous publication. Since apoE is mainly produced by astrocytes in the brain, we assessed the presence of astrocytes at different time points by immunostains for glial fibrillary acidic protein (GFAP). We found that small clusters of GFAP-positive astrocytes started to emerge in some VZ area at week 4. GFAP-positive astrocytes showed an immature morphology with short processes, which were separated from surrounding TuJ1-positive neuronal cells (Fig. 1e). At week 12 of differentiation, GFAP-positive astrocytes increased in number and migrated within the neuronal layers, displaying typical mature astrocyte morphology with long processes (Fig. 1f). Furthermore, to determine the influence of the technical difference to the differentiation of cerebral organoids across different rounds, we compared the levels of GFAP and TuJ1 in the cerebral organoids from two rounds of differentiation. We found no significant differences in the expression of GFAP and TuJ1 at week 12 between the first and second round of experiments (Supplementary Fig. 9). These results indicate the successful development of iPSCs to cerebral organoids with cortical like organization composed of abundant mature neurons and astrocytes.

**APOE4 and AD status exacerbate neurodegeneration.** With the establishment of the cerebral organoid culture system, we generated four groups of iPSC-derived cerebral organoids (Con-E3, Con-E4, AD-E3, and AD-E4). Cerebral organoid size was monitored and there were no evident differences observed among different groups at 2 and 12 weeks (Supplementary Fig. 2). To evaluate the effect of APOE4 and disease status on neuronal apoptosis/regeneration in the cerebral organoids, cleaved caspase-3 (CASP3) was analyzed by immunostaining at week 12 (Fig. 2a). To avoid the possible influences of necrosis observed in...
the core portion of organoids, the immunoreactivity was measured only in the surface neuronal layers. We found the increased cleaved CASP3 immunoreactivity in the AD-E4 organoids compared to other groups with an interactive effect between APOE4 and AD status (Fig. 2b). Consistently, western blotting also revealed higher cleaved CASP3/CASP3 ratio in AD-E4 organoids (Fig. 2c, d). These results indicate that APOE4 and AD status synergistically exacerbate apoptosis in late stage of organoid development. Presynaptic synaptophysin and postsynaptic PSD95 were decreased in AD organoids groups compared to healthy subject-derived cerebral organoids, whereas no significant APOE4 effect was observed (Fig. 2c, e, and f). In contrast, on week 4, reverse transcription-quantitative PCR (RT-qPCR) revealed that cerebral organoids from AD patients exhibited higher mRNA levels of mature neuronal markers including MAP2, CTIP2, and SATB2, but not GFAP, an astrocyte marker (Supplementary Fig. 3A–D). Synaptophysin and PSD95 were also upregulated in AD organoid groups by week 4 (Supplementary Fig. 3E–G). While synaptophysin levels were increased in AD-E4 organoids (Supplementary Fig. 3F), APOE4 did not influence PSD95 levels (Supplementary Fig. 3G). Together, these results suggest that organoid maturation and synaptic formations are accelerated in cerebral organoids from AD patients at the early stage.

Increased Aβ amounts in cerebral organoids from AD-iPSCs.

To investigate the impacts of APOE4 and disease status on Aβ accumulation and deposition, iPSC-derived cerebral organoids
were utilized for the analyses at different time points (weeks 4, 8 and 12). Organoids were sequentially lysed in RIPA buffer and formic acid (FA), and subjected to the measurements for Aβ40 and Aβ42. Using ELISA, we detected Aβ at an earlier time point than that reported by Lin et al.28, in which they assessed Aβ accumulation by western blotting. Higher levels of Aβ40 at week 8 (Supplementary Fig. 4A) and Aβ42 at weeks 4 and 8 (Supplementary Fig. 4B) were observed in the RIPA-soluble fraction from AD organoids as compared to those from healthy subject-derived cerebral organoids. The increases of Aβ40 (Fig. 3a), Aβ42 (Fig. 3b), and Aβ42/Aβ40 ratio (Fig. 3c) in AD organoid groups were magnified at week 12 independent of APOE4. Aβ40 and Aβ42 in detergent-insoluble FA fraction were undetectable at any time points. In addition, neither APOE4 nor disease status affected APP derivatives including sAPPα, sAPPβ, and CTF-β at week 12 (Fig. 3d–f), whereas APOE4 but not AD status was

Fig. 2 APOE4 enhances apoptosis and synaptic loss in cerebral organoids from AD patients. Cerebral organoids were subjected to immunostaining and western blotting at week 12. a Representative images and quantification of cellular apoptosis evaluated by immunostaining of cleaved CASP3. Scale bar: 100 μm. b Cleaved CASP3 immunoreactivities were quantified from 5 cerebral organoids per line, and the averaged values were compared among the groups (APOE4: p = 0.032, AD: p = 0.0569, APOE4 x AD: p = 0.018, Con-E3 vs. AD-E4: p = 0.0523, Con-E4 vs. AD-E4: p = 0.0112, AD-E3 vs. AD-E4: p = 0.014). All data are expressed as mean ± SEM (N = 5). c–f Cleaved CASP3, CASP3, synaptophysin, PSD95, and Tuj1 levels in the lysates of 4–5 cerebral organoids per line were analyzed by western blotting and quantified. All data are expressed as mean ± SEM (N = 5). d Cleaved CASP3 levels were normalized to total CASP3 levels and compared among groups (APOE4: p < 0.0001, AD: p < 0.0001, APOE4 x AD: p = 0.0020, Con-E3 vs. Con-E4: p = 0.009, Con-E3 vs. AD-E3: p = 0.0206, Con-E4 vs. AD-E4: p < 0.0001, AD-E3 vs. AD-E4: p < 0.0001). Synaptophysin and PSD95 levels were normalized to Tuj1 levels and compared among groups (APOE4: p = 0.5841, AD: p = 0.0002, APOE4 x AD: p = 0.0453, Con-E3 vs. AD-E4: p = 0.0069, Con-E3 vs. AD-E4: p = 0.0002, Con-E4 vs. AD-E3: p = 0.0077, APOE4: p = 0.8794, AD: p = 0.0025, APOE4 x AD: p = 0.0551, Con-E4 vs. AD-E3: p = 0.0404, Con-E4 vs. AD-E4: 0.0019). ANCOVA for APOE4, AD status, and APOE4 x AD status was performed by including sex, sampling age, and source of iPSCs as co-variables, which was followed by two-sided Tukey-Kramer tests to compare between the groups with two factors (APOE4 and AD status). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
associated with lower levels of full-length APP (Fig. 3g, h). These results suggest that Aβ clearance mechanism rather than APP processing is altered in cerebral organoids from AD patients regardless of APOE4 status, resulting in enhanced Aβ accumulation and increased Aβ42/Aβ40 ratio.

**Exacerbated tauopathy in cerebral organoids from APOE4-iPSCs.** To examine the tau pathology in the healthy subject-derived and AD patient-derived cerebral organoids carrying different APOE genotypes, we stained cerebral organoids with anti-phosphorylated tau (p-tau) AT8 at week 12 and found more p-tau accumulation in organoids with APOE4 (Fig. 4a). Consistent with the results, western blotting with AT8 revealed that APOE4 and AD status were correlated with higher p-tau/tau ratio in the organoids (Fig. 4b–d). When RIPA lysates (Fig. 4e) and FA fractions (Fig. 4f) were subjected to ELISA, APOE4, and AD status were independently associated with p-tau upregulation in the organoids at week 12. While RIPA-soluble p-tau increased in all groups in time, APOE4 or AD patient-derived organoids showed higher p-tau levels as early as week 4 (Supplementary Fig. 4C). Together, these results indicate that APOE4 and AD status aggravate tau pathology in iPSC-derived organoids without interactive effects.

When apoE levels were measured in the organoids by ELISA, higher soluble apoE levels in RIPA fraction was associated with APOE4 at week 12 (Fig. 5a); however, AD status rather than APOE4 was associated with increased insoluble apoE in the FA fraction at week 12 (Fig. 5b). Both APOE4 and AD status were associated with the increased soluble apoE levels at week 4 (Supplementary Fig. 4D). No significant differences in GFAP/Tuj1 ratio were observed among the four groups of cerebral organoids by immunostaining (Supplementary Fig. 5A–B, RT-qPCR (Supplementary Fig. 5C), and western blotting (Supplementary Fig. 5D), suggesting that APOE4 or AD status leads to the higher apoE level without increasing astrocyte population in the cerebral organoids. Among all groups, significant positive correlations were observed between p-tau and apoE levels in RIPA (Fig. 5c) and FA fractions (Fig. 5d). Although there were no
evident correlations between Aβ and apoE levels (Fig. 5e, f), Aβ levels (Fig. 5g, h) were also positively correlated with p-tau levels in RIPA fraction. These results suggest that Aβ and apoE might associate with p-tau through different molecular pathways.

**Altered transcriptomes in cerebral organoids from AD-iPSCs.**

To further address the impact of APOE4 and/or AD status on transcriptional profiles in iPSC-derived organoids, we performed RNA-sequencing (RNA-seq) at week 12. We identified 1331 and 717 differentially expressed genes (DEGs) between Con-E3 and Con-E4, and between AD-E3 and AD-E4, respectively, with an overlap of 302 genes (Supplementary Data 1, Supplementary Fig. 5A). Among the overlapped genes, 265 genes changed in the same direction. When comparing between healthy subject-derived and AD patient-derived cerebral organoids, we identified 1188 DEGs between Con-E3 and AD-E3, and 501 DEGs between Con-E4 and AD-E4, with an overlap of 317 genes (Supplementary Data 1, Supplementary Fig. 6B). Furthermore, pathway analysis revealed “Amyloid proteins” as the top-ranked network affected by the disease status and APOE4 interaction (Supplementary Fig. 6C). The top-ranked pathways enriched by DEGs were

\[ \text{AT8AT8/Tuj1 Con-E3 Con-E4 AD-E3 AD-E4} \]

\[ \text{p-tau in RIPA (pg/mg protein)} \]

\[ 0 \quad 100 \quad 200 \quad 300 \quad 400 \]

\[ 0 \quad 200 \quad 400 \quad 600 \]

\[ \text{AT8/Tau (ratio to Con-E3)} \]

\[ 0 \quad 1 \quad 2 \quad 3 \quad 4 \]

\[ \text{PHF-1/Tau (ratio to Con-E3)} \]

\[ 0 \quad 1 \quad 2 \quad 3 \quad 4 \]

\[ \text{p-tau in FA (pg/mg protein)} \]

\[ 0 \quad 50 \quad 100 \quad 150 \quad 200 \]

\[ 0 \quad 50 \quad 100 \quad 150 \quad 200 \]

**Fig. 4** APOE4 and AD status enhance p-tau levels in iPSC-derived cerebral organoids. Cerebral organoids were subjected to analyses by immunostaining, western blotting, and ELISA at week 12. a Representative images of the immunostaining for p-tau with AT8 (Ser202/Thr205) antibody. Scale bar: 100 μm. b-d Total tau and p-tau levels in the RIPA lysates of 4-5 cerebral organoids per line were analyzed by western blotting and quantified. The p-tau levels detected by AT8 antibody (c, APOE4: \( p = 0.0037 \), AD: \( p = 0.0010 \), APOE4 x AD \( p = 0.6128 \), Con-E3 vs. AD-E3: \( p = 0.0290 \), Con-E3 vs. AD-E4: \( p = 0.0010 \), Con-E4 vs. AD-E4: \( p = 0.0273 \) and PHF1 (Ser396/Ser404) (d, APOE4: \( p = 0.3742 \), AD: \( p = 0.0404 \), APOE4 x AD: \( p = 0.1823 \) were normalized to total tau. e, f Amounts of p-tau in the RIPA fraction (e, APOE4: \( p < 0.0001 \), AD: \( p < 0.0001 \), APOE4 x AD: \( p = 0.7063 \), Con-E3 vs. Con-E4: \( p = 0.0029 \), Con-E3 vs. AD-E3: \( p = 0.0015 \), Con-E3 vs. AD-E4: \( p < 0.0001 \), Con-E4 vs. AD-E4: \( p = 0.0001 \), AD-E3 vs. AD-E4: \( p = 0.0010 \) and the FA fraction (f, APOE4: \( p < 0.0001 \), AD: \( p < 0.0001 \), APOE4 x AD: \( p = 0.4038 \), Con-E3 vs. Con-E4: \( p = 0.0121 \), Con-E3 vs. AD-E3: \( p < 0.0001 \), Con-E3 vs. AD-E4: \( p = 0.0024 \), Con-E4 vs. AD-E4: \( p = 0.0012 \)) from 4 to 5 cerebral organoids per line were measured by ELISA. Data were normalized to individual total protein concentration. All data are expressed as mean ± SEM (\( N = 5 \)). ANCOVA for APOE4, AD status, and APOE4 x AD status was performed by including sex, sampling age, and source of iPSCs as co-variables, which was followed by two-sided Tukey-Kramer tests to compare between the groups with two factors (APOE4 and AD status). *\( p < 0.05 \), **\( p < 0.01 \), ****\( p < 0.0001 \).
In all comparisons (Supplementary Fig. 7D–F), no significant differences in neuron and astrocyte proportion were observed among population (Supplementary Fig. 7A). No significant gene expression changes were found by RT-qPCR and western blotting, and confirmed decreased mRNA and protein levels in AD-E4 organoids (Fig. 6D, f, j–l), suggesting that neurons predominantly contributed to the phenotypes of cerebral organoids from different groups compared to astrocytes.

Weighted Gene Co-expression Network Analysis (WGCNA) identified two gene modules that were significantly associated with disease status: module magenta was positively correlated with AD status, while module yellow was positively correlated with healthy subject-derived cerebral organoids (negatively correlated with AD) (Fig. 6a). Genes in the yellow module were enriched for RNA metabolism dysregulation (Fig. 6b), which included ERCC4, DGCR8, POLR3A, CLP1, HSPA4, PNO1, VPS18, RAD17, LCMT2, and RPUSD2 as top-ranked hub genes (Fig. 6c). We further validated expressions of selected genes by RT-qPCR and western blotting, and confirmed decreased mRNA and protein levels in AD-E4 organoids (Fig. 6d, f, j–l). Genes in the magenta module were enriched for those related to DNA and nucleosome metabolism pathways (Supplementary Fig. 8A). CHIC1, H2BC7, H2BC8, ASB3, H2BC18, H2BC21, H1-3, ARRDC3, and TBL1XR1-AS1 were identified as top-ranked hub genes (Supplementary Fig. 8B). We further confirmed the increased mRNA levels of CHIC1, ASB3, and ARRDC3 by RT-qPCR in AD organoids, in particular those with APOE4 (Supplementary Fig. 8C–E). Since some of the hub genes in the yellow module (PNO1, DGCR8, VPS18, and HSPA4) are...
closely related to lysosomal stress granules formation under cellular stress conditions.\(^29,30\), we immunostained the cerebral organoids for a stress granule marker Ras GTPase-activating protein-binding protein 1 (G3BP) in the cerebral organoids at week 12, and found an increase of G3BP-positive punctates in AD organoids (Fig. 6e). We further quantified G3BP, endosome marker protein early endosome antigen 1 (EEA1), and lysosome marker protein lysosomal-associated membrane protein 1 (LAMP1) by western blotting, and found the interactive effect between APOE4 and AD status; G3BP

\[
\text{ERCC4} \quad \text{DGCR8} \quad \text{POLR3A} \quad \text{CLP1} \quad \text{HSPA4} \quad \text{PNO1}
\]

| Gene Ontologies Enriched in the Module |
|----------------------------------------|
| ncRNA metabolic process                 |
| ncRNA processing                       |
| tRNA metabolic process                 |
| tRNA processing                        |
| RNA processing                         |
| organic cyclic compound binding        |
| heterocyclic compound binding          |
| RNA modification                       |
| RNA binding                            |
| intracellular membrane-bounded organelle |

**mRNA expression (ratio to Con-E3)**

**ERCC4/β-actin**

**POLR3A/β-actin**

**HSPA4/β-actin**

**Con-E3**
**Con-E4**
**AD-E3**
**AD-E4**

- **Con-E3**
- **Con-E4**
- **AD-E3**
- **AD-E4**

**Gene expressions**

- G3BP
- EEA1
- LAMP1
- β-actin

**Con-E3**
**Con-E4**
**AD-E3**
**AD-E4**
significantly increased in organoids from AD patients carrying APOE4 (Fig. 6f, g). No significant changes were observed in EEA1 (Fig. 6h) and LAMP1 levels (Fig. 6i). These results imply that the disruption of RNA metabolism in cerebral organoids from AD patients accelerates stress granule formation especially in the presence of APOE4.

**Discussion**

While AD is neuropathologically diagnosed through postmortem assessments in patients with dementia\(^\text{31}\), recent research framework has established antemortem AD classification using biomarkers for Aβ deposition (A), pathologic tau (T), and neurodegeneration (N)\(^\text{32}\). Thus, to explore the complex AD pathogenesis and develop therapeutic interventions for the disease, the establishment of human models system recapitulating ATN phenotypes has become essential. Toward this, we have comprehensively investigated AD-related pathogenic pathways using iPSC-derived 3-D cerebral organoids from sporadic AD patients with or without APOE4. Cerebral organoids not only simulate intrinsic spatial patterning, but also display acquisition of cell identity in a timed manner that closely mimics the temporal patterning with sequential neuronal layer formation, accompanied with matured astrocytes\(^\text{21,22,33}\). Furthermore, the phenotypes related to aberrant extracellular protein aggregation can be recapitulated only in 3-D culture systems\(^\text{28,34,35}\). Using iPSC-derived cerebral organoid system, we identified multiple APOE4- and/or AD disease status-dependent pathogenic pathways, revealing complex etiology associated with APOE4, the strongest genetic risk factor for late-onset AD.

Consistent with results from 2-D cultures of iPSC-derived neurons\(^\text{13,18,28,36}\), we found the elevated Aβ40 and Aβ42 levels in RIPA-soluble fraction in AD patient-derived organoids regardless of APOE status. Since APP processing was not altered, AD brain organoids may have compromised machineries for enzymatic Aβ degradation and/or cellular Aβ clearance. Of note, a significant reduction of soluble Aβ levels was observed in the cerebral organoids from a sporadic AD patient upon isogenic conversion of APOE4 to APOE3. Thus, it is possible that APOE4 and other gene variants synergistically facilitate Aβ accumulation in AD organoids. Notably, insoluble Aβ and amyloid plaque pathology was undetectable by ELISA and immunostaining in our organoid models from sporadic AD patients at week 12. While Aβ plaque deposition is detected 2–4 months after the differentiation in iPSC-organoid models from familial AD and Down syndrome patients\(^\text{35,37}\), those from sporadic AD patients likely have Aβ deposition 6 months after differentiation\(^\text{26}\). Thus, longer differentiation duration may be required to assess amyloid deposition in iPSC-derived organoids from sporadic AD cases.

Another pathological hallmark of AD is the abnormal phosphorylation, mislocalization, and aggregation of tau\(^\text{18,39}\), although tauopathy is also detected in non-AD neurodegenerative diseases including frontotemporal dementia (FTD) and progressive...
Furthermore, APOE4 apoE amounts in positive correlations in both RIPA and FA fractions, which is consistent with the results from mouse studies showing that APOE4 deficiency ameliorates tauopathy. In addition, Wang et al. has shown that p-tau levels were increased in AD patient carrying APOE ε4/ε4 (Par-E4/4) and the APOE ε3/ε3 isogenic line (Iso-E3/3) were measured by ELISA. Data were normalized to individual total protein concentrations. The ratio of Aβ42/Aβ40 was calculated accordingly. Amounts of Aβ in RIPA (d) and FA (e) were measured by ELISA. Data were normalized to individual total protein concentrations. Representative images of the immunostaining for p-tau with AT8 antibody. Scale bar: 100 μm. Total tau and p-tau in RIPA lysates were analyzed by western blotting and quantified. The p-tau detected by AT8 antibody and PHF1 antibody were normalized to total tau (AT8: Aβ40: PHF1: p = 0.0152, PHF1: p = 0.0043). Amounts of p-tau in RIPA (k) and FA (l) were measured by ELISA. Data were normalized to individual total protein concentration. G3BP, levels in RIPA lysates were analyzed by western blotting and quantified. The levels of G3BP were normalized to β-actin (p = 0.0022). Lysates of 4 cerebral organoids were analyzed as one sample. All data are expressed as mean ± SEM (n = 6). Two-sided Mann-Whitney U tests were performed to determined statistical significance, *p < 0.05, **p < 0.01.

Supranuclear palsy (PSP) may accelerate the p-tau accumulation in an Aβ-dependent manner. Of note, apoE and p-tau levels show positive correlations in both RIPA and FA fractions, which is consistent with the results from mouse studies showing that APOE4 deficiency ameliorates tauopathy. In addition, Wang et al. has shown that p-tau levels were increased in APOE-deficient iPSC-derived neurons when treated with lysates of iPSC-derived neurons with APOE4. Thus, although further studies are necessary, APOE4 likely induces a gain-of-toxic effect on p-tau accumulation in iPSC-derived cerebral organoids.

Intriguingly, we found marked increase in cellular apoptosis and reductions of synaptic proteins in mature organoids from AD patients compared to healthy subject-derived cerebral organoids. In supporting our results, Wang et al. has shown that p-tau levels were increased in AD patient carrying APOE ε4/ε4 (Par-E4/4) and the APOE ε3/ε3 isogenic line (Iso-E3/3) were measured at week 12. The perimeters of cerebral organoids were measured (n = 10). Representative images of cellular apoptosis with the immunostaining for cleaved CASP3. Scale bar: 100 μm. Levels of cleaved CASP3 and CASP3 levels in the lysates were analyzed by western blotting and quantified (p = 0.0043). Amounts of p-tau in RIPA (d) and Aβ40 (e) were measured by ELISA. Data were normalized to individual total protein concentrations. The ratio of Aβ42/Aβ40 was calculated accordingly. Amounts of apoE in RIPA (f) and FA (g) were measured by ELISA. Data were normalized to individual total protein concentrations. Representative images of the immunostaining for p-tau with AT8 antibody. Scale bar: 100 μm. Total tau and p-tau in RIPA lysates were analyzed by western blotting and quantified. The p-tau detected by AT8 antibody and PHF1 antibody were normalized to total tau (AT8: Aβ40: PHF1: p = 0.0152, PHF1: p = 0.0043). Amounts of p-tau in RIPA (k) and FA (l) were measured by ELISA. Data were normalized to individual total protein concentration. G3BP, levels in RIPA lysates were analyzed by western blotting and quantified. The levels of G3BP were normalized to β-actin (p = 0.0022). Lysates of 4-5 cerebral organoids were analyzed as one sample. All data are expressed as mean ± SEM (n = 6). Two-sided Mann-Whitney U tests were performed to determined statistical significance, *p < 0.05, **p < 0.01.
reduced neurite length in comparison to those from healthy individuals. Wang et al. also found expression of apoE4 was associated with increased GABAergic neuron degeneration. More supportively, the gain-of-toxic-function of apoE4 could be ameliorated when apoE4 was converted to apoE3 by gene editing, which is highly consistent with what we found in the cerebral organoid model. In contrast, at the early neuronal differentiation stage, increased mature neuronal markers and synaptic proteins were detected in AD-derived organoids. Given that similar observations were reported in iPSC-derived neurons from AD subjects, specific factors facilitating excess neuronal maturation in the early stage may be commonly preserved in iPSCs from AD patients, resulting in enhanced neurodegeneration in the late stage. While APOE4 likely leads to early maturation of neurons partially consistent with our results, we demonstrated that APOE4 further exacerbates the effects of AD status on apoptosis induction and synaptic loss in cerebral organoids at week 12. Thus, it is tempting to speculate that accelerated neuronal differentiation/maturity is synergistically affected by AD-related factors and APOE4 in the pre-symptomatic stage, and that the mechanistic exhaustion followed by neuronal dysfunction may contribute to disease development, although further studies are needed.

Our transcriptomic analysis demonstrated that an enrichment of gene sets involving RNA metabolisms is predominantly affected by AD status in the iPSC-derived cerebral organoids; while APOE4 did not have a strong effect on the profiles. RNA metabolism process is highly dynamic and requires a complex interplay among RNA-binding proteins (RBPs). Thus, the disruption of RBP homeostasis is frequently involved in the pathology and genetics of neurodegenerative diseases including FTD and amyotrophic lateral sclerosis (ALS), where aggregation-prone RBPs often co-localize with stress granules. Interestingly, we also found a significant increase in stress granules in organoids from AD patients. While various RBPs have been shown to deposit in stress granules, internalized tau sensitizes cells to stress by promoting formation and stability of stress granules. Thus, enhanced p-tau accumulation may trigger transcriptional changes and stress granule formation in organoids from AD patients. There were also reductions of mRNAs in genes related to DNA repair (ERCC4 and DSGR8) and anti-apoptosis (PON1 and HSPA4) in the organoids from AD patients. However, further studies are necessary to determine if those are causatively or consequently involved in neurodegeneration.

Although our results demonstrate that iPSC-derived cerebral organoid is a useful modeling system to investigate AD- and APOE4-related phenotypes, we should state several limitations. First, the core of cerebral organoids shows necrosis-like changes after week 12, likely due to the lack of vascular systems containing proper supplies of nutrition and gas exchange as well as immune cells to eliminate cell debris and toxic molecules. To address this caveat, emerging technology has further developed cerebral organoid models in which vascular cells or microglia are incorporated. Thus, in future studies, we plan to utilize the iPSC-derived cerebral organoid system with neuroimmune and/or neurovascular system to establish a more advanced platform for AD research. Second, there was heterogeneity in organoid size and growth rate even though identical iPSC lines were used. We also noticed that maturation of astrocytes and the detection of Aβ or apoE in cerebral organoids may vary depending on differentiation protocols. For example, Lin et al. found lower soluble apoE levels in cerebral organoids with APOE4 compared to those with APOE3, which is different from our results. The differences in the medium used for differentiation and the time points of medium change may potentially contribute to the different results under the stress conditions induced by APOE4 or AD status. The technical differences and the intrinsic variability using iPSC-derived cerebral organoid models should be considered whenever comparing results from different studies. Third, because of the limited availability of patient-derived iPSC lines, we could not match sex among our study groups in our study. Nonetheless, we did adjust sex as a variable in our analysis. Further optimization and standardization in the iPSC-derived cerebral organoid modeling system should be able to overcome these weaknesses.

In conclusion, our study established a true 3-D human cerebral organoid system to address AD pathogenesis. We successfully recapitulated AD-related pathologies related to ATN classification in our model system. APOE4 predominantly aggravates p-tau accumulation, while AD status is associated with higher levels of Aβ and p-tau, apoptosis, synaptic loss and increased stress granule formation. Importantly, APOE4 synergistically accelerates apoptosis and stress granule formation with AD status in our 3D model. Thus, exploring molecular mechanisms in the interaction between APOE4 and AD-related pathways should provide clues as to how APOE4 vastly increases AD risk. Because isogenic conversion of APOE4 into APOE3 reverses much of the AD-related phenotypes in cerebral organoids from AD, APOE4 might be a promising therapeutic target for AD.

Methods

Generation of iPSCs from human skin fibroblasts. Human skin biopsies from normal individuals and AD patients with APOE ε4/ε4 or ε4/ε3 genotype were obtained from Mayo Clinic patients under IRB protocols with patient consent for research, which was approved by the Mayo Clinic Institutional Review Board. APOE genotype was confirmed by Sanger sequencing using DNA samples from fibroblast lines. Cells were cultured in fibroblast medium containing 10% fetal bovine serum (FBS) (Gemini Bio-Products). The iPSCs were generated by electrotransformation of three epimolar vectors into the fibroblasts using the NHDF nucleofector kit (Lonza). Three microgramms of expression plasmid mixtures were electroporated into 6 × 10^6 fibroblasts with 100 μl transfection reagents. After transfection, fibroblasts were plated onto a 100 mm Matrigel (Corning) coated dish. After 5 days of culture, the fibroblast medium was replaced with TeSR-E7 complete medium (Stemcell Technologies) and changed every day. iPSC colonies were isolated and expanded after 3–4 weeks in culture. The iPSC colonies were passaged using Dispase (Stemcell Technologies) and subjected to rock inhibitor Y27632 (Sigma-Aldrich) treatment for the first 24 h.

Trilineage differentiation of human iPSCs. Three germ layer differentiation was used to confirm the pluripotency of iPSCs using the STEMediif Trilineage Differentiation kit (Stemcell Technologies) according to manufacturer’s instructions with some modification. When cells were ~70% confluent, iPSCs were passaged using Accutase (Stemcell Technologies) and plated into an AggreWell™800 plate (Stemcell Technologies) to form embryonic bodies (EB) in mTeSR medium for 2 days. EBs were washed out from the AggreWell plate and transferred to 6-well non-tissue culture treated plates (Corning) in specific differentiation medium for each lineage. EBs were subjected to differentiation into mesoderm and endoderm lineages for 5 days or an ectoderm lineage for 7 days, then seeded onto Matrigel-coated plates for further analysis. Differentiation was assessed by immunostaining for germ layer-specific markers (Endoderm: SOX17; Mesoderm: Brachyury; Ectoderm: Nestin/Sex2).

Cerebral organoid culture. Cerebral organoids were generated according to manufacturer’s instructions of the commercial STEMediif Cerebral Organoid Kit (Stemcell Technologies). On day 0, any pre-differentiated cells in the hiPSCs culture were removed by scraping under a microscope. Human iPSC colonies were dissociated into single-cell suspension with Accutase. In total, 9000 cells were then plated into each well of a U-bottom ultra-low-attachment 96-well plate in EB formation media (medium A) supplemented with 10 μM Y27632. An extra 100 μl EB medium was added on day 2 and day 4, respectively. EBs were moved to 24-well low attachment plates in neural induction medium (medium B) for another 4 days. EBs were further embedded into 20 μl of matrigel and cultured in neural expansion medium (medium C + D) for 3 days in 6-well low attachment plates for organoid formation. In the final stage, the organoids were transferred to 10-cm dishes with neural culture medium (medium E), and moved to an orbital shaker for further culture. Medium E was replaced with neuronal maturation medium after 4 days which was composed of DMEM/F12 + Neuro (in Medium (1:1) supplemented with N2, B27, BDNF (20 ng/ml), GDNF (20 ng/ml), ascorbic acid (200 μM), and dbcAMP (100 mM) (Sigma-Aldrich). Cerebral organoids were
collected at different time points for immunostaining and other biochemical analysis. We differentiated 20 lines of iPSCs into cerebral organoids within a close time framework, and generated a comprehensive list of all RNA samples for our experiments.

Immunostaining. At pre-defined time points, cerebral organoids were fixed in 4% paraformaldehyde for 30 min and washed with PBS three times. After fixation, organoids were dehydrated with 30% sucrose in PBS at 4 °C. Organoids were then embedded with optical cutting temperature (OCT) compound (VWR) and frozen on dry ice. Frozen tissue was sectioned at 30 μm using a cryostat and collected on ultra-frosted glass microscope slides. Sections were stored at −20 °C. For immunostaining, sections were permeabilized in 0.1% Triton X-100 and blocked with blocking buffer containing 4% normal donkey serum, 2% BSA, and 1% PBS. Sections were then incubated with primary antibodies in blocking buffer overnight at 4 °C. The information of primary antibodies and their dilutions used in this study are as follows: Cleaved caspase-3 (Cell Signaling Technology, 9662, 1:2000), Cleaved PARP (Bio-Rad, 2367006, 1:500) for 1 h at 4 °C. Supernatants (soluble fraction) were collected and the pellet was re-suspended in 50 μl of 70% formic acid (FA), sonicated, and neutralized with 2.5 M Tris buffer (pH 8.5) (insoluble fraction). Total protein concentration in the soluble fraction was determined using a Pierce BCA Protein Assay Kit.

Tissue processing. Cerebral organoids were harvested at pre-defined time points and lysed with RIPA Lysis and Extraction Buffer supplemented with Protease and Phosphatase Inhibitor Cocktails for Cell Lysis (Roche). Lysed samples were sonicated and incubated for 60 min on ice. Samples were centrifuged in an ultra-centrifuge (Beckman Coulter) at 100,000 × g for 1 h at 4 °C. Supernatant (soluble fraction) was collected and the pellet was resuspended in 50 μl of 70% formic acid (FA), sonicated, and neutralized with 2.5 M Tris buffer (pH 8.5) (insoluble fraction). Total protein concentration in the soluble fraction was determined using a Pierce BCA Protein Assay Kit.

Western blotting. Samples in soluble fraction were loaded into a 4–20% sodium dodecyl sulfate-polyacrylamide gel (Bio-Rad), and transferred to PVDF Immobilon FL membranes (Millipore). After blocking with 5% non-fat milk in PBS, membranes were blotted overnight with primary antibodies in 5% non-fat milk containing 0.1% Tween-20, and then probed with Li-COR IRDye secondary antibodies or horseradish peroxidase-conjugated secondary antibody, detected by SuperSignal West Femto Chemiluminescent Substrate (Pierce). The information of primary antibodies and their dilutions used in this study are as follows: Cleaved caspase-3 (Cell Signaling Technology, 9662, 1:1000), Immunoglobulin G (Bio-Rad, 61126, 1:1000), and α-tubulin (Abcam, ab6286, 1:300). The reaction was stopped and read at 450 nm with a microplate reader (Biotek). Plates were coated overnight with an apoE antibody (WUE4) in carbonate buffer at 4 °C. Because oligodendrocytes compose negligible proportion of the cell population in all but one sample, oligodendrocyte was not considered in the following analysis.

RNA-seq, quality control, and normalization. Twenty mRNA samples were sequenced at Mayo Clinic using Illumina HiSeq 4000. Reads were mapped to the human reference genome hg38. Raw gene read counts and sequencing quality control were generated using the Mayo Clinic RNA-seq analytic pipeline: MAP-Rseq Version 3.09.2. Conditional quantile normalization (CQN) was performed on the raw gene counts to correct for gene length differences, GC bias, global technical variance, and to obtain similar quantities and log2-transformed values across samples2. Normalized expression values, genes with an average of log2 RPKM ≥ 3 in at least one group were considered expressed. Using this selection threshold, 18,291 genes were included in the downstream analysis.

Differential gene expression and pathway analysis. Differential gene expression analyses were performed using the Partek Genomics Suite (Partek Inc., St. Louis, MO). Gene expression between AD patient- and healthy subject-derived cerebral organoids, between APOE genotypes, and the interaction between AD disease status and APOE genotype were calculated using analyses of variance models (ANOVA). Pathway analysis of the Geneset overview was performed using MetaCore and IPA. To correct for false discovery rate, p < 0.05 and fold change ≥1.5 were used for performing MetaCore pathway analysis (© MetaCore (Feb 2020) of Clarivate Analytics. All rights reserved).

DEGs cell-type distribution analysis of RNA-seq data. Cell proportion was estimated using marker genes described in BRETIGEA6, a published reference dataset. For human apoE ELISA, 96-well plates were coated overnight with an apoE antibody (WUE4) in carbonate buffer at 4 °C. The plates were blocked with 1% milk in PBS, and washed three times with PBS. Recombinant apoE3 and apoE4 (Fitzgerald) were used as standards for the ELISA. Samples were diluted and incubated at 4 °C overnight. The plates were washed and incubated with biotin-conjugated goat anti-apoE antibody (Meridian Life Science) for 2 h at room temperature. After incubation with Horseradish Peroxidase Avidin D (Vector Laboratories) for 90 min at room temperature, the plate was developed by adding tetramethylbenzidine Super Slow substrate (Sigma). The reaction was stopped and read at 450 nm with a microplate reader (Biotek). Results were normalized to total protein concentration of the cell lysate.

RT-qPCR. RNA was extracted via the Trizol/chloroform method, followed by DNase and cleanup using the RNase-Free DNase Set and the RNeasy Mini Kit (Qiagen). The ddPCR method was used to determine the relative expression of each gene with ACTB gene β-actin as a reference. The primers used to amplify target genes by RT-qPCR are as follows: ACTB F (5′-CTGGAACACCCACCTCTTACAAGT-3′) and R (5′-AATTGACTGACCAGATGCTATTTGTA-3′), PDMF F (5′-CAGGTTGCGACCCTGAAGAAAAT-3′) and R (5′-CACTCTGGAATCTGCTGCGAGG-3′), ACTB F (5′-GTTTGTGGAATCTGCTGCGAGG-3′) and R (5′-GGAATGACCCGTAGGAACTAAA-3′) and R (5′-GATGACCCGCTGCTC-3′), SATB2 F (5′-CCCTAGGCAATAGAATGCG-3′) and R (5′-CCAGAGATG GAGGTT-3′). The reaction was stopped and read at 450 nm with a microplate reader (Biotek). Real-time qPCR was conducted with Universal SYBR Green SuperMix (Bio-Rad) using an iCycler thermocycler (Bio-Rad). The ΔΔCt method was used to determine the relative expression of each gene with ACTB gene β-actin as a reference. The primers used to amplify target genes by RT-qPCR are as follows: ACTB F (5′-CTGGAACACCCACCTCTTACAAGT-3′) and R (5′-AATTGACTGACCAGATGCTATTTGTA-3′), PDMF F (5′-CAGGTTGCGACCCTGAAGAAAAT-3′) and R (5′-CACTCTGGAATCTGCTGCGAGG-3′), ACTB F (5′-GTTTGTGGAATCTGCTGCGAGG-3′) and R (5′-GGAATGACCCGTAGGAACTAAA-3′) and R (5′-GATGACCCGCTGCTC-3′), CLPI F (5′-CAGCTTTCTGAGGGCCAGCAATG-3′) and R (5′-CCCTGAGCAGATCTCGCCACTC-3′), CACG F (5′-CCCTGAGCAGATCTCGCCACTC-3′) and R (5′-CGTCCAGATCTCGCCACTC-3′), NR2F6 F (5′-TTTGAGTGTGCTACTCGTGG-3′) and R (5′-ATGTTCTTACACGCTGGCTACCGTGC-3′). The reaction was stopped and read at 450 nm with a microplate reader (Biotek).
(WGCNA) using the log2-transformed, CQN-normalized gene expression values. We used the soft power of 16, hybrid dynamic tree cutting, a minimum module size of 50 genes, and a minimum height for merging modules at 0.3 to build a signed hybrid co-expression networks. Each gene module was summarized by the first principal component of the scaled module expression profiles (module eigengene). Each module was assigned a unique color identifier, and genes that did not fulfill these criteria for any of the modules were assigned to the gray module. To assess the correlation of modules to AD disease status and APOE genotype, we defined healthy subject-derived cerebral organoids as 0 and AD patient-derived cerebral organoids as 1; and defined the APOE3 genotype as 0, and the APOE4 genotype as 1. Modules were annotated using R package anRichment (https://horryholland.genetics.ucla.edu/html/CoexpressionNetwork/GeneAnnotation). The connection among the top hub genes in the yellow module was visualized using VisANT.

Statistical analyses and reproducibility. ANCOVA for APOE4, AD status, sex, sampling age and source of iPSCs was performed to determine the interaction effect between APOE4 and AD status on each continuous variable for western blotting, ELISA, or RT-qPCR from four groups of cerebral organoids, followed by Tukey-Kramer tests to compare between selected two groups using IMP software version 15.0. For batch difference and isogenic cerebral organoids comparison, the Mann–Whitney U test was performed to determine the significance using GraphPad Prism version 8.0. Spearman correlation analysis was used to analyze the association between APOE4 and GraphPad. Experiments were repeated in two independent differentiation batches. Data were presented as mean ± SEM. A p value of <0.05 was considered statistically significant.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
Full scans of the gels and blots are available in Source data file. All relevant data are available from the corresponding author upon reasonable request. All the other data supporting the findings of this study are available within the article and its supplementary information files and from the corresponding author upon reasonable request. A reporting summary for this article is available as a Supplementary Information file. The RNA-seq data are available via the AD Knowledge Portal (https://adknowledgeportal.synapse.org). The AD Knowledge Portal is a platform for accessing data, analyses, and tools generated by the Accelerating Medicines Partnership (AMP-AD) Target Discovery Program and other National Institute on Aging (NIA)-supported programs to enable open-science practices and accelerate translational learning. The data, analyses, and tools are shared early in the research cycle without a publication embargo on secondary use. Data are available for general research use according to the following requirements for data access and data attribution (https://adknowledgeportal.synapse.org/DataAccessInstructions). For access to content described in this manuscript see [https://doi.org/10.7303/syn22307008]. Source data are provided with this paper.

Received: 27 December 2019; Accepted: 2 October 2020.

Published online: 02 November 2020.

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Acknowledgements

We are grateful to Dr. Yadong Huang and Chenghong Wang for providing the APOE isogenic lines. This work was partially supported by Mayo Clinic Center for Regenerative Medicine, Neuroregeneration Lab. Lab Funding. This work was supported by NIH grants RFAAG051504, RFAAG046205, RFAAG057181, R01AG066395, P01NS074969, P30AG062677 (to G.B.), Younkin Fellowship and Alzheimer’s Association Research Fellowship 2018-AARF-592302 (to J.Z.), and R01AG061796 (to N.E.-T.). Dr. Zbigniew K. Wszolek is partially supported by the Mayo Clinic Center for Regenerative Medicine, the gifts from The Sol Goldman Charitable Trust, the Donald G. and Jodi P. Heeringa Family, the Haworth Family Professorship in Neurodegenerative Diseases fund, and by Albertson Parkinson’s Research Foundation.

Author contributions

J.Z., C.L., F.S., N.E.T., T.K., and G.B. conceived and designed the project, and wrote the paper. M.D., L.J., S.G.Y., N.R.G.R., Z.W., and D.B. helped with collecting human skin biopsies and generating iPSC lines. J.Z., Y.F., Y.Y., W.L., Y.M., K.C., L.J., and T.N. executed the experiments and analyzed the data. Y.R., X.W., Y.C., and Y.A. performed analysis for RNA-sequencing data.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information is available for this paper at https://doi.org/10.1038/s41467-020-19264-0.

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Peer review information Nature Communications thanks Tracy Young-Pearse and the other anonymous reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.

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