Chimeric cerebral organoids reveal the essentials of neuronal and astrocytic APOE4 for Alzheimer’s tau pathology

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The apolipoprotein E4 (APOE4) genotype is one of the strongest genetic risk factors for Alzheimer’s disease (AD), and is generally believed to cause widespread pathological alterations in various types of brain cells. Here, we developed a novel engineering method of creating the chimeric human cerebral organoids (chCOs) to assess the differential roles of APOE4 in neurons and astrocytes. First, the astrogenic factors NFIB and SOX9 were introduced into induced pluripotent stem cells (iPSCs) to accelerate the induction of astrocytes. Then the above induced iPSCs were mixed and cocultured with noninfected iPSCs under the standard cultivating condition of cerebral organoids. As anticipated, the functional astrocytes were detected as early as 45 days, and it helped more neurons matured in chCOs in comparison of the control human cerebral organoids (hCOs). More interestingly, this method enabled us to generate chCOs containing neurons and astrocytes with different genotypes, namely APOE3 or APOE4. Then, it was found in chCOs that astrocytic APOE4 already significantly promoted lipid droplet formation and cholesterol accumulation in neurons while both astrocytic and neuronal APOE4 contributed to the maximum effect. Most notably, we observed that the co-occurrence of astrocytic and neuronal APOE4 were required to elevate neuronal phosphorylated tau levels in chCOs while Aβ levels were increased in chCOs with neuronal APOE4. Altogether, our results not only revealed the essence of both neuronal and astrocytic APOE4 for tau pathology, but also suggested chCOs as a valuable pathological model for AD research and drug discovery.

Signal Transduction and Targeted Therapy (2022) 7:176 ; https://doi.org/10.1038/s41392-022-01006-x

INTRODUCTION
Alzheimer’s disease (AD) is the most common kind of dementia that is characterized by deteriorating memory and other cognitive functions.1 In AD, the aggregation of amyloid-β (Aβ) and phosphorothioate-associated protein tau leads to the formation of senile plaques and neurofibrillary tangles (NFT), which are considered as two major neuropathological hallmarks of AD.2 These pathogenic aggregates of Aβ and tau induce the destruction of synapses, thus cause memory and cognitive impairment during the early phase of AD pathogenesis.3 The majority (>95%) of AD cases are late-onset sporadic AD (LO-SAD) and <5% are early-onset familial AD (EO-FAD).4 Accumulating evidences indicate that EO-FAD is associated with various mutations in genes linked to Aβ biogenesis, such as the amyloid precursor protein (APP) and the presenilins (PS1 and PS2), suggesting abnormal Aβ metabolism in EO-FAD.5 Compared with EO-FAD, the etiology of LO-SAD is likely to be more complicated and much less clear. A number of genetic factors are reported to be associated with LO-SAD, including but not limited to apolipoprotein E (APOE), clusterin (CLU), phosphatidylinositol binding clathrin assembly protein (PICALM), triggering receptor expressed on myeloid cells 2 (TREM2), complement component 3b/4b receptor 1 (CR1), ATP-binding cassette transporter member 7 (ABCA7), bridging integrator 1 (BIN1), and sialic acid binding Ig-like lectin 3 (CD33). Among them, APOE is the highest ranked susceptibility gene for LO-SAD.6

APOE gene is strongly associated with the onset of Alzheimer’s disease. There are three polymorphic alleles of APOE, namely APOE2, APOE3, and APOE4. Compared with the APOE3 homozygotes, people carrying two copies of APOE4 have a roughly 14-fold increase in the risk of developing AD, while APOE2 allele is protective.7 ApoE is an apolipoprotein, which play critical roles in cholesterol transport and lipid homeostasis.8 In the brain, ApoE is mainly synthesized and secreted by astrocytes, but is also expressed by other brain cell types including neurons.8,9 Studies demonstrate lipid metabolic defects and mitochondrial dysfunction in APOE4 astrocytes, which has a positive correlation of AD pathogenesis.10,11 Additionally, astrocytic-specific removal of APOE4 reduced tau-mediated neurodegeneration and phosphorylated tau (p-tau) pathology.12 Similarly, APOE4 neurons also display multiple AD-linked molecular and cellular alterations, including increased levels of p-tau, diminished synaptic plasticity, and cholesterol dysregulation.13,14 Although the role of APOE4 on different brain cell types has been studied separately, the overall effects of different APOE4-carrying cell types on AD pathogenesis remain largely unexplored. A recent study on mouse models with different humanized APOE alleles has revealed that APOE4 astrocytes cause disease-associated changes on various brain

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Received: 3 November 2021 Revised: 29 March 2022 Accepted: 14 April 2022
Published online: 13 June 2022

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cells including neurons, oligodendrocytes, and microglia.\textsuperscript{15} Be aware of the anatomical and physiological differences between humans and animals, it is necessary to evaluate the effects of different APOE4 cells in human cell-based disease models in parallel with animal studies.

Human cerebral organoids (hCOs) recapitulate architectural and functional features of human brain and provide good in vitro models for studying neurological disorders. Astrocytes play important roles in modulating neurogenesis, synapse formation and metabolic homeostasis.\textsuperscript{17} Important roles in modulating neurogenesis, synapse formation and astrocytes, respectively. We showed that APOE4 astrocytes elevated neuronal lipid droplet formation and cholesterol accumulation. Notably, we also found that the co-occurrence of APOE4 astrocytes and neurons were required for the elevation of neuronal p-tau levels in chCOs, indicating the essentials of both neuronal and astrocytic APOE4 for the tau pathology.

RESULTS

Accelerated induction of astrocytes in iPSC-derived chCOs

We hypothesized that expression of the astrogenic factors induce astrocyte differentiation in human cerebral organoids. We first introduced the astrogenic factors NFIB and SOX9 into induced pluripotent stem cells (iPSCs) to accelerate the induction of astrocytes. The immunostaining of NFIB and SOX9 showed that nearly all cells were transduced, although no selection procedure is applied (Supplementary Fig. 1a). Then the above induced iPSCs were mixed and cocultured with noninfected iPSCs under the standard condition of cerebral organoids to generate the chCOs (Fig. 1a). We next evaluated the astrocytic differentiation in these chCOs and control hCOs. By immunostaining, we found that GFAP\textsuperscript{+} astrocytes already appeared in chCOs at day 20 (D20) while very few GFAP\textsuperscript{+} cells were detected in control hCOs (Fig. 1b, c). By extending the time of culturing to D45, we observed that the ratio of GFAP\textsuperscript{+} astrocytes in chCOs further increased to 10–15%, and these cells got longer in morphology (Fig. 1d, e). In addition, we examined another astrocyte-specific marker S100B by immunostaining, and found that S100B\textsuperscript{+} cells also significantly increased in chCOs compared with control hCOs (Fig. 1f, g). Efficient induction of CD44 (cell surface marker for astrocytes) in chCOs was also confirmed by flow cytometry analysis (Fig. 1h).

We also purified astrocyte-lineage cells from chCOs on day 45 by immunopanning and found that these isolated cells expressed GFAP as well as astrocyte marker S100B (Fig. 1i). In agreement with immunostaining and FACs results, qRT-PCR analysis revealed a robust increase in expression of astrocyte markers (GFAP, S100B and GLAST) and mature astrocyte genes (AGXT2L1, RANBP2L5, IGFBP7, GLT1 and ALDOC) in chCOs on day 45 (Supplementary Fig. 1b, j).\textsuperscript{20,21} However, no significant alteration in fetal astrocyte genes was observed, since the increased number of all astrocytes in chCOs may possibly result in comparable number of fetal astrocytes in chCOs and hCOs even though these fetal astrocytes comprise a smaller percentage of total astrocytes in chCOs (Fig. 1j). In addition, to verify the efficiency of astrocyte generation, we co-stained of SOX9 with GFAP and MAP2, and found that nearly 90% SOX9\textsuperscript{+} cells were GFAP\textsuperscript{+}, while only ~8% SOX9\textsuperscript{+} cells were MAP2\textsuperscript{+}, suggesting that most transduced iPSCs differentiated to astrocytes (Supplementary Fig. 1c). Collectively, our results indicated that astrogliogenesis was accelerated in chCOs.

Enhanced neurogenesis and neuronal maturation in chCOs

Astrocytes maintain brain homeostasis by clearing neurotransmitters such as glutamate. To determine whether astrocytes from chCOs enable neurotransmitter clearing, we measured glutamate uptake by astrocytes purified from D45 chCOs. These astrocytes took up glutamate at a rate of about 0.14 μM/min, which was largely reduced by DL-threo-β-benzylxoxaspartic acid (TBOA, a competitive blocker of excitatory amino acid transporters) treatment (Fig. 2a), suggesting astrocytes from chCOs enabled neurotransmitter clearing. Astrocytes contributes to the process of neurogenesis.\textsuperscript{22} To analyze the levels of neurogenesis in chCOs and control hCOs, we labeled the dividing neural progenitor cells by 5-ethyl-2'-deoxyuridine (EdU) and analyzed their neuronal differentiation 5 days after EdU labeling by stained the marker of newborn neurons doublecortin (DCX). We found that the density of newborn neurons, marked as EdU/DCX-double-positive cells, was increased significantly in chCOs, suggesting that the neurogenesis process was promoted in chCOs (Fig. 2b, c). Consistently, we also observed a marked increase in the number of MAP2\textsuperscript{+} neurons in chCO on D30 and D45 (Fig. 2d, e), suggesting elevated neurogenesis in these organoids. We next assessed whether the synapse formation was enhanced in chCOs by immunostaining for the pre- and postsynaptic proteins Synapsin I (SYN) and PSD95, the results showed that chCOs exhibited more synaptophysin and PSD95 puncta than control hCOs, indicating the increased number of astrocytes enhanced synapse formation in chCOs (Fig. 2f, g). Besides, less apoptotic cells were observed in chCOs by immunostaining with cleaved caspase-3 antibody, suggesting improved cell viability of chCOs (Fig. 2h, i). To further compare the difference between chCOs and hCOs in neuronal maturation, we performed electrophysiological recordings to characterize neuronal activity. Electrophysiological analysis with whole-cell recording in slices from D60 chCOs showed that 11 of 25 cells produced multiple high-amplitude action potentials (APs). In contrast, only 3 of 25 cells from D60 control hCOs produced low-amplitude APs, which is indicative of more mature neurons in chCOs (Fig. 2j, k). Since it was reported that accelerated neuronal maturation increased AD-associated Aβ secretion, we measured the level of secreted Aβ in chCO and hCO cultures by enzyme-linked immunosorbent assay (ELISA).\textsuperscript{24} In consistent with our results, we found that the Aβ level was higher in D30 organoid cultures compared with their D20 counterparts. Notably, there was a larger increase of Aβ level in chCO supernatant while extending the time of culturing from D20 to D30, supporting the accelerated neuronal maturation in chCOs (Fig. 2l). In conclusion, these results indicated enhanced neurogenesis and neuronal maturation in chCOs.

APOE4 chCOs exhibit elevated levels of lipid droplets, Aβ and p-tau than APOE4 hCOs

We next determined whether increased proportion of astrocytes in chCOs affected AD modeling. First, APOE4 induced pluripotent stem cells (iPSCs) were generated from their parental APOE3 cells by CRISPR/Cas9 gene editing. Then the APOE4 iPSCs and their APOE3 parental cells were used to generate hCOs or chCOs with APOE3 or APOE4 genotype. We analyzed lipid droplet content in these organoids by Nile red staining and found that APOE4 increased lipid droplet levels in both hCOs and chCOs compared with their APOE3 counterparts, and APOE4 chCOs showed more lipid droplets than APOE4 hCOs (Supplementary Fig. 2a, b). Similar alterations were also observed in Aβ and p-tau levels (Supplementary Fig. 2c, d). Taken together, these results indicated that chCOs exhibited elevated level of disease-associated pathologies than conventional hCOs while modeling AD.
Astrocytic and neuronal APOE4 elevate cholesterol and lipid droplets in neurons

Our results showed that astrocytes barely appeared in D45 hCOs, so that most astrocytes in chCOs originated from NFIB/SOX9 virus-infected iPSCs. To address the distinct role of neuronal and astrocytic APOE4, we generated four kinds of chCOs with different APOE genotypes in neurons and astrocytes by mixing NFIB/SOX9 virus-infected and noninfected APOE3 or APOE4 iPSCs (Fig. 3a). The percentage of MAP2+ and GFAP+ cell was similar between differentiation kinds of chCOs, indicating that neuron/astrocyte...
Differentiation in these organoids was not affected by APOE genotype (Supplementary Fig. 3a, b). We also verified APOE3 and APOE4 expression in chCOs or neurons purified from them by real-time PCR with published APOE4 and APOE3-specific primers (Supplementary Fig. 3c, d).\textsuperscript{25,26} The identities of the neurons in chCOs were analyzed by immunostaining with vGLUT1 (glutamatergic), GAD1 (GABAergic) and TH (dopaminergic). The results showed that there were more vGLUT1-positive cells than GAD1-positive or TH-positive cells in chCOs (Supplementary Fig. 3e, f). The neuronal cholesterol levels in these chCOs were analyzed by filipin staining. We noticed that astrocytic or neuronal APOE4 alone was able to elevate the cholesterol levels in neurons, while both of them contributed to the maximum effect, indicating that astrocytic and neuronal ApoE is involved in the regulation of neuronal cholesterol homeostasis (Fig. 3b, c). We also detected neuronal lipid droplet content in the chCOs and similar results were observed (Fig. 3d, e). Overall, these results showed that both astrocytic and neuronal APOE4 exacerbated the lipid burden in neurons.

Astrocytic and neuronal APOE4 are both indispensable for the elevation of neuronal p-tau levels in chCOs

Next, we investigated the Aβ and tau pathology in these four kinds of chCOs. ELISA analyses of different Aβ species in the D45 chCO culture supernatants revealed that compared with chCOs containing APOE3 neurons and APOE3 astrocytes (NE3 + AE3), the levels of Aβ40 and Aβ42 were significantly increased in chCOs containing APOE4 neurons (NE4 + AE3 and NE4 + AE4). However, no obvious elevation of Aβ levels was observed in chCOs containing APOE3 neurons and APOE4 astrocytes (NE3 + AE4), suggesting that APOE4 neurons were required for alteration of Aβ levels (Fig. 4a, b, c). In addition, neither astrocytic nor neuronal APOE4 affected the protein levels of immature and mature APP, indicating that they exerted little effect on expression regulation of APP (Supplementary Fig. 4a, b, c). Similarly, no significant changes in the levels of Nicastrin (NCT), ADAM Metallopeptiase Domain 10 (ADAM10) and beta-secretases1 (BACE1) in chCOs were observed, suggesting that secretases were not altered in these chCOs (Supplementary Fig. 4d, e, f, g). We also examine the expression of Aβ degrading enzymes in four kinds of chCOs and found that MMP3 and IDE expression were reduced in NE4 + AE3 and NE4 + AE4 chCOs, suggesting that Aβ cleaving mechanism may be impaired in these chCOs (Fig. 4d and Supplementary Fig. 4h). To examine the tau pathology in these chCOs, we co-stained them with anti-p-tau (AT8) and anti-MAP2 antibodies and found that there was no significant difference in neuronal p-tau levels between NE3 + AE3, NE3 + AE4 and NE4 + AE3 chCOs. However, the neuronal p-tau levels were significantly increased in NE4 + AE4 chCOs compared with the other three kinds of chCOs, suggesting that astrocytic and neuronal APOE4 were both required for elevated neuronal tau phosphorylation (Fig. 4e, f, g). Similar results were obtained by immunostaining with another p-tau antibody (PHF1) (Fig. 4h, i, j). The change in p-tau levels was also verified by western blotting (Fig. 4k, l). It is reported that excess cholesterol is related to Alzheimer’s Aβ and tauopathies.\textsuperscript{27,28} Thus, we hypothesized that cholesterol may mediate the effect of APOE on tau pathology in chCOs. To test this, we treated chCOs with two inhibitors of cholesterol synthesis: atorvastatin and simvastatin. The results demonstrate that atorvastatin and simvastatin both significantly reduce the levels of p-tau in NE4 + AE4 chCOs, indicating that APOE4 may induce tau pathology by enhancing cholesterol level (Fig. 4m, n). Besides, we also performed functional studies in these chCOs including electrophysiological recordings and synapse formation analysis. The SYN and PSD95 staining were similar between different kinds of chCOs, and the frequency of action potentials between these four kinds of chCOs was also not significant (Fig. 4o, p and Supplementary Fig. 4i, j). To validate the findings from iPSC-derived chCOs, we also examined the Aβ and tau levels in H9-ESC-derived chCOs and similar results were obtained (Fig. S4k, i, m, n, o). Altogether, these results indicated that APOE4 neurons were required for alteration of Aβ levels, while the elevation of neuronal p-tau required both astrocytic and neuronal APOE4.

**DISCUSSION**

Three-dimensional human cerebral organoid culture provides a novel system to study the pathogenesis of AD and to evaluate potential therapeutic interventions. In order to better recapitulate the features of neurological diseases including AD, efforts have been made to improve hCO generation. Astrocytes are critically involved in the pathogenesis of AD, however their differentiation and maturation in hCOs is slow compared with neurons.\textsuperscript{29,30} In the study, we developed a protocol to boost astroglial differentiation in part of chCOs by virus-mediated expression of two gliogenic transcription factors NFIB and SOX9. The virus-mediated expression of gliogenic transcription factors NFIB and SOX9 has been applied for rapid and reproducible acquisition of astrocytes in two-dimensional (2D) culture.\textsuperscript{29} However, they are not good representation of the complicated in vivo environment. ChCOs allow more interactions between different neural cells and provide better spatial architectures, but they also have limitations, such as batch-to-batch variability and difficulty for downstream analysis. Astrocytes are known to play a critical role in the formation of neuronal networks.\textsuperscript{31,32} Our data demonstrates accelerated generation of functional astrocytes as well as more immature and mature neurons in chCOs, suggesting that the increased number of astrocytes may promote the neuronal differentiation and maturation in chCOs. The findings indicate that chCOs could serve as a useful platform to study neurological diseases involving both neurons and astrocytes. Cakir et al.\textsuperscript{33} reported that ectopic expression of ETV2 drove vascularization in hCOs. Our study together with their findings suggest that forced expression of lineage-specific differentiating factors is a promising approach to improve the organoid generation for complex organs such as brain, heart and liver. Microglia-astrocyte crosstalk and microglia-neuron crosstalk have been reported to be critical in the pathology of AD by modulating clearance of Aβ/tau and neuronal survival.\textsuperscript{34,35} Since microglia originate from the mesoderm lineage and other CNS cells are derived from neuroectodermal
progenitors, most cerebral organoids generated by classical protocols lack the integration of microglia. Ormel et al. reported that microglia developed in cerebral organoids by in the absence of SMAD inhibition. Following their findings, we will further optimize the current protocol to develop microglia in chCOs. Moreover, with these chimeric organoids, we are able to assess the intricate and cell type-dependent effects of various disease risk genes in a more physiologically relevant context. 

APOE4 has a profound impact on dysregulation of lipid metabolism associated with AD pathogenesis. We observed that...
the levels of neuronal lipid droplets and cholesterol were higher in chCoCs with APOE4 astrocytes than those with APOE3 astrocytes, suggesting that APOE4 astrocytes were associated with dysregulated lipid and cholesterol in neurons. Consistent with our findings in chCoCs, a recent study has also reported diminished capacity of APOE4 astrocytes in eliminating neuronal lipids. 14, 15

Studies have reported that APOE4 worsens tau pathology in both animal and human cell models. 13,36 Selective removal of astrocytic APOE4 in the P301S tau/ apoE4 mice reduces cortical and hippocampal phosphorylated tau levels, suggesting an important role of astrocytic APOE4 in tau pathology. 15 Moreover, co-culturing P301S tau-expressing-neurons with APOE4 astrocytes also significantly elevates neuronal tau phosphorylation and cell death.37 Consistently, although the neurons didn’t express pathological tau mutants, we were able to observe increased neuronal p-tau levels in chCoCs containing both APOE4 neurons and astrocytes. However, the raise in neuronal levels of p-tau was not detected in other chCoCs. These results suggested that neuronal and astrocytic APOE4 were both critical for the increased phosphorylation of tau. Notably, a recent study demonstrates that neuronal tau can be transported to astrocytes in vivo.15 In addition, ApoE is observed to interact with tau in an allele-dependent manner.17 These findings together with our data support the idea that the phosphorylation process of tau in neurons may be accelerated by APOE4 astrocytes, and the neuron-astrocyte transportation of p-tau is possibly impaired by neuronal APOE4. Besides, APOE2 is reported to exert protective effects against AD, such as reducing APP transcription and Aβ secretion.40,41 However, it is also associated with increased tau pathology, suggesting the intricate role of ApoE2 in regulating tau and Aβ.42 It will be very interesting to verify the effect of APOE2 neurons or astrocytes on AD-associated pathologies in chCoCs. Moreover, in consistent with other studies in 2D culture, we observed that the ptau level was also significantly reduced in in NE4 + AE4 chCoCs after inhibiting cholesterol synthesis by atorvastatin and simvastatin, suggesting targeting cholesterol metabolism is a valuable strategy in developing therapeutic agents for AD patients carrying APOE4.39 Wang et al.37 recently report that cholesterol is synthesized in astrocytes and transferred to neurons in apoE lipoprotein particles. These cholesterol intergates into the neuronal membranes and forms lipid rafts to enhancing the interation of APP with β- and γ-secretase, thus promotes Aβ production. In addition to their findings, our results showed that neither astrocytic nor neuronal APOE4 affected the expression of APP and secreazetes. Meanwhile, expression of some Aβ degrading enzymes in neurons were reduced in NE4 + AE3 and NE4 + AE4 chCoCs, suggesting that Aβ clearing mechanism may be impaired by APOE4. Taken together, these findings indicates that apoE may paticipate in multiple steps of Aβ metabolism, and further investigation on the underlying molecular mechanism is intriguing. Given the essential role of APOE4 in the pathogenesis of AD, our findings not only provide a deeper understanding between different APOE4 cells and tauopathy, but also help to develop potential therapeutic strategies for patients who carry the APOE4 allele.

MATERIALS AND METHODS

hPSC culture

The human induced PSC line were generated from a 66-year-old healthy female by iXCells Biotechnologies (catalog no. 30HU-002) and cultured on Matrigel (BD Biosciences) coated dishes in mTeSR1 medium (Stem Cell Technologies). Cultures were passaged every 5–7 days with Gentle Cell Dissociation Reagent (Stem Cell Technologies). The APOE3 and APOE4 hESCs were kindly provided by Dr. Ru Zhang, Tongji University. All cells were confirmed negative for mycoplasma.

Generation of chimeric human cerebral organoids (chCoCs) with NFI B and SOX9 induction

STEMdiff™ Cerebral Organoid Kit (Stem Cell Technologies) was applied for generation of chCoCs. The hPSCS or H9-ESCs were dissociated with Gentle Cell Dissociation Reagent (Stem Cell Technologies), infected with FUGW-NFIB and FUGW-SOX9 lentivirus, mixed with equal amount of uninfected hPSCS (4500 infected cells and 4500 uninfected cells), and plated into each well of a 96-well round-bottom ultra-low-attachment plate containing EB Formation Medium to generate EBs. On day 5, EBs were transferred into a 24-well ultra-low attachment plate containing Induction Medium and cultured for an additional 2 days. After embedded in Matrigel, these organoids transferred to Expansion Medium, and cultured for 3 days. On day 10, they were transferred to Maturation Medium and placed on an orbital shaker for extended periods of culture.

Immunofluorescence staining and quantification

Remaining Matrigel was removed by washing with phosphate-buffered saline (PBS) before fixation. Then organoids were fixed with 4% PFA in PBS for 30 min, washed three time with PBS, and submerged in 30% sucrose for 1 – 2 days until samples sank. After embedded in OCT, organoids were cryosectioned at 20 µm. Sections were incubated overnight at 4 °C with primary antibodies diluted in PBS containing 0.3% Triton X-100 and 3% donkey serum. After washing with PBS for three times; sections were incubated with secondary antibodies at room temperature for 1 h. The information of primary antibodies used were as follows: Gfap (rabbit, DAKO Z033401, 1:1000),MAP2 (rabbit, Millipore AB5622, 1:500), S100B (rabbit, Beyotime AF1945, 1:200), DCX (goat, Santa Cruz sc-8066, 1:500), MN1020, 1:500), PHF1 (rabbit, Abcam ab184951, 1:500), hNuclei (mouse, Millipore AB1281, 1:200), NFlb (rabbit, Abcam ab186738, 1:100), SOX9 (mouse, Abcam ab76997, 1:100). Secondary antibodies were used as follows: donkey anti-rabbit Cy3 (Jackson ImmunoResearch 711-165-152, 1:1000), donkey anti-rabbit Alexa Fluor 488 (Thermo Fisher Scientific, A11001, 1:500). Immunofluorescence signal was analyzed by ImageJ.
Fig. 3  Astrocytic and neuronal APOE4 elevated cholesterol and lipid droplet levels in neurons. a Experiment procedure for generation of chCOs with neurons and astrocytes carrying APOE3 or APOE4 allele. b Representative images of different chCOs on D45 stained with filipin, MAP2, and human nuclei. Scale bar: 50 µm. c Quantification of mean filipin fluorescence density in the neurons. Data represent the mean ± s.e.m. (n = 6–8 organoids, from three different batches; ***p < 0.001). d Representative images of chCOs and control hCOs on D45 stained with LipidTox and MAP2. Scale bar: 30 µm. e Quantification of the lipid droplet density in the neurons. Data represent the mean ± s.e.m. (n = 8–9 organoids, from four different batches; *p < 0.05, ***p < 0.001)
488 (Molecular probes A21206, 1:1000), donkey anti-mouse cy3 (Molecular Probes 715-165-150, 1:1000), donkey anti-goat cy3 (Jackson ImmunoResearch 705-165-147, 1:1000). For filipin staining, sections were washed three times with PBS and incubated with 1.5 mg/ml filipin (MCE, HY-N6716) in PBS containing 10% FBS for 2 h and rinsed three times with PBS. For lipid droplet staining, sections were washed three times with PBS and stained with LipidTox (Thermo Fisher Scientific, H34477) according to the manufacturer’s protocols. EdU staining were performed with 0.05 mg/ml filipin (MCE, HY-N6716) in PBS containing 10% FBS for 2 h and rinsed three times with PBS. For lipid droplet staining, sections were washed three times with PBS and stained with LipidTox (Thermo Fisher Scientific, H34477) according to the manufacturer’s protocols. EdU staining were performed with
Fig. 4 The co-occurrence of APOE4 astrocytes and neurons are required for the elevation of neuronal p-tau levels. a A404 levels in the supernatants of different chCOs on D45. Data represent the mean ± s.e.m. (n = 6 different batches; **p < 0.001). b A42 levels in the supernatants of different chCOs on D45. Data represent the mean ± s.e.m. (n = 6 different batches; ***p < 0.001). c The ratio of A42/A40 in the supernatants of different chCOs on D45. Data represent the mean ± s.e.m. (n = 6 different batches; **p < 0.01). d qPCR analysis of Aβi degrading enzymes expression in different chCOs. Data represent the mean ± s.e.m. (n = 5 different batches; **p < 0.01, ***p < 0.001). e Immunostaining of different chCOs on D45 for p-tau (AT8) and MAP2. Scale bar: 50 µm. f Quantification of the p-tau (AT8) intensity in the neurons. Data represent the mean ± s.e.m. (n = 8–9 organoids, from three different batches; ***p < 0.001). g The percentage of the p-tau (AT8)-positive area in the neurons. Data represent the mean ± s.e.m. (n = 8–9 organoids, from three different batches; **p < 0.01, ***p < 0.001). h Immunostaining of different chCOs on D45 for p-tau (PHF1) and TUJ1. Scale bar: 30 µm. i Quantification of the p-tau (PHF1) intensity in the neurons. Data represent the mean ± s.e.m. (n = 8–9 organoids, from three different batches; *p < 0.05, **p < 0.01, ***p < 0.001). j The percentage of the p-tau (PHF1)-positive area in the neurons. Data represent the mean ± s.e.m. (n = 8–9 organoids, from three different batches; **p < 0.01, ***p < 0.001). k p-tau (PHF1) levels of different chCOs on D45 analyzed by western blotting. l Quantification of p-tau (PHF1) levels normalized to total tau. Data represent the mean ± s.e.m. (n = 3 different batches), m chCOs were analyzed for p-tau (AT8) and MAP2 after 10-day-treatment of simvastatin (10 µM) and atorvastatin (10 µM) (D35–D45). Scale bar: 30 µm. n Quantification of the p-tau (AT8) intensity in the neurons of chCOs from m. Data represent the mean ± s.e.m. (n = 6 organoids, from three different batches; **p < 0.01, compared with NE4 + AE4 vehicle group). o Current clamp recordings of one cell in different chCOs on D60. p Action potential (AP) of different chCOs on D60.
(ANOVA) followed by Bonferroni test was used. A p-value less than 0.05 was considered statistically significant.

DATA AVAILABILITY
The datasets in this study are available from the corresponding author upon reasonable request.

ACKNOWLEDGEMENTS
We thank Longfei Gao, Yongfeng Yang, and the core facility for cell biology for technical assistance. We are grateful to Naimei Jing, Jianwei Jiao, and Yang Liu for helpful discussions. We thank all members of the two laboratories for sharing reagents and advice. This project was supported by “Strategic Priority Research Program” of the Chinese Academy of Sciences [XDA16010309], National Key Research and Development Programs of China [2018YFA0108003], the National Science Foundation for Young Scientists of China [B1901094], and grants from the Ministry of Science and Technology of China [2017YFA0104002].

AUTHOR CONTRIBUTIONS
S.H., Y.Y., and G.P. designed the projects and supervised this study. S.H. and Z.Z. analyzed the experimental data and drafted the manuscript. J.C. performed the experimental work and analyzed the data. Y.T. and G.P. revised the manuscript. All authors have read and approved the article.

ADDITIONAL INFORMATION
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41392-022-01006-x.

Competing interests: The authors declare no competing interests.

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