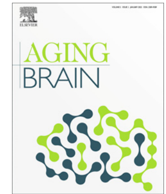
Aging Brain 2 (2022) 100055

Contents lists available at ScienceDirect

Aging Brain

journal homepage: www.elsevier.com/locate/nbas



Apolipoprotein E loss of function: Influence on murine brain markers of physiology and pathology

Heather Buchanan [1], Claire Hull [2], Maria Cacho Barraza, Mirela Delibegovic, Bettina Platt *

Institute of Medical Sciences, School of Medicine, Medical Sciences & Nutrition, Foresterhill, University of Aberdeen, Aberdeen AB25 2ZD, Scotland, UK

ARTICLE INFO

Article history:
Received 23 June 2022
Revised 11 October 2022
Accepted 13 October 2022

Keywords:
Apolipoprotein E
Synaptic
Neuroinflammation
Tau
Amyloid-β
Alzheimer's disease

ABSTRACT

The canonical role of Apolipoprotein E (ApoE) is related to lipid and cholesterol metabolism, however, additional functions of this protein have not been fully described. Given the association of ApoE with diseases such as Alzheimer's Disease (AD), it is clear that further characterisation of its roles, especially within the brain, is needed.

Therefore, using protein and gene expression analyses of neonatal and 6-month old brain tissues from an ApoE knockout mouse model, we examined ApoE's contribution to several CNS pathways, with an emphasis on those linked to AD. Early neonatal changes associated with ApoE−/− were observed, with decreased soluble phosphorylated tau (p-tau, −40 %), increased synaptophysin (+36 %) and microglial Iba1 protein levels (+25 %) vs controls. Progression of the phenotype was evident upon analysis of 6-month-old tissue, where decreased p-tau was also confirmed in the insoluble fraction, alongside reduced synaptic and increased amyloid precursor protein (APP) protein levels. An age comparison further underlined deviations from WT animals and thus the impact of ApoE loss on neuronal maturation.

Taken together, our data implicate ApoE modulation of multiple CNS roles. Loss of function is associated with alterations from birth, and include synaptic deficits, neuroinflammation, and changes to key AD pathologies, amyloid-β and tau.

© 2022 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

1. Introduction

Apolipoprotein E forms a vital component of lipoproteins and helps facilitate the transport of lipids and cholesterol around the body. In humans, the ApoE gene is polymorphic such that this protein can be expressed as three different isoforms: ApoE2, ApoE3 and ApoE4. These isoforms, produced primarily by hepatocytes in the periphery and astrocytes in the central nervous system (CNS), not only differentially affect the structure and function of ApoE physiologically, but also confer risk for various diseases [29]. For instance, ApoE2 and ApoE4 are linked to the development of dysbetalipoproteinemia and atherosclerosis, respectively. ApoE4 has been identified as the greatest genetic risk factor for sporadic Alzheimer's disease (AD); while ApoE2 has been shown to protect against AD, and those who are homozygous for ApoE4 are up to 15 times more likely to develop the disease, and at a younger age [10,11,17]. Though cardiovascular diseases may be attributed to the well documented role for ApoE in lipid metabolism and cholesterol homeostasis, it is far less clear how ApoE affects the CNS. Indeed, the striking nature of its influence on neurological disease has highlighted signifi-

* Corresponding author at: Chair in Translational Neuroscience, School of Medicine, Medical Sciences & Nutrition, University of Aberdeen, Foresterhill, Aberdeen AB25 2ZD, Scotland, UK.

E-mail address: b.platt@abdn.ac.uk (B. Platt).

[1] Present address: Department of Pathology and Cell Biology, Columbia University Medical Centre, New York, NY, USA.

[2] Present address: TauRx Pharmaceuticals Ltd, Aberdeen, Scotland, UK.

https://doi.org/10.1016/j.nbas.2022.100055
2589-9589/© 2022 The Authors. Published by Elsevier Inc.
This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

cant gaps in our understanding of functions it may hold beyond its canonical role, especially within the CNS.

Research investigating ApoE in the brain has so far largely been conducted in an AD-related context. This has benefited from the generation of ApoE transgenic models such as the targeted replacement mice, in which the endogenous ApoE gene has been replaced by a human isoform [28,63,62]. This has allowed a more thorough investigation into the pleiotropic functions of this protein and a large body of literature now exists highlighting the effect of ApoE isoforms, and in particular, ApoE4 on amyloid-β (Aβ) deposition [3,75], neuroinflammation [71,77] and synaptic function [13,56] to name a few. Importantly, this research has not only provided insight into pathophysiological mechanisms but also provided an indication of the extent to which ApoE plays a role in normal CNS physiology and in different cellular contexts. As an appealing drug target for AD (see Williams et al. [73] for a review of ApoE-related therapeutics), it is therefore of the utmost importance to answer unresolved questions regarding how this protein influences brain physiology and development.

One model which can offer further insight is the ApoE knockout (ApoE−/−) model [49,51]. This model, which is globally deficient for the ApoE protein, presents with elevated levels of plasma cholesterol and triglycerides [51] and has been used extensively as a model of atherosclerosis. Despite the apparent roles of ApoE on both sides of the BBB, there has been little convergence between the fields, and surprisingly, this loss-of-function model has not been widely explored for brain-related research, even though it provides the unique opportunity to examine the neuronal consequences of ApoE deficiency.

Few CNS-related studies have implicated ApoE−/− in BBB impairment [20], vascular dysfunction [2], and increased susceptibility to oxidative stress [59,69]. Given ApoE's role in AD, it is of particular interest that cholinergic deficits [24,60] and an enhanced neuroinflammatory profile [27,68] have been commonly observed in its absence. Significant synaptic dysfunction and impaired ability to recover following brain injury [9,40,68] were also reported, suggesting that ApoE is required for normal synaptic and neuronal health and its loss may result in neurodegeneration. Additionally, ApoE−/− has been linked to tau hyperphosphorylation [22,23] and cognitive deficits, especially with regards to spatial learning and memory [19,47].

Nevertheless, a more comprehensive characterisation of the ApoE−/− brain is required since there are several contrasting reports to the above-mentioned findings. For example, synaptic dysfunction [1] and tau hyperphosphorylation [43,55] have been disputed. Furthermore, in relation to AD, it is not yet clear whether ApoE loss of function is linked to amyloid precursor protein (APP) processing and Aβ pathology. It is also noteworthy that existing CNS studies in this model have been conducted at an age when the prominent peripheral atherogenic phenotype was fully developed. While this allows analyses of comorbidities associated with disease, the CNS phenotypes previously observed may thus be consequential to and/or affected by confounding peripheral factors.

The main objective of this study was therefore to examine the contribution of ApoE to neuronal function and specifically to AD-associated pathways and mechanisms. Using the ApoE−/− mouse model offers advantages as it 1) allows detailed investigation into the physiological role of ApoE, and 2) offers an opportunity to investigate sporadic risk factors of AD and 3) may uncover novel therapeutic targets in pathological states relevant to the disease. Markers were here examined in neonatal tissue to establish early emerging phenotypes in the absence of extensive peripheral pathology, and in adult (6 months of age) mice. We report that loss of ApoE altered a wide range of pathways, with emergence of phenotypic profiles already occurring from birth. Together, our findings emphasise the importance of this protein in neuronal physiology and implicate ApoE in the maintenance or modulation of multiple CNS processes relevant to disease.

2. Materials & methods

2.1. Animals

All animal procedures were performed in accordance with European (FELASA) and UK Home Office regulations under the EU Directive 63/2010EU and Animal (Scientific Procedures) Act 1986. Six-month old female ApoE−/− mice on a C57BL/6J background [49] and all neonatal (post-natal day 1–5, male and female) mice were derived from mice purchased from the Jackson Labs and then bred in-house at our facility while C57BL/6J wild-type (hereafter, WT) controls were supplied by a commercial vendor (Charles River). Mice were group housed with *ad libitum* access to food and water in a climate-controlled environment (20–21 °C, 60–65 % humidity) and standard 12-hour day/ night cycle. Animals were sacrificed by cervical dislocation and brain tissue was snap frozen in liquid nitrogen and stored at −80 °C until needed. Experimental protocols were not blinded or randomised.

2.2. Tissue preparation and Western blotting

Western blots were carried out as described previously [7,30]. In brief, brain tissue was homogenised in NP40 lysis buffer supplemented with protease and phosphatase inhibitors (Roche Life Science, Burgess Hill, UK). Homogenates were centrifuged (12,000 rpm, 4 °C, 20 min) and the soluble supernatant was collected and stored at −80 °C. Heat stable fractions for tau analysis were isolated by further heating the NP40 lysate to 90 °C for 10 min followed by centrifugation (14,000 rpm, 4 °C, 10 min) and supernatant collection. Total tau (AT5) levels were investigated using IgG depleted samples whereby IgGs were removed by incubating the soluble NP40 supernatant with an equal volume of Dynabeads™ Protein G (Fisher Scientific, Loughborough, UK) overnight at 4 °C. Insoluble tau fractions were obtained by resuspending and incubating the soluble NP40 pellet 1:1 in 70 % formic acid with agitation at 4 °C overnight. The formic acid suspension was then centrifuged (14,000 rpm, 4 °C, 20 min) prior to collection of the supernatant the following day. Prior to use, formic acid samples were neutralised with 4 parts neutralising buffer (2 M Tris, 2 M NaH2P04).

Samples were normalised to 3 μg/μl in lithium dodecyl sulphate. Due to the strong denaturing conditions of formic acid, which prevented protein concentration determination, a set volume of insoluble neutralised samples was used. For APP and Aβ analysis, samples were assessed in non-denatured conditions. Proteins were separated on 4–12 % Bis-Tris gels and transferred onto nitrocellulose membranes using either wet or dry standard transfer conditions. Membranes were blocked in Tris-buffered saline with Tween (TBST) containing 5 % milk powder for 1 h at RT and then incubated with primary antibody overnight at 4 °C. For full details of antibodies used see Table S1. The following day, membranes were treated with secondary antibody and then visualised using enhanced chemiluminescence and captured with a Vilber-Fusion-SL camera (Vilber, Eberhardzell, Germany). Membranes were either re-probed for total protein (for phospho-markers) or subsequently stained with Coomassie Blue total protein.

Immunoreactivity was quantified using ImageJ (V1.47, NIH, USA) software according to area under the curve (AUC) measurements. Values were either adjusted to respective total protein values (in the case of phospho-markers) or to the loading control, Coomassie Blue total protein stain [50]. Adjusted values were then expressed relative to control groups within blots.

Note, the effect of genotype was probed per age group, while age-dependent changes were determined within each genotype, based on independent blots.

2.3. Quantitative polymerase chain reaction (qPCR)

RNA from brain tissue was isolated using TRI reagent® (Sigma, Dorset, UK)) according to the manufacturers' instructions. cDNA was synthesis from 1 μg of RNA using a Tetro cDNA synthesis kit (Bioline, London, UK) and a GS-1 G-Storm thermocycler (Labtech International, Sussex, UK). Genes of interest (see Table S1 for primer sequences) were quantified using quantitative PCR (qPCR). Target genes were amplified using a GoTaq qPCR Master Mix (Promega, Southampton, UK) run in a Roche LightCycler® 480 System (Roche Life Science). Gene expression was determined by the Pfaffl method [48] and normalised to the geometric mean of the three most stable reference genes (from Y-Whaz, NoNo, 18S, GAPDH or β-Actin). Values were then expressed relative to control groups.

2.4. Statistics

Statistical analysis was conducted using GraphPad Prism (V7). Normal distribution was probed for (Shapiro-Wilk normality test), prior to analysis via either a Student's unpaired two-tailed *t*-test, with Welsh correction if appropriate, or a Mann Whitney *U* test. For all data, the level of significance was set as $p \leq 0.05$.

3. Results

The global knockout of mouse ApoE (see Fig. S1 for confirmation of brain ApoE knockout) has so far been primarily used to study atherosclerosis. In line with the physiological role of ApoE and the published phenotype, we first confirmed highly elevated levels of plasma cholesterol and an altered lipoprotein profile but noted that these differences were limited to the periphery, as brain cholesterol was found unchanged (Fig. S2).

3.1. Tau species in ApoE−/− mice

3.1.1. Soluble tau

In order to determine whether ApoE loss was associated with AD-relevant primary pathologies, tau markers were assessed in brain tissue from ApoE−/− and WT mice, firstly in the soluble fraction [30]. Using immunoblotting, PHF-1 phosphorylated tau (p-tau; epitope: Ser396/ 404) and total tau protein levels (AT5 antibody) were found unaffected by ApoE−/− at the neonatal age (Fig. 1A & B). In contrast, early changes were established upon analysis of CP-13 p-tau reactive species (epitope: Ser202) with significantly decreased levels being detected in ApoE−/− compared to WT neonatal mice (Fig. 1B; p = 0.0002, −40 %). At 6 month of age, the opposite trend was seen with significantly decreased PHF-1 labelled p-tau being detected for all but the 55 kDa p-tau isoform (Fig. 1C; 50 kDa, p = 0.0054; 60 kDa, p = 0.0003; total, p = 0.0011, −41 %), while CP-13 labelled species were unchanged (Fig. 1D). AT5 total tau was also found comparable between groups (Fig. 1E). Therefore, an epitope-specific decrease in soluble p-tau was established in ApoE−/− mice compared to WT controls in both neonatal and 6-month old brain tissue.

Next, to assess age-related changes relevant for development and maturation, protein levels were examined on separate blots using 6-month old WT and ApoE−/− tissue (Fig. 1F), here compared and normalised to their respective neonatal controls, with changes in WT levels taken as a reference (control). As expected, an age-dependent shift in the molecular weight of tau species was observed, likely a reflection of age-specific isoform expression. As tau is developmentally regulated, at the neonatal age the predominant form of murine tau detected is 3R, whereas by 6-month old, the major isoform present is 4R [64]. Thus, to measure overall (total) tau levels, the sum of all tau immunoreactivity was analysed. Western blot analysis of PHF-1 soluble phosphorylation levels found no changes across ageing in WT mice (Fig. 1G), however, ApoE−/− mice displayed significantly decreased levels at 6-month old compared to their respective neonatal controls (Fig. 1G; p = 0.0472, −22 %). P-tau detected by CP-13, on the other hand, revealed a similar decline in phosphorylation over ageing for both genotypes (Fig. 1G; WT, p < 0.0001, −67 %; ApoE−/−, p = 0.001, −60 %). Analysis of AT5 total tau levels found that protein levels were unaffected by age in WT mice, while levels declined in ApoE−/− mice (Fig. 1G, p = 0.015, −29 %). Taken together, these data confirm an overall, general age-dependent decrease in soluble tau levels for both genotypes, with specific p-tau levels (PHF-1) declining more dramatically in ApoE−/− mice.

3.1.2. Insoluble tau

Tau levels in the insoluble fraction were explored next. No neonatal differences between genotypes were

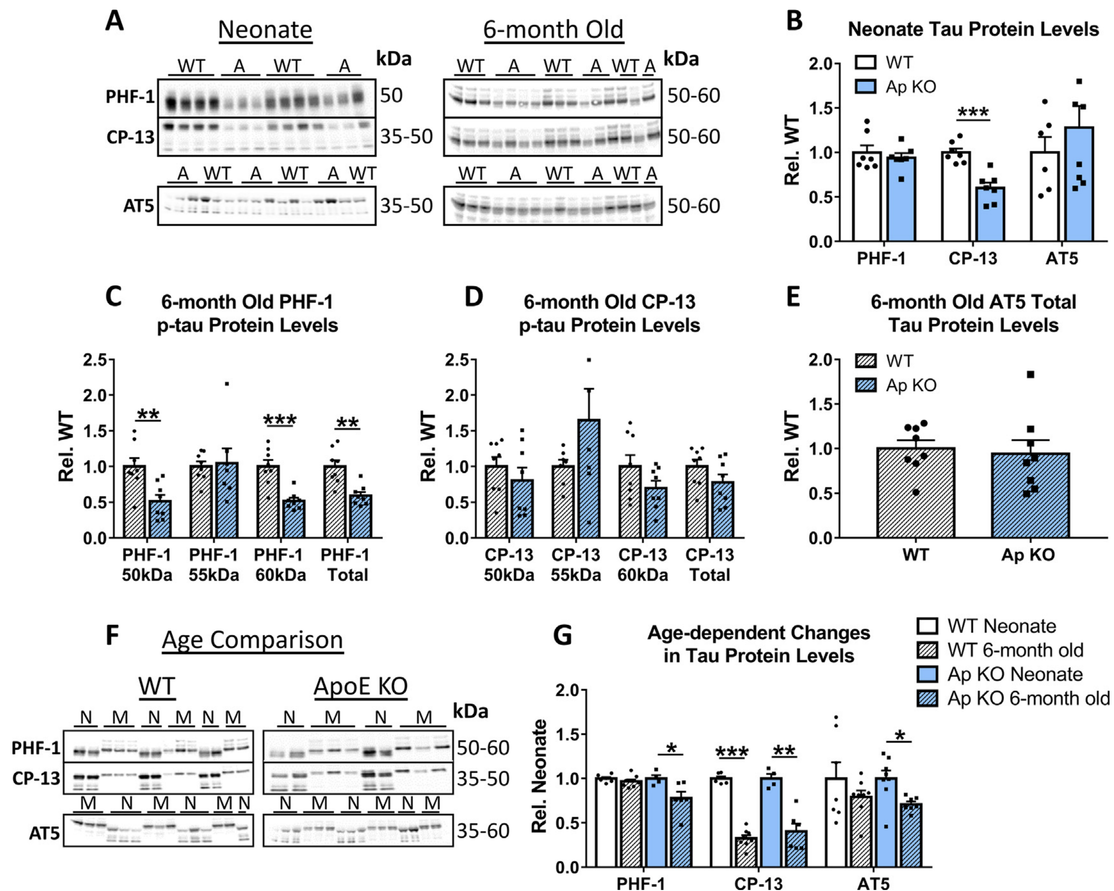


Fig. 1. Decreased soluble tau levels in ApoE-/- vs. WT mice.
(A) Representative Western blots of neonate and 6-month old WT and ApoE-/- (A) brain tissue with molecular weights shown. **(B)** Quantification of ApoE-/- (Ap KO; n = 8) neonatal tissue probed for soluble phosphorylated (PHF-1, CP-13) and total (AT5) tau according to WT (n = 7) groups. 6-month old WT (n = 8) and ApoE-/- (n = 7) **(C, D)** phospho-tau (p-tau; 50kDa, 55kDa, 60kDa, total) and **(E)** total tau analysis. **(F)** Representative Western blots of WT (N: n = 6, M: n = 8) and ApoE-/- (N: n = 4, M: n = 6) mice assessed over ageing using both neonatal (N) and 6-month old (mature, M) samples. **(G)** WT and ApoE-/- age-related changes in tau protein levels according to neonatal levels. Phospho markers were normalised to total tau (AT5) and total tau was quantified from an IgG depleted fraction and adjusted to Coomassie total protein stain. Data are presented as scatter as well as mean + SEM. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

observed for protein levels of PHF-1 and CP-13 reactive p-tau (Fig. 2A & B). Nonetheless, by 6 months of age, significantly lower levels were detected in the brains of ApoE−/− vs WT mice for both p-tau markers (Fig. 2B; PHF-1, p = 0.0002, −48 %; CP-13, p = 0.0039, −54 %), thus confirming that ApoE deficiency resulted in an age-dependent decrease in insoluble p-tau levels compared to controls.

We further assessed age-related changes in 6-month old mice compared to respective neonatal controls (independent blots). Similar to the soluble fraction, distinct immunoreactive banding was seen between the two age groups. Immunoblotting analysis also detected similar developmental and ageing profiles for both groups, with a marked decrease in PHF-1 (Fig. 2C; WT, p = 0.0007, −60 %; ApoE−/−, p < 0.0001, −54 %) and CP-13 (Fig. 2C; WT, p = 0.0002, −86 %; ApoE−/−, p < 0.0001, −77 %). These age-related changes were also confirmed with additional qPCR analysis of tau gene expression, i.e. reduced expression was detected at 6 month compared to younger controls for both genotypes (Fig. 2D; WT, p = 0.0099, −71 %; ApoE−/−, p = 0.0011, −50 %).

3.2. Aβ-related markers in ApoE−/− mice

Following on from our investigation of tau levels, we subsequently explored the impact of ApoE loss on Aβ-related markers. Using immunoblotting, basal alterations in these markers were assessed in neonatal ApoE−/− and WT brain tissue. No differences in full length APP, as analysed by 6E10, or sAPPα were observed between ApoE−/− and WT mice (Fig. 3A & B). Notably, monomeric Aβ species were also probed for, however, no signal was detected in this age group. In contrast, levels of full length APP were found significantly increased in 6-month old ApoE−/− groups compared to controls (Fig. 3B; p = 0.0251, + 116 %), although these changes were found without alterations to monomeric Aβ levels as measured by MOAB-2 or sAPPα which remained consistent across genotypes (Fig. 3B).

Furthermore, we used immunoblotting to assess age-related changes in protein levels of Aβ-related markers, again in 6-month old animals compared to their respective neonatal controls. In line with our finding that APP levels were increased in 6-month old ApoE−/− animals, full-

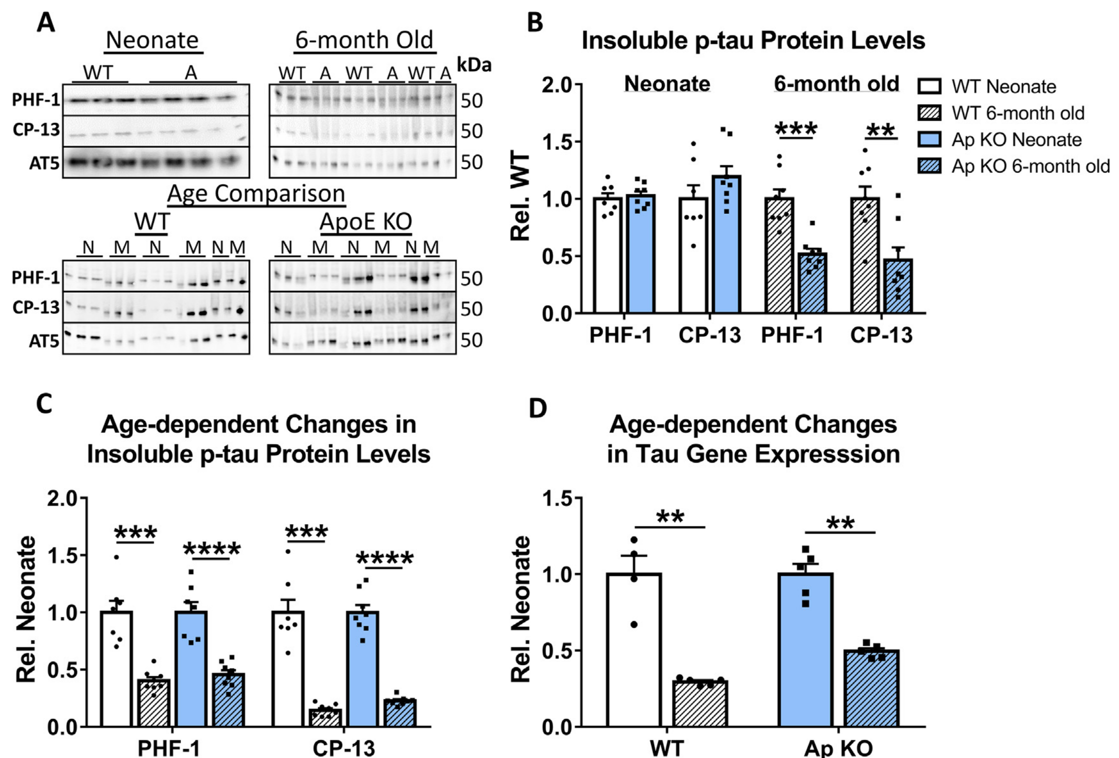


Fig. 2. Decreased insoluble tau levels in 6-month old ApoE-/- compared to WT mice.
(A) Representative Western blots of insoluble tau assessed in neonate (WT, n = 7; ApoE-/- (A), n = 8) and 6-month old (WT, n = 8; A, n = 7) brain tissue and across both ages (neonate (N), n = 7; 6-month old (mature, M), n = 8) in an age comparison. **(B)** Insoluble tau phosphorylation (p-tau; PHF-1, CP-13) levels in ApoE-/- (Ap KO) neonate and 6-month old tissue compared to WT controls. **(C)** Changes in WT and ApoE -/- p-tau at 6-months old quantified according to neonatal controls. P-tau levels were normalised to total tau (AT5) protein. **(D)** Tau gene expression over ageing with quantification at 6-months old compared to neonatal groups for both WT and Ap KO mice (n = 5 for all). Data are illustrated as scatter plus mean+ SEM. ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$.

length APP (measured by 6E10) was significantly increased at the older age in ApoE−/− animals compared to their neonatal controls, but not for WT groups (Fig. 3C; p = 0.0014, + 195 %). Protein levels of sAPPα, on the other hand, were unchanged for both WT and ApoE−/− mice (Fig. 3C). Monomeric Aβ species were also examined across ageing for both genotypes. However, immunoreactivity was only detected at the older age, therefore, the increase in protein levels at 6 months of age was not quantified and has been plotted as adjusted raw values for visual reference only (Fig. 3C). Age-related changes in APP gene expression, explored using qPCR analysis, revealed an increased expression at 6 months for both WT (Fig. 3D; p < 0.0001, + 174 %) and ApoE−/− (Fig. 3D; p < 0.0001, + 192 %) groups. Thus, taken together our results indicate that while Aβ-related markers were unchanged at birth, ApoE deficiency promoted levels of full length APP at 6 months of age.

3.3. Synaptic protein levels in ApoE−/− mice

To examine the association of ApoE and synaptic changes, we next assessed levels of two markers: the presynaptic vesicular marker, synaptophysin (SYP), and the postsynaptic scaffolding protein, PSD95. Surprisingly, in neonatal brain tissue, while there was no difference in protein levels of PSD95 across genotypes (Fig. 4A & B), levels of SYP were found to be significantly higher in ApoE−/− mice (Fig. 4B; p = 0.0059, + 36 %). In contrast, by 6 months of age, reduction of SYP levels approached significance (Fig. 4B; p = 0.0544); here, PSD95 levels were also lower in ApoE−/− compared to WT controls (Fig. 4B; p = 0.0114, −15 %). Hence, our data are in agreement with previous suggestions of an age-dependent synaptic reduction in KO vs WT mice [32,41]. Further investigations of age-related changes in synaptic markers (by measuring 6-month protein levels compared to their respective neonatal controls in separate blots), indicated higher protein levels of presynaptic SYP at 6 months for both ApoE−/− (Fig. 4C; p < 0.0001, +593 %) and WT (Fig. 4C; p = 0.0006, +761 %) mice. This developmental adjustment also coincided with increasing protein levels of PSD95 in WT brain tissue (Fig. 4C; p = 0.0005, +227 %). Interestingly, however, this was not seen in ApoE−/− tissue, where PSD95 levels remained constant across neonatal and 6-month old groups (Fig. 4C), further indicative of synaptic adjustments in ApoE−/− animals.

3.4. Neuroinflammation in ApoE−/− mice

Neuroinflammation is another well-characterised feature of neurodegeneration, and loss of ApoE has previously

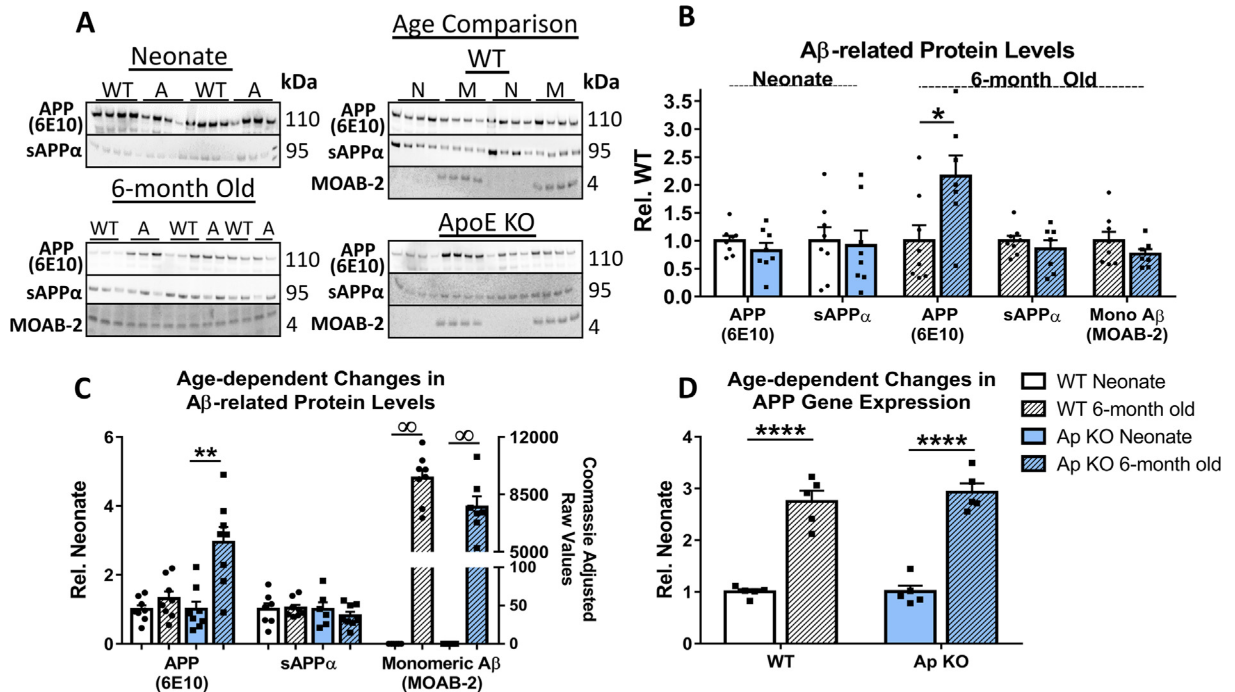


Fig. 3. ApoE-/- mice have increased full length APP levels at 6 months.
(A) Representative Western blots of WT and ApoE-/- (A) neonate and 6-month old brain tissue with molecular weights indicated. WT and ApoE-/- examination over ageing using neonate (N) and 6-month (mature, M) old samples is also shown. **(B)** Quantification of ApoE-/- (Ap KO) neonate and 6-month old samples probed for full-length APP (APP, 6E10), sAPP α and monomeric (mono) A β (MOAB-2), expressed relative to WT controls. **(C)** Age-dependent changes assessed in WT and ApoE KO mice at 6-month were quantified according to respective neonatal levels. All markers were normalised and adjusted to the loading control (Coomassie Blue total protein stain). For reference, age-related changes in monomeric A β (MOAB-2) were plotted as Coomassie adjusted raw values. N = 8 for all. **(D)** Analysis of APP gene expression across ageing and development in 6-month old compared to neonatal WT and Ap KO mice (n = 5). Individual data are shown as scatter, plus mean+ SEM. * = p < 0.05, ** = p < 0.01, **** = p < 0.0001.

been associated with neuroinflammatory pathologies [27,70]. To explore respective markers in our cohorts, WT and ApoE−/− brain tissue was probed for Iba1, a marker of microglia, and GFAP, a marker of astrogliosis. Early phenotypic changes were firstly assessed using brain tissue from neonatal mice, where Iba1 protein levels, measured by immunoblotting, were significantly elevated in ApoE−/− compared to WT controls (Fig. 5A & B; p = 0.0395, +25 %). At this age, total levels of GFAP and its isoforms remained unchanged across groups (Fig. 5B). Similarly, by 6-months of age, an increase in Iba1 protein levels in ApoE−/− brains approached significance (Fig. 5C; p = 0.055), again alongside unaltered GFAP levels (Fig. 5C). Collectively, our data determined that ApoE−/− mice present with an inflammatory phenotype, specifically related to microglia, and that this inflammatory profile emerged at birth.

Subsequent immunoblots to specifically confirm age-related adjustments in neuro-inflammatory markers were assessed in ApoE−/− and WT brain tissue. As expected, analysis of 6-month old WTs revealed a significant decline in Iba1 compared to their neonatal controls (Fig. 5D; p = 0.0001, −49 %), a likely reflection of glial numbers which are high during the first weeks of postnatal development and then decline and plateau [39,46]. Similar changes were not seen in ApoE−/− groups; microglial levels remained constant between birth and adulthood (Fig. 5D). For the astrocytic marker, GFAP, lower levels of the 45 kDa isoform were confirmed for both WT (Fig. 5E; p < 0.0001, −82 %) and ApoE−/− (Fig. 5E; p = 0.0031, −76 %) at 6-months old vs neonates. Yet, only WT mice displayed alterations in the 50 kDa isoform, here, levels significantly decreased at 6 months compared to neonatal controls (Fig. 5E; p = 0.0275, −57 %). Consequently, reliably decreased total GFAP protein levels at 6 months old vs younger controls were only confirmed for WT (Fig. 5E; p < 0.0001, −77 %) mice, while the ApoE−/− group remained unchanged (Fig. 5E). Taken together, these findings demonstrate an enhanced inflammatory profile, and also altered age-related adjustments in inflammatory proteins, in ApoE−/− mice.

3.5. Tau kinases and protein translation in ApoE−/− mice

Lastly, in order to investigate whether post-translational changes in kinase activity could account for differences seen in p-tau status, or if protein translation was overall affected by the loss of ApoE, immunoblotting analysis for these markers was conducted (Fig. 6A). Neonatal tissue was firstly assessed to determine whether early changes were occurring. Levels of phospho-JNK (the active form of the kinase) were significantly increased in ApoE−/− animals compared to controls (Fig. 6B; p = 0.0289, + 46 %). ApoE absence was confirmed to only

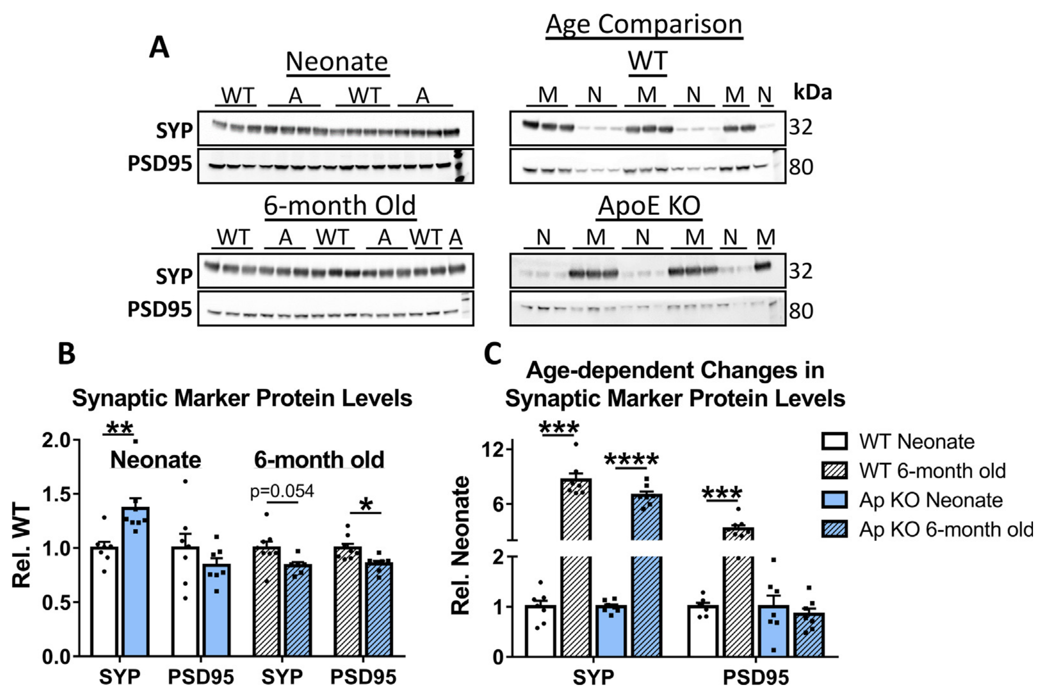


Fig. 4. ApoE-/- mice present with an age-related synaptic decline.
(A) Representative Western blots of WT and ApoE-/- (A) neonatal (WT, n = 7; A, n = 8) and 6-month old (WT, n = 8; A, n = 7) brain tissue. Western blots of neonate (N) and 6-month old (mature, M) samples for an age comparison analysis are also presented. **(B)** Quantification of synaptophysin (SYP) and PSD95 protein levels in WT and ApoE-/- (Ap KO) neonate and 6-month old mice. **(C)** SYP and PSD95 protein levels in 6-month old WT and Ap KO brain tissue expressed according to respective neonatal controls. All markers were normalised to Coomassie-stained total protein. Data are shown as scatter, plus mean + SEM. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$.

affect phosphorylation status, as total JNK levels were unchanged (Fig. 6B). On the other hand, analysis of GSK3β, the major tau kinase, revealed that levels of phospho-GSK3β (the inactive form) and total GSK3β were unaffected by genotype (Fig. 6B).

Furthermore, we also analysed protein levels of ribosomal S6 (rpS6). While the exact details of rpS6‘s role is largely unknown, the phosphorylated and active form of this protein is associated with increased protein translation [6,12] and can therefore be used as an indicator of protein synthesis. We report that ApoE−/− neonatal animals presented with lower levels of p-rpS6 compared to WT mice; this decrease occurred in the absence of a difference in total levels (Fig. 6B; $p = 0.0497$, −11 %). Thus, altered kinase activity and reduced protein synthesis rates may be an early occurring phenotype in the absence of ApoE.

Analysis of 6-month old tissue, however, revealed that in contrast to the younger tissue, phosphorylation levels of JNK, and thus the active isoform of JNK, were markedly decreased in ApoE−/− mice compared to WTs (Fig. 6C; $p = 0.0274$, −34 %). Additionally, analysis of GSK3β suggested an increase in its activity due to the decline of inactive p-GSK3β (Fig. 6C; $p = 0.015$, −78 %). For both kinases, total levels of the protein were consistent across ApoE−/− and WT tissue, confirming the effect of ApoE loss on phosphorylation only (Fig. 6C). Analysis of rpS6 (both phosphorylated and total) also yielded no statistical difference between analytical groups (Fig. 6C). Together, these data demonstrate comparable protein translation at the older age with alterations to tau-associated kinases.

4. Discussion

This study used ApoE−/− mice to examine the impact of ApoE loss on CNS markers of brain physiology and pathology. Several pathways relevant to AD states (tau, Aβ species, synaptic pathways, neuroinflammation and protein turnover activity) were interrogated in neonatal and 6-month old tissues. Collectively, our findings highlight the importance of this protein to brain maturation and function and demonstrate that absence of ApoE, or loss of its function, causes alterations in several key processes with phenotypic manifestations already emerging in early development.

Abnormal phosphorylation of the microtubule binding protein, tau, is a key event in tauopathies. While ApoE has been associated with its stabilisation and the formation of microtubules [61], previous studies remained conflicted on whether tau phosphorylation is increased [22], 2000) or unchanged [43,55]. We here provide evidence that phosphorylation is in fact reduced in ApoE−/− mice, largely in an age-dependent manner. ApoE interaction with cytoskeletal proteins is not limited to tau, but also includes proteins such as microtubule associated protein 2 (MAP2), actin and tubulin [18,25]. ApoE3 binding of tau *in vitro* has been proposed to prevent its self-assembly / phosphorylation [61]. Hence, we suggest that the decreased phosphorylation observed here may be related to a broader role for ApoE in maintaining the cytoskeleton. Indeed, Veinbergs et al. [68] observed reduced levels of the dendritic marker, MAP2, in the frontal cortex and hippocampus of ApoE−/−

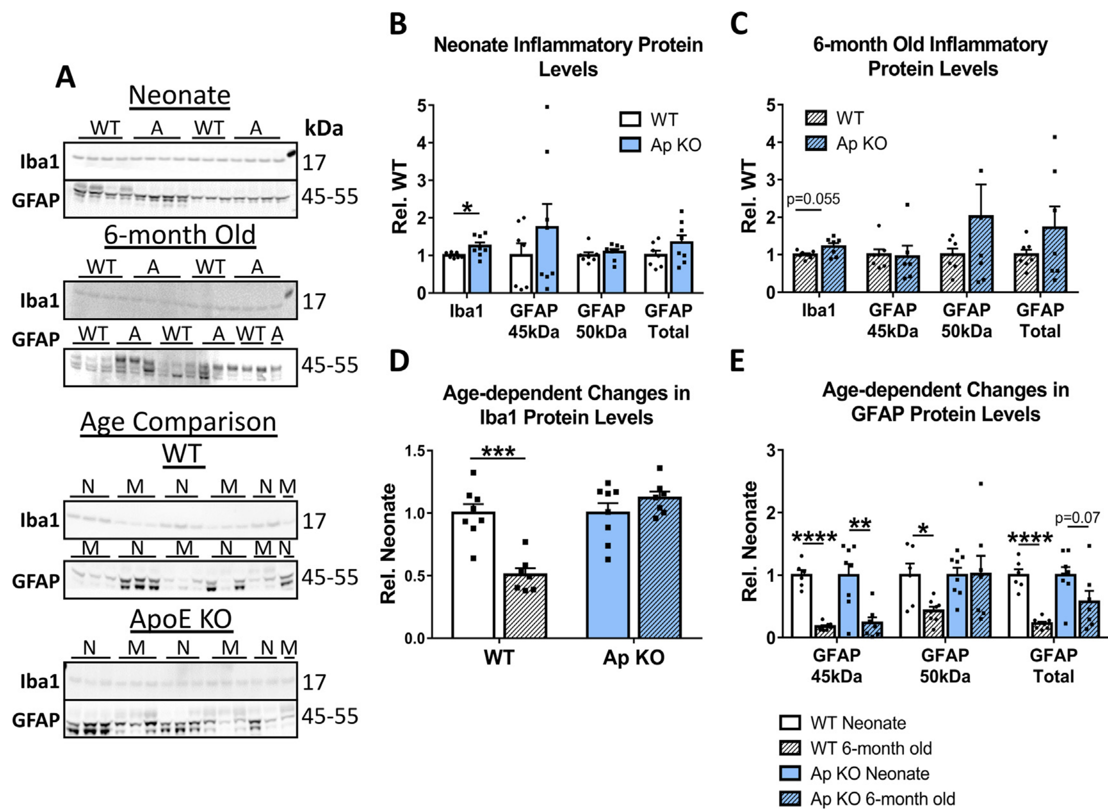


Fig. 5. Increased inflammatory protein levels and altered age-related changes in ApoE-/- compared to WT mice.
(A) Representative Western blots of ApoE-/- and WT neonatal (WT, n = 7; A, n = 8) and 6-month (WT, n = 8; A, n = 7) old brain tissue with molecular weights shown. Western blots of neonatal (N) and 6-month (mature, M) samples used for an age-comparison are also included. Quantification of ApoE-/- (Ap KO) Iba1 and GFAP (45kDa, 50kDa, total) protein levels in **(B)** neonate and **(C)** 6-month old brain tissue compared to WT levels. Age-related changes in WT and ApoE-/- **(D)** Iba1 and **(E)** GFAP protein levels expressed relative to respective neonatal levels. All markers were normalised to Coomassie total protein stain. Data are displayed as scatter and mean+SEM. * = p < 0.05, ** = p < 0.01, *** = p < 0.001, **** = p < 0.0001.

mice, while extensive disruption to the dendritic compartments and reduced levels of α and β tubulin have also been seen [40]. Together, these data suggest that ApoE is required to maintain the stability of the somatodendritic apparatus. While tau phosphorylation is physiologically essential for e.g. axonal transport [58], this is subject to a continuous and dynamic regulation. Abnormally low or ineffective ApoE levels could therefore alter cytoskeletal integrity [35]. It is noteworthy that p-tau differences were already prominent at the neonatal age. Tau phosphorylation is linked to neurogenesis and is highest during early development [21], while ApoE3 can promote neurite extension, linked to microtubule stability [14,45]. In view of our observations, reduced p-tau levels are indicative of deficits in these processes caused by absence of ApoE at birth, which consequently could have wide-ranging damaging as well as compensatory implications.

In light of the alterations to tau, we also examined tau-associated kinases. The active isoform of JNK was indeed decreased in 6-month old ApoE deficient mice, yet analysis of GSK3β, the major tau kinase, indirectly pointed towards elevated levels of the active kinase isoform. Increased tau kinase activity has been shown previously, with enhanced activation of JNK and GSK3β detected in ApoE−/− mice on high cholesterol diet; the authors suggested this to contribute to diet-induced tau hyper-phosphorylation [55]. Therefore, similarly to previous studies, we here identify a close link between ApoE and altered kinase activation although it is unlikely that the alterations seen here are directly responsible for the observed decrease in tau phosphorylation. Nevertheless, the mechanisms behind these differences require further examination as JNK and GSK3β have numerous other neuronal functions [5,76].

In relation to the other hallmark of AD, Aβ, the consequence of global ApoE−/− on brain pathology is less clear. Previous studies utilising ApoE−/− animals crossed with Aβ-transgenic models suggested that ApoE facilitates [4,66] but is not required [15] for Aβ fibrillization and deposition. In the present study, we found that lack of ApoE at 6 months of age and across ageing is sufficient to promote an increase in full length APP, the precursor of Aβ. We note that these changes were without alterations to levels of soluble sAPPα and monomeric Aβ, however, given the observed age-dependent increase in APP it is plausible that downstream effects may not be apparent yet at 6 months. In view of the notion that ApoE deficiency

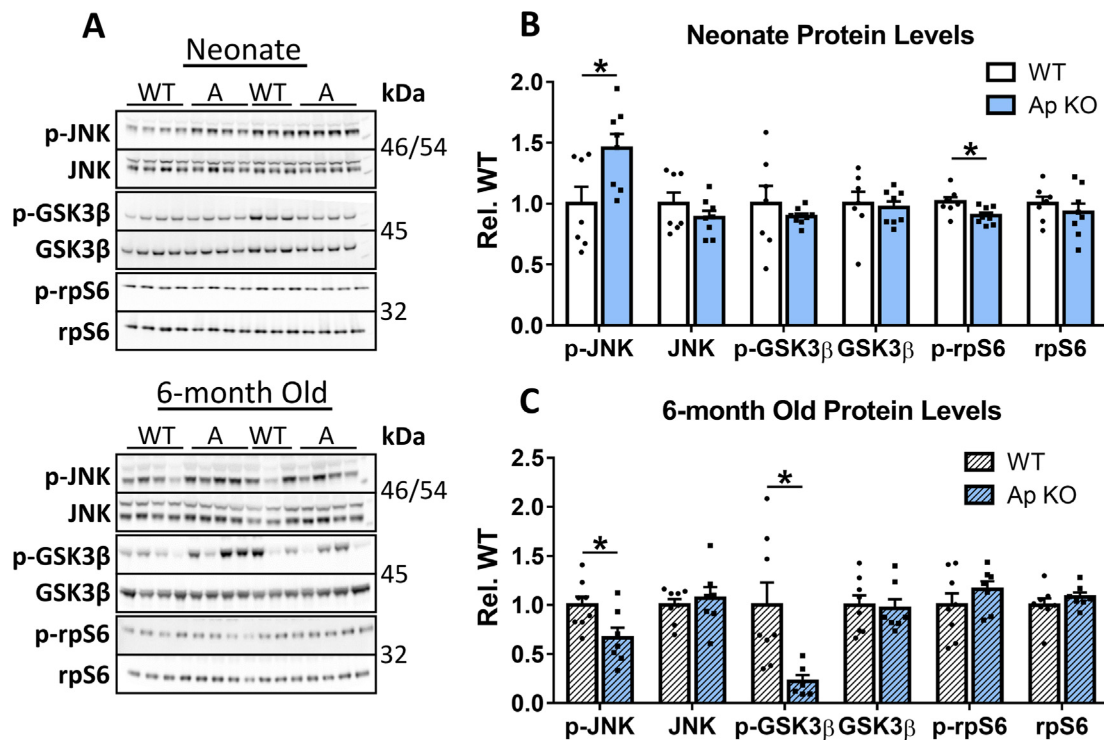


Fig. 6. ApoE-/- mice display altered kinase activity and protein translation.
(A) Representative Western blots of neonate (WT, n = 7; A, n = 8) and 6-month old (WT, n = 8; A, n = 7) brain tissue with molecular weights shown. Quantification of p-JNK, JNK, p-GSK3 β, GSK3 β, p-rpS6 and rpS6 protein levels in **(B)** neonate and **(C)** 6-month old ApoE-/- (Ap KO) tissue according to WT controls. Phospho (p) proteins were normalised to their respective total protein levels and total proteins were adjusted to Coomassie blue total protein stain. Data are presented as scatter plus mean+ SEM. * = $p < 0.05$.

can be considered to some extent similar to a loss of function caused by ApoE4, it is of interest that Huang et al. [26] discovered that ApoE was able to increase APP mRNA levels in an isoform-dependent (ApoE4 > ApoE3 > ApoE2) manner resulting in corresponding increases in Aβ species. Similarly, Lee et al. [34] reported that levels of APP were increased in neuronal human iPSCs when cultured in conditioned media from ApoE4 compared to ApoE3 astrocytes. A recent study has also connected ApoE and neuronal APP via a shared mechanism whereby ApoE employs astrocyte-derived cholesterol to shuttle APP in and out of lipid clusters [72]. Taken together, there are clear implications for ApoE in the regulation of APP, and we posit that the ApoE−/− model may provide valuable insights that could be followed up regarding ApoE4-mediated loss of function diseased states.

The impact of ApoE−/− on synaptic function has not been conclusively determined with data of both an age-related decline [32,41] and no change reported [1]. Our results offer agreement for age-associated synaptic adjustments, with increased synaptophysin levels observed in neonates as a potential manifestation of early compensatory mechanisms to sustain synaptic function. Somewhat in line with this, we also found reduced p-rpS6 levels in younger mice, suggestive of a lower protein synthesis and translational activity in neonates. Notably, in support of ApoE's absence affecting the somatodendritic compartment to a larger extent [41,40,68], we found a greater decline in postsynaptic compared to presynaptic protein levels in 6-month old animals. When examining age-related developmental changes, we also showed that ApoE−/− PSD95 levels remained constant across maturation, highly indicative of a postsynaptic deficit since WT showed a substantial increase with age. Thus, we concur with the existing literature which strongly implies that CNS ApoE is required to maintain synaptic morphology, and its loss is detrimental to neuronal function. What causes this synaptic deficit remains to be answered, and functional and behavioural tests should determine the ultimate impact on neuronal communication. Importantly, ApoE is a vital carrier of cholesterol that is required for normal neuronal physiology, including axonal growth, synaptic formation, synaptic re-modelling [42,57] and neuronal reinnervation following injury [9,53], which may underlie the changes observed here.

Furthermore, ApoE−/− animals also exhibited neuroinflammatory changes (neonatal mice displaying increased Iba1 levels and 6-month old animals demonstrating a strong trend), indicative of microgliosis. This phenotype was evident here upon assessing age-related changes and align with the consistently documented neuroinflammation in this model [27,68], as well as the observation that these mice display an increased glial and cytokine response following immune stimulation [36,77]. Early

studies have described that ApoE-mimetic peptides derived from the receptor-binding region of ApoE can suppress microglia and astrocytic responses [33,37]. Therefore, together these data highlight the anti-inflammatory nature of ApoE, with loss of this protein assumably associated with loss of its protective effect. The mechanism behind ApoE's long assumed role in immunomodulation is not clear but inhibition of JNK [52], modulation of microglial phagocytosis via TREM2 interaction [31] and acting as a checkpoint inhibitor of the complement cascade [74] have all be proposed. It is interesting that the different GFAP isoforms displayed variations across ageing in ApoE−/− animals compared to controls. CNS ApoE is primarily produced by astrocytes, so it is plausible that loss of this protein would in some way alter the function and activity of these glial cells. Up to 10 different GFAP isoforms have been identified [44], and the ApoE genotype can influence glial activation [77]; therefore, our findings indicate a mutual modulation of specific isoforms.

Ultimately, it is clear that ApoE has a myriad of functions across the CNS and deficiencies as well as loss of function of this protein impacts several brain processes. However, when assessing the translational relevance of ApoE-related studies, disparities between murine and human lipoprotein biology should be considered. These include but are not limited to differences in major cholesterol carrier, ApoE expression in which mice express only one form of the ApoE protein compared to the three in humans, and gene homology [38,73]. In addition, neuronal disturbances may not necessarily be directly due to the loss of ApoE per se, but rather a secondary disruption. This model also displays several alterations such as brain oxidative stress [59] and cholinergic deficits [24], which equally may underpin functional deterioration. Similarly, peripheral ApoE loss which induces a susceptibility to atherosclerosis, may also impact the findings generated here in the CNS; we found no differences to brain cholesterol levels in ApoE−/− mice while serum levels were dramatically altered. While peripheral and CNS ApoE have historically been considered as discrete pools, accumulating evidence suggests an interplay and crosstalk between the two compartments as exemplified by Lane-Donovan et al. [32] who strongly implied that peripheral and CNS ApoE can exert independent effects on brain function. This builds on previous observations that hypertriglyceridemia and hypercholesteremia can negatively impact cognition and AD-associated pathologies [16,65]. On this note, only 6-month old female ApoE−/− animals were investigated here, primarily due to the ease at which female mice can be group-housed. However, previous work has shown that female ApoE−/− animals display elevated serum cholesterol levels and an increased susceptibility to developing atherosclerosis vs males [8,67], a phenotype which may contribute to the documented worsened cognitive dysfunction also seen in this sex [32,54]. Therefore, it would be interesting for future studies to also conduct a gender comparison on the pathways investigated here. How lack of ApoE may transduce across the BBB remains to be shown, but ApoE deficiency leads to a BBB impairment which may permit easier access of neurotoxic agents into the brain [20] and here (Fig. S3). Thus, confounding factors arising from the development of atherosclerosis and whether the origin of CNS changes are a consequence of peripheral or central pathology remains to be confirmed. Importantly, however, we also assessed neonatal brains to mitigate peripheral influence and found that absence of ApoE even at this age was sufficient to induce CNS changes, thus confirming the importance of ApoE to normal physiology and CNS development.

4.2. Conclusion

In conclusion, the ApoE−/− mouse model is an important tool to investigate the role of ApoE and the consequences of its absence. Together, we here demonstrate that loss of ApoE results in alterations to several processes and pathways across the brain. Phenotypes emerged from early age, further highlighting the importance of this protein to normal processes. Questions remain as to the contribution of peripheral vs CNS ApoE yet given the far-reaching roles of this protein in the CNS, our data have several implications for how it may influence AD disease states. ApoE is strongly linked to the prevalence of AD and advances in our understanding of ApoE CNS biology will help identify pathological mechanisms and additional therapeutic targets.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

HB was funded by a studentship from the Alzheimer's Society, awarded to BP and colleagues as part of a Doctoral training Centre grant (code AS-DTC-2014-017). The antibodies, CP-13 and PHF-1 were generously provided by the Peter Davies lab.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nbas.2022.100055.

References

[1] Anderson R, Barnes JC, Bliss TVP, Cain DP, Cambon K, Davies HA, et al. Behavioural, physiological and morphological analysis of a line of apolipoprotein E knockout mouse. Neuroscience 1998. doi: https://doi.org/10.1016/S0306-4522(97)00598-8.

[2] Ayata C, Shin HK, Dileköz E, Atochin DN, Kashiwagi S, Eikermann-Haerter K, et al. Hyperlipidemia disrupts cerebrovascular reflexes and worsens ischemic perfusion defect. J Cereb Blood Flow Metab 2013. doi: https://doi.org/10.1038/jcbfm.2013.38.

[3] Bales KR, Liu F, Wu S, Lin S, Koger D, DeLong C, et al. Human APOE isoform-dependent effects on brain β-amyloid levels in PDAPP transgenic mice. J Neurosci 2009. doi: https://doi.org/10.1523/JNEUROSCI.0887-09.2009.

[4] Bales KR, Verina T, Dodel RC, Du Y, Altstiel L, Bender M, et al. Lack of apolipoprotein E dramatically reduces amyloid β-peptide deposition. Nat Genet 1997;17:263–4. doi: https://doi.org/10.1038/ng1197-263.

[5] Beurel E, Grieco SF, Jope RS. Glycogen synthase kinase-3 (GSK3): Regulation, actions, and diseases. Pharmacol Ther 2015. doi: https://doi.org/10.1016/j.pharmthera.2014.11.016.

[6] Biever A, Valjent E, Puighermanal E. Ribosomal protein S6 phosphorylation in the nervous system: From regulation to function. Front Mol Neurosci 2015. doi: https://doi.org/10.3389/fnmol.2015.00075.
[7] Buchanan H, Mackay M, Palmer K, Tothová K, Katsur M, Platt B, et al. Synaptic Loss, ER Stress and Neuro-Inflammation Emerge Late in the Lateral Temporal Cortex and Associate with Progressive Tau Pathology in Alzheimer's Disease. Mol Neurobiol 2020. doi: https://doi.org/10.1007/s12035-020-01950-1.
[8] Caligiuri G, Nicoletti A, Zhou X, Törnberg I, Hansson GK. Effects of sex and age on atherosclerosis and autoimmunity in apoE-deficient mice. Atherosclerosis 1999. doi: https://doi.org/10.1016/S0021-9150(99)00081-7.
[9] Chen Y, Lomnitski L, Michaelson DM, Shohami E. Motor and cognitive deficits in apolipoprotein E-deficient mice after closed head injury. Neuroscience 1997. doi: https://doi.org/10.1016/S0306-4522(97)00007-9.
[10] Corder EH, Saunders AM, Risch NJ, Strittmatter WJ, Schmechel DE, Gaskell PC, et al. Protective effect of apolipoprotein E type 2 allele for late onset Alzheimer disease. Nat Genet 1994. doi: https://doi.org/10.1038/ng0694-180.
[11] Corder, E.H., Saunders, A.M., Strittmatter, W.J., Schmechel, D.E., Gaskell, P.C., Small, G.W., Roses, A.D., Haines, J.L., Pericak-Vance, M. A., 1993. Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families. Science (80-.). 10.1126/science.8346443.
[12] Dekeryte, R., Franklin, Z., Hull, C., Croce, L., Kamli-Salino, S., Helk, O., Hoffmann, P.A., Yang, Z., Riedel, G., Delibegovic, M., Platt, B., 2021. The BACE1 inhibitor LY2886721 improves diabetic phenotypes of BACE1 knock-in mice. Biochim. Biophys. Acta - Mol. Basis Dis. 10.1016/j.bbadis.2021.166149.
[13] Dumanis SB, Tesoriero JA, Babus LW, Nguyen MT, Trotter JH, Ladu MJ, et al. ApoE4 decreases spine density and dendritic complexity in cortical neurons in vivo. J Neurosci 2009. doi: https://doi.org/10.1523/JNEUROSCI.4026-09.2009.
[14] Fagan AM, Bu G, Sun Y, Daugherty A, Holtzman DM. Apolipoprotein E-containing high density lipoprotein promotes neurite outgrowth and is a ligand for the low density lipoprotein receptor-related protein. J Biol Chem 1996. doi: https://doi.org/10.1074/jbc.271.47.30121.
[15] Fagan AM, Watson M, Parsadanian M, Bales KR, Paul SM, Holtzman DM. Human and murine apoE markedly alters Aβ metabolism before and after plaque formation in a mouse model of Alzheimer's Disease. Neurobiol Dis 2002. doi: https://doi.org/10.1006/nbdi.2002.0483.
[16] Farr SA, Yamada KA, Butterfield DA, Abdul HM, Xu L, Miller NE, et al. Obesity and hypertriglyceridemia produce cognitive impairment. Endocrinology 2008. doi: https://doi.org/10.1210/en.2007-1722.
[17] Farrer LA, Cupples LA, Haines JL, Hyman B, Kukull WA, Mayeux R, et al. Effects of age, sex, and ethnicity on the association between apolipoprotein E genotype and Alzheimer disease: A meta-analysis. J Am Med Assoc 1997. doi: https://doi.org/10.1001/jama.278.16.1349.
[18] Fleming LM, Weisgraber KH, Strittmatter WJ, Troncoso JC, Johnson GVW. Differential binding of apolipoprotein E isoforms to tau and other cytoskeletal proteins. Exp Neurol 1996. doi: https://doi.org/10.1006/exnr.1996.0064.
[19] Fuentes D, Fernández N, García Y, García T, Morales AR, Menéndez R. Age-related changes in the behavior of apolipoprotein e knockout mice. Behav Sci (Basel) 2018. doi: https://doi.org/10.3390/bs8030033.
[20] Fullerton SM, Shirman GA, Strittmatter WJ, Matthew WD. Impairment of the blood-nerve and blood-brain barriers in apolipoprotein E knockout mice. Exp Neurol 2001. doi: https://doi.org/10.1006/exnr.2001.7631.
[21] Fuster-Matanzo A, de Barreda EG, Dawson HN, Vitek MP, Avila J, Hernández F. Function of tau protein in adult newborn neurons. FEBS Lett 2009. doi: https://doi.org/10.1016/j.febslet.2009.08.017.
[22] Genis I, Gordon I, Sehayek E, Michaelson DM. Phosphorylation of tau in apolipoprotein E-deficient mice. Neurosci Lett 1995. doi: https://doi.org/10.1016/0304-3940(95)12007-Q.
[23] Genis L, Chen Y, Shohami E, Michaelson DM. Tau hyperphosphorylation in apolipoprotein E-deficient and control mice after closed head injury. J Neurosci Res 2000;60:559–64. doi: https://doi.org/10.1002/(SICI)1097-4547(20000515)60:4<559::AID-JNR15>3.0.CO;2-K.
[24] Gordon I, Grauer E, Genis I, Sehayek E, Michaelson DM. Memory deficits and cholinergic impairments in apolipoprotein E-deficient mice. Neurosci Lett 1995. doi: https://doi.org/10.1016/0304-3940(95)12006-P.
[25] Huang DY, Goedert M, Jakes R, Weisgraber KH, Garner CC, Saunders AM, et al. Isoform-specific interactions of apolipoprotein E with the microtubule-associated protein MAP2c: implications for Alzheimer's disease. Neurosci Lett 1994. doi: https://doi.org/10.1016/0304-3940(94)90204-6.
[26] Huang YWA, Zhou B, Wernig M, Südhof TC. ApoE2, ApoE3, and ApoE4 Differentially Stimulate APP Transcription and Aβ Secretion. Cell 2017. doi: https://doi.org/10.1016/j.cell.2016.12.044.
[27] Janssen CIF, Jansen D, Mutsaers MPC, Dederen PJWC, Geenen B, Mulder MT, et al. The effect of a high-fat diet on brain plasticity, inflammation and cognition in female ApoE4-knockin and ApoE-knockout mice. PLoS ONE 2016. doi: https://doi.org/10.1371/journal.pone.0155307.
[28] Knouff C, Hinsdale ME, Mezdour H, Altenburg MK, Watanabe M, Quarfordt SH, et al. Apo E structure determines VLDL clearance and atherosclerosis risk in mice. J Clin Invest 1999;103:1579–86. doi: https://doi.org/10.1172/JCI6172.
[29] Kockx M, Traini M, Kritharides L. Cell-specific production, secretion, and function of apolipoprotein E. J Mol Med 2018. doi: https://doi.org/10.1007/s00109-018-1632-y.
[30] Koss DJ, Dubini M, Buchanan H, Hull C, Platt B. Distinctive temporal profiles of detergent-soluble and -insoluble tau and Aβ species in human Alzheimer's disease. Brain Res 2018;1699:121–34. doi: https://doi.org/10.1016/j.brainres.2018.08.014.
[31] Krasemann S, Madore C, Cialic R, Baufeld C, Calcagno N, El Fatimy R, et al. The TREM2-APOE Pathway Drives the Transcriptional Phenotype of Dysfunctional Microglia in Neurodegenerative Diseases. Immunity 2017. doi: https://doi.org/10.1016/j.immuni.2017.08.008.
[32] Lane-Donovan C, Wong WM, Durakoglugil MS, Wasser CR, Jiang S, Xian X, et al. Genetic restoration of plasma apoe improves cognition and partially restores synaptic defects in ApoE-deficient mice. J Neurosci 2016. doi: https://doi.org/10.1523/JNEUROSCI.1054-16.2016.
[33] Laskowitz DT, Thekdi AD, Thekdi SD, Han SKD, Myers JK, Pizzo SV, et al. Downregulation of microglial activation by apolipoprotein E and apoE-mimetic peptides. Exp Neurol 2001. doi: https://doi.org/10.1006/exnr.2001.7541.
[34] Lee S-I, Jeong W, Lim H, Cho S, Lee H, Jang Y, et al. APOE4-carrying human astrocytes oversupply cholesterol to promote neuronal lipid raft expansion and Aβ generation. Stem Cell Rep 2021;16:2128. doi: https://doi.org/10.1016/J.STEMCR.2021.07.017.
[35] Lindwall G, Cole RD. Phosphorylation affects the ability of tau protein to promote microtubule assembly. Chem: J. Biol; 1984.
[36] Lynch JR, Morgan D, Mance J, Matthew WD, Laskowitz DT. Apolipoprotein E modulates glial activation and the endogenous central nervous system inflammatory response. J Neuroimmunol 2001. doi: https://doi.org/10.1016/S0165-5728(00)00459-8.
[37] Lynch JR, Tang W, Wang H, Vitek MP, Bennett ER, Sullivan PM, et al. APOE genotype and an ApoE-mimetic peptide modify the systemic and central nervous system inflammatory response. J Biol Chem 2003. doi: https://doi.org/10.1074/jbc.M306923200.
[38] Maloney B, Ge YW, Alley GM, Lahiri DK. Important differences between human and mouse APOE gene promoters: Limitation of mouse APOE model in studying Alzheimer's disease. J Neurochem 2007. doi: https://doi.org/10.1111/j.1471-4159.2007.04831.x.
[39] Mamber C, Kamphuis W, Haring NL, Peprah N, Middeldorp J, Hol EM. GFAPδ Expression in Glia of the Developmental and Adolescent Mouse Brain. PLoS ONE 2012. doi: https://doi.org/10.1371/journal.pone.0052659.
[40] Masliah E, Mallory M, Ge N, Alford M, Veinbergs I, Roses AD. Neurodegeneration in the central nervous system of apoE-deficient mice. Exp Neurol 1995. doi: https://doi.org/10.1006/exnr.1995.1088.
[41] Masliah E, Samuel W, Veinbergs I, Mallory M, Mante M, Saitoh T. Neurodegeneration and cognitive impairment in apoE-deficient mice is ameliorated by infusion of recombinant apoE. Brain Res 1997. doi: https://doi.org/10.1016/S0006-8993(96)01420-5.
[42] Mauch, D.H., Nägier, K., Schumacher, S., Göritz, C., Müller, E.C., Otto, A., Pfrieger, F.W., 2001. CNS synaptogenesis promoted by glia-derived cholesterol. Science (80-.). 10.1126/science.294.5545.1354.
[43] Mercken L, Brion JP. Phosphorylation of tau protein is not affected in mice lacking apolipoprotein E. NeuroReport 1995. doi: https://doi.org/10.1097/00001756-199511270-00025.
[44] Moeton M, Stassen OMJA, Sluijs JA, van der Meer VWN, Kluivers LJ, van Hoorn H, et al. GFAP isoforms control intermediate filament network dynamics, cell morphology, and focal adhesions. Cell Mol Life Sci 2016. doi: https://doi.org/10.1007/s00018-016-2239-5.

[45] Nathan, B.P., Bellosta, S., Sanan, D.A., Weisgraber, K.H., Mahley, R.W., Pitas, R.E., 1994. Differential effects of apolipoproteins E3 and E4 on neuronal growth in vitro. Science (80-.). 10.1126/science.8171342.
[46] Nikodemova M, Kimyon RS, De I, Small AL, Collier LS, Watters JJ. Microglial numbers attain adult levels after undergoing a rapid decrease in cell number in the third postnatal week. J Neuroimmunol 2015. doi: https://doi.org/10.1016/j.jneuroim.2014.11.018.
[47] Oitzl MS, Mulder M, Lucassen PJ, Havekes LM, Grootendorst J, De Kloet ER. Severe learning deficits in apolipoprotein E-knockout mice in a water maze task. Brain Res 1997. doi: https://doi.org/10.1016/S0006-8993(96)01448-5.
[48] Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 2001. doi: https://doi.org/10.1093/nar/29.9.e45.
[49] Piedrahita JA, Zhang SH, Hagaman JR, Oliver PM, Maeda N. Generation of mice carrying a mutant apolipoprotein E gene inactivated by gene targeting in embryonic stem cells. Proc Natl Acad Sci U S A 1992. doi: https://doi.org/10.1073/pnas.89.10.4471.
[50] Plucińska K, Crouch B, Koss D, Robinson L, Siebrecht M, Riedel G, et al. Knock-in of human BACE1 cleaves murine APP and reiterates alzheimer-like phenotypes. J Neurosci 2014. doi: https://doi.org/10.1523/JNEUROSCI.0433-14.2014.
[51] Plump AS, Smith JD, Hayek T, Aalto-Setälä K, Walsh A, Verstuyft JG, et al. Severe hypercholesterolemia and atherosclerosis in apolipoprotein E-deficient mice created by homologous recombination in ES cells. Cell 1992. doi: https://doi.org/10.1016/0092-8674(92)90362-G.
[52] Pocivavsek A, Burns MP, Rebeck GW. Low-density lipoprotein receptors regulate microglial inflammation through c-Jun N-terminal kinase. Glia 2009. doi: https://doi.org/10.1002/glia.20772.
[53] Poirier J, Baccichet A, Dea D, Gauthier S. Cholesterol synthesis and lipoprotein reuptake during synaptic remodelling in hippocampus in adult rats. Neuroscience 1993. doi: https://doi.org/10.1016/0306-4522(93)90456-P.
[54] Raber J, Wong D, Buttini M, Orth M, Bellosta S, Pitas RE, et al. Isoform-specific effects of human apolipoprotein E on brain function revealed in ApoE knockout mice: Increased susceptibility of females. Proc Natl Acad Sci U S A 1998. doi: https://doi.org/10.1073/pnas.95.18.10914.
[55] Rahman A, Akterin S, Flores-Morales A, Crisby M, Kivipelto M, Schultzberg M, et al. High cholesterol diet induces tau hyperphosphorylation in apolipoprotein e deficient mice. FEBS Lett 2005. doi: https://doi.org/10.1016/j.febslet.2005.10.024.
[56] Rodriguez GA, Burns MP, Weeber EJ, Rebeck GW. Young APOE4 targeted replacement mice exhibit poor spatial learning and memory, with reduced dendritic spine density in the medial entorhinal cortex. Learn Mem 2013. doi: https://doi.org/10.1101/lm.030031.112.
[57] Saher G, Brügger B, Lappe-Siefke C, Möbius W, Tozawa RI, Wehr MC, et al. High cholesterol level is essential for myelin membrane growth. Nat Neurosci 2005. doi: https://doi.org/10.1038/nn1426.
[58] Sato-Harada R, Okabe S, Umeyama T, Kanai Y, Hirokawa N. Microtubule-associated proteins regulate microtubule function as the track for intracellular membrane organelle transports. Cell Struct Funct 1996. doi: https://doi.org/10.1247/csf.21.283.
[59] Shea TB, Rogers E, Ashline D, Ortiz D, Sheu MS. Apolipoprotein E deficiency promotes increased oxidative stress and compensatory increases in antioxidants in brain tissue. Free Radic Biol Med 2002. doi: https://doi.org/10.1016/S0891-5849(02)01001-8.
[60] Siegel JA, Benice TS, Van Meer P, Park BS, Raber J. Acetylcholine receptor and behavioral deficits in mice lacking apolipoprotein E. Neurobiol Aging 2011. doi: https://doi.org/10.1016/j.neurobiolaging.2008.12.006.
[61] Strittmatter WJ, Saunders AM, Goedert M, Weisgraber KH, Dong LM, Jakes R, et al. Isoform-specific interactions of apolipoprotein E with microtubule-associated protein tau: Implications for Alzheimer disease. Proc Natl Acad Sci U S A 1994. doi: https://doi.org/10.1073/pnas.91.23.11183.
[62] Sullivan PM, Mezdour H, Aratani Y, Knouff C, Najib J, Reddick RL, et al. Targeted replacement of the mouse apolipoprotein E gene with the common human APOE3 allele enhances diet-induced hypercholesterolemia and atherosclerosis. J Biol Chem 1997;272:17972–80. doi: https://doi.org/10.1074/jbc.272.29.17972.
[63] Sullivan PM, Mezdour H, Quarfordt SH, Maeda N. Type III hyperlipoproteinemia and spontaneous atherosclerosis in mice resulting from gene replacement of mouse Apoe with human APOE*2. J Clin Invest 1998;102:130–5. doi: https://doi.org/10.1172/JCI2673.
[64] Takuma H, Arawaka S, Mori H. Isoforms changes of tau protein during development in various species. Dev Brain Res 2003. doi: https://doi.org/10.1016/S0165-3806(03)00056-7.
[65] Thirumangalakudi L, Prakasam A, Zhang R, Bimonte-Nelson H, Sambamurti K, Kindy MS, et al. High cholesterol-induced neuroinflammation and amyloid precursor protein processing correlate with loss of working memory in mice. J Neurochem 2008. doi: https://doi.org/10.1111/j.1471-4159.2008.05415.x.
[66] Ulrich JD, Ulland TK, Mahan TE, Nyström S, Peter Nilsson K, Song WM, et al. ApoE facilitates the microglial response to amyloid plaque pathology. J Exp Med 2018. doi: https://doi.org/10.1084/jem.20171265.
[67] van Ree JH, van den Broek WJAA, Groot PHE, Frants RR, Wieringa B, Hofker MH, et al. Diet-induced hypercholesterolemia and atherosclerosis in heterozygous apolipoprotein E-deficient mice. Atherosclerosis 1994. doi: https://doi.org/10.1016/0021-9150(94)93037-6.
[68] Veinbergs I, Mallory M, Mante M, Rockenstein E, Gilbert JR, Masliah E. Differential neurotrophic effects of apolipoprotein E in aged transgenic mice. Neurosci Lett 1999. doi: https://doi.org/10.1016/S0304-3940(99)00243-8.
[69] Veinbergs I, Mallory M, Sagara Y, Masliah E. Vitamin E supplementation prevents spatial learning deficits and dendritic alterations in aged apolipoprotein E-deficient mice. Eur J Neurosci 2000. doi: https://doi.org/10.1046/j.1460-9568.2000.01308.x.
[70] Veinbergs, I., Mante, M., Jung, M.W., Van Uden, E., Masliah, E., 1999b. Synaptotagmin and synaptic transmission alterations in apolipoprotein E- deficient mice. Prog Neuro-Psychopharmacology Biol Psychiatry 10.1016/S0278-5846(99)00013-5.
[71] Vitek MP, Brown CM, Colton CA. APOE genotype-specific differences in the innate immune response. Neurobiol Aging 2009. doi: https://doi.org/10.1016/j.neurobiolaging.2007.11.014.
[72] Wang H, Kulas JA, Wang C, Holtzman DM, Ferris HA, Hansen SB. Regulation of beta-amyloid production in neurons by astrocyte-derived cholesterol. Proc Natl Acad Sci U S A 2021. doi: https://doi.org/10.1073/pnas.2102191118.
[73] Williams T, Borchelt DR, Chakrabarty P. Therapeutic approaches targeting Apolipoprotein e function in Alzheimer's disease. Mol Neurodegener 2020. doi: https://doi.org/10.1186/s13024-020-0358-9.
[74] Yin C, Ackermann S, Ma Z, Mohanta SK, Zhang C, Li Y, et al. ApoE attenuates unresolvable inflammation by complex formation with activated C1q. Nat Med 2019. doi: https://doi.org/10.1038/s41591-018-0336-8.
[75] Youmans KL, Tai LM, Nwabuisi-Heath E, Jungbauer L, Kanekiyo T, Gan M, et al. APOE4-specific changes in Aβ accumulation in a new transgenic mouse model of alzheimer disease. J Biol Chem 2012. doi: https://doi.org/10.1074/jbc.M112.407957.
[76] Zeke A, Misheva M, Reményi A, Bogoyevitch MA. JNK signaling: regulation and functions based on complex protein-protein partnerships. Microbiol Mol Biol Rev 2016. doi: https://doi.org/10.1128/mmbr.00043-14.
[77] Zhu Y, Nwabuisi-Heath E, Dumanis SB, Tai LM, Yu C, Rebeck GW, et al. APOE genotype alters glial activation and loss of synaptic markers in mice. Glia 2012. doi: https://doi.org/10.1002/glia.22289.