FEATURED ARTICLE

Apolipoprotein E4 disrupts the neuroprotective action of sortilin in neuronal lipid metabolism and endocannabinoid signaling

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Abstract

Introduction: Apolipoprotein E (apoE) is a carrier for brain lipids and the most important genetic risk factor for Alzheimer's disease (AD). ApoE binds the receptor sortilin, which mediates uptake of apoE-bound cargo into neurons. The significance of this uptake route for brain lipid homeostasis and AD risk seen with apoE4, but not apoE3, remains unresolved.

Methods: Combining neurolipidomics in patient specimens with functional studies in mouse models, we interrogated apoE isoform–specific functions for sortilin in brain lipid metabolism and AD.

Results: Sortilin directs the uptake and conversion of polyunsaturated fatty acids into endocannabinoids, lipid-based neurotransmitters that act through nuclear receptors to sustain neuroprotective gene expression in the brain. This sortilin function requires apoE3, but is disrupted by binding of apoE4, compromising neuronal endocannabinoid metabolism and action.

Discussion: We uncovered the significance of neuronal apoE receptor sortilin in facilitating neuroprotective actions of brain lipids, and its relevance for AD risk seen with apoE4.

KEYWORDS
Alzheimer's disease, apolipoprotein E, brain lipoprotein metabolism, DHA, endocannabinoids, lipoprotein receptors, polyunsaturated fatty acids, VPS10P domain receptors

1 | BACKGROUND

Apolipoprotein E (apoE) is the major carrier for lipids in the brain. It is secreted by astrocytes and microglia and delivers essential lipids to neurons that take up apoE-bound cargo via apoE receptors (reviewed in ref 1). Apart from its role in lipid homeostasis, apoE also bears significance as the most important genetic risk factor for the sporadic form of Alzheimer's disease (AD) because carriers of the *APOE ε4* gene variant are at a significantly higher risk of AD than carriers of the common *APOE ε3* allele.[2] A large body of work confirmed the potential of apoE4, as compared with apoE3, to accelerate neurodegenerative processes (reviewed in ref 3). Many hypotheses have been advanced about how apoE may affect brain health; still, the mechanism(s) that distinguish apoE3 and apoE4 functions in brain lipid homeostasis and progression of AD remain controversial.

Previously, we identified the lipoprotein receptor sortilin as a major endocytic route for the uptake of apoE-containing lipoproteins in neurons in vitro and in vivo. In gene-targeted mice, loss of sortilin

impaired neuronal clearance of murine apoE and was associated with enhanced accumulation of amyloid beta (Aβ) peptides and senile plaque formation.[4] However, the relevance of the sortilin-dependent uptake of apoE for brain lipid homeostasis and for the risk of AD seen in carriers of the human *APOE ε*4 genotype remained unclear.

Combining mass spectrometry (MS)-based lipidomics in patient specimens with functional studies in humanized mouse models expressing apoE3 or apoE4, we uncovered a unique role for sortilin and apoE3 in facilitating the neuronal metabolism of polyunsaturated fatty acids (PUFAs) into endocannabinoids (eCBs) that signal an anti-inflammatory gene expression profile in the brain. The ability of sortilin to sustain neuroprotective eCB signaling is disrupted by binding of apoE4, increasing pro-inflammatory markers, and possibly aggravating the amyloidogenic burden in the brain.

2 | METHODS

2.1 | Materials and general methods

AD specimens were collected from donors from whom written informed consent for the use of the material for research purposes had been obtained (see supplementary methods). Mouse strains and details on animal experimentation are also given in supplementary methods. Determination of transcript or protein levels in tissue were performed by quantitative reverse transcription PCR (qRT-PCR) and SDS-PAGE, respectively, using standard protocols. Cell culture experiments involving Chinese hamster ovary (CHO) cells are detailed in supplementary methods.

2.2 | Lipid analyses

Quantification of levels of total cholesterol, triglycerides, and free fatty acids (FAs) in plasma was performed using commercial kits (Biovision, Roche, Cayman). Mouse plasma lipoprotein profiles were established by TNO Biosciences (Leiden, The Netherlands) using fast protein liquid chromatography (FPLC). Targeted lipidomics was performed on brain cortex specimens from human subjects or mice, or on apoE-containing lipoproteins from human cerebrospinal fluid (CSF) or mouse plasma using liquid chromatography-mass spectrometry (LC-MS) as detailed in supplementary methods.

2.3 | Statistical analysis

For all in vivo experiments, an indicated number *n* is the number of mice per group used in an experiment. For primary cell culture experiments, an indicated number *n* is the number of independent glial preparations (biological replicates) used for western blotting or qRT-PCR analyses. For co-localization studies in CHO cells, *n* is the number of cells analyzed in replicate experiments. Each mouse (or biological replicate in a cell culture experiment) represents a statistically independent experimental unit, which was treated accordingly as an independent

RESEARCH IN CONTEXT

1. Systematic review: The authors reviewed the available literature using scientific databases (eg, PubMed). Although the role of sortilin in systemic lipid metabolism and cardiovascular disease is firmly established in several articles (properly cited herein), the relevance of this neuronal apolipoprotein E (apoE) receptor for brain lipid metabolism and the risk of Alzheimer's disease (AD) is unclear.

2. Interpretation: Our findings provide a molecular explanation for the neuroprotective actions of apoE3 in brain lipid metabolism, and why this protective lipid pathway is lost in carriers of the *APOE ε*4 genotype. These findings represent a major conceptual advance in the understanding of pathophysiology of apoE4, the most important genetic risk factor for sporadic AD known to date.

3. Future directions: Future efforts will elucidate the molecular details of a sortilin-dependent neuronal metabolism of lipids, and how this protective lipid pathway may be restored in carriers of the *APOE ε*4 genotype.

value in the statistical analyses. Statistical analyses were performed using GraphPad Prism software. For all data with two independent variables, two-way analysis of variance (ANOVA) with Bonferroni or Tukey multiple comparison test was applied. When comparing apoE3- and apoE4-targeted replacement mice, either wild-type (WT) or knockout (KO) for *Sort1*, *P* values for apoE indicate the impact of *APOE* genotype, irrespective of Sort1 genotype, whereas *P* value for sortilin indicates the impact of *Sort1* genotype, irrespective of *APOE* genotype. The given *P* value for interaction indicates whether the effect of the *Sort1* genotype depends on the *APOE* genotype, or vice versa.

3 | RESULTS

3.1 | Sortilin acts as brain clearance receptor for apoE3 and apoE4

In mice, inactivation of *Sort1*, the gene encoding sortilin, results in brain accumulation of murine apoE due to impaired clearance of the protein by neurons lacking this apoE receptor. Accumulation of murine apoE coincides with increased levels of Aβ peptides in the brain of receptor-deficient mice.[4] Although these findings implicated sortilin as an apoE receptor in AD-related processes, they failed to address the relevance of this receptor for the risk of AD seen in humans expressing apoE4 as compared with apoE3.

Now, we introduced the *Sort1* defect (KO) into mice carrying a targeted replacement of the murine *Apoe* locus with genes encoding human apoE3 (E3;KO) or apoE4 (E4;KO).[5] As shown for murine apoE before,[4] loss of sortilin also caused accumulation of human apoE3
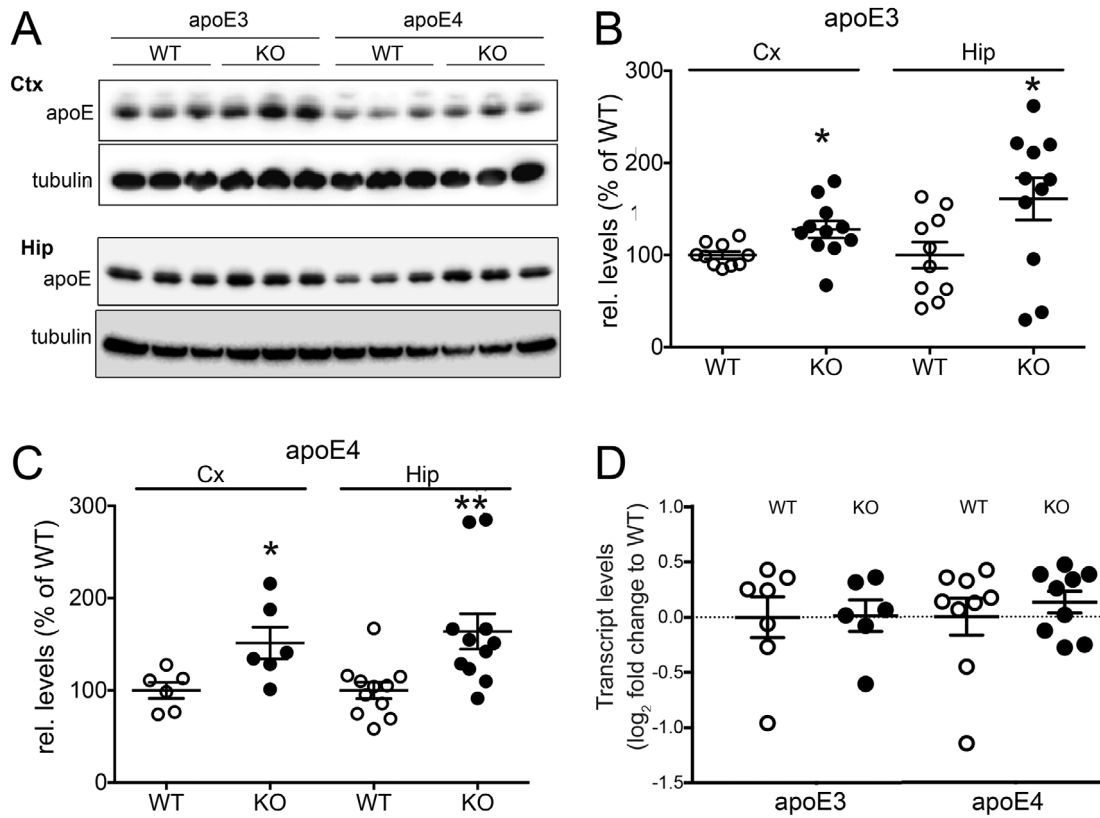
FIGURE 1 Sortilin deficiency causes brain accumulation of apoE3 and apoE4. (A-C) Levels of apoE3 and apoE4 in cortex (Ctx) and hippocampus (Hip) of sortilin WT and KO mice (n = 6-11 mice per group, 3 months of age) were determined by western blot analysis (A) and densitometric scanning of replicate blots (B and C). Values are mean ± standard error of the mean (SEM) given as percent of WT control (mean set to 100%). Accumulation of apoE3 and apoE4 in KO as compared with WT tissues was determined by Student's t test ($^*P$ < .05; $^{**}P$ < .01). Detection of tubulin served as loading control in A. (D) Quantitative RT-PCR analysis of transcript levels for apoE3 and apoE4 in brain extracts of sortilin WT and KO mice at 3 months of age (n = 6-9 mice per group). Values are mean ± SEM given as $\log_2$ fold change compared with levels in the respective WT animals (mean set to 0)

(Figure 1A, B) and apoE4 (Figure 1A, C) in the cortex and hippocampus of KO mice compared with WT. Sortilin deficiency did not impact transcript levels for apoE3 or apoE4 in the brain (Figure 1D), or the levels of apoE3 and apoE4 protein (Figures S1A, B) or transcripts (Figure S1C) in primary astrocytes from (E3;KO) or (E4;KO) mice as compared with WTs. Thus increased brain levels of human apoE3 and apoE4 in KO mice were likely due to impaired neuronal clearance rather than increased astrocytic production of the apolipoproteins. These findings substantiated sortilin as a major brain clearance receptor for murine and human apoE variants alike.

3.2 | Interaction of sortilin and apoE3 controls brain levels of PUFAs and eCBs

Next, we queried an interaction of sortilin with apoE in brain lipid homeostasis that may distinguish between apoE3 and apoE4 actions. Our strategy was based on the documented roles for apoE and the lipoprotein receptor sortilin in control of lipid metabolism,[4,6] and on the importance of neuronal lipid homeostasis for risk of AD (reviewed in ref 7).

Initially, we used LC-MS-based lipidomics to determine the levels of various lipid classes in brain cortices of (E3;KO) and (E4;KO) mice. When compared with WTs, we observed a distinct impact of genotypes on brain levels of total FAs and ω-3 PUFAs, with levels being lower in (E3;KO) as compared with (E3;WT) mice (Figure 2A). By contrast, levels of these lipids were always low in E4 compared with (E3;WT) animals, irrespective of the presence or absence of sortilin. This interaction of *Sort1* and *APOE* in control of brain lipids was seen for ω-3 but not for ω-6 PUFAs (Figure 2A), in line with the neuroprotective actions of ω-3 PUFAs in AD.[8-10]

To delineate the relevance of sortilin and apoE3 for brain PUFA metabolism, we determined levels of bioactive PUFA derivatives in the brains of our four mouse strains (Figure 2B). We focused on eCBs, lipid-based neurotransmitters produced from PUFAs in neurons. Most eCBs act anti-inflammatory and neuroprotective, and alterations in eCB levels have been associated with AD.[11] Of the six eCBs and related lipids tested (Figure 2B), levels of anandamide and linoleoyl-ethanolamide (LEA) were not impacted by genotypes. Levels of oleyl-ethanolamide (OEA) and palmitoyl-ethanolamide (PEA) were reduced in animals lacking sortilin, but irrespective of *APOE* genotype.
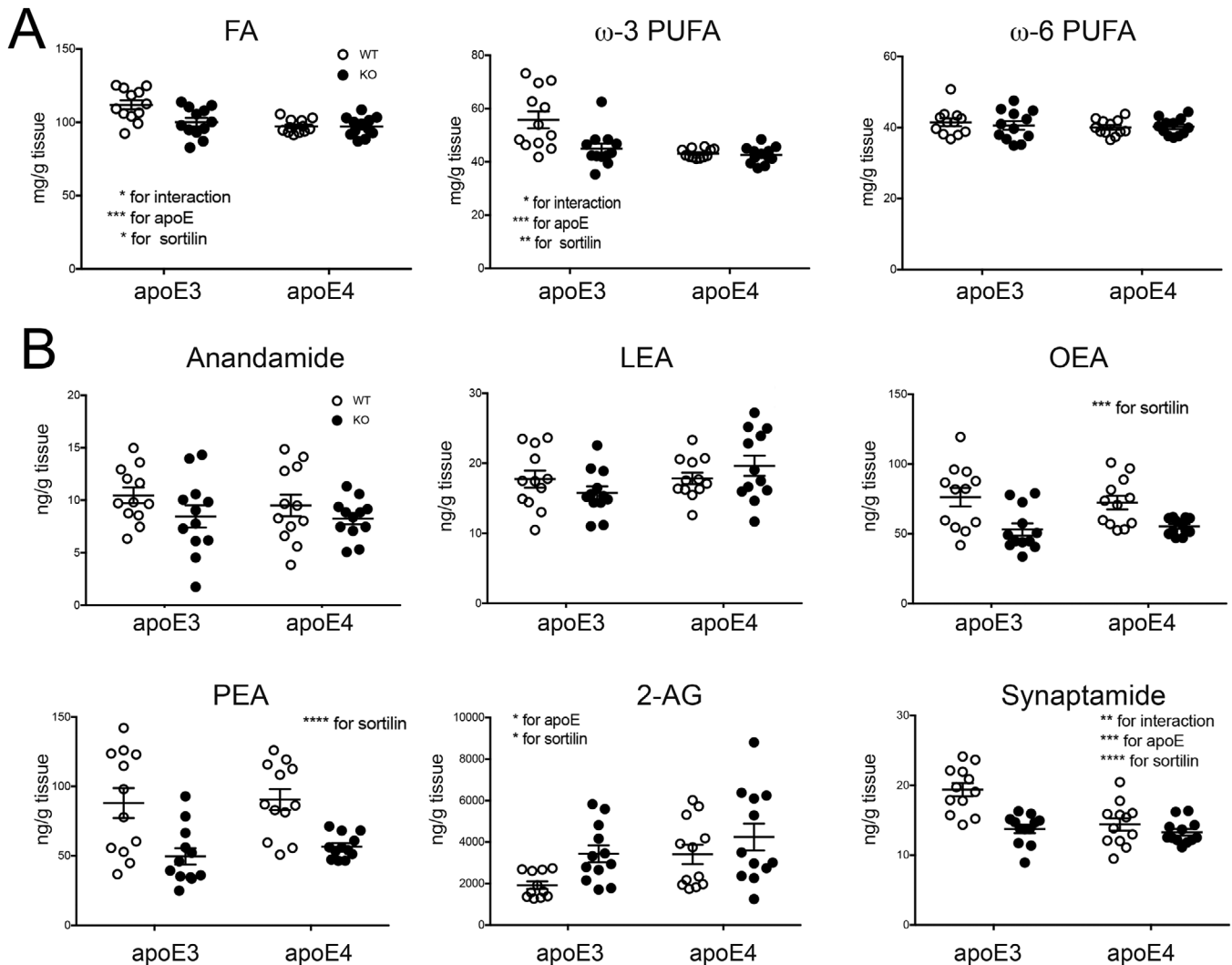
FIGURE 2 Sortilin and apoE3 interact in control of brain levels of polyunsaturated fatty acids (PUFAs) and endocannabinoids. (A) Levels of total FAs as well as of ω-3 and ω-6 PUFAs were determined in brain cortices of apoE3- and apoE4-targeted replacement mice either wild-type (WT) or homozygous for the *Sort1* null allele (KO) (3 months of age, n = 12 per genotype). (B) Levels of endocannabinoids and endocannabinoid-like lipids were determined in brain cortices of mice of the indicated *APOE* and *Sort1* genotypes (3 months of age, n = 11-12 per genotype). Data are the mean ± SEM. The significance of data was determined by two-way analysis of variance (ANOVA) ($^*P < .05$; $^{**}P < .01$; $^{***}P < .001$; $^{****}P < .0001$). 2-AG, 2- arachidonoylglycerol; LEA, linoleoyl-ethanolamide; OEA, oleyl-ethanolamide; PEA, palmitoyl-ethanolamide

However, levels of 2- arachidonoylglycerol (2-AG) and the eCB-like lipid synaptamide showed an interaction between *APOE* and *Sort1*. In detail, physiological 2-AG levels were low in (E3;WT) mice but increased in (E3;KO) animals. By contrast, levels in E4 mice were always high compared with (E3;WT), irrespective of *Sort1* genotype. The converse pattern was seen for synaptamide, with physiological levels being high in (E3;WT) but lower in all other genotype combinations. The dependency of eCB levels on *APOE* genotype was substantiated in brain specimens of AD patients homozygous for *APOE ε3* or *APOE ε4* (Figure 3). As in mice, levels of synaptamide were lower (P < .05) while levels of 2-AG tended to be higher (P = .057) in *APOE ε4/ε4* as compared with *APOE ε3/ε3* carriers. Levels of anandamide were also decreased in the *APOE ε4/ε4* genotype (P < .05), whereas OEA, LEA, and PEA levels were not impacted.

3.3 | *Sort1* and *APOE ε3* safeguard an anti-inflammatory gene expression profile signaled by peroxisome proliferator-activated receptors (PPARs)

eCBs exert their actions by signaling via G protein–coupled cannabinoid receptors CB1 and CB2 or by acting as ligands for peroxisome proliferator-activated receptors (PPARs). Because synaptamide does not engage CB1/2,[12] we explored the relevance of sortilin and apoE3 interaction in eCB metabolism by focusing on PPARs. Using a microarray-based strategy to assess transcript levels of 84 PPAR target genes, we identified 12 brain transcripts that showed dependence on *Sort1* and *APOE*, being either higher or lower in (E3;WT) as compared with the other three genotypes (Figure 4A). Affected transcripts included PPARγ (*Pparg*) and the retinoic X receptor (*Rxrg*), which
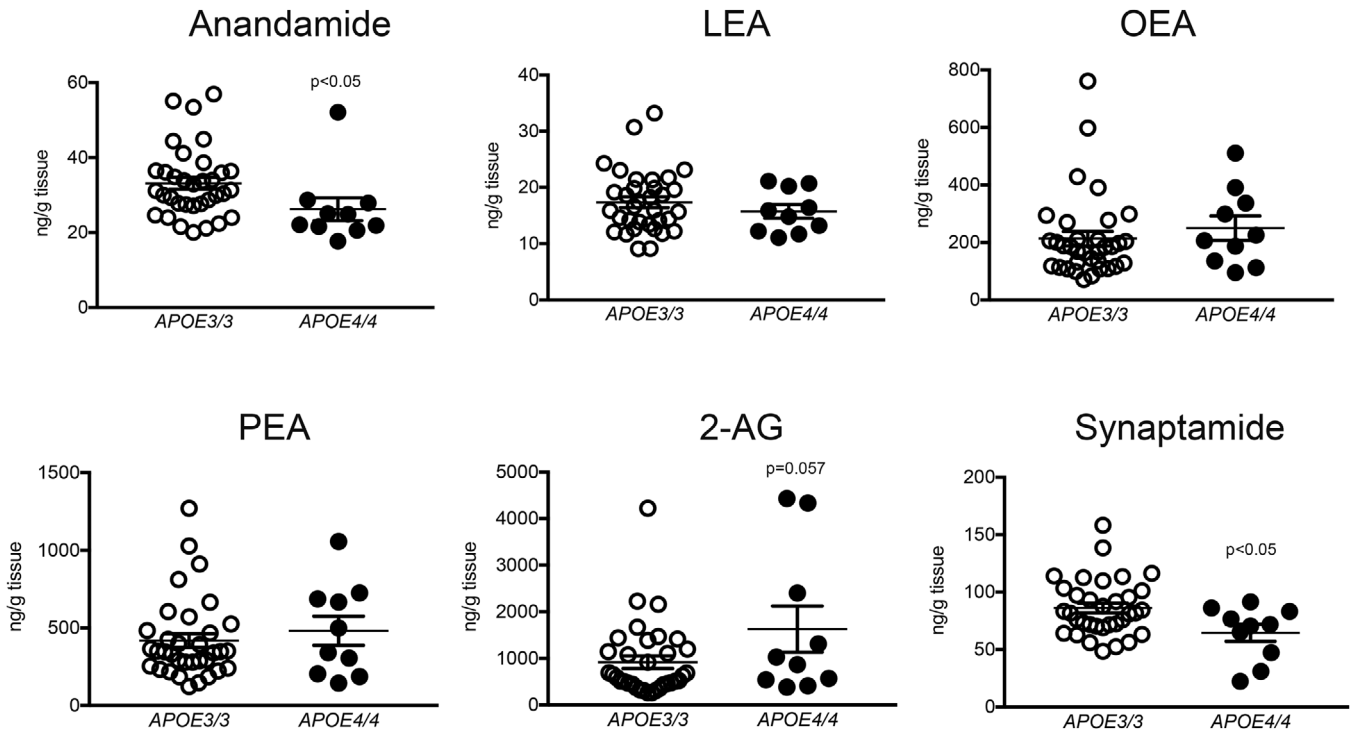
FIGURE 3 ApoE-dependent changes in endocannabinoid (eCB) levels in the human brain. Levels of the indicated eCBs and eCB-like lipids were determined in prefrontal cortex specimens of AD patients homozygous for *APOE ε3* or *APOE ε4* (n = 10 for *APOE ε4/ε4*, n = 34 for *APOE ε3/ε3.*) Values are mean ± standard error of the mean (SEM). The significance of data was determined by Student's *t* test. 2-AG, 2- arachidonoylglycerol; LEA, linoleoyl-ethanolamide; OEA, oleyl-ethanolamide; PEA, palmitoyl-ethanolamide

form heterodimers with PPARs; but also factors in lipid homeostasis, such as fatty acid transporter Slc27a4 and very long-chain acyl-CoA synthetase Slc27a5. Using qRT-PCR for PPAR targets *Pparg, Mmp9,* and *Klf10,* we substantiated an effect of sortilin on eCB-dependent gene transcription in E3 but not in E4 mice (Figure 4B). PPARs are lipid sensors that suppress inflammatory responses by inducing an anti-inflammatory gene expression profile, an activity reducing neurodegeneration.[13,14] In line with interaction of *Sort1* and *APOE ε3* in promoting PPAR activities, loss of sortilin in apoE3 mice or the presence of apoE4 (irrespective of *Sort1* genotype) resulted in alterations in the mouse brains consistent with a pro-inflammatory state. These changes included decreased transcription of *Vegf,* but elevated transcript levels of *Tnfα* and *Gfap* in (E3;KO), (E4;WT), and (E4;KO) mice as compared with (E3;WT) (Figure 4C). A potential pro-inflammatory state in the three mouse strains as compared with (E3;WT) was supported by increased immunosignals for glial fibrillary acidic protein (GFAP) in the brain (Figure 4D, E).

Our data indicated interaction of *Sort1* and *APOE ε3* in safeguarding a neuroprotective metabolism and action of eCBs, potentially protecting the brain from inflammatory insults. This neuroprotective eCB action is lost in apoE3 mice that lack sortilin. By contrast, this neuroprotective action of sortilin in brain lipid metabolism is not supported by apoE4, as PPAR activities are decreased in E4 mice irrespective of *Sort1* genotype.

3.4 | Sortilin and apoE3 do not impact systemic metabolism of PUFAs

Anandamide and 2-AG are produced from arachidonic acid (ARA), while synaptamide is derived from docosahexaenoic acid (DHA). Mainly, ARA and DHA are supplied to the brain from the bloodstream as FA or esterified to phospholipids. In the brain, ARA and DHA are re-esterified to membrane phospholipids or converted into bioactive metabolites, such as eCBs (reviewed in ref 15). To query the impact of sortilin and apoE on PUFA transport into the brain, we quantified lipid levels in brain tissue, and in plasma and brain lipoproteins. Levels of DHA, but not of ARA, were reduced in the brains of (E3;KO), (E4;WT), and (E4;KO) compared with (E3;WT) mice (Figure S2A). A similar trend in reduction of DHA ($P = .1$) but not ARA levels was seen in brain tissues from AD patients with *APOE ε4/ε4* as compared with *APOE ε3/ε3* (Figure S2B). Altered brain levels of DHA were not due to differential association of lipids with lipoproteins containing apoE3 or apoE4, as plasma lipoprotein profiles were indistinguishable comparing all four mouse genotypes (Figure S3A), as were the levels of DHA and ARA in apoE-containing lipoproteins isolated from plasma of these mice (Figure S3B). In addition, levels of DHA and ARA were comparable in apoE3- and apoE4-containing brain lipoproteins isolated from human CSF (Figure S3C-F). Finally, transcript levels of enzymes in neuronal metabolism of 2-AG and synaptamide were not affected by *Sort1*
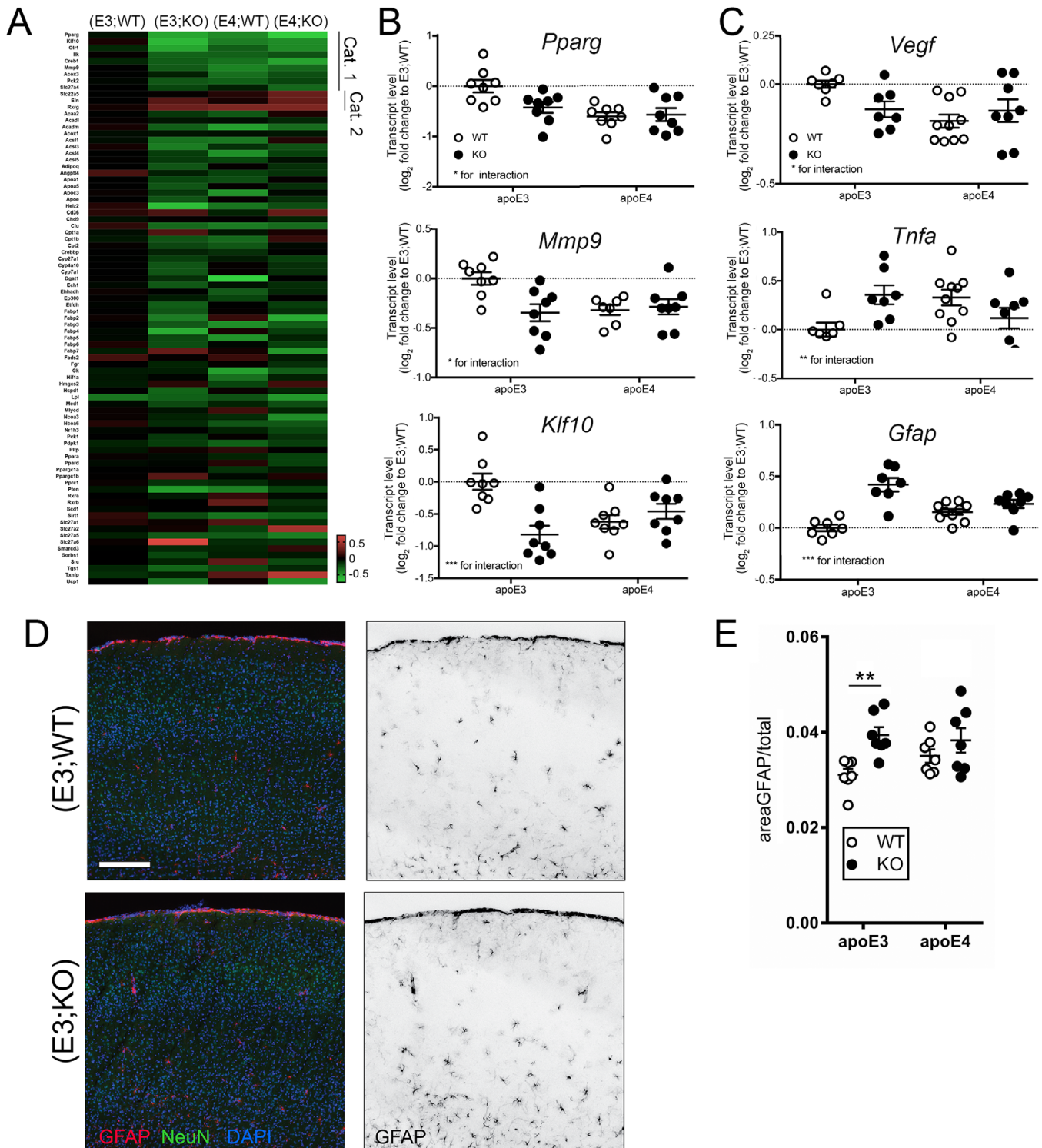
FIGURE 4　Interaction of *Sort1* and *APOE* ε3 controls peroxisome proliferator-activated receptors (PPAR)–dependent gene expression. (A) Heatmap of expression levels of target genes of PPARs in the brain of apoE3- or apoE4-targeted replacement mice either WT or homozygous for the *Sort1* null allele (KO) (Mouse PPAR Targets RTš Profiler PCR Array, Qiagen). Transcripts up (Cat. 1) or down (Cat. 2) in (E3;WT) as compared with the other three genotype groups are highlighted (n = 3-4 mice per group, 3 months of age). (B) Quantitative RT-PCR analysis of transcript levels of the indicated PPAR target genes in brain cortices of apoE3 and apoE4 mice either WT or KO for *Sort1*. Log$_2$ fold changes as compared to (E3;WT) mice set to value 0 are given (n = 7-8 mice per group, 3 months of age; two-way ANOVA). (C) Quantitative RT-PCR analysis of vascular endothelial growth factor (*Vegf*), tumor necrosis factor α (*Tnfa*), and glial fibrillary acidic protein (*Gfap*) in brain cortices of mice of the indicated *Sort1* and *APOE* genotypes. Log$_2$ fold changes as compared to (E3;WT) mice set to value 0 are given (n = 7-10 mice per group; two-way ANOVA). Mice were 18 months of age. (D) Immunodetection of GFAP (red) on exemplary cortical brain sections of (E3;WT) and (E3;KO) mice. Sections were also stained for neuronal marker NeuN (green) and DAPI (blue). Both merged color images (left panels) and single GFAP channels in gray scale

or *APOE* genotype as shown by qRT-PCR on mouse brain extracts (Figure S4).

3.5 | ApoE4 disrupts cell-surface recycling of sortilin

So far, our studies failed to identify changes in biosynthesis or extracellular transport that may explain why alterations in DHA and eCB metabolism in E4 mice resembled defects in apoE3 animals lacking sortilin. Thus, we focused on the hypothesis that apoE4 may disrupt the activity of sortilin, rendering E4 brains essentially sortilin depleted. This hypothesis was based on the propensity of apoE4 to impair intracellular sorting and thereby the activity of several cell surface receptors.[16-18]

Both apoE variants interact with sortilin equally well, as shown previously using surface plasmon resonance analysis.[19] To query whether receptor trafficking, rather than binding, was differentially impacted by apoE variants, we tested co-localization of sortilin with apoE3 or apoE4 in CHO cells using proximity ligation assays (PLAs). Co-localization of sortilin with apoE3 was seen in a scattered vesicular pattern throughout the cytoplasm, whereas co-localization with apoE4 showed a distinct pattern close to the cell membrane (Figure 5A). Altered localization of sortilin complexed to apoE4 was substantiated by co-immunostaining the PLA signal with transferrin (Tf), commonly used to mark the early endocytic and the recycling compartments of cells.[20] The extent of co-localization of sortilin/apoE complexes (PLA signal) with Tf was significantly higher with apoE4 as compared with apoE3 (Figure 5C, D). These findings indicated extended retention of sortilin and apoE4 complexes in endocytic and/or recycling compartments.

To further substantiate the differential impact of apoE4 on sortilin sorting, we established the trafficking path of the receptor in CHO cells by labeling sortilin molecules on the cell surface with antibodies and following their subsequent intracellular route using immunocytochemistry (Figure 6A). Within 15 minutes, labeled receptors internalized from the cell surface (Figure 6A, panel surface labeling) into intracellular compartments (Figure 6A, panel internalization). To interrogate recycling of these internalized receptors, we subsequently treated the cells with dynasore, an inhibitor of endocytosis, to block continuous re-entry of recycled receptors into the cells. Application of dynasore resulted in the accumulation of labeled sortilin molecules at the plasma membrane, confirming cell-surface recycling of internalized (antibody-labeled) receptors (Figure 6A, panel recycling). Application of dynasore did not alter levels of expression of sortilin as shown by qRT-PCR (Figure S5A).

The recycling path of sortilin established in CHO cells was not impacted by the presence of apoE3 in the culture medium (Figure 6B). However, in the presence of apoE4, internalized receptors failed to re-appear on the cell surface and remained largely intracellular (Figure 6B). To more accurately quantify the extent of sortilin recycling in the presence of apoE3 versus apoE4, we biotinylated sortilin molecules on the surface of CHO cells and followed their internalization and recycling fate as schematized in Figure 6C. Recycled receptors accumulating at the cell surface were stripped of their biotin tag by glutathione treatment to only retain a biotin label in the intracellular (non-recycling) receptor pool. The extent of receptor recycling was determined by subtracting the amount of biotinylated receptors purified on streptavidin beads after 60 minutes of recycling from the total amount of biotinylated receptors internalized at the beginning of the experiment. In these studies, the amount of receptors recycling back to the cell surface was reduced by 50% in apoE4-treated as compared to apoE3-treated cells (Figures 6D, E). The detrimental effect of apoE4 was specific for the recycling step of the sorting path as the extent of internalization of sortilin (Figure S5B) or its ligand apoE (Figures S5C, D) was not different comparing apoE3 and apoE4 treatment conditions.

4 | DISCUSSION

We propose a novel concept for the neuroprotective metabolism of PUFA in the brain. It involves interaction of sortilin with apoE3 to support neuronal uptake and action of DHA and eCBs, providing anti-inflammatory gene expression and suppressing noxious insults that possibly deteriorate brain health in AD. Loss of sortilin in apoE3 mice compromises this neuroprotective PUFA uptake pathway, thereby increasing pro-inflammatory gene expression. Similar detrimental effects are seen in WT mice in the presence of apoE4 as it disrupts receptor trafficking.

In mice, loss of sortilin reduces neuronal uptake of apoE and causes alterations in brain lipid homeostasis also seen in apoE KO animals (ie, accumulation of sulfatides).[4] These data suggested sortilin as a key player in apoE-dependent lipid metabolism in the brain. Our new results substantiate this role by identifying the interaction of sortilin with apoE3 in control of neuronal DHA and eCB metabolism and action. It is notable that the same effect of *APOE ε4* on DHA and eCBs levels as in our mouse models is also seen in AD patients, substantiating the clinical relevance of our findings from mouse models.

DHA and its bioactive metabolites are regulators of brain health, linking inflammation with neurodegeneration. DHA is the most abundant brain ω-3 PUFA. It regulates numerous cellular processes, especially the resolution of brain inflammation.[15,21] Low levels of DHA in plasma or brain correlate with human AD,[22-24] and dietary DHA intake lowers Aβ levels in mice.[8-10] A similar role in the control of inflammation in the aging brain has been suggested for anandamide and 2-AG. Anandamide levels decrease in the brains of AD patients[25,26]

(right panels) are given. Scale bar: 250 μm. (E) Quantification of the GFAP-immunoreactive area in the cortex of apoE3 and apoE4 mice either WT or KO for *Sort1* is shown (n = 7 mice per group; two-way ANOVA with Tukey's multiple comparisons test). Mice were 18 months of age. $^*P < .05$; $^{**}P < .01$; $^{***}P < .001$
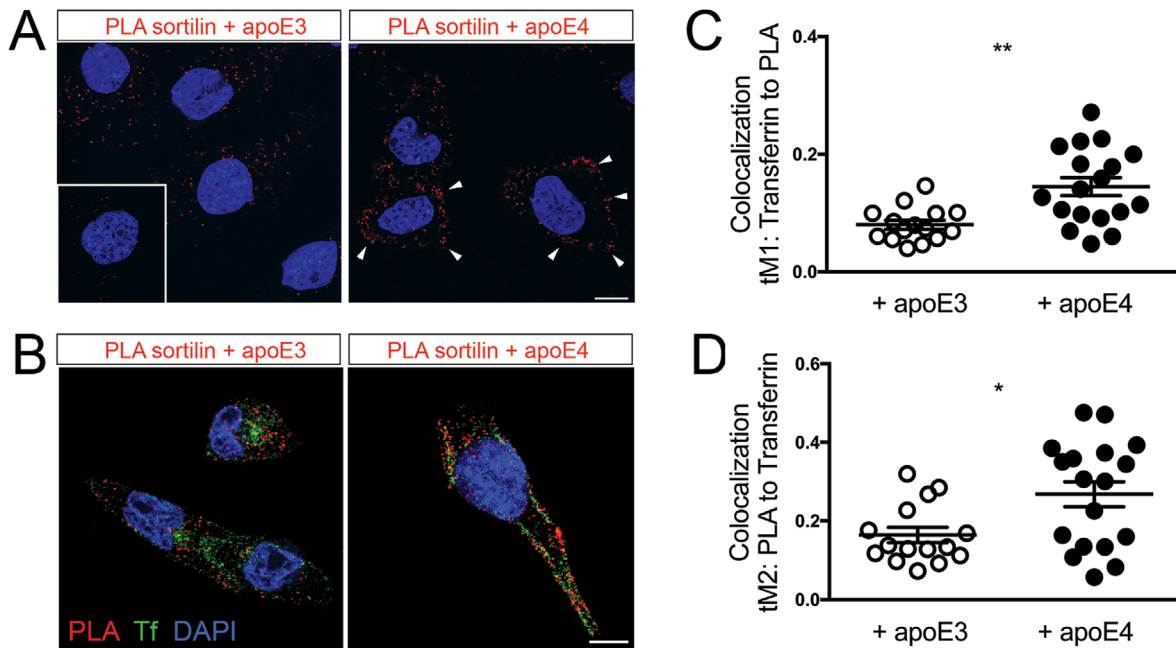
FIGURE 5 ApoE4 alters the intracellular localization of sortilin/ligand complexes. (A) CHO cells stably expressing murine sortilin (CHO-S) were treated for 24 hours at 37 °C with 5 µg/mL of myc-tagged apoE3 or apoE4 produced in HEK293 cells.[4] Co-localization of sortilin and apoE was tested by proximity ligation assay (PLA) using primary antisera directed against sortilin and myc, respectively. PLA signals (red) for colocalization showed a dispersed vesicular pattern with apoE3 but a juxtamembrane pattern with apoE4 (white arrowheads). The inset documents the absence of PLA signals in cells not treated with apoE. Cell nuclei were counterstained with DAPI (blue). (B) CHO-S cells were treated for 2 hours with 5 µg/mL of myc-tagged apoE3 or apoE4, followed by incubation with 25 µg/mL of Alexa Fluor 647-conjugated transferrin (Tf) for another 20 minutes. Subsequently, complex formation between sortilin and apoE was detected by PLA (red signal), while fluorescent Tf conjugates (green signal) marked early endocytic and recycling compartments. Cell nuclei were counterstained with DAPI (blue). (C, D) Co-localization of sortilin and apoE complexes (PLA signal) with Tf was quantified as thresholded Manders' values (tM) in experiments exemplified in panel B. Colocalization of receptor/ligand complexes in Tf-positive cell compartments was significantly higher in apoE4 as compared with apoE3 treated cells as shown by Manders' values tM1 (C) and tM2 (D). Data are given as mean ± SEM (n = 15-18 cells for each condition; Student's t test). *P < .05; **P < .01. Scale bars in A and B: 10 µm

and mouse models of AD.[27] Conversely, levels of 2-AG increase in AD patients[28] and mouse models,[29] and increased 2-AG signaling exacerbates synaptic failure.[30] Although little is known about synaptamide, it is considered a bioactive mediator of DHA, as it recapitulates the protective actions seen with DHA.[31] We document a pathological increase in brain levels of 2-AG and a concomitant decrease in DHA, anandamide, and synaptamide in humans and mice with apoE4, and in apoE3 mice lacking sortilin, identifying the relevance of *Sort1* and *APOE* genotypes for brain PUFA homeostasis.

We focused on the relevance of sortilin and apoE3 interaction for DHA and eCB signaling by investigating PPARs. Transcript levels of PPAR-γ increase in AD patients to counteract neuroinflammatory insults.[32] Non-steroidal anti-inflammatory drugs ameliorate AD-related processes due to their ability to stimulate PPAR and inhibit inflammatory responses.[33,34] In line with a function for sortilin and apoE3 in PPAR actions, we observed a concordant dysregulation of transcriptional targets in E4 mice and in E3 animals lacking sortilin (Figure 4A, B). The relevance of *Sort1* and *APOE ε3* to counteract brain inflammation was supported by an increase in pro-inflammatory markers GFAP and tumor necrosis factor α (TNFα) (Figure 4C–E). GFAP indicates brain inflammation in rodent AD models,[35] and increases in GFAP

levels in rat models of AD are reverted by eCB application.[36] Increased levels of TNFα are also linked to AD pathology.[37,38] Another sign of a potentially pro-inflammatory state in E4 mice and in (E3;KO) animals is downregulation of vascular endothelial growth factor (VEGF) (Figure 4C). VEGF suppresses inflammatory brain response[39] and reduces neurodegeneration in mouse models of AD.[40,41]

Sortilin facilitates cellular uptake of apoE3 and apoE4 equally well, both in vitro (Figure S5C, D) and in vivo (Figure 1A–C). In addition, levels of PUFA bound to apoE3- or apoE4-containing lipoprotein in plasma or brain are comparable (Figure S3). Thus, apoE variants likely do not discriminate the types of PUFA taken up into neurons via sortilin. Rather it is the detrimental effect of apoE4 on trafficking of sortilin that impairs this neuronal uptake route for lipids. Following endocytic uptake, apoE3 recycles to the cell surface for re-secretion. This route is not taken by apoE4 that accumulates in the endocytic pathway.[16,17,42] Impaired recycling of apoE4 disrupts cell surface re-exposure of apoE binding proteins, as shown for APOER2[18] or the insulin receptor,[20] mechanisms believed to contribute to impaired synaptic plasticity and altered brain insulin signaling in AD, respectively. Consequently, reversal of impaired recycling has been suggested as therapeutic approach for AD risk imposed by apoE4.[43] We now show a similar
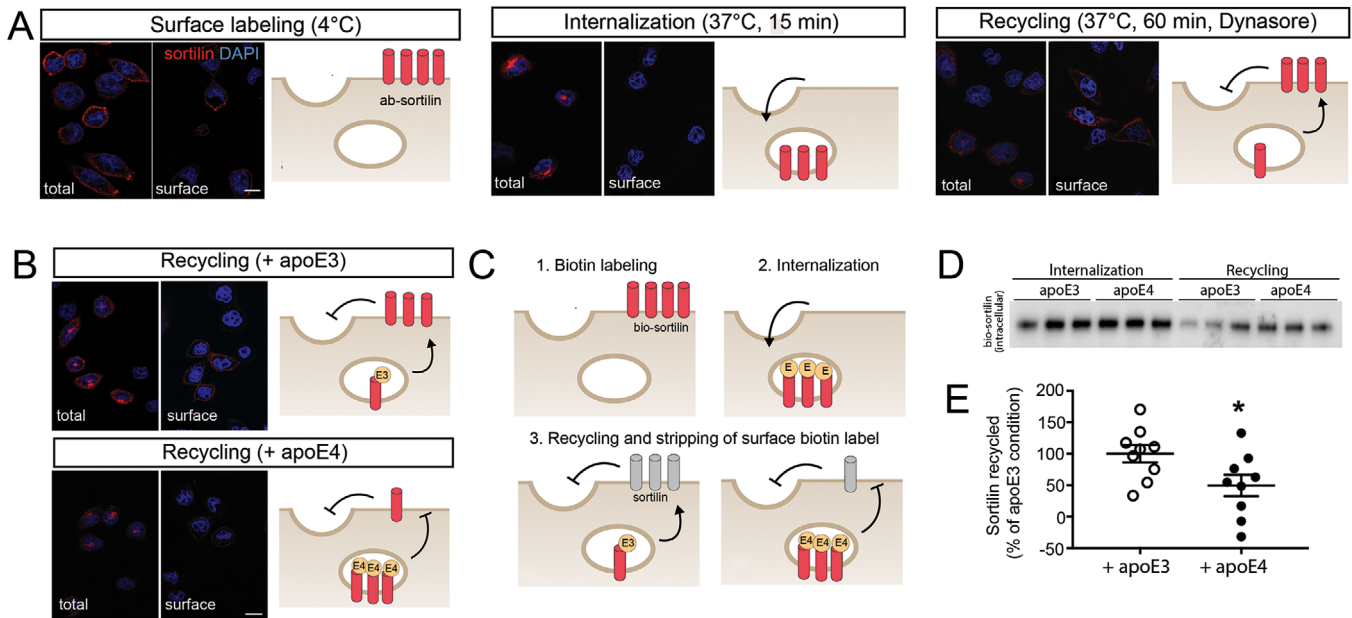
FIGURE 6 ApoE4 disrupts cell surface recycling of sortilin. (A) Immunodetection of sortilin (red) in CHO-S cells under the indicated treatment conditions. Cells were either permeabilized (total) or non-permeabilized (surface) to distinguish between total and cell-surface receptor pools. Cells were counterstained with DAPI (blue). Scale bar: 10 μm. Schematics visualize the proposed trafficking fate of sortilin in the respective images. *Panel Surface labeling*: Receptor molecules at the cell surface were decorated with primary antibody (ab-sortilin, red symbols) in CHO-S cells kept at 4 °C to disrupt membrane trafficking. Immunosignals for sortilin in non-permeabilized cells (surface) confirm plasma membrane localization of the labeled receptors. *Panel Internalization*: Following shift to 37 °C for 15 minutes, labeled receptors move to an intracellular localization as evidenced by immunosignals in the permeabilized (total) but not in the non-permeabilized (surface) condition. *Panel Recycling*: To confirm re-appearance of internalized receptors at the cell surface, cells were subsequently treated with dynasore for 60 minutes at 37 °C. When endocytosis was blocked with dynasore to prevent re-uptake of surface localized receptors, labeled sortilin molecules accumulated at the cell surface as evidenced by immunosignals in non-permeabilized cells (surface), substantiating a recycling path for the receptor. (B) Experiment as in (A) but cells were incubated in apoE3- or apoE4-conditioned medium. Antibody-tagged sortilin molecules recycled to cell surface in the presence of apoE3 as documented by immunosignals in non-permeabilized cells (upper panels). No receptor recycling was observed in the presence of apoE4 as evidenced by the absence of immunosignals in the non-permeabilized condition (lower panels). (C) Experimental strategy to quantify sortilin recycling in the presence of apoE3 or apoE4. Sortilin molecules at the surface of CHO-S cells kept at 4 °C were biotinylated (panel labeling; bio-sortilin, red receptor symbols). Following shift to 37 °C for 15 minutes in the presence of 5 μg/mL of apoE3 or apoE4 (yellow symbols), receptors remaining at the cell surface were stripped of their biotin label using glutathione (GSH) treatment (panel 2, Internalization). Finally, cells were incubated for 60 minutes at 37 °C in the presence of dynasore and then with GSH, stripping all recycled receptors accumulating at the surface of biotin label (panel 3, Recycling; gray receptor symbols). (D) Western blot analysis of biotinylated sortilin molecules affinity-purified on streptavidin beads from CHO-S cells after internalization and recycling phases as described in (C, steps 2 and 3). (E) The amount of biotinylated (bio-)sortilin was determined by densitometric scanning of replicate western blots as exemplified in (D). The percentage of recycled receptors was determined by subtracting the amount of biotinylated (intracellular) receptors at the recycling phase (step 3) from that at internalization (step 2). Mean values for apoE3 were set to 100%. Significantly fewer receptors recycled in the presence of apoE4 than apoE3 (49.56 ± 16.92% vs 100 ± 13.84%). Data are given as mean ± SEM (n = 9 biological replicates from three independent experiments; Student's *t* test). $^*P$ <.05

deleterious impact of apoE4 on sortilin trafficking (Figures 5 and 6), likely explaining the loss of anti-inflammatory actions of this receptor in eCB metabolism in the apoE4 brain.

In conclusion, we identify a novel pathway linking neuronal apoE handling by sortilin with the well-known neuroprotective actions of PUFA in the brain, and we provide a possible molecular explanation for the risk of AD seen with apoE4.

CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

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SUPPORTING INFORMATION

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