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FEATURED ARTICLE

Protein phosphatase 2A and complement component 4 are linked to the protective effect of *APOE* ε2 for Alzheimer's disease

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Abstract

Introduction: The apolipoprotein E (*APOE*) ε2 allele reduces risk against Alzheimer's disease (AD) but mechanisms underlying this effect are largely unknown.

Methods: We conducted a genome-wide association study for AD among 2096 ε2 carriers. The potential role of the top-ranked gene and complement 4 (C4) proteins, which were previously linked to AD in ε2 carriers, was investigated using human isogenic *APOE* allele-specific induced pluripotent stem cell (iPSC)–derived neurons and astrocytes and in 224 neuropathologically examined human brains.

Results: *PPP2CB* rs117296832 was the second most significantly associated single nucleotide polymorphism among ε2 carriers ($P = 1.1 \times 10^{-7}$) and the AD risk allele

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increased *PPP2CB* expression in blood ($P = 6.6 \times 10^{-27}$). *PPP2CB* expression was correlated with phosphorylated tau231/total tau ratio ($P = .01$) and expression of C4 protein subunits *C4A/B* ($P = 2.0 \times 10^{-4}$) in the iPSCs. PPP2CB (subunit of protein phosphatase 2A) and C4b protein levels were correlated in brain ($P = 3.3 \times 10^{-7}$).

Discussion: PP2A may be linked to classical complement activation leading to AD-related tau pathology.

KEYWORDS
Alzheimer's disease, apolipoprotein E, C4B, human induced pluripotent stem cells, PPP2CB, tau protein

1 | INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disorder and accounts for 60% to 80% of all cases of dementia. None of the prescribed medications for symptomatic treatment of AD retard or stop neuronal degeneration.[1] AD currently affects about 5.8 million Americans age 65 and older and will increase to 13.8 million by 2050 if current trends continue.[1] AD is the sixth leading cause of death in the United States and its mortality rate increased 146% between 2000 and 2018.[1] The apolipoprotein E (*APOE*) genotype is the strongest risk factor for the common form of AD that occurs after age 65 years.[2] Combinations of amino acid residues at 112 (rs429358) and 158 (rs7412) determine three common *APOE* alleles (ε2, ε3, and ε4), where ε4 increases but ε2 decreases AD risk.[3,4] Lifetime risk of AD among female ε4 homozygotes is ≈60% and 10 times higher than for ε2 carriers matched for sex and age.[3,4]

Neuropathological hallmarks of AD are neurofibrillary tangles (NFTs) consisting of decomposed microtubules and phosphorylated tau (p-tau) and neuritic plaques (NPs) containing toxic amyloid beta (Aβ) peptides.[1] The protective effect of ε2 on tangle burden is independent from that on plaque burden and specific to AD pathology.[5] An exaggerated modifying effect of *APOE* alleles on clinical and pathological manifestations of AD was demonstrated by the recent discovery of the rare *APOE* Christchurch (APOEch) mutation on a ε3 chromosome background in a carrier of the deleterious presenilin 1 (*PSEN1*) E280A mutation that causes early onset AD typically between ages 30 and 60 among members of a large kindred with autosomal dominant AD. Notably, the E280A mutation carrier presented with delayed cognitive impairment in her seventies, and showed profound plaque burden but limited NFT involvement by positron emission tomography (PET) imaging.[6] In fact, accumulation of tau protein (the primary constituent of tangles) measured by PET is strongly associated with memory decline and most prominent in the medial temporal lobe.[7] Although these studies confirm that ε2 and other rare *APOE* mutations attenuate AD risk and AD-related pathology, the underlying mechanisms are unknown.

Previously we reported genome-wide association study (GWAS) findings in non-Hispanic Whites[8] and multi-ethnic populations[9] assembled by the Alzheimer's Disease Genetics Consortium (ADGC) stratified by *APOE* ε4 carrier status. In this study, we used a series of epidemiological, computational, and experimental approaches to identify genetic factors contributing to the protective effect of ε2 (Figure 1). First, we conducted a GWAS for AD among ε2 carriers in a large cohort assembled by the ADGC. Top-ranked genes from the GWAS that were previously associated with Aβ and tau were selected to identify ε2-related biological networks. Next, the functional relevance to AD of the gene appearing most central to AD-related pathways was investigated experimentally in human isogenic *APOE* induced pluripotent stem cell (iPSC)–derived neurons and astrocytes, and in brain tissue of neuropathologically confirmed AD cases and controls.

RESEARCH IN CONTEXT

1. **Systematic review**: The authors reviewed the literature using traditional (e.g., PubMed) as well as preprinted (e.g., medRxiv) sources on genetic association, human induced pluripotent stem cells (iPSCs), and neuropathology in human autopsied brains for Alzheimer's disease (AD).
2. **Interpretation**: We show that the mechanism underlying the protective effect of apolipoprotein E ε2 against AD is linked to the classical complement protein modulation on tau pathology. A protein phosphatase 2A (PP2A) catalytic subunit protein, PPP2CB, and complement 4B, C4b, are key proteins in this mechanism. This provides a new insight on the relationship between complement-mediated neuroinflammation and tau pathology.
3. **Future directions**: Follow-up functional genomics experiments of the top-ranked variant in the human iPSC neurons and astrocytes validation are warranted. Future research can extend to establish a novel blood biomarker for complement and astrocytic phenotypes linking to neuroinflammation in the brain. This could lead to novel drug development or repurposing existing PP2A- or complement-targeted drugs for treating AD.

2 | METHODS

2.1 | Subjects and phenotypic evaluation

The study included 34 ADGC cohorts containing 14,031 subjects meeting clinical or neuropathological criteria for probable AD and 14,471 cognitively unimpaired controls for whom *APOE* genotype and genome-wide single nucleotide polymorphism (SNP) array data were available (Table S1 in supporting information).[2] We excluded subjects who were younger than 65 years of age at the time of censoring (onset of symptoms for AD cases or last examination/death for controls), ε2/ε4 subjects, and entire cohorts containing fewer than 10 cases and 10 controls from analyses in the total sample or within *APOE* genotype subgroups. Characteristics of the remaining 15,132 subjects (4970 AD cases; 10,162 controls) from 29 cohorts with *APOE* genotypes ε2/ε2 or ε2/ε3 (485 AD cases; 1611 controls) and ε3/ε3 (4485 AD cases; 8551 controls) who were included in the analysis are provided in Table S2 in supporting information.

A diagnosis of probable AD among clinically evaluated subjects in ADGC datasets was established by Diagnostic and Statistical Manual of Mental Disorders 4th Edition (DSM-IV) criteria or the National Institute of Neurological and Communicative Disorders and Stroke/Alzheimer's Disease and Related Disorders Association criteria for probable AD.[10] Controls were cognitively unimpaired based on an assessment using a cognitive screening test. The ADGC sample included 5007 neuropathologically examined subjects (4018 cases; 989 controls).[5]

2.2 | Genotyping, quality control, and imputation

We applied quality control filters to exclude individuals with genome-wide genotype call rate < 95%, discordance between reported and genetically determined sex, and individuals of non-European ancestry.[2] Within each dataset, SNPs with a call rate < 95%, Hardy-Weinberg equilibrium $P < 10^{-6}$, and minor allele frequency (MAF) < 0.01 were excluded. Principal components (PC) of ancestry were determined separately in each cohort using EIGENSOFT.[11] The first three PCs were included as covariates in all subsequent analyses to correct for population substructure. SNP genotype probabilities were imputed using the Haplotype Reference Consortium (HRC) reference panel (https://imputationserver.sph.umich.edu/). SNPs with MAF < 0.01 or

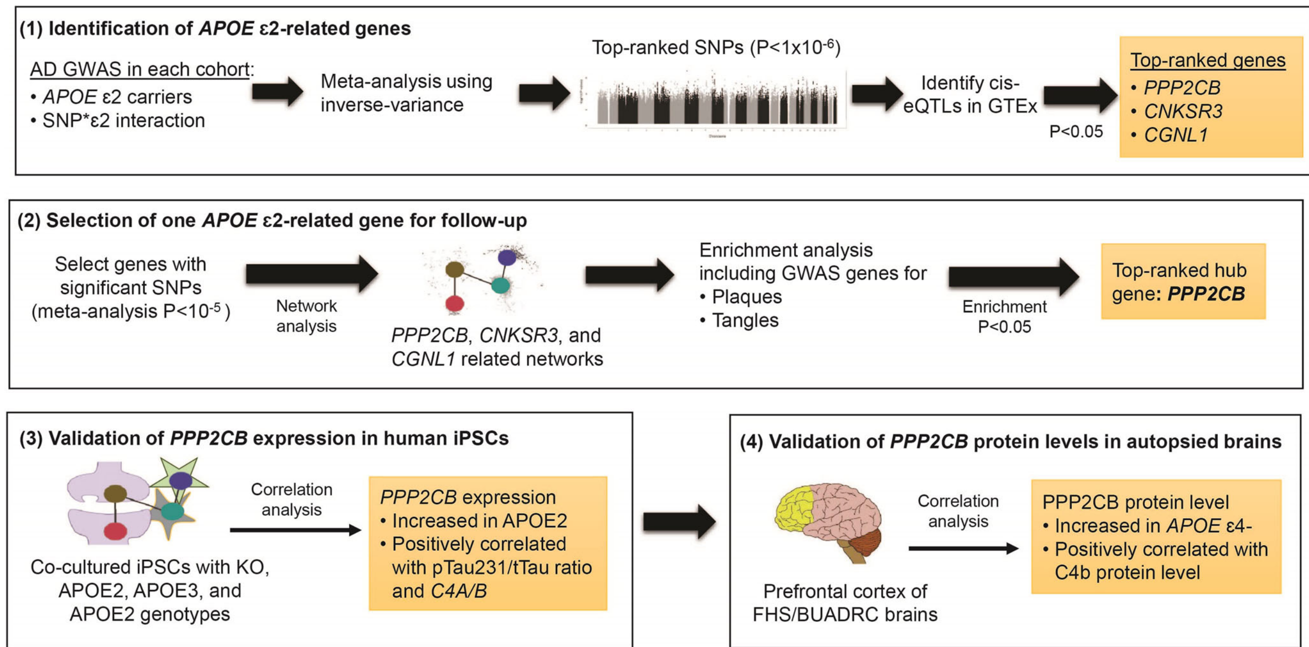

FIGURE 1 Study design. AD, Alzheimer's disease; *APOE*, apolipoprotein E; BUADRC, Boston University Alzheimer's Disease Research Center; eQTL, expression quantitative trait loci; FHS, Framingham Heart Study; GWAS, genome-wide association study; SNP, single nucleotide polymorphism

imputation quality (R^2) < 0.8, and SNPs missing in > 20% of the total sample were excluded.

2.3 | Genome-wide association analyses

The association of AD was tested with each SNP using a logistic regression model including a quantitative estimate between 0 and 2 representing the probability of the effect allele to incorporate the uncertainty of the imputation estimates and covariates to adjust for age, sex, and PCs. The MIRAGE and NIA-LOAD cohorts were analyzed using generalized estimating equation (GEE) models to account for family structure. Genome-wide association (GWA) analysis for the main effect of a SNP was conducted separately in each cohort and *APOE* ε2 carriers including ε2/ε2 and ε2/ε3 genotypes. To assess interaction between a SNP and ε2, models included a main effect for dose of ε2 coded as 0, 1, or 2 and a term for the interaction between the SNP and ε2 dosage in ε2 non-carriers comprising ε2/ε2, ε2/ε3, and ε3/ε3 genotypes (Table S2). Odds ratios (ORs) and 95% confidence intervals (95% CIs) were computed for the SNP or *APOE* x SNP interaction. The results were combined by meta-analysis after correcting for genomic inflation within each dataset using the inverse variance method in METAL.[12] Results for SNPs that were missing in > 20% of the constituent datasets were excluded.

2.4 | Gene expression and pathway analyses

We evaluated genotype-specific differential expression of the top-ranked SNPs ($P < 10^{-5}$) in the GWAS using GTEx data (https://www.gtexportal.org). Analyses were limited to SNPs that modulate expression of nearby genes, that is, cis-eQTLs (expression quantitative trait loci), in any tissue. The modifying potential of the top-ranked SNPs on expression and methylation in neuropathologically examined human brains was assessed using data generated from Religious Orders Study and Memory and Aging Project (ROSMAP) participants.[13] Biologically correlated gene networks were constructed using the Ingenuity Pathway Analysis (IPA; QIAGEN) software and seed genes containing SNPs that were significantly associated with AD ($P < 10^{-5}$) among ε2 carriers or through interaction with ε2. Networks that contained one of three top-ranked genes and more than five seed genes were selected for further validation by enrichment analysis including genes containing a SNP previously associated ($P < 10^{-3}$) with measures of NPs or NFTs.[14] We followed up the top-ranked gene, *PPP2CB*, as well as *C4A* and *C4B*, which were recently linked to *APOE* ε2 by transcriptome analysis.[15]

2.5 | Differentiation of *APOE* genotype-specific human iPSC-derived neurons and astrocytes

The human parental iPSC line for *APOE* ε4/ε4, isogenic iPSC lines for *APOE* ε2/ε2 or *APOE* ε3/ε3, and an *APOE* knock-out (KO) iPSC line were purchased from ALSTEM (ALSTEM Inc.). These *APOE* isogenic iPSCs were then differentiated into neurons and astrocytes as previously described.[16–18] Briefly, iPSCs were rapidly induced into human excitatory neurons over 24 days via a doxycycline-inducible neurogenin2 (NGN2) system by lentiviral infection of pLV-TetO-hNGN2-eGFP-Puro (Addgene plasmid #79823) and FUdeltaGW-rtTA (Addgene plasmid #19780). Human iPSCs were differentiated to neural progenitor cells (NPCs) using the STEMdiff SMADi Neural Induction Kit (STEMCELL Technologies) and subsequently to astrocytes in astrocyte medium

(ScienCell). Human iPSC-derived astrocytes after day 54 of differentiation were co-cultured with the differentiated neurons at day 15. After 9 days upon co-culture, neurons were collected for subsequent experimental assays. Details of iPSC characterization and differentiation, as well as the generation of the neuron/astrocyte co-culture system, are described in the supporting information.

2.6 | Measurement of PPP2CB and C4A/B expression in co-culture system

Expression levels of *PPP2CB* and *C4A/B* among *APOE* isogenic cells were measured using quantitative reverse transcription polymerase chain reaction (qRT-PCR). Total tau (t-tau), p-tau, and Aβ levels from the co-cultured system were evaluated by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions. Additional details of procedures for RNA isolation, qRT-PCR, and ELISA measurements are provided in the supporting information.

2.7 | Evaluation of PPP2CB and C4a/b proteins in human brain tissue

The association of protein levels for catalytic subunit of the PP2A enzyme complex encoded by *PPP2CB* and *PPP2CA* with AD, AD-related biomarkers (including Aβ_{42}, phosphorylated tau 181 [p-tau181], phosphorylated tau 231 [p-tau231], and t-tau), and C4a and C4b proteins was assessed in dorsolateral prefrontal cortex (Brodmann area 8/9) tissue obtained from 224 participants in the Framingham Heart Study (FHS) and Boston University Alzheimer's Disease Research Center (BUADRC). Details about ascertainment and clinical examination of these subjects were previously reported.[15] Proteins C1q and CRP were also measured because recent studies demonstrated ApoE isoforms bind C1q protein and modulate classical complement-dependent synapse loss and neuroinflammation,[19] and plasma C-reactive protein (CRP) levels modulate *APOE* genotype-dependent onset of AD.[20] Procedures for preparing frozen tissues and lysates, and immunoassay measurements, are provided in the supporting information. All immunoassay measures were adjusted for age at death and sex. The residuals of these measures were rank-transformed for subsequent association analyses. We tested associations of PPP2CA and PPP2CB levels with measures of Aβ_{42}, p-tau181/t-tau ratio, p-tau231/t-tau ratio, C1q, C4a, C4b, and CRP as quantitative outcomes using linear regression models including covariates for age at death and sex. Analyses were conducted in the total sample as well as in *APOE* genotype ε2/ε3, ε3/ε3, and ε3/ε4 groups.

3 | RESULTS

3.1 | Novel *APOE* ε2-related associations with AD

Genome-wide scans for AD revealed moderate evidence of genomic inflation in the ε2 group ($\lambda < 1.13$) and based on the model including a term for the interaction between ε2 and each SNP ($\lambda < 1.07$; Figure S1 in supporting information). Four SNPs showed suggestive evidence for association using ε2 carriers (Table 1; Figure S2 in supporting information), among which *PPP2CB* SNP rs117296832 was nearly genome-wide significant ($P = 1.1 \times 10^{-7}$, Figure S3a in supporting information). Only one of these SNPs was associated with AD at a nominal significance level in other *APOE* genotype groups (rs1708845 in *RIC8B* in ε4/ε4 subjects, $P = 4.0 \times 10^{-4}$), but the effect was in the opposite direction (Table S3 in supporting information). Significant associations were also observed for interactions of ε2 with *CGNL1* SNP rs17239735 ($P = 4.8 \times 10^{-9}$, Figure S3b) and *CNKSR3* SNP rs17239735 ($P = 4.6 \times 10^{-7}$, Figure S3c). The significant interaction between rs17239735 and ε2 is explained in part by a nominally significant association of this SNP in ε3/ε3 subjects in the opposite direction

TABLE 1 Novel associations ($P < 10^{-6}$) for Alzheimer's disease risk among *APOE* ε4 non-carriers

						APOE ε2 carriers[a]			SNP x ε2 Interaction[b]		
SNP	Locus	CH	A1	A2	Freq1	OR	95% CI	*P*	OR	95% CI	*P*
rs117296832	*PPP2CB*	8	A	G	0.03	3.94	2.37–6.54	1.1E-07	2.46	1.56–3.86	9.8E-05
rs76084405	*STAT5B*	17	A	G	0.03	4.84	2.68–8.72	1.6E-07	4.27	2.33–7.80	2.5E-06
rs77786537	*RIPOR2*	6	G	A	0.11	1.97	1.51–2.56	5.8E-07	1.94	1.52–2.49	1.1E-07
rs78802006	*KDM4C*	9	C	G	0.04	3.79	2.25–6.40	6.0E-07	2.66	1.53–4.63	5.6E-04
rs17038845	*RIC8B*	12	A	G	0.03	3.55	2.16–5.86	6.7E-07	2.55	1.58–4.13	1.3E-04
rs57056064	*RBFOX1*	16	C	T	0.06	2.34	1.67– 3.28	7.7E-07	1.88	1.37–2.57	7.8E-05
rs17239735	*CGNL1*	15	C	T	0.19	1.72	1.37–2.16	3.8E-06	1.83	1.49–2.24	4.8E-09
rs9478555	*CNKSR3*	6	A	G	0.17	1.53	1.22–1.92	2.4E-04	1.70	1.38–2.08	4.6E-07

Abbreviations: *APOE*, apolipoprotein E; CH, chromosome; A1, risk allele; CI, confidence interval; Freq1, risk allele frequency; OR, odds ratio; PCs, principal components; SNP, single nucleotide polymorphism.
[a]SNP association results among ε2/ε2 and ε2/ε3 subjects.
[b]Association results for the interaction of the SNP with ε2 carrier status.
Notes: OR, 95% CI, and *P*-value (*P*) were calculated in each study using a logistic regression model including covariates for age, sex, and PCs. Meta-analysis was conducted using the inverse variance option with correction of the genomic inflation factor in each study.

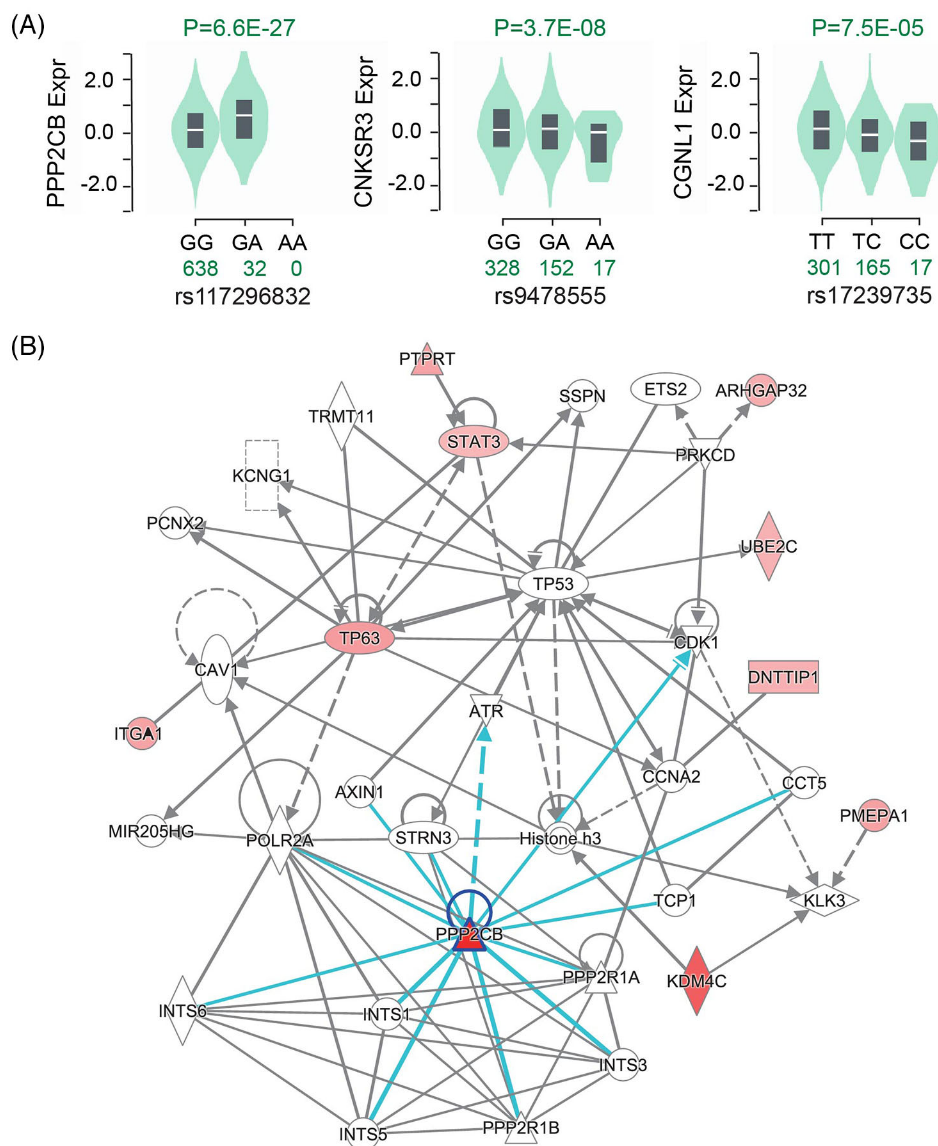

FIGURE 2 Apolipoprotein E (*APOE*) ε2-related genes. A, The most significant tissue-specific cis-eQTLs (expression quantitative trait loci) involving the top-ranked single nucleotide polymorphisms (SNPs) from the genome-wide association study. SNP genotypes are shown on the X-axis and the normalized gene expression level is indicated on the Y-axis. Genotype-specific results are shown for influence of rs117296832 on *PPP2CB* expression in whole blood (left), rs9478555 on *CNKSR3* expression in esophagus-mucosa (middle), and rs17239735 on *CGNL1* expression in cultured fibroblasts (right). B, Top-ranked *APOE* ε2-related network derived from the genome-wide association results in *APOE* ε2 carriers. Gradient for red color of the seed genes represents significance level for association with Alzheimer's disease (AD) among *APOE* ε2 carriers. *PPP2CB* is a hub gene that is also the most significantly AD-associated gene in this network ($P = 1 \times 10^{-7}$)

(Table S3). None of these six SNPs were significant at $P < 6.0 \times 10^{-3}$ in subjects lacking the ε2 allele (Table S3).

Three of the eight SNPs associated with AD in ε2-related analyses were also significant cis-eQTL SNPs (Figure 2A). AD risk allele for rs117296832 (*A*) significantly increased *PPP2CB* expression in whole blood ($P = 6.6 \times 10^{-27}$), whereas the AD risk alleles for rs9478555 (*A*) and rs17239735 (*C*) significantly decreased expression of *CNKSR3* in esophagus-mucosa tissue ($P = 3.7 \times 10^{-8}$) and *CGNL1* in cultured fibroblast cells ($P = 7.5 \times 10^{-5}$), respectively. None of these SNPs were associated with expression or methylation levels in brain tissue.

The ε2-related gene network containing *PPP2CB* (Table S4 in supporting information) was significantly enriched with genes previously associated with density of NPs and NFTs[14] and includes nine suggestive GWAS genes in ε2 carriers ($P < 10^{-5}$): *ARHGAP32*, *DNTTIP1*, *ITGA1*, *KDM4C*, *PMEPA1*, *PTPRT*, *STAT3*, *TP63*, and *UBE2C* (Figure 2B). Genes in this network are involved in several pathways including RNA polymerase II transcription, stress response, regulation of TP53, Rho GTPases, wingless/integrated signaling, protein folding, apoptosis, and mitotic checkpoint (Table S5 in supporting information).

3.2 | *PPP2CB* and *C4A/B* expression are linked to *APOE* ε2 in iPSC-derived neurons and astrocytes

We derived neurons (iNeurons) and astrocytes (iAstrocytes) using isogenic *APOE* KO, ε2/ε2 (APOE2), ε3/ε3 (APOE3), and ε4/ε4 (APOE4) human iPSC lines (Figure S4a in supporting information) and generated rapidly induced excitatory iNeurons within 4 weeks by overexpression of neuronal transcriptional factor neurogenin-2 (Figure 3A, red). To generate iAstrocytes, iPSCs were differentiated into NPCs (Figure S4b), and subsequently induced into iAstrocytes. Human iNeurons with isogenic *APOE* genotypes were co-cultured with the isogenic iAstrocytes for 9 days starting from day 15 using cell inserts assembled in the six-well plates (Figure 3B).

Because astrocytes mainly produce and secrete brain ApoE to the extracellular space,[21,22] we measured the amount of intracellular and secreted ApoE levels from APOEKO-, APOE2-, APOE3-, or APOE4-carrying iAstrocytes in the neuron–astrocyte co-culture system. APOE2 iAstrocytes exhibited the highest intracellular ApoE level compared to other iAstrocytes. There was no discernable ApoE production in APOE KO iAstrocytes and ApoE secretion was significantly reduced in conditioned media from APOE2 and APOE4 compared to APOE3 iAstrocytes (Figure 3C). The influence of *APOE* genotypes on AD-related proteins including t-tau, p-tau181, p-tau231, Aβ_{40}, and Aβ_{42} were quantified in iNeurons derived from each iPSC line. We observed the highest level of p-tau231/t-tau ratio in APOE2 iNeurons and the p-tau181/t-tau ratio was lowest in APOE4 iNeurons, compared to APOEKO or other *APOE* genotype iNeurons (Figure 3D). There were no significant differences of Aβ_{40} or Aβ_{42} levels among various *APOE* iNeurons (Figure S4c).

Co-expression of *PPP2CB* with *C4A/B* was investigated in isogenic *APOE* iNeurons in the neuron–astrocyte co-culture system. *PPP2CB* expression was significantly increased in APOE2 compared to APOE3 and APOE4 iNeurons, whereas there were no significant differences in *C4A/B* expression among the APOE-defined groups of iNeurons (Figure 2E). We identified a positive correlation of *PPP2CB* expression with p-tau231/t-tau ratio ($P = .013$) but not with p-tau181/t-tau ratio ($P = .74$; Figure 3F and Figure S4d). *C4A/B* expression was inversely correlated with Aβ_{42} level (Figure 2G), but not correlated with levels of p-tau231 or p-tau181 (Figure S4d). Taken together, we validated the positive correlations between *PPP2CB* (by GWAS) and *C4A/B* (by transcriptome analysis[15]) with AD in human brain tissue by establishing a correlation between *PPP2CB* and *C4A/B* expression ($P = 2.0 \times 10^{-4}$) in iNeurons co-cultured with isogenic iAstrocytes regardless of the *APOE* genotype of the iNeuron (Figure 3H).

3.3 | PPP2CB and C4b protein levels are correlated in the brain

The level of PPP2CB protein was significantly lower ($P = .008$) and the Aβ_{42} level was significantly higher ($P = .005$) among ε4 carriers compared to subjects lacking ε4, noting that the mean PPP2CB level trended higher in ε2 carriers compared to subjects with other *APOE* genotypes (Table 2 and Figure S5a in supporting information). Levels of other proteins including PPP2CA (Figure S5c) were not different between ε4 carriers and non-carriers (Table 2). As expected, protein levels of PPP2CA and PPP2CB were significantly correlated ($P = 2 \times 10^{-7}$) and this pattern was very similar across *APOE* genotype groups (Table 3). Both PPP2CA and PPP2CB levels were negatively correlated with Aβ_{42} level among ε3/ε4 subjects ($P < .01$; Table 3 and Table S6 in supporting information). The levels of PPP2CA and PPP2CB were inversely associated with the Aβ_{42} level ($P < 0.01$), while the level of PPP2CA, but not PPP2CB, was positively associated with the p-tau231 level among ε3/ε4 subjects ($P = .0053$, Table S6). These results suggest that in the presence of *APOE* ε4 both PPP2CA and PPP2CB may have a role in processing Aβ, and PPP2CA may also be involved in tau phosphorylation. We observed a significant correlation between PPP2CB and C4b levels in the entire sample ($P = 3.3 \times 10^{-7}$), a pattern which was evident among subjects with and without ε4 (Table 3). In contrast, PPP2CA and PPP2CB levels were uncorrelated with C4a level (Table 3 and Table S6). We also observed significant association between PPP2CA and CRP levels in the total sample ($P = 6.4 \times 10^{-4}$), whereas the association of PPP2CB and CRP levels was not nominally significant ($P > .05$) but trended in the same direction (Table 3 and Table S6). C4b and Aβ_{42} levels were correlated ($P = .047$), whereas p-tau231 level was significantly correlated with levels of C4a ($P = .023$) and CRP ($P = .0024$; Table S7 in supporting information).

4 | DISCUSSION

4.1 | Key findings

This study provides evidence that the mechanism underlying the protective effect of *APOE* ε2 is distinct from the deleterious effect of *APOE* ε4 on AD risk. Using a genome-wide approach, we identified SNPs in eight loci that are significantly associated with AD risk among *APOE* ε2 carriers or through interaction with the ε2 allele among persons lacking the ε4 allele. The fact that the top-ranked SNPs were not associated with AD among individuals lacking ε2 suggests that the effect of these SNPs on AD risk are dependent upon the presence of at least one copy of the ε2 allele. Three of these SNPs are linked to significant cis-eQTLs involving *PPP2CB*, *CNKSR3*, and *CGNL1*. Network analysis seeded with genes showing at least suggestive evidence of association with AD identified biological networks containing *PPP2CB*, *CNKSR3*, and *CGNL1*. One of the networks containing *PPP2CB* as the top-ranked hub gene was significantly enriched with genes previously associated with plaques and tangles. A role for *PPP2CB* and its connection to the complement 4 protein was established by experiments conducted in iNeurons and iAstrocytes differentiated from iPSCs co-cultured with cells containing a single ApoE isoform or *APOE* KO, as well as assessment of proteins measured in neuropathologically examined brain tissue from AD cases and controls.

Substantial evidence suggests that ε4 binds Aβ more aggressively than ε3 leading to greater deposition and less effective clearance of Aβ.[23,24] A previous study showed that ε4 in the presence of

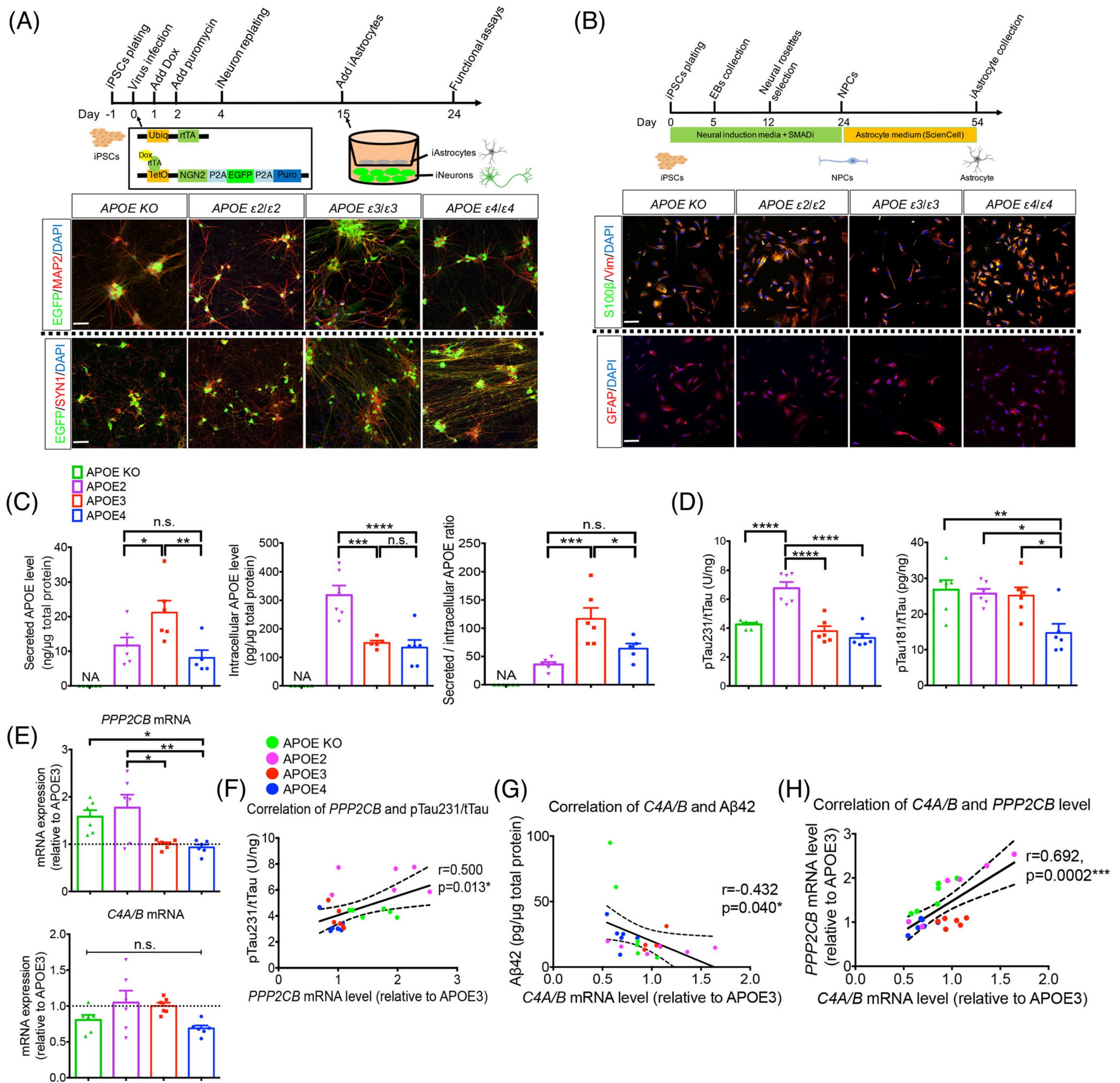

FIGURE 3 Correlation of *PPP2CB* with *C4A/B* gene expression and Alzheimer's disease (AD)-related protein levels in isogenic apolipoprotein E (*APOE*) human induced pluripotent stem cell (iPSC)-derived neurons co-cultured with astrocytes. A, Scheme for generating iPSC-induced neurons and immunocytochemistry with MAP2 and synapsin1 (SYN1) antibodies in neurons. Scale bar, 75 μm. B, Scheme for generating iPSC-derived astrocytes and immunocytochemistry with astrocytic markers (S100β, Vimentin, and GFAP). Scale bar, 75 μm. C, Secreted ApoE in conditioned media from iPSC-neurons/astrocytes co-cultures and intracellular ApoE from iAstrocytes were measured by enzyme-linked immunosorbent assay. n = 5~6 independent cultures per cell line. D, Levels of phosphorylated tau (p-tau)231/total tau (t-tau) and p-tau181/t-tau in iPSC-derived neurons were measured by quantitative ELISA with six independent cultures per cell line. E, Expression of *PPP2CB* and *C4A/B* determined by quantitative reverse transcription polymerase chain reaction and normalized to APOE3 neurons with six independent cultures per cell line. Correlations of (F) *PPP2CB* expression with pT231/t-tau, (G) *C4A/B* expression with amyloid beta (Aβ)42, and (H) co-expression of *PPP2CB* with *C4A/B* in total iPSC-neurons samples. The dashed line indicates 95% confidence band of the best-fit line. n = 24. Data expressed as mean ± standard error of the mean, one-way analysis of variance with Tukey's post hoc test, two-sided. Pearson correlation coefficients are shown. $^*P < .05$, $^{**}P < .01$, $^{***}P < .001$, $^{****}P < .0001$

TABLE 2 Protein levels measured in brain in the FHS and BUADRC datasets by *APOE* genotype

	ALL			ε2/ε3			ε3/ε3			ε3/ε4			ε4− versus ε4+		
Protein	N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD	β	SE	*P*-value
Aβ42	224	0.01	1.00	44	−0.39	1.11	112	0.03	0.94	32	0.43	1.01	0.53	0.19	5.3×10^{-3}
p-tau181	165	−0.04	0.97	32	−0.32	0.97	84	0.03	0.97	24	0.08	0.97	0.20	0.21	0.36
p-tau231	221	0.00	0.98	44	−0.03	0.93	109	0.01	0.98	32	0.17	1.10	0.18	0.19	0.33
t-tau	221	−0.01	0.99	44	0.25	1.21	109	−0.15	0.96	32	−0.14	0.85	−0.07	0.19	0.71
p-tau181/t-tau ratio	157	0.00	1.00	35	−0.09	1.26	83	0.04	0.95	26	−0.04	0.90	0.03	0.21	0.90
p-tau231/t-tau ratio	219	−0.01	0.98	44	−0.20	0.96	107	0.06	0.93	32	0.27	0.96	0.34	0.18	0.06
PPP2CA	203	0.00	1.00	38	−0.14	1.19	104	0.01	0.97	31	−0.01	0.97	0.00	0.20	0.99
PPP2CB	203	0.00	1.00	38	0.07	0.96	104	0.06	1.06	31	−0.45	0.92	−0.52	0.19	8.0×10^{-3}
C1q	184	−0.01	1.00	24	0.00	1.27	107	0.01	0.99	32	−0.18	0.85	−0.22	0.19	0.25
C4a	203	0.00	1.00	38	−0.16	0.99	104	0.01	0.96	31	0.17	1.08	0.18	0.19	0.33
C4b	203	0.00	1.00	38	0.04	1.23	104	0.03	0.94	31	−0.14	1.02	−0.22	0.19	0.26
CRP	214	0.02	0.98	41	0.00	0.82	110	0.16	0.92	32	0.18	0.99	0.02	0.17	0.92

Abbreviations: Aβ, amyloid beta; *APOE*, apolipoprotein E; BUADRC, Boston University Alzheimer's Disease Research Center; N, sample size; p-tau, phosphorylated tau; SD, standard deviation; SE, standard error; t-tau, total tau.

TABLE 3 Association of PPP2CB protein level with classical complement and AD-related proteins by *APOE* genotype

	ALL			ε2/ε3				ε3/ε3				ε3/ε4			
Outcome	β	SE	*P*-value	N	β	SE	*P*-value	N	β	SE	*P*-value	N	β	SE	*P*-value
Aβ42	−0.11	0.07	0.13	53	−0.19	0.18	0.31	118	0.13	0.09	0.13	45	−0.58	0.18	$\mathbf{2.4 \times 10^{-3}}$
p-tau181	−0.11	0.08	0.20	34	−0.11	0.20	0.60	90	−0.05	0.11	0.63	30	−0.31	0.22	0.17
p-tau231	−0.06	0.07	0.38	53	−0.15	0.17	0.38	115	−0.05	0.09	0.61	45	−0.11	0.22	0.63
t-tau	0.12	0.07	0.10	53	0.13	0.21	0.53	115	0.20	0.09	0.03	45	−0.19	0.16	0.25
p-tau181/t-tau	0.13	0.08	0.10	30	0.46	0.23	0.05	82	0.12	0.10	0.25	26	0.05	0.20	0.81
p-tau231/t-tau	−0.13	0.07	0.07	53	−0.05	0.16	0.74	113	−0.13	0.09	0.13	45	−0.04	0.19	0.83
PPP2CA	0.35	0.07	$\mathbf{2.4 \times 10^{-7}}$	53	0.66	0.17	$\mathbf{4.9 \times 10^{-4}}$	118	0.31	0.09	$\mathbf{4.4 \times 10^{-4}}$	45	0.32	0.18	0.09
CRP	0.12	0.07	0.09	48	0.37	0.15	0.02	113	0.14	0.09	0.10	42	0.22	0.19	0.27
C1q	0.10	0.07	0.16	30	0.35	0.26	0.20	107	0.05	0.10	0.60	39	0.18	0.17	0.28
C4a	0.11	0.07	0.12	53	0.14	0.17	0.42	118	0.10	0.09	0.25	45	−0.03	0.22	0.89
C4b	0.35	0.07	$\mathbf{3.3 \times 10^{-7}}$	53	0.49	0.19	0.01	118	0.28	0.08	$\mathbf{8.7 \times 10^{-4}}$	45	0.59	0.17	$\mathbf{1.8 \times 10^{-3}}$

Abbreviations: Aβ, amyloid beta; AD, Alzheimer's disease; *APOE*, apolipoprotein E; β, effect size; p-tau, phosphorylated tau; SD, standard deviation; SE, standard error; t-tau, total tau.
Note: Significant after multiple testing correction (0.05/11 outcomes = 0.0045) are highlighted in bold.

Aβ promotes tangle pathology while ε2 is associated with fewer tangles, but both ε4 and ε2 are not associated with tangle pathology in the absence of Aβ.[25] Importantly, the role of *APOE* ε2 in AD has not been intensively studied and is often assumed to mirror the action of ε4 because it is associated with less Aβ.[25] Rather, our findings suggest that ε2 may regulate the interaction between the catalytic subunit of protein phosphatase 2A (PP2A) and tau phosphorylation. This idea is consistent with the correlation of PPP2CB protein with p-tau181/t-tau measured in the brain in the small sample of ε2 carriers ($P = .05$) for which the effect size was approximately four times and nine times larger than observed for ε3/ε3 and ε3/ε4 subjects, respectively (Table 3). In addition, a *PPP2CB* SNP allele that was significantly associated with AD risk in ε2 carriers increased *PPP2CB* expression, which in turn correlated with p-tau231/t-tau and *C4A* and *C4B* expression in iNeurons co-cultured with isogenic iAstrocytes. The correlation of *PPP2CB* and *C4B* expression is consistent with the correlation of their

corresponding protein levels in neuropathologically examined human brains. Previously, we reported *C4A* and *C4B* are the most significantly differentially expressed genes in brain tissue from AD cases and controls with the *APOE* ε2/ε3 genotype.[15] These findings suggest the possibility that PP2A dysfunction triggers activation of the classical complement cascade leading to increased tau phosphorylation,[26] whereas the ApoE2 isoform may attenuate the effect of the classical complement cascade on events leading to AD.[19] However, correlations of two catalytic subunit proteins, PPP2CA and PPP2CB, with C4a or C4b proteins seem distinct with stronger correlation between PPP2CB and C4b levels.

We also identified a genome-wide significant interaction of ε2 with *CGNL1* SNP rs17239735. *CGNL1* encodes a member of the cingulin family, which localizes to adherens and tight cell–cell junction and regulates the activity of the small GTPases RhoA and Rac1 (http://genecards.org). In mice, the Cgnl1 protein is enriched in central nervous system endothelial cells in the blood–brain barrier[27] and isi perturbed during traumatic brain injury.[28]

4.2 | PP2A and classical complement proteins

Previously, it was shown that *PPP2CA* expression in the hippocampus[29] and PP2A activity in frontal and temporal areas[30] are significantly reduced in persons with AD. PP2A/Bα (brain PP2A enzyme) binds to tau at residues 221-396 with greater affinity for tau isoforms containing the adult four repeat (4R) than fetal tau.[31] PP2A enzymatic activity is negatively correlated with tau phosphorylation levels at multiple sites in the human brain and this association is greater at tau position 231 than 181.[32] Tau phosphorylation at the 231 site significantly decreases binding of tau to PP2A leading to poor dephosphorylation activity by the brain PP2A enzyme.[33,34] These reports are consistent with our finding of significant correlation of the amount of p-tau231 but not p-tau181 with mRNA expression of *PPP2CB* in iPSCs regardless of *APOE* genotype. However, the correlation of p-tau231 with PPP2CA and PPP2CB protein levels seemed largely dependent on *APOE* genotype, indicating the role of the PPP2CA and PPP2CB isozymes may be distinct in brain. Among ε4 carriers, the level of PPP2CA, but not PPP2CB, protein level was correlated with p-tau231. Plasma p-tau231 level differentiates the clinical stages of AD and tau pathology earlier than the plasma p-tau181 level.[35] The level of p-tau231, but not p-tau181, in cerebrospinal fluid is dependent on *APOE* ε4 carrier status.[36]

Complement 4 is a key component of the classical complement pathway, an innate immune system. *C4A* and *C4B* are among the group of major histocompatibility complex (MHC) III genes located between MHC I and MHC II gene clusters. The MHC locus has been consistently reported as one of the most significant association signals for late onset AD.[2,37] However, it has been challenging to disentangle variants in this region that are in high linkage disequilibrium. A previous study showed a statistically significant increase in the repeat length of copy number variants in both *C4A* and *C4B* in AD compared to control subjects.[38] Nerl et al. reported a high relative risk (RR = 8.8) of AD associated with a *C4B* variant in a study of 42 AD cases and 59 age-matched controls,[39] but other studies were unable to confirm this finding.[40,41] A recent analysis of UK Biobank data found that *C4A* expression is associated with cognitive performance and brain atrophy.[42] ApoE isoforms bind C1q protein and modulate classical complement-dependent synapse loss and neuroinflammation.[19] Although we did not observe significant correlations of C1q with p-tau and Aβ_{42} levels in the brain, C1q and CRP levels were highly correlated, suggesting that C1q-mediated neuroinflammation may not be AD-specific.

4.3 | Limitations

Our findings should be considered in light of several caveats. First, the sample of ε2 carriers especially among AD cases is small and thus reduced power for a GWAS in this *APOE* genotype group. This problem also hampered opportunities for replication. To mitigate this limitation, we also evaluated a model including a term for the interaction between ε2 and SNP in a much larger sample including ε2 carriers and ε3/ε3 subjects. Second, arguably the significance of our association findings is less than reported because of moderate genomic inflation (i.e., $\lambda = 1.07$-1.13). However, this degree of inflation does not meaningfully impact the highlighted results. Third, our co-culture system may not represent a true microenvironment of neurons and astrocytes in *post mortem* brains due to a lack of microglia. In fact, we did not detect *APOE*-genotype-dependent effects of Aβ in the co-culture system. In addition, we tested one isogenic line for iPSC experiments, as this was the only isogenic line available with the required *APOE* KO, ε2/ε2, ε3/ε3, and ε4/ε4 backgrounds for the experiments. Nonetheless, our newly developed co-culture system using isogenic *APOE* genotype-specific human iPSC-derived neurons and astrocytes demonstrated that this system is robust for assessing effects of *APOE* and *APOE*-genotype-dependent changes related to AD. Fourth, we were unable to compare *PPP2CB* allele-specific differences on expression and protein levels in iPSC-derived neurons and astrocytes because all of these cells were derived from the same isogenic parental lines. Fifth, the small number of brains from *APOE* ε2 carriers, especially in the FHS/BUADRC dataset, restricted our ability to evaluate fully *APOE*-genotype-specific associations with PP2A catalytic subunits, complement cascade proteins, and AD-related proteins. The small sample size may also explain the lack of significant cis-eSNPs in the ROSMAP brain dataset. Furthermore, information about cognitive performance and medication use prior to death was unavailable for many of the subjects in the autopsy sample, and thus we could not adjust for these variables in brain tissue analyses. Sixth, results of the follow-up analyses in iPSCs are not corrected for multiple testing, which was difficult to determine because independence of many comparisons is unclear. However, using a very conservative threshold, most of the comparisons with PPP2CB protein level (Table 3) remain significant after correction. Seventh, our focus on mechanisms underlying the protective effect of ε2 obscured potentially important findings from other *APOE* genotype groups. Finally, we are aware of a recent study showing that p-tau217 measured in plasma accurately discriminated AD from other neurodegenerative

disorders,[43] but were unable to assess this metabolite in our sample due to lack of a reliable assay tool.

4.4 | Conclusions

Efforts in ApoE-targeted therapeutics including modification of the ApoE4 structure, modulation of ApoE lipidation, inhibiting ApoE and Aβ interaction, and development of ApoE-mimetic peptides have been largely unsuccessful.[44] Given the well-documented high correlation between tau and cognitive impairment in humans,[7] our study suggests that modifying the interaction between PP2A and complement pathway components may reduce tau phosphorylation. Thus, extensive follow-up studies validating this novel link are warranted and may lead to novel drug development or repurposing existing PP2A- or complement-targeted drugs for treating AD.[45–47]

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CONFLICTS OF INTEREST

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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