

# Phospholipid dysregulation contributes to ApoE4-associated cognitive deficits in Alzheimer's disease pathogenesis

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**The apolipoprotein E4 (ApoE4) allele is the strongest genetic risk factor for developing sporadic Alzheimer's disease (AD). However, the mechanisms underlying the pathogenic nature of ApoE4 are not well understood. In this study, we have found that ApoE proteins are critical determinants of brain phospholipid homeostasis and that the ApoE4 isoform is dysfunctional in this process. We have found that the levels of phosphoinositol biphosphate (PIP<sub>2</sub>) are reduced in postmortem human brain tissues of ApoE4 carriers, in the brains of ApoE4 knock-in (KI) mice, and in primary neurons expressing ApoE4 alleles compared with those levels in ApoE3 counterparts. These changes are secondary to increased expression of a PIP<sub>2</sub>-degrading enzyme, the phosphoinositol phosphatase synaptojanin 1 (synj1), in ApoE4 carriers. Genetic reduction of synj1 in ApoE4 KI mouse models restores PIP<sub>2</sub> levels and, more important, rescues AD-related cognitive deficits in these mice. Further studies indicate that ApoE4 behaves similar to ApoE null conditions, which fails to degrade synj1 mRNA efficiently, unlike ApoE3 does. These data suggest a loss of function of ApoE4 genotype. Together, our data uncover a previously unidentified mechanism that links ApoE4-induced phospholipid changes to the pathogenic nature of ApoE4 in AD.**

Alzheimer's disease | apolipoprotein E4 | phospholipid | dysregulation | cognitive deficits

The apolipoprotein E4 (ApoE4) allele is a primary genetic risk factor for sporadic Alzheimer's disease (AD) (1). Although only ~20% of humans are ApoE4 carriers, these individuals account for up to 65% of all AD cases. Elucidation of the contribution of ApoE4 to AD pathogenesis has been a considerable challenge. The mechanisms that underlie the link between ApoE4 genotype and AD are not yet well understood, but have been suggested to involve reduced clearance of brain A $\beta$  (2, 3). Other A $\beta$ -independent mechanisms have been implicated (4), including alterations in brain membrane lipid composition and metabolism (5–7). For example, evidence suggests that in postmortem AD brains, certain alterations in brain membrane lipid composition are exaggerated by the ApoE4 genotype (5–8). In addition, a recent paper reported changes in serum levels of 10 phospholipids as predicting phenotype conversion from normal aging to either amnesic mild cognitive impairment (MCI) or early AD in 2–3-y intervals with more than 90% accuracy (9). These findings suggest that specific phospholipid homeostasis may play an important role in the pathogenesis of AD, and possibly in ApoE4-increased susceptibility. In the present study, we test the hypotheses that ApoE proteins are critical determinants of brain phospholipid homeostasis and that the ApoE4 isoform is dysfunctional in this process.

## Results

**Changes in Phosphoinositol Metabolites Correlate with AD Disease Development, and ApoE4 Genotype Exacerbates Phosphoinositol Biphosphate Reduction.** We first analyzed phospholipid composition of human parietal cortex tissues of both ApoE4 carriers and non-ApoE4 subjects (Fig. S1A). There was significant correlation between changes in phosphoinositol metabolites [PI; phosphoinositol phosphate (PIP) and phosphoinositol biphosphate (PIP<sub>2</sub>)] and AD disease development and progression (Fig. 1A). A specific pattern of changes including reduction of PIP<sub>2</sub> and PI levels, as well as a reciprocal elevation in PIP levels, was observed along with disease progression from normal aging to MCI and early AD [clinical dementia rating (CDR) scale from 0 to 1].

Interestingly, ApoE4 genotype specifically exacerbates the changes in PIP<sub>2</sub> levels along with disease progression. A reduction of PIP<sub>2</sub> was observed in ApoE4<sup>+/-</sup> tissue in both CDR 0 (Fig. 1B) and CDR 0.5–1 cohorts (Fig. 1C). PIP<sub>2</sub> levels were much lower in the ApoE4<sup>+/-</sup> brain tissues of normal aged subjects (0.15% of total phospholipids measured) compared with ApoE4<sup>-/-</sup> counterparts (0.42%;  $P = 0.026$ ). A trend of elevation in substrate PI levels in ApoE4<sup>+/-</sup> brains was seen but did not achieve statistical significance. No changes were seen in other phospholipid species such as PIP, phosphoserine, phosphatidic acid, and cardiolipin (Fig. S1B and C). Reduction in PIP<sub>2</sub> levels was also observed in patients with early AD with the ApoE4 genotype, using a cohort of brain tissues from patients with a clinical diagnosis of MCI or early AD (CDR ranges from 0.5 to 1; Fig. 1C). Brain PIP<sub>2</sub> levels in ApoE4<sup>+/-</sup> patients (0.19%) were

## Significance

**The apolipoprotein E4 (ApoE4) genotype is the strongest genetic risk factor for developing Alzheimer's disease (AD). However, the mechanisms that underlie this link between ApoE4 genotype and AD are not well understood. Our data in this paper are the first mechanistic studies to our knowledge that link ApoE4 genotype-specific changes in brain phospholipid homeostasis to ApoE4-increased susceptibility to develop AD. Our studies indicate previously unidentified therapeutic options for the treatment of AD, targeting ApoE4's pathogenic nature.**

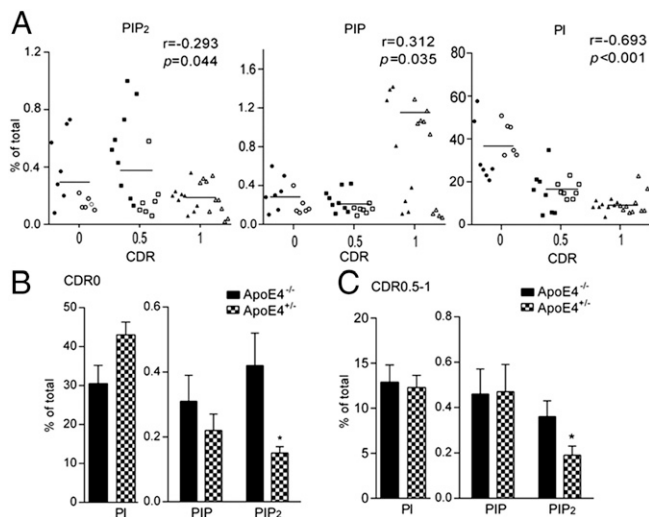
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**Fig. 1.** Changes in PI, PIP, and PIP<sub>2</sub> correlate with AD disease development and progression. Reductions in PIP<sub>2</sub> levels are exacerbated in ApoE4 carriers. (A) Correlation of PI/PIP/PIP<sub>2</sub> and AD stages. Closed symbols, ApoE4<sup>-/-</sup> subjects; open symbols, ApoE4<sup>+/-</sup> carriers. Phospholipid profile of ApoE4<sup>-/-</sup> brain samples (B) with CDR 0 and (C) with CDR 0.5–1. \**P* < 0.05 with ANOVA tests.

significantly lower than those in ApoE4<sup>-/-</sup> patients (0.36%; *P* = 0.036). There were no changes seen in PI or PIP levels in ApoE4<sup>+/-</sup> versus ApoE4<sup>-/-</sup> brain samples. Concomitantly, the amounts of amyloid plaque and neurofibrillary tangles in ApoE4<sup>+/-</sup> brains were much higher than those in ApoE4<sup>-/-</sup> brains, even in the normal aging population (Fig. S1C), suggesting that ApoE4 effects on amyloid burden may exacerbate its effects on PIP<sub>2</sub> reduction, as implicated by a previous report that oligomeric Aβ can reduce PIP<sub>2</sub> levels in mouse cortical neuronal cultures (10). It should be noted, however, that in advanced AD stages (a cohort of CDR 3 subjects; Fig. S2A and B), there was a trend of reduction in PIP<sub>2</sub> levels in ApoE4<sup>+/-</sup> brains compared with ApoE4<sup>-/-</sup> brains, but it did not achieve statistical significance as a result of large sample variations. The PI, in contrast, was statistically reduced in ApoE4<sup>+/-</sup> brains compared with ApoE4<sup>-/-</sup> counterparts at this stage, possibly because of a significant disruption of cell membrane integrity by the ApoE4 genotype at the advanced disease stage.

Interestingly, ANOVA analysis of all samples reveals a main effect of sex on brain PIP<sub>2</sub> homeostasis independent of CDR stage and ApoE genotype (*P* = 0.039). Brain PIP<sub>2</sub> levels of female subjects were lower than those of male subjects in cohorts with CDR 0.5–1 and 3 (Fig. S2C). The reduction of PIP<sub>2</sub> levels over the course of disease development and progression was more closely correlated in female subjects (Fig. S2C; *r* = -0.530) than in the overall population (Fig. 1A; *r* = -0.293). In addition, ApoE4 genotype-associated effects on PIP<sub>2</sub> levels can be observed in female subjects from cohorts with CDR 0.5–1 and 3, whereas these effects can only be seen in the cohort of CDR 0.5–1 male subjects (Fig. S2D). Together, these results suggest that sex-specific effects on brain PIP<sub>2</sub> homeostasis may, in addition to ApoE4 genotype, synergistically contribute to AD pathogenesis and disease progression.

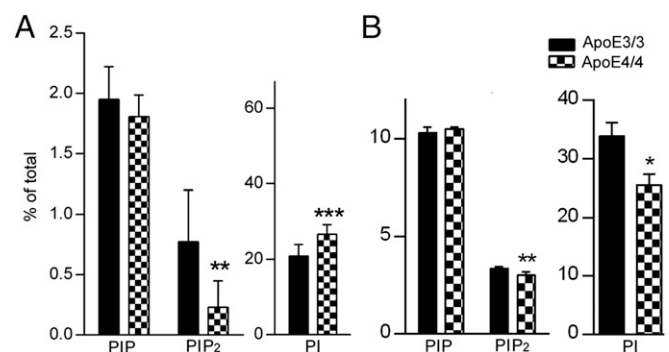
Reduction of PIP<sub>2</sub> in ApoE4 human brains was further recapitulated in animal models carrying the human ApoE4 genotype (11–13), as well as in neurons and astrocytes expressing ApoE4. As shown in Fig. 2, the amount of PIP<sub>2</sub> was significantly reduced in male ApoE4/4 homozygous KI mouse hippocampal regions at 9 mo of age (0.23%) if compared with ApoE3/3 tissues (0.77%; *P* = 0.002). PI levels in these mice were elevated (26.72% vs. 20.93% in ApoE3 mice; *P* < 0.001), similar to what we

observed in human brain tissues derived from CDR 0 group (Fig. 1B). No statistically significant changes were seen in levels of other phospholipids. A decrease in PIP<sub>2</sub> levels was also seen in 9-month-old female ApoE4/4 hippocampal brain tissues (Fig. S3A). Trends of PI/PIP<sub>2</sub> changes were seen in ApoE4/4 mouse brains at as early as 3 mo of age (whole-brain lipid analysis; Fig. S3B), suggesting the intrinsic effects of ApoE4 on PI metabolism, particularly PIP<sub>2</sub> levels, which become more prominent with the ApoE4-induced accelerated aging processes (14–19). Therefore, we speculate that ApoE4-induced PIP<sub>2</sub> changes may contribute to ApoE4-induced exacerbation of neurodegenerative processes.

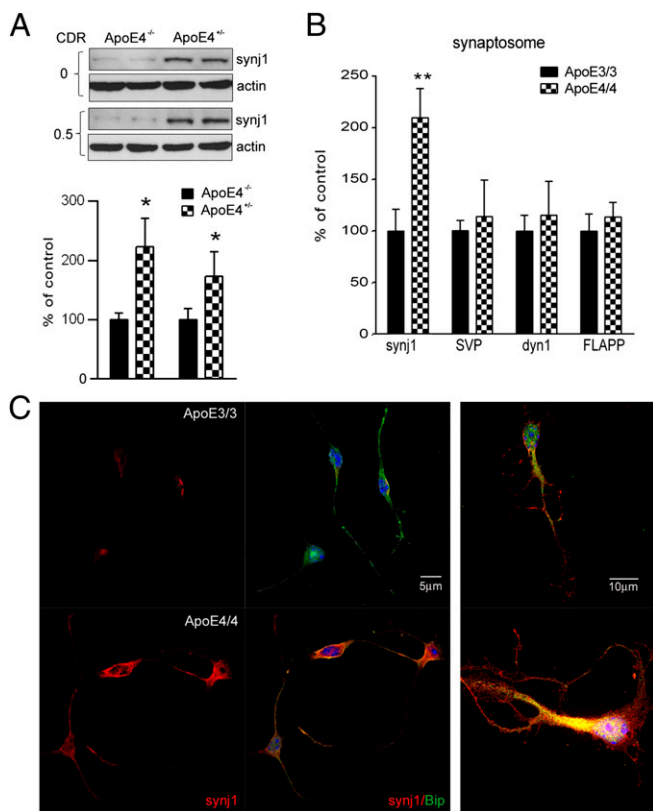
Moreover, a reduction in PIP<sub>2</sub> levels was observed in cultured hippocampal neurons (Fig. 2B) and astrocytes (Fig. S3C) derived from mice expressing human ApoE4 genotype compared with ApoE3 counterparts. A reduction of PIP<sub>2</sub> levels was seen in ApoE4 neurons (3.02%) compared with ApoE3 neurons (3.36%; *P* = 0.008). However, the levels of PI were dramatically reduced in ApoE4 neurons (25.45%) compared with ApoE3 (33.9%; *P* = 0.03), suggesting neuronal-specific changes in PI/PIP<sub>2</sub>. A statistically significant reduction of PIP<sub>2</sub> was seen in ApoE4 astrocytes compared with in ApoE2 astrocytes, with a lesser decrease of reduction compared with in ApoE3 astrocytes (Fig. S3C; ApoE2, 1.23%; ApoE3, 0.74%; and ApoE4, 0.58%). Together, our data suggest that ApoE4 specifically induces reduction in PIP<sub>2</sub> levels.

#### Expression Levels of a PIP<sub>2</sub>-Degrading Enzyme, Synaptojanin 1, Are Elevated in ApoE4 Brains and Neurons.

Next, we investigated whether the reduction of PIP<sub>2</sub> levels in ApoE4 brain is secondary to increased expression and/or enzymatic activities of the rate-limiting enzyme of brain PIP<sub>2</sub> pathway, synaptojanin 1 (synj1). Synj1 is a PIP<sub>2</sub>-degrading enzyme that is highly enriched in the brain (20, 21). As shown in Fig. 3A, synj1 protein levels in ApoE4<sup>+/-</sup> subjects were much higher than those in ApoE4<sup>-/-</sup> subjects. The differences can be seen in both the normal aged cohort (with CDR 0; synj1 levels in ApoE4<sup>+/-</sup> brains were 223.1% of those in ApoE4<sup>-/-</sup> brains) and the early AD cohort (with CDR 0.5–1; synj1 levels in ApoE4<sup>+/-</sup> brains were 173.3% of those in ApoE4<sup>-/-</sup> brains). However, at the advanced stage (CDR 3), this difference in synj1 protein levels was diminished (Fig. S4A; synj1 levels in ApoE4<sup>+/-</sup> are 104.1% of levels in ApoE4<sup>-/-</sup>). There were no differences seen in levels of another endocytic adaptor protein dynamin 1 (dyn1) between ApoE4<sup>+/-</sup> and ApoE4<sup>-/-</sup> groups at all stages of AD, suggesting the changes



**Fig. 2.** Changes in PIP<sub>2</sub> levels observed in mouse brain and primary neurons in ApoE4/4 mice. (A) Amounts of PIP<sub>2</sub> in hippocampus of male ApoE4/4 homozygous KI mice at 9 mo of age; \*\**P* < 0.01, \*\*\**P* < 0.001 compared with ApoE3/3 male homozygous KI mice with independent-sample *t* tests. (B) PIP<sub>2</sub> levels in primary hippocampal neurons derived from ApoE4/4 or ApoE3/3 homozygous KI mice. \**P* < 0.05, \*\*\**P* < 0.001 versus ApoE3/3 neurons with independent-sample *t* tests. The levels of PI were dramatically reduced in ApoE4 neurons (25.45 ± 1.95%) compared with ApoE3 (33.9 ± 2.3%; *P* = 0.03), suggesting neuron-specific changes in PI/PIP<sub>2</sub>.



**Fig. 3.** Expression levels of synaptojanin 1 are elevated in ApoE4 brains. (A) The synj1 protein levels in ApoE4<sup>+/-</sup> brains from CDR 0 and CDR 0.5–1 cohorts. \**P* < 0.05 versus ApoE4<sup>-/-</sup> brains with ANOVA tests. (B) Synj1 and dyn1 protein levels in ApoE4/4 mouse hippocampal synaptosomes. \**P* < 0.05, \*\**P* < 0.01 versus ApoE3/3 with independent-sample *t* tests. (C) Immunofluorescence staining of synj1 (red) and Bip (green) in ApoE4/4 or ApoE3/3 neurons. (Right) Magnified image.

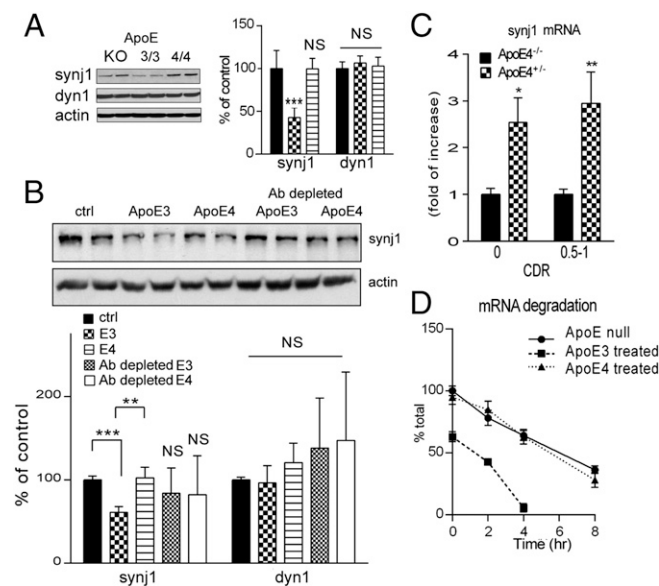
in synj1 are specific (Fig. S4B). Consistently, protein levels of synj1, but not dyn1, in ApoE4/4 mouse neocortical and hippocampal tissues were significantly elevated compared with in ApoE3/3 brains (Fig. S4C). It should be noted that the differences in synj1 expression levels are not yet seen in 3-mo-old ApoE4/4 and E3/3 brains, suggesting a possible compensatory mechanism on regulation of synj1 expression levels at early ages of animals (Fig. S4D).

Because synj1 is enriched at the synapse (20, 21), we then analyzed synj1 levels in synaptosomes [validated by a synaptosome marker, synaptic vesicle protein (SVP)] extracted from ApoE4/4 mouse hippocampal brain tissues. As shown in Fig. 3B, the amount of synj1 proteins in ApoE4/4 mouse hippocampal synaptosomes was elevated versus that in ApoE3/3 brains (193.1% of controls; *n* = 4; *P* = 0.003; this was more robust compared with changes in ApoE4/4 brain total lysates shown in Fig. S4C). There were no significant changes in dyn1 or total amyloid precursor protein (holoAPP) protein levels in synaptosomes of ApoE4/4 mouse hippocampal brain tissues compared with in ApoE3/3 counterparts. These data suggest that neuronal synj1/PIP<sub>2</sub> levels at synaptic terminals are probably more sensitive to changes induced by ApoE isoforms, which could contribute to ApoE4-induced synaptic dysfunction.

Interestingly, we observed a more prominent increase in synj1 protein levels induced by ApoE4 in neurons (Fig. 3C) than that in astrocytes (Fig. S5A). The amount of synj1 (red fluorescent signals) was increased in ApoE4/4 primary neurons compared with that in ApoE3/3 cells. A magnified image of individual cells

showed dramatic differences in synj1 levels at synaptic terminals and neuronal processes, as well as in cell bodies of ApoE4/4 and E3/3 neurons. An endoplasmic reticulum marker, pre-B cell immunoglobulin heavy chain-binding protein Bip, was stained as a control (green fluorescent signals). Similar differences in fluorescent intensity are seen when comparing synj1 levels in ApoE4/4 and E3/3 astrocytes (Fig. S5A). Consistently, the levels of synj1 protein in ApoE4/4 primary neurons were 253.8% of those in ApoE3/3 neurons, whereas synj1 levels in ApoE4/4 astrocytes were 176% of those in ApoE3/3 astrocytes (Fig. S5B). Although the differences in synj1 expression levels in ApoE3/3 versus ApoE4/4 astrocytes were less robust, they were still significant; these changes may play a functional role in differential regulation of astrocyte functions by various ApoE isoforms.

**Loss-of-Function Effects on Synj1 Expression by ApoE4 Genotype.** We next investigated whether the differences in synj1 expression levels between ApoE4 versus E3 alleles are secondary to ApoE3-induced down-regulation or ApoE4-induced up-regulation of synj1 expression. First, we compared synj1 levels in ApoE3/3 or ApoE4/4 brains to that of ApoE null mouse brains and found that the levels of synj1 were comparable in ApoE4/4 and ApoE<sup>-/-</sup> brains, whereas ApoE3/3 brain synj1 levels were much lower (Fig. 4A). These results suggest a loss-of-function effect on synj1 expression by ApoE4 genotype, similar to ApoE knockout conditions. No differences were detected in the amount of dyn1 (Fig. 4A)



**Fig. 4.** Loss-of-function effects on synaptojanin 1 expression by ApoE4 genotype. (A) The synj1 levels were comparable between ApoE4/4 and ApoE<sup>-/-</sup> mouse brains (E4 99.5 ± 12.3% of controls, with ApoE<sup>-/-</sup> synj1 levels as 100%), whereas ApoE3/3 mouse brain synj1 levels were much lower (42.8 ± 11.5%; \*\*\**P* < 0.001 versus ApoE<sup>-/-</sup> synj1 levels with ANOVA tests). No significant differences were seen in dyn1 levels. (B) Synj1 and dyn1 protein levels in ApoE<sup>-/-</sup> neurons after exposure to conditioned media containing no ApoE, ApoE3, or ApoE4 with or without immune depletion by an anti-ApoE antibody. E3, 61.1 ± 7.6% of controls; \*\*\**P* < 0.001 versus ApoE<sup>-/-</sup> synj1 levels; E4, 102.6 ± 13.3% of controls; \*\**P* = 0.01 with ANOVA tests. Antibody-depleted E3 media 83.9 ± 14.2% of controls; *P* = 0.86; antibody-depleted E4 media 82.3 ± 21.9% of controls; *P* = 0.60. (C) Synj1 mRNA levels for ApoE4<sup>+/-</sup> and ApoE4<sup>-/-</sup> brains from CDR 0 and 0.5–1 cohorts. \**P* < 0.05, \*\**P* < 0.01 versus ApoE4<sup>-/-</sup> with ANOVA tests. (D) Synj1 mRNA in ApoE<sup>-/-</sup> neurons exposed to ApoE3 or E4 conditioned media over time after the addition of actinomycin. The slope of synj1 mRNA degradation in ApoE3-treated cells (*y* = -15.73*x* + 66.58; *R*<sup>2</sup> = 0.96) was much steeper than that in ApoE4-treated (*y* = -8.62*x* + 97.88; *R*<sup>2</sup> = 0.99) or ApoE<sup>-/-</sup> conditions (*y* = -7.77*x* + 96.82; *R*<sup>2</sup> = 0.99).

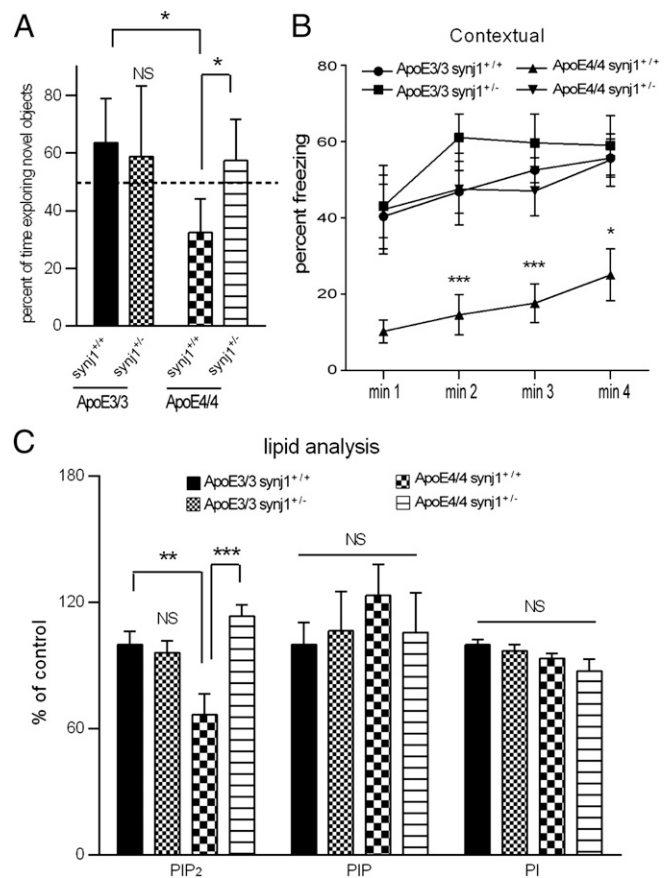
or holoAPP (Fig. S6A) in ApoE4 versus E3 mouse brain lysates compared with ApoE KO brain lysates. There were modest elevations in endogenous mouse A $\beta$  levels of ApoE4 hippocampal brain lysates compared with ApoE3 counterparts (Fig. S6B). These data are consistent with previous reports that ApoE genotypes do not affect APP processing but instead regulate A $\beta$  degradation (2, 3).

We next studied the dynamic changes in synj1 expression levels using ApoE null neurons incubated with conditioned media derived from ApoE4 or E3 astrocytes. As shown in Fig. 4B, the baseline synj1 levels in ApoE<sup>-/-</sup> neurons were rather high, similar to what we observed in ApoE<sup>-/-</sup> brain tissues (Fig. 4A). Incubation of ApoE<sup>-/-</sup> neurons with conditioned media derived from ApoE3, but not ApoE4 or ApoE<sup>-/-</sup> astrocytes, reduced synj1 protein levels (Fig. 4B). The inhibitory effects of ApoE3-containing media on synj1 were almost completely abolished after conditioned media was depleted of ApoE (Fig. 4B). There were no differences in dyn1 levels in neurons treated with various conditions, suggesting the specific regulation of synj1 expression by ApoE isoforms. These results indicate that the ApoE3 isoform suppresses synj1 expression. In contrast, the ApoE4 allele has no effect on synj1, indicating a loss of function for this allele.

The amount of synj1 mRNA was also elevated in ApoE4 human (Fig. 4C) and mouse (Fig. S6C) brains compared with their ApoE3 counterparts, whereas dyn1 mRNA levels were unchanged (Fig. S6D). Conditioned media from ApoE3-expressing astrocytes accelerated the degradation of synj1 mRNA in ApoE<sup>-/-</sup> neurons (Fig. 4D). The rate of synj1 mRNA degradation, however, was similar in neurons treated with conditioned media from ApoE4 or ApoE<sup>-/-</sup> astrocyte cultures. These data suggest that ApoE3 down-regulates synj1 expression by promoting degradation of synj1 mRNA. In contrast, ApoE4 lacks this activity and is unable to reduce neuronal synj1 levels.

**Synj1 Reduction Can Rescue Cognitive Deficits and Restore PIP<sub>2</sub> Homeostasis in ApoE4 KI Mice.** Mice with a human ApoE4 KI background (11–13) spent less time exploring a novel object than ApoE3 KI mice (Fig. 5A), indicating that ApoE4 mice had significant memory impairments including an inability to discriminate novel from familiar objects. This deficit of the ApoE4 KI mice was abolished by genetic knockdown of synj1. The ApoE3<sup>+/+</sup> synj1<sup>+/-</sup> animals behaved similarly to wild-type littermates (ApoE3<sup>+/+</sup> synj1<sup>+/+</sup>). The total amount of time spent in exploring objects (seconds) was comparable among all four genotype groups (Fig. S7A). In the fear conditioning test, all four groups responded similarly in the training phase, showing increased freezing after the foot shock ( $P < 0.001$  baseline vs. postshock; Fig. S7B). On day 2 in the contextual phase, ApoE4<sup>+/+</sup> synj1<sup>+/-</sup> animals froze less compared with ApoE3<sup>+/+</sup> synj1<sup>+/+</sup> mice (Fig. 5B), suggesting impaired hippocampal function of ApoE4<sup>+/+</sup> synj1<sup>+/-</sup> mice. Importantly, this defect was not observed in ApoE4<sup>+/+</sup> synj1<sup>+/-</sup> mice. Furthermore, there were no significant differences in freezing behavior between ApoE3<sup>+/+</sup> synj1<sup>+/+</sup> and ApoE3<sup>+/+</sup> synj1<sup>+/-</sup> groups. On day 3 in the cued phase, all groups exhibited increased freezing when the tone was presented with the novel context, a result consistent with intact amygdala function (Fig. S7C;  $P < 0.001$  baseline vs. posttone).

The synj1<sup>+/-</sup> genotype reversed the decreased PIP<sub>2</sub> levels in the ApoE4<sup>+/+</sup> synj1<sup>+/-</sup> mice (Fig. 5C). The amount of synj1 in ApoE3<sup>+/+</sup> synj1<sup>+/+</sup> mice was significantly lower than that in ApoE4<sup>+/+</sup> synj1<sup>+/+</sup> mice (Fig. S7D), which is consistent with our prior observations (Figs. 3 and 4). Synj1 haploinsufficiency in ApoE4 mice (ApoE4<sup>+/+</sup> synj1<sup>+/-</sup>) reduced the levels of synj1 to comparable levels in ApoE3<sup>+/+</sup> synj1<sup>+/-</sup> mouse brains. No changes were seen in dyn1 or holoAPP levels with synj1 reduction in ApoE4 or E3 mice. Together, these results suggest that genetic reduction of synj1 in ApoE4 KI mouse models can restore impaired PIP<sub>2</sub> homeostasis and rescue AD-related cognitive deficits in these mice.



**Fig. 5.** Reduced synj1 rescues cognitive deficits and restores PIP<sub>2</sub> homeostasis in ApoE4 KI mice. (A) The amount of total time spent exploring a novel object ( $n = 6$ /group) and (B) the frequency of freezing behavior during the contextual phase of a fear conditioning test ( $n = 9$ /group) in four groups of mice: ApoE4<sup>+/+</sup> synj1<sup>+/+</sup>, ApoE4<sup>+/+</sup> synj1<sup>+/-</sup>, ApoE3<sup>+/+</sup> synj1<sup>+/+</sup>, and ApoE3<sup>+/+</sup> synj1<sup>+/-</sup>. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  versus ApoE3<sup>+/+</sup> synj1<sup>+/+</sup> with ANOVA tests. (C) The levels of PI/PIP/PIP<sub>2</sub> in mouse hippocampus.

## Discussion

Accumulating evidence indicates that phospholipid regulators including synj1 (22–24), phospholipase D (25), and PI-binding clathrin assembly protein (26) play important roles in pathways leading to neurodegenerative processes such as lipid granule accumulation, endosomal/lysosomal degradation, and regulation of microglia and astrocyte functions. Our data show that ApoE3 increases brain PIP<sub>2</sub> levels through promotion of synj1 mRNA degradation and suppression of synj1 expression. In contrast, ApoE4 has lost the ability to regulate synj1 mRNA degradation and protein expression, and thereby ApoE4 reduces brain PIP<sub>2</sub> levels. Our findings reveal a previously unidentified molecular mechanism underlying the strong association of ApoE4 with sporadic AD, whereby ApoE4 genotype-specific changes in brain phospholipid homeostasis predispose to AD susceptibility.

Several ApoE functions including A $\beta$ -dependent and A $\beta$ -independent pathways have been proposed for ApoE4-related AD pathogenesis (27). One such pathway may be aberrant metabolism of membrane phospholipids. Recent literature, including data from our laboratory, suggests that PI homeostasis, particularly PIP<sub>2</sub>, plays an important role in AD (10, 22, 24, 28, 29). For example, one study demonstrated PIP<sub>2</sub> reduction in the prefrontal cortex of patients with AD (29). Here, our phospholipid analysis of human brain tissues is the first to our knowledge to demonstrate a specific pattern of changes in PI metabolism (including reduction of PI and PIP<sub>2</sub>, and a reciprocal increase in PIP) that correlates

with disease conversion from normal aging to MCI and early AD. More important, our data suggest that ApoE4 genotype exacerbates the changes in PIP<sub>2</sub> levels in particular, along with disease development and progression. It is interesting that reduced PIP<sub>2</sub> is found even in normal aged ApoE4 carriers (CDR 0; Fig. 1), suggesting that impaired brain phospholipid metabolism precedes the development of overt AD clinical symptoms. Reduced synj1 rescues cognitive deficits in ApoE4 KI mice, which lack amyloid pathology (Fig. 5), suggesting an A $\beta$ -independent mechanism underlying ApoE4-induced cognitive impairments. Sex-specific effects on brain PIP<sub>2</sub> homeostasis are also observed in our analysis (Fig. S2 C and D), consistent with recent evidence showing that compared with men, women's brains are more vulnerable to AD pathological processes, and cognitive functions decline faster (30, \*<sup>†</sup>).

A recent paper reported changes in serum levels of 10 phospholipids (including PI) as predicting phenotypic conversion to either amnesic MCI or early AD in 2–3 y intervals with more than 90% accuracy (9). Despite the fact that this study was performed in a rather small-sized patient cohort and did not have cerebrospinal fluid analysis of lipidomics or brain imaging studies to correlate with their serum biomarker studies, our findings in addition to this report suggest a potential importance of assessing specific phospholipid profiling as AD biomarkers in predicting conversion and progression of disease. In contrast, the reduction of PI levels has a much stronger correlation with AD disease development and progression, suggesting a putative functional role for this phospholipid in the structure and function of cell membranes, which are most likely disrupted at early stages of AD. However, it should be noted that the changes in PI metabolism are prominently seen in early disease progression (from CDR 0 to 1; Fig. 1), but not much so at the advanced stage (CDR 3; Fig. S2). It is possible that at advanced AD stages, the severity of disrupted membrane integrity may confound any specific patterns of changes in phosphoinositol metabolism and become less indicative for disease progression at later stages.

Our data showing a reduction of PIP<sub>2</sub> and a reciprocal increase in substrate PIP/PI levels along AD phenotype conversion suggest a possibility of accelerated PIP<sub>2</sub> degradation processes (such as elevated PIP<sub>2</sub> degrading enzyme synj1 levels), rather than impaired PIP<sub>2</sub> synthesis. PIP<sub>2</sub> is a signaling lipid involved in ion channel regulation, exocytosis, endocytosis, actin cytoskeleton rearrangement, and cell signaling (20). PIP<sub>2</sub> dephosphorylation at the synapse is mediated primarily by synj1, a type 2 polyphosphate-5-phosphatase encoded by a gene present on human chromosome 21. Synj1 is expressed and enriched at the synapse and has been shown to play a role in endocytosis, presynaptic vesicle recycling, and postsynaptic receptor trafficking (20, 21). A potential link between AD and synj1 was suggested by studies demonstrating that familial AD mutations in the phosphoserine 1 result in a perturbation of PIP<sub>2</sub> metabolism. Overexpression of synj1 led to an increase in A $\beta$  production (23). In addition, one of the yeast orthologs of synj1, INP52, was identified in an unbiased genome-wide screen for modifiers of A $\beta$  toxicity (31). We have recently shown that genetically reducing synj1 promoted A $\beta$  degradation through lysosomes and ameliorated memory deficits in an AD transgenic mouse model (22). Here our data are the first, to our knowledge, to show specific changes in PIP<sub>2</sub> and synj1 levels induced by ApoE4 genotype. More important, we demonstrate that differential expression in synj1 levels may contribute to ApoE4-induced AD pathological process. We speculate that one pathogenic mechanism of ApoE4 is to perturb neuronal, and in particular

synaptic, PIP<sub>2</sub> homeostasis by altering synj1 expression at synapse (Fig. 3), which could contribute to the development of ApoE4-induced cognitive deficits and synaptic dysfunction.

Interestingly, the regulation of synj1 expression levels by ApoE isoforms is mediated by differential regulation of synj1 mRNA degradation rates in cells expressing various ApoE genotypes. As shown in Fig. 4, ApoE3 degrades synj1 mRNA rapidly (its half-life is ~2 h), whereas ApoE4 fails to do so. In contrast, synj1 protein degradation rates are comparable between different ApoE isoforms, given the fact that synj1 protein half-life is usually long (~16 h). The mRNA stability is often regulated by microRNA binding to a 3'-UTR regions (32). We have performed microRNA array studies and found distinct changes in certain microRNA levels between ApoE4 and ApoE3 samples. These results give credence to the potential involvement of and regulation of synj1 expression by ApoE isoforms through microRNA.

Overall, our studies implicate a novel therapeutic strategy targeted at PIP<sub>2</sub> homeostasis to modify the ApoE4 pathogenic phenotypes. As demonstrated by genetic reduction of synj1 in ApoE4 mice, we are able to restore impaired PIP<sub>2</sub> homeostasis and rescue cognitive deficits in an ApoE4 mouse model. Therefore, pharmacological interventions to reduce synj1 expression levels and/or phosphatase activities could achieve similar functional outcomes in ApoE4 conditions. Several current therapeutic strategies have been focused on removing or ablating ApoE4, based on the observations that ApoE4 contributes to AD-related neurodegenerative processes and that total absence of ApoE proteins has no significant neurocognitive deficits (33, 34). However, our data suggest that ApoE is critical to maintain brain phospholipid homeostasis and that ApoE4 exerts less of this effect than other isoforms. For example, ApoE4 demonstrates a loss-of-function effect on regulating synj1 mRNA stability and protein expression, similar to ApoE null condition. These data raise a concern for therapeutic strategies directed at reduction of brain ApoE levels, which may not be sufficient to rescue ApoE4-related phospholipid dysregulation because of loss-of-function effects on phospholipid homeostasis induced by ApoE4 isoform.

In summary, our findings represent the first mechanistic studies to our knowledge that link ApoE4 genotype-specific changes in brain phospholipid homeostasis with ApoE4-dependent increased susceptibility to develop AD. These studies may uncover new therapeutic directions for the treatment of sporadic AD.

## Methods

**Human Brain Sample Selection and Preparation.** Equal amounts of postmortem brain tissues (50  $\mu$ g by net weight) from parietal cortex regions were obtained from the Icahn School of Medicine at Mount Sinai Brain Bank and used for lipid analysis and mRNA/protein expression studies.

**Phospholipid Analysis.** Human or mouse brain samples were used for lipid extraction, followed by the quantification by anion-exchange HPLC, as described previously (22, 35).

**Generation of Synj1 Haploinsufficiency Mice with Human ApoE Background.** The human ApoE4 or ApoE3 KI mouse models (12, 13, 36) were mated with heterozygous synj1 null mice (synj1<sup>+/-</sup>) (37). Double heterozygous F1s were then bred with F0 ApoE4/4<sup>+/+</sup> or ApoE3/3<sup>+/+</sup> mice to generate offspring that express human ApoE4/4 or E3/3 in the synj1<sup>+/-</sup> background. Genotypes were determined by PCR amplification, as described (37).

**Immunocytochemical Studies.** Hippocampal neurons were obtained from 17-d-old mouse embryos and grown for 7 d in vitro, as described (38), before being fixed and stained for confocal microscopy analysis (LSM510) (39).

**Brain and Neuronal Lysate Preparation and Analysis.** Snap-frozen mouse hemibrains or cultured neurons were harvested in lysis buffer (40) and processed via step-wise solubilization (40, 41), followed by SDS/PAGE to determine levels of synj1, dyn1, holoAPP, and CTFs. Levels of A $\beta$ <sub>42</sub> were determined by high-sensitive mouse A $\beta$ <sub>42</sub> ELISA kits (Wako). Alternatively,

\*Yaffe K, et al. Alzheimer's Association International Conference, July 24–28, 2015, Washington, DC, 3121 (abstr).

<sup>†</sup>Tosun D, et al. Alzheimer's Association International Conference, July 24–28, 2015 Washington, DC, 5556 (abstr).

homogenates of mouse brains were fractionated via sucrose gradient to collect synaptosomes according to Huttner et al. (42).

**mRNA Extraction and Degradation.** Synj1 mRNA synthesis were determined by quantitative real time-PCR. The half-life of synj1 mRNA was determined by treatment of actinomycin at 50 µg/mL for various periods before being subjected to RNA extraction (43, 44).

**Behavior Studies.** Six- to 9-month-old male ApoE4 synj1<sup>+/+</sup> and synj1<sup>+/-</sup> mice were tested with the novel object recognition memory task (45) and fear conditioning studies, as described (46).

**Antibodies and Reagents.** The anti-synj1 (mouse human Ab, Novus), anti-Bip and β actin (Santa Cruz), anti-MAB348 and clone 41 (Millipore), anti-mouse and rabbit HRP, Texas-Red or Alexa<sup>488</sup> conjugated anti-mouse and rabbit IgG (Vector Laboratories Inc.) were purchased. pAb369 (C-terminal APP antibody) was used to detect human and mouse holo-APP and CTFs (47).

**Statistical Analysis.** Levels of synj1, dyn1, and holoAPP were normalized to β-actin levels and expressed as percentage of control. Absolute Aβ<sub>42</sub> concentrations were quantitatively determined by ELISA (Wako) and expressed as percentage of control. Independent sample *t* tests were used to determine

significant mean differences (the threshold for significance set at *P* < 0.05). The ANOVA with post hoc tests were used to determine group differences for multiple comparisons. The Pearson correlation coefficients were measured to determine the linear relationship between two variables. All statistical analysis was performed using SPSS v21.0.

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