Title

APOE4 induces site-specific tau phosphorylation through calpain-CDK5 signaling pathway in EFAD-Tg mice.

Running title

ApoE4 accelerates site-specific tau phosphorylation.

Byline

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Keywords

ApoE4; Alzheimer’s disease; tau; phosphorylation; calpain-CDK5; transgenic mice.
APOE4 induces tau hyperphosphorylation through calpain-CDK5 signaling pathway in EFAD-Tg mice

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**ABSTRACT**

*APOE4* is the greatest genetic risk factor for Alzheimer’s disease (AD), particularly associated with increased levels of amyloid-β (Aβ) peptide and amyloid deposition. However, it remains unclear whether *APOE4* is associated with greater tau phosphorylation and neurofibrillary tangle formation, a hallmark of AD leading to structural disruption of the neuronal cytoskeleton. The current study used 3 and 7 month old EFAD mice, which express human *APOE* and over-express specifically human Aβ42 via 5 familial-AD (FAD) mutations, to investigate *APOE* genotype-specific effects on site-specific tau phosphorylation. The results reveal that AD-like site-specific tau phosphorylation was increased in E4FAD mice, accompanied by disrupted cortical neuronal morphology, compared to E3FAD mice. Further analysis demonstrated that the levels of CDK5, its regulatory subunits (p35 and p25) and calpain (including calpain1 and calpain2), but not GSK3β, were significantly increased in E4FAD mice compared to E3FAD mice. These results suggest that the *APOE4* genotype contributes to increased site-specific tau phosphorylation via activation of the calpain-CDK5 signaling pathway.

**Keyword:** Apolipoprotein E; amyloid beta; Alzheimer’s disease; tau; phosphorylation; calpain-CDK5; EFAD mice.
INTRODUCTION

As the most common form of dementia in individuals over 60, Alzheimer’s disease (AD) affects millions worldwide. AD pathology is characterized by extracellular aggregation of the neurotoxic peptide amyloid-beta (Aβ) into amyloid plaques and the intracellular aggregation of site-specific phosphorylated tau (p-tau) into neurofibrillary tangles (NFTs) (1, 2).

The primary genetic risk factor for sporadic AD is inheritance of the APOE4 gene for apolipoprotein E (apoE) (3-13). As the protein component of lipoproteins, apoE is a major lipid transport protein and the only apolipoprotein expressed in the brain, where it is expressed primarily by astrocytes. Thus, apoE is crucial for brain lipid homeostasis, as cholesterol and phospholipids are required for neuronal growth, repair and synaptogenesis (14-16). In humans, apoE is a 299 amino acid protein that has three naturally occurring isoforms that differ by a single amino acid change: apoE2 (Cys112,158), apoE3 (Cys112Arg158), and apoE4 (Arg112,158) (16). Among the general population, APOE3 is the most common, accounting for 77% of alleles, while APOE4 accounts for 15%, and APOE2 8% (16-18). Although APOE4 is expressed in a smaller portion of the population, genetic and epidemiological evidence suggests that individuals with the APOE4 allele have a ~3- and ~12-fold greater risk of developing AD with a single or double allele, respectively, compared to APOE3 and APOE2 (3-13). Further, 40% of AD patients carry at least one copy of the APOE4 allele (16, 18), and APOE4 is associated with the onset of cognitive deficits at an earlier age compared to APOE3 (8-13). Although the cellular mechanism underlying this epidemiological phenomenon has remained the focus of intensive research,
how APOE4 exerts its risk effects on AD remains unclear.

Previous studies demonstrate that APOE4 is associated with increased levels of amyloid-β (Aβ) peptide and amyloid deposition, with plaques appearing earliest with apoE4, compared to apoE3 and apoE2 (19-21). While no studies have conclusively demonstrated an effect of apoE isoform on in vivo Aβ production, one hypothesis is based on the apoE isoform-specific binding to soluble Aβ (E2 > E3 > E4), suggesting a role for isoform-specific differences in Aβ clearance (18). This diminished ability of apoE4 to form apoE/Aβ complex results in increased levels of soluble Aβ, which may remain soluble as oligomeric Aβ (oAβ), or precede to form fibrils and then amyloid (for review (22, 23)). Using novel ELISAs for both oAβ and apoE/Aβ, we have demonstrated that increased oAβ and decreased apoE/Aβ are associated with AD and APOE4 in human CSF and brain tissue, and EFAD mouse brain (for review (22), (20, 21)).

Unlike apoE interactions with Aβ, less is known about interactions between apoE and tau. The co-localization of apoE with NFTs in AD brains was first reported in 1991(24), two years prior to the discovery that APOE4 was the primary genetic risk factor for AD (25). Phosphorylated tau (p-tau) levels are greater in APOE-knockout (KO) mice compared to wild type (wt) mice expressing mouse-APOE (m-APOE), suggesting a direct role for apoE in the prevention of site-specific p-tau, and thus NFT formation (26). However, the apoE isoform-specific effect on p-tau remain unclear, due in part to the use of different APOE-Tg mouse models. In neuron-specific enolase-APOE (NSE-APOE)-Tg mice that express h-APOE in CNS neurons via the NSE promoter, p-tau is greater in NSE-APOE4 compared to NSE-APOE3-Tg mice (27, 28). In glial fibrillary acidic protein-APOE (GFAP-APOE)-Tg
mice that express h-APOE isoforms in astrocytes under control of the GFAP promoter, no difference in p-tau was reported between apoE3 and apoE4 isoforms (27, 29). There are obvious limitations in these two APOE-Tg models: 1) In NSE-APOE-Tg mice, apoE expression is regulated by a heterologous promoter and limited to CNS neurons rather than astrocytes; 2) in the GFAP-APOE-Tg mice, apoE expression is again regulated by a heterologous promoter known to be up-regulated by astrocyte-specific neuroinflammation (27-31). Thus, investigators have concentrated their attention on APOE-targeted replacement (APOE-TR) mice that express h-APOE under control of the endogenous m-APOE promoter as being a more physiologically relevant model of APOE expression. However, even results from APOE-TR are apparently contradictory, with reports that APOE4-TR mice have greater levels of p-tau than APOE3-TR mice (30) or that there is no significant difference in the levels of phosphorylation at 9 of 13 tau epitopes between APOE4- and APOE3-TR mice (31). Interesting, induction of the amyloid cascade via intracerebroventricular (ICV) infusion of thiorphan resulted in an increase in p-tau in APOE4-TR mice, but not APOE3-TR mice (32). Potential confounds in comparing these studies include the different ages of the mice, an important factor when investigating age-related pathological changes, and multiple lines of evidence that indicate the overall levels of apoE4 are lower in APOE4-TR mice compared to APOE3-TR mice (33), an observation consistent with measure of apoE in human brain (34).

To further investigate apoE isoform-specific effect on p-tau, we turned our attention to APOE-TR mice on a background of familial AD (FAD) mutations. Specifically EFAD-Tg mice are 5xFAD+/human APOE+/+ (E2FAD, E3FAD, E4FAD; for derivation (20)). Male EFAD mice accumulate significant amyloid in the subiculum and deep layer of the frontal
cortex from 2-6 months (M), with E4FAD > E3FAD = E2FAD (20). Importantly, soluble and oligomeric Aβ42 are also greater in E4FAD mice compared to E3FAD at 6M. As well, E4FAD mice exhibit greater microgliosis and astrogliosis compared to E3FAD, particularly in the subiculum and deep layers of the frontal cortex (35). In addition, the genotype-specific neuroinflammatory response is supported by increased expression of the proinflammatory markers and decreased expression of the anti-inflammatory markers in E4FAD mice compared to E3FAD (36). Recent data comparing 7M male and female E3FAD and E4FAD mice revealed both cerebral amyloid angiopathy (CAA) and amyloid in the cortex were significantly greater with APOE4 and in females (37). In previous we demonstrate that, from 2-6M, postsynaptic proteins are reduced and cognition impaired (Morris water maze and Y-maze) in 5xFAD/APOE-KO and E4FAD mice compared to E3FAD and E2FAD mice (38).

While the EFAD-Tg mice are an ideal model to study APOE genotype-specific effects on AD pathology, effects on p-tau and NFT formation in EFAD-Tg mice has not been investigated. Normally, tau plays a key role in regulating microtubule dynamics, axonal transport and neurite outgrowth. Site-specific phosphorylation of tau leads to pathological self-aggregation and microtubule instability, eventually resulting in disruptions of the neuronal cytoskeleton and nerve fibers, reformation of these fibrils into paired helical filaments (PHF) and eventually NFTs (39-41). Although multiple kinases can affect this aberrant phosphorylation state of tau, the major kinases thought to be involved are glycogen synthase kinase 3 beta (GSK3β) and cyclin-dependent kinase-5 (CDK5) (39, 42), particularly its neuron-specific activators, p35 and p25 (43, 44). Calpain, a calcium activated protease, is
an upstream regulator of CDK5 kinase, and, under certain pathological conditions, activated calpain cleaves p35 to p25, forming an active CDK5/p25 complex (45). Calpain has two prototypical isoforms, µ-calpain (calpain 1) and m-calpain (calpain 2). The CDK5/p25 complex is both more stable and active relative to CDK5/p35 (45, 46).

Using EFAD mice, the current study is designed to determine the effect of APOE genotype on p-tau, and identify the primary kinase pathway that regulates site-specific tau phosphorylation. Briefly, p-tau is increased in E4FAD mouse cortex at 3M and 7M compared to E3FAD mice. Importantly, we found that the calpain-CDK5, not GSK3β, signaling pathway is involved in site-specific p-tau.

MATERIALS AND METHODS

Animals

All experiments were conducted in accordance with the rules and regulations of the Institutional Animal Care and Use Committee at Fujian Medical University, in compliance with international guidelines for the ethical treatment and use of animals. Investigators conducting sample processing and analysis were blinded to APOE genotype and age. Male 3M and 7M E3FAD and E4FAD mice were used in the described experimental procedures. EFAD-Tg mice are 5xFAD+/−/human APOE+++/− (E3FAD and E4FAD; for derivation (20)).

Tissue Harvest

Animals were anesthetized via intraperitoneal (i.p.) injection of 10% chloral hydrate (3ml/kg) and transcardially perfused with ice-cold 0.1M phosphate-buffered saline (PBS). Directly following perfusion, brains were removed and bisected along the mid-sagittal line.
For immunohistochemistry (IHC), left hemi-brains were drop-fixed in 4% paraformaldehyde (PFA) for 4–6 hours and then placed in 30% sucrose in PBS at 4°C. When the hemi-brains sank to the bottom of the sucrose solution (indicating dehydration), they were immediately snap-frozen in liquid nitrogen and transferred to -80°C for storage. For Western blot analysis, cortices were dissected from right hemi-brains and immediately snap-frozen in liquid nitrogen and transferred to -80°C for storage.

**Western Blotting**

Cortices were homogenized in ice-cold lysis buffer (0.1M PBS, 1M Na₃VO₄, 2mM NaF, 2.5mM Na₄P₂O₇, 1% Triton X-100 and 1% protease inhibitor cocktail). Lysed cortices were centrifuged at 16,000 x g at 4°C for 25 min, and the supernatants were collected. Supernatant protein concentrations were determined and each sample was adjusted to 2.0mg/mL protein with lysis buffer and 6X sample buffer (containing 125mM Tris, pH 6.8, 0.006% bromophenol blue, 130mM dithiothreitol, 10% sodium dodecyl sulfate and 10% glycerol). An equal amount of protein from each sample was heated at 80°C for 10min, and separated by SDS-PAGE, and transferred to PVDF membranes. The following primary antibodies were used: tau [pT205] (1:10000, Invitrogen/Biosource), tau [pT231] (1:20000, Invitrogen/Biosource), tau [pS396] (1:200000, Invitrogen/Biosource), tau [Ps404] (1:3000, Invitrogen/Biosource), tau5 (1:3000, Invitrogen/Biosource), β-actin (1:4000, Abcam), CDK5 (1:1000, Santa Cruz), GSK3β (1:1000, Sigma), phospho-GSK-3α/β (pTyr279/216) (1:1000, Sigma), p35/p25 (1: 1000, Cell Signaling), calpain 1 (1:1000, Abcam), and calpain 2 (1:1000, Abcam). HRP-conjugated secondary antibodies (1:2000, KPL), enhanced chemiluminescence kit (Millipore) and Image J software were used to quantify
immunoreactive band densities relative to β-actin.

**Immuohistochemistry (IHC)**

Frozen left hemi-brains from each mouse were sectioned coronally at 30µm on a freezing microtome (Leica) and were stored at -20°C in cryoprotectant solution (containing 30% glycerol, 30% ethylene glycol, 40% 0.1M PBS). Immediately prior to antibody staining, tissue sections were washed in Tris-buffered saline (TBS; 6 x 10min), incubated in 3% hydrogen peroxide (H₂O₂; 10min) to quench endogenous peroxidase. After additional washing in TBS (3 x 10min), tissue sections were blocked with 0.3% Triton X-100, 0.25% bovine serum albumin (BSA), and 5% normal goat serum (GS) in TBS for 1 hour at room temperature. Free-floating sections were subsequently incubated with an anti-p-tau antibody (tau [pT205] (1:2000; Invitrogen/Biosource), tau [pT231] (1:2000; Invitrogen/Biosource), tau [pS396] (1:2000; Invitrogen/Biosource) or tau [pS404] (1:1000; Invitrogen/Biosource)) diluted in TBS containing 0.25% BSA, 2% GS, and 0.3% Triton X-100 for 48 hours on an oscillatory rotator at 4°C. Next, tissue sections were washed in TBS + 1% Triton X-100 (TBS-X) (6 x 10min), and incubated for 90min at room temperature in biotinylated isotype-specific secondary antibodies (Vector Laboratories) diluted 1:600 in TBS + 0.25% BSA, 2% GS and 0.3% Triton X. Tissue sections were then washed in TBS-X (6 x 10min), and incubated in Vector Elite avidin–peroxidase (1:200; Vector Laboratories) for 60min at room temperature, washed again in TBS-X (3 x 5min) and washed in sodium acetate solution (3 x 5min). Immunoreactivity was detected using diaminobenzidine (DAB) before tissue sections were gently washed with TBS-X (3 x 10min). Tissue samples were mounted on poly-L-lysine-coated glass slides before air drying, dehydration in a series of increasing
concentrations of ethanol, clearing in xylene, and coverslipping with permanent mounting medium (Vector Laboratories).

**Modified Marsland’s Silver Staining**

Frozen left hemi-brains were sectioned coronally at 30µm thickness. For the modified Marsland’s silver staining process, tissue sections were washed in TBS (3 x 10min) and double distilled water (ddH₂O; 3 x 10min) prior to staining with 20% silver nitrate at 37°C. Sections were then incubated in an oven at 37°C for 1 hour. Following incubation, sections were quickly washed in ddH₂O, and further fixed in 10% formalin (2 x 10sec). Next, sections were treated with ammonia-silver solution for 1min, and washed for 1min in 10% formalin until sections turned brownish-yellow in color and the staining solution turned black. Sections were then washed in ddH₂O before incubation in 5% sodium thiosulfate for 1min to stop the ammonia-silver reaction. The resulting stained sections were rinsed in ddH₂O and mounted on poly-L-lysine coated glass slides. Mounted slides were air-dried, dehydrated in increasing concentrations of ethanol, cleared in xylene and cover slipped with permanent mounting medium (Vector Laboratories) (47). Following this modified procedure, nerve fibers were identified by their black staining.

**Statistical Analysis**

Quantified results are expressed as mean ± standard error of the mean (S.E.M.). Statistical analysis was performed using SPSS (version 11.0 for Windows). Student's t-tests were performed between the E3FAD and E4FAD groups. Statistical significance was established at $p < 0.05$. 
RESULTS

Tau phosphorylation in the cortex of E3FAD and E4FAD mice

In order to assess the impact of *APOE3* and *APOE4* on p-tau, the cortices of male E3FAD and E4FAD mice were analyzed at 3M and 7M. Total tau and p-tau levels were measured at sites T205, S396, S404 and T231 by Western blotting and IHC analysis of coronal sections. As quantified from Western blot analysis, although the levels of total tau and p-tau at sites S396, S404 and T231 at 3M were not significantly different between E3FAD and E4FAD mice, p-tau at T205 was significantly greater in E4FAD compared to E3FAD mice (*p* < 0.001). As measured by tau5, no significant difference in total tau levels were observed at 3M in E3FAD compared to E4FAD mice (Figure 1A). IHC findings revealed a similar increase in p-tau at T205 in E4FAD compared to E3FAD (Figure 1A). In 7M EFAD mice, the levels of p-tau at all four sites were significantly higher in E4FAD compared to E3FAD (*p* < 0.001), as quantified from Western blots and observed by IHC (Figure 1B). Similar to that found in 3M mice, no significant difference in total tau levels were observed at 7M in E3FAD compared to E4FAD mice (Figure 1B).

Effect of apoE4 on GSK3β and CDK5

GSK3β and CDK5 are the primary kinases thought to phosphorylate tau, inducing pathological phosphorylation. Given that phosphorylation of GSK3β at Y216 increases GSK3β activity (43), we measured p-GSK3β (Y216) and total GSK3β in the cortex of EFAD mice by Western blot. At 3M, the ratio p-GSK3β (Y216) to GSK3β (p-GSK3β/GSK3β) was not significantly different between E4FAD and E3FAD mice (Figure 2A), with similar
results observed at 7M (Figure 2B). We also measured the levels of CDK5 and its neuron-specific activators, p35 and p25 (43, 44) in the cortex of EFAD mice by Western blot. At 3M, the levels of CDK5, p25, and p35 were significantly increased in E4FAD compared to E3FAD mice ($p < 0.001$) (Figure 2A), with similar results observed at 7M (Figure 2B). Importantly, the level of p25 was significantly increased compared to p35, evidence of calpain cleavage of p35 to p25 to form the active CDK5/p25 complex. These findings suggest that CDK5, but not GSK3β, plays an important role in the increased p-tau observed in E4FAD mice.

**Effect of apoE4 on calpain**

Calpain, an upstream regulator of CDK5 kinase, has two main isoforms, calpain 1 and calpain 2. To investigate potential *APOE* genotype-specific differences, we measured the levels of calpain 1 and calpain 2 in the cortex of EFAD mice by Western blot. At 3M, levels of calpain 1 and calpain 2 were significantly higher in E4FAD compared to E3FAD mice ($p < 0.001$) (Figure 3A); with similar results observed at 7M ($p < 0.001$ and $p < 0.05$, respectively) (Figure 3B).

**Morphology of the nerve fibers**

Site-specific phosphorylation of tau eventually results in disruptions of the neuronal cytoskeleton, characterized by swelling, irregular and even ruptured nerve fibers (39-41). This pathological degeneration of nerve fibers is characterized by heavy staining with Marsland’s silver stain. As shown in Figure 4, a modified protocol was used to examine neuronal morphology in the cortex of EFAD mice. At 3M, cortical nerve fibers of E3FAD and E4FAD mice were both directionally regular and morphologically normal, although
those of E4FAD mice appear to be more heavily stained, indicative of cellular swelling (Figure 4A). At 7M, cortical nerve fibers were morphologically irregular and even ruptured in E4FAD, but not in E3FAD mice (Figure 4B). These data are consistent with apoE4 association with damaged neuronal fibers that increases with age.

Discussion

The mechanisms mediating APOE4-induced AD risk are multifactorial. While the effects of APOE4 on Aβ-mediated AD pathology is an established field of research, the effects of APOE genotype on p-tau levels during the development and progression of AD remains understudied. The present study utilized the AD-relevant EFAD mice to define the effect of APOE genotype on tau phosphorylation. APOE genotype-specific changes in p-tau were significant, although these mice express m-TAU and not h-TAU. Compared to E3FAD mice, E4FAD mice exhibited increased p-tau and disrupted neuronal morphology in the cortex, a region of early Aβ deposition and neuroinflammation, E4FAD > E3FAD youmans 2012 (20, 35). Further, the calpain-CDK5 signaling pathway (upstream of tau phosphorylation), but not GSK3β, was activated to a greater degree in E4FAD compared to E3FAD mice. These findings suggest that apoE4 contributes to aberrant site-specific p-tau, likely due to alterations in the calpain-CDK5 signaling pathway.

In AD brains, p-tau is significantly elevated, aggregating into PHFs and eventually NFTs. The number of neocortical NFTs correlates with the severity of cognitive impairment and dementia (48-52). As the cortex is a brain region subject to AD-induced damage, including NFT formation (1, 52, 53), our current study focused on the cortex in E3FAD and E4FAD mice. Interestingly, we demonstrate p-tau at sites T205, S396, S404 and T231 is
greater in 7M E4FAD mice compared to age-matched E3FADs. It is well established that neuronal microtubule stability is closely related to the p-tau levels in the brain, particularly at T205, S396, S404 and T231 (54, 55). As such, we used Marsland’s silver staining to examine nerve fiber morphology as an index of apoE4-modulated p-tau-induced microtubule destabilization in cortical neurons from E3FAD and E4FAD mice. Our findings demonstrate heavy staining of swollen nerve fibers in E4FAD mice at 3M that become irregular and ruptured at 7M, whereas E3FAD mice showed normal staining and morphology at both 3M and 7M. Thus, given that the changes observed in nerve fiber morphology are consistent with significantly increased p-tau levels in E4FAD mice, our data suggest that APOE4 contributes to microtubule instability and damage, and eventually synaptotoxicity and neuronal loss. These findings are consistent with previous studies in EFAD mice that demonstrate E4FAD mice exhibit enhanced age-induced loss of synaptic proteins and increased cognitive impairment compared to E3FAD mice (38). These results that apoE4 can exacerbate cognitive impairment via p-tau are consistent with studies in humans (56-58) and APOE-Tg mice (30, 59, 60).

Although tau is phosphorylated by a number of candidate protein kinases, GSK-3β and CDK5 are the two kinases that receive intensive research attention (43, 44, 54, 61, 62). The neuron-specific CDK5 activator p35 can be proteolytically cleaved to p25 by calpain, particularly during a variety of pathological conditions. Compared to p35, p25 does not contain a membrane localization sequence and has a longer half-life, leading to increased kinase activity of CDK5 (44, 63-65). Additionally, the calpain-CDK5 signaling pathway plays a prominent role in the activation of AD pathology (66-69), but the role of apoE isoform in
this context has not been tested. Herein we report that calpain, p25, p35 and CDK5 all increased in E4FAD mice compared E3FAD mice at both 3M and 7M, suggesting that calpain-CDK5 signaling may play a role in apoE4-induced damage to cortical neurons via increases in p-tau in EFAD-Tg mice and AD patients. As a ubiquitous calcium-sensitive protease, calpain is activated by increases in intracellular calcium (70). ApoE isoform-specific regulation of intracellular calcium can occur via: 1) Ca$^{2+}$ channel modulation (71); 2) apoE4-mediated dysregulation of calcium homeostasis (72); and 3) apoE4-mediated dysregulation of intracellular calcium levels via abberant modulation of neuronal NMDA receptors (73, 74). Additional studies will explore the impact of apoE4 on increased intracellular calcium-mediated enhancement of the calpain-CDK5 signaling pathway.

APOE4 likely increases AD risk through various pathways, both Aβ-dependent and –independent (for review (75)). For example as an Aβ-dependent pathway, we have demonstrated the both amyloid deposition and soluble Aβ42 and oAβ levels are increased in E4FAD mice compared with E3FAD mice (20, 35, 76, 77). The observed Aβ-induced glial activation and resultant neuroinflammation in APOE4 can lead to increased intracellular calcium (36, 78). Therefore, increased p-tau and microtubular impairment in E4FAD mice may be attributed to increased calcium-induced calpain activation. On the other hand, Aβ-independent mechanisms are also believed to contribute to the APOE-induced risk for AD. Studies demonstrate age- and sex-dependent differences in cognitive impairment in APOE4-TR mice (59, 79, 80), the mechanism of which may be due to the loss positive function caused by reduced levels of apoE4 (81), or the gain of toxic function caused by apoE4 fragmentation by proteolytic processing (82). Our previous work supports the reduced
levels of apoE in E4FAD compared E3FAD mice (20, 38). Despite the interesting and informative findings this study provides, it remains unclear whether the increased p-tau associated with E4FAD was due to overall reduced apoE levels (loss of positive function) or to a gain of toxic function of apoE4, perhaps via apoE4 fragmentation. Future studies employing 5xFAD/APOE-KO in conjunction with EFAD mice will help shed light on this critical unanswered question.
Abbreviations

AD  Alzheimer's disease
FAD  familial AD
5xFAD Tg mice expressing 5 FAD mutations
EFAD mice Tg-mice expressing 5xFAD mutations and human APOE3 or APOE4
Aβ  amyloid-beta
apoE apolipoprotein E (protein)
APOE apolipoprotein E (gene)
CDK5 Cyclin-dependent kinase-5
GFAP glial fibrillary acidic protein
GSK3β Glycogen synthase kinase 3 beta
h  human
m  mouse
M  month
min  minute
NFTs neurofibrillary tangles
NSE neuron-specific enolase
sec second
TR targeted replacement
Tg transgenic

Conflict of Interest

The authors declare that they have no competing interests.

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Fig. 1. Tau levels and phosphorylation in the cortex in EFAD-Tg mice. A. Tau phosphorylation levels were increased in 3M E4FAD mice at T205 site by Western blotting and immunohistochemistry. B. Tau phosphorylation levels were increased in 7M E4FAD mice at T205, S396, S404 and T231 sites by Western blotting and immunohistochemistry. The total tau levels were not significantly different between the two groups. The protein level in E4FAD mice was quantified as densitometry values which were normalized to the ratio of those in E3FAD mice.

Data are presented as mean ± SEM, n=6. *p<0.05 E4FAD vs. E3FAD. Scale bar = 200 µm.
Fig. 2 The effect of apoE4 on tau kinases. The expressions of p-GSK3β (Tyr216), GSK3β, subunit p35, subunit p25 and subunit cdk5 in cortex were detected by Western blotting. A. At 3M, the ratio of p-GSK3β (Tyr216) to GSK3β was not significantly different between E4FAD mice and E3FAD mice. But p35, p25 and CDK5 were significantly increased in E4FAD mice. B. Similar results were observed at 7M. The protein level in E4FAD mice was quantified as densitometry values which were normalized to the ratio of those in E3FAD mice. Data are presented as mean ± SEM, n=6. p<0.05: *E4FAD vs. E3FAD, #p25 vs. p35.
Fig. 3 ApoE4 increased the calpain level. The expression levels of calpain in the cortex of E3FAD mice and E4FAD mice were analyzed by Western blotting. Calpain 1 and calpain 2 were increased in E4FAD mice at A. 3M and B. 7M. The protein level in E4FAD mice was quantified as densitometry values which were normalized to the ratio of those in E3FAD mice. Data are presented as mean ± SEM, n=6. *p<0.05 E4FAD vs. E3FAD.
Fig.4 The effect of apoE4 on nerve fibers. The nerve fibers (black) were stained by modified Marsland’s silver staining. A. At 3M, the nerve fibers in E3FAD mice and E4FAD mice were integrated and regularly shaped, but the nerve fibers had a heavy staining in E4FAD mice. B. At 7M, the nerve fibers became irregular and ruptured in E4FAD mice. Nerve fibers are indicated by white arrow. Scale bar = 10 μm.


27. Brecht WJ, Harris FM, Chang S, Tesser I, Yu GQ, Xu Q, Dee Fish J, Wyss-Coray T, Buttini M, Mucke L, Mahley RW, Huang Y. Neuron-specific apolipoprotein e4 proteolysis is associated with increased tau...


