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## Reduced cortical excitatory synapse number in *APOE4* mice is associated with increased calcineurin activity

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### Abstract

Synaptic loss is a symptom of Alzheimer's disease (AD) that is associated with the onset of cognitive decline and the loss of executive function. The strongest genetic risk factor for AD is the *APOE4* allele, which results in both a greater risk of developing AD as well as an earlier age of onset of AD. Dendritic spines, the anatomical substrate of the excitatory synapse, are reduced in the cortex of humanized *APOE4* mice but the reason for this synaptic decline is unknown.

Calcineurin, a calcium/calmodulin dependent phosphatase, is a mediator of dendritic spine retraction. We used humanized *APOE* mice to examine how *APOE* genotype altered calcineurin activity and found that *APOE4* mice have 35% higher cortical calcineurin activity compared to *APOE3* mice. This occurred in the absence of any increase in calcineurin protein levels or mRNA expression. The elevation in calcineurin was associated with 10% fewer dendritic spine number in Layer II/III of the cortex. Treatment with the calcineurin inhibitor FK506 reduced calcineurin activity by 64% and resulted in normalization of dendritic spine numbers in *APOE4* mice.

In conclusion we find that the *APOE4* gene in mice is associated with elevated calcineurin activity and fewer dendritic spine numbers compared to *APOE3* mice. Importantly, calcineurin in *APOE4* remains sensitive to pharmacological inhibition and spine density can be rescued by treatment with FK506.

### Keywords

*APOE* genotype; PP2B; dendritic spine; FK506; Alzheimer's disease

## INTRODUCTION

Dendritic spines are the physical structure that makes up the post-synaptic synapse. They contain glutamate receptors, and therefore are recognized as excitatory synapses. Dendritic spines can be visualized in the central nervous system (CNS) by examining the small membranous protrusions arising from the dendritic arbors of neurons that provide the anatomical architecture for synaptic transmission. Dendritic spines also serve to increase the

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surface area of the postsynaptic density and the number of possible contacts between neurons.<sup>1</sup> Dendritic spines are plastic during neurodevelopment, arising and retracting frequently, but in adult brains, spines remain very stable, especially in the cortex.<sup>2</sup> However, *in vivo* live imaging studies have revealed that spine turnover continues into adult life.<sup>3</sup> While one-month old mice display a net loss of cortical spine density over a 2-week period, adult mice display a consistent 3–5% turnover of cortical dendritic spines.<sup>3</sup> Over the course of 18 months 26% of spines in adult mice that were present at the start of the experiment were eliminated, and 19% new spines had formed, indicating that while the majority of cortical synapses are stable there remains a significant proportion that show continuous plasticity throughout life.<sup>3</sup> Synaptic plasticity can also occur through changes in spine morphology, and there is a strong correlation between the size of the spine and the strength of the synapse. Dendritic spines that are short and stubby are considered to be more mature than those that are long and thin.<sup>4</sup>

Changes in spine morphology are associated with developmental disorders such as autism spectrum disorders and fragile X syndrome. In these cases, dendritic spines display long, thin, filopodia-type structure associated with immature spines.<sup>5</sup> Changes in dendritic spine density are associated with neurodegenerative diseases including Alzheimer's disease.<sup>6</sup> Interestingly the apolipoprotein E (*APOE*) epsilon 4 (*APOE4*) gene has been associated with AD for over 20 years<sup>7</sup> with the *APOE4* allele conveying higher risk of developing AD, as well as earlier age of onset.<sup>7</sup> The *APOE4* allele impacts multiple pathways relevant to Alzheimer's disease pathogenesis including impaired amyloid clearance from the brain,<sup>8</sup> neuroinflammation,<sup>9</sup> reduced synaptic activity,<sup>10</sup> and reduced dendritic spine density in Layer II/III of the cortex.<sup>11</sup> Dendritic spine loss is an early event in *APOE4* mice and is apparent by 3 months of age,<sup>11</sup> although the mechanisms remain unknown. While the apoE protein is produced in astrocytes in the brain,<sup>12</sup> many apoE receptors are located on the post-synaptic synapse.<sup>13,14</sup> These apoE receptors can modify synaptic plasticity by mediating NMDA receptor activation and activation of subcellular signaling cascades.<sup>15</sup>

One of the major regulators of dendritic spine stability is the calcium/calmodulin dependent phosphatase, calcineurin (PP2B). Activation of calcineurin results in dephosphorylating of the GluR1 subunit of the AMPA receptor, and consequential collapse of the post-synaptic density can occur.<sup>16</sup> In this study we aimed to explore the mechanisms underlying the reduction in dendritic spine density in *APOE4* mice. We examined dendritic spine number and morphology in *C57Bl/6*, *APOE3*, and *APOE4* mice with a focus on calcineurin activity. We used the calcineurin inhibitor FK506 to reduce the activity of this phosphatase and quantify the downstream effects on dendritic spines. We found that *APOE4* mice have elevated calcineurin activity that correlates with reduced spine numbers compared to *APOE3* mice. We further found that *APOE4* mice are responsive to calcineurin inhibition with spine number normalizing after 4 days of treatment with FK506.

## EXPERIMENTAL PROCEDURES

### Ethical Statement

Procedures were carried out in accordance with protocols approved by Georgetown University Animal Care and Use Committee, and adhered to all federal regulations.

## Study Design

**Study 1**—Four-month old male C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) were randomly treated with vehicle or FK506 for 4 days (n = 4). Bisected PBS perfused brains were used for calcineurin assay or Golgi staining. For Golgi counts an average of 18 neurons were counted for each animal.

**Study 2**—Four-month old *APOE3* and *APOE4* targeted replacement mouse brains were probed for calcineurin activity or fixed for Golgi staining and dendritic spine counts as described below (n = 5 for each genotype). For Golgi Counts an average of 11 neurons were counted for each animal. A separate cohort of mice (n = 5 for each genotype) were used for mRNA and protein studies.

**Study 3**—Littermates from humanized *APOE3* and *APOE4* mice were randomly assigned to either a vehicle or FK506 group (n=10 for each group). Mice were treated with vehicle or FK506 for 4 days, and were euthanized by CO<sub>2</sub> inhalation 24h after the final drug administration. PBS perfused brains were bisected and assigned to either calcineurin assay (n = 10), assigned for mRNA and protein (n= 5), or fixed for Golgi staining and dendritic spine counts (n = 5). For Golgi Counts an average of 26 neurons were counted for each animal. For spine width studies, an average of 173 individual spines were assessed per group.

## Experimental Procedures

**Experimental Animals**—4–6 month-old male *APOE* targeted replacement mice express human *APOE* alleles in place of murine *ApoE*, under the control of the endogenous murine *ApoE* promoter.<sup>17</sup> Levels of apoE protein and dendritic spine counts in these mice have been previously characterized.<sup>11,18</sup>

**Drug Administration**—FK506 (#3631, Tocris, Minneapolis, MN) was dissolved in 10% ethanol (Fisher Scientific, Hampton, NH) and suspended with 1% Tween 80 (Fisher) in phosphate buffered saline (Fisher). Mice received 10mL/kg bodyweight of a 10mg/mL FK506 solution for a final dose of 100 mg/kg. Mice received a single i.p. injection once a day for 4 days and were euthanized 24h after the final administration.<sup>19</sup>

**Calcineurin Activity Assay**—Calcineurin activity in the brain was measured using a Calcineurin Cellular Activity Assay Kit (#BML-AK816, Enzo Life Sciences, Farmingdale, NY). Entire cortex was isolated from lysed and free phosphates removed. Phosphatase activity was detected using a colorimetric phosphopeptide substrate. A standard curve was constructed using recombinant human calcineurin. Calcineurin activity was calculated using the following formula:  $Phosphatase\ Activity_{(total)} - Phosphatase\ Activity_{(calcium\ independent)} = Calcineurin\ Activity$ .

**Golgi Staining**—For detailed characterization of dendritic spines, we stained brain hemispheres with Golgi stain using a Rapid Golgi Stain Kit (#PK401, FD NeuroTechnologies, Columbia, MD), as previously described.<sup>20,21</sup>

For the layer II/III neurons, we performed two separate counts: dendritic spines on basal shaft (BS) dendrites, and dendritic spines on the apical oblique (AO) dendrites. BS dendrites project directly off the cell soma, and our counts incorporated dendritic spines along a 20  $\mu\text{M}$  section of the shaft between 30–100  $\mu\text{M}$  away from the soma. AO dendrites project off the apical dendrite and our counts only incorporated primary AO dendrites at least 30  $\mu\text{M}$  away from the apical dendrite. Different neurons were used to quantify AO and BS segments of healthy pyramidal neurons of cortical layers II/III. We focused on Layer II/III neurons in the frontal and parietal cortex between Bregma 0.00mm to -5.60mm. Brains were coded prior to Golgi staining and dendritic spine images were captured blind to code, then counted blind to code using Image J Software (National Institute of Health, Bethesda, MD).

**Calcineurin protein and mRNA analysis**—Calcineurin protein detection was performed as previously described,<sup>22</sup> using anti-calcineurin subunit A (#PA5-15579, Fisher) and secondary (anti-rabbit IgG, Jackson ImmunoResearch, West Grove, PA) antibodies.

RNA was extracted from cortex brain tissue using TRIzol® reagent (Invitrogen) and RT-QPCR was performed as described previously<sup>23</sup> using the Prism 7900HT fast sequence detection system (Applied Biosystems). Taqman probes were used to analyse GAPDH (Mm99999915\_m1), Ppp3ca (Mm01317678\_m1) and Ppp3cb (Mm00920265\_m1) under the following cycle conditions, 50°C for 2 minutes, 95°C for 20seconds, (95°C for 1 second, 60°C for 20 seconds)× 40 repeats. The fold change in mRNA expression was calculated using the  $2^{-\text{Ct}}$  method.<sup>24</sup>

### Statistical Analysis

Data were analyzed using either an unpaired t-test for two groups or two-way ANOVA for multiple comparisons with Bonferroni multiple comparisons test. All statistical tests were performed using GraphPad Prism software, version 5.0f (GraphPad Software, Inc., San Diego, CA), and *p* values of less than 0.05 were considered statistically significant.

## RESULTS

### Peripheral administration of FK506 inhibits brain calcineurin activity and increases dendritic spine number in C57Bl/6 mice

Peripheral administration of FK506 for 4 days resulted in a 50.1% reduction in calcineurin activity in C57Bl/6 mice compared to vehicle-treated mice ( $P < 0.05$ ; Fig 1a). Dendritic spine analysis revealed that FK506 caused a significant increase in the number of apical oblique spines (10.7% increase;  $P < 0.05$ ; Fig 1B). We found a 7.4% increase in spine density on basal shaft dendrites however this did not attain significance (Fig 1C). Overall these changes resulted in a 9.1% increase in total spine density ( $P < 0.01$ ; Fig 1D).

### APOE4 mice have elevated basal brain calcineurin activity compared to APOE3 mice, and fewer cortical dendritic spines

Calcineurin activity was measured in isolated cortices from *APOE3* and *APOE4* mice. We found that basal levels of brain calcineurin activity in *APOE4* mice was increased by 36.7% compared to *APOE3* mice ( $P < 0.05$ ; Fig 2A). This increase in activity was not due to an

increase in mRNA expression of the catalytic A subunit (encoded by PP3CA or PP3CB; Fig 2B) or an increase in protein levels of calcineurin A (Fig 2C). The elevated calcineurin activity was associated with decreased dendritic spine number on apical oblique dendrites (10.3% decrease,  $P < 0.05$ ; Fig 2B) and basal shaft dendrites (10.0%,  $P < 0.05$ ; Fig 2C) in APOE4 mice. Total spine number decreased by 10.1% ( $P < 0.05$ ; Fig 2D).

### Dendritic spine loss in APOE4 mice can be rescued with FK506 treatment

We treated APOE3 and APOE4 mice with either vehicle or FK506 for 4 days, and euthanized the mice for analysis 24h after the final dose. Again, we found that sham APOE4 mice had elevated levels of brain calcineurin activity compared to sham APOE3 mice (34.9% increase;  $P < 0.05$ ; Fig 3A). FK506 administration reduced calcineurin activity by 36.6% in APOE3 mice ( $P < 0.05$ ; Fig 3A) and by 64.2% in APOE4 mice ( $P < 0.0001$ ; Fig 3A).

Lastly, Golgi staining revealed that vehicle-treated APOE4 mice had less apical oblique dendritic spines compared to vehicle-treated APOE3 mice (9.8% reduction;  $P < 0.01$ ; Fig 3B). FK506 treatment resulted in a 9.3% increase in spine number in APOE3 mice ( $P < 0.05$ ; Fig 3B), and a 30.6% increase in spine number in APOE4 mice ( $P < 0.0001$ ; Fig 3B).

Apical oblique spine length was not impacted in FK506-treated APOE3 mice, but FK506 caused a 7.8% decrease in the width of the dendritic spine head ( $P < 0.05$  vs APOE4 vehicle; Fig 3C).

## DISCUSSION

In this study we investigated potential mechanisms to explain why APOE4 mice have reduced dendritic spine numbers compared to APOE3 mice. We first validated previously published work to show that peripheral administration of the calcineurin inhibitor FK506 could successfully reduce calcineurin activity in the brain, resulting in increased spine density in the cortex. Here, we report that APOE4 mice have elevated basal calcineurin activity and that this corresponds to decreased spine density in the cortex of these mice. Moreover, we demonstrate that calcineurin in APOE4 mice remains responsive to FK506 treatment, which normalized dendritic spine numbers in APOE4 mice.

Calcineurin activation results in a dephosphorylation of the ser(845) site on the GluR1 subunit of the AMPA receptor. This leads to AMPA receptor endocytosis and an overall reduction in total number AMPA receptors, and ultimately a decrease in dendritic spines.<sup>16</sup> FK506 is an immunosuppressant that binds to a family of proteins called the FK506 binding proteins (FKBP), with the resultant FKBP-FK506 complex directly inhibiting calcineurin activity.<sup>25</sup> Previous studies have found that seven day administration of FK506 in mice results in an increase in the number of dendritic spines in Layer II/III of the cortex.<sup>26</sup> Our data in C57Bl/6 mice confirmed that peripherally administered FK506 can reduce brain calcineurin activity and subsequently increase cortical dendritic spine density. It is an important distinction that FK506 itself does not cause spine growth, but rather prevents the degradation of spines leading to a greater overall spine number.

Dendritic spines are reduced in disease states such as Alzheimer's disease,<sup>6</sup> where synapse number is known to correlate with cognitive decline.<sup>27</sup> Dendritic spine loss is also seen in animal models of Alzheimer's disease, where calcineurin inhibition by FK506 has been shown to ameliorate spine loss.<sup>19</sup> The *APOE4* allele, first identified as a genetic risk factor for late onset Alzheimer's disease over 20 years ago,<sup>7</sup> remains one of the strongest genetic risk-factors identified to date.<sup>28</sup> Early synapse loss and cognitive deficits are characteristic of humanized homozygous *APOE4* mice<sup>11,29</sup> and *APOE* transgenic mice,<sup>30</sup> and there is a *APOE4* allele-dependent loss of dendritic spines in normal aging and Alzheimer's disease brain.<sup>30</sup> The humanized *APOE* mice were used in the present study, and here we have confirmed that *APOE4* mice have reduced spine density in the cortex compared to *APOE3* mice. The major novel finding in this report is that we examined subcellular mechanisms that regulate dendritic spine loss and found that calcineurin activity is higher in *APOE4* mice compare age-matched *APOE3* mice. This change appears to be a change in enzyme kinetics, as neither mRNA nor protein levels were altered by *APOE4*.

To our knowledge this is the first report of a change in calcineurin activity in *APOE4* mice, however published data is supportive of a role for differential regulation of this phosphatase by *APOE* genotype. In an in vitro study, neurons co-cultured with glial cells from humanized *APOE4* mice displayed delayed spine formation and early synaptic loss due to impaired regulation of the AMPA receptor in dendritic spines.<sup>31</sup> As discussed above, calcineurin is a key regulator of AMPA dynamics in dendritic spines.<sup>16</sup> Multiple factors can influence how calcineurin is activated including the frequency, duration, and amplitude of calcium input following NMDA receptor stimulation.<sup>32</sup> When rat hippocampal neurons are treated with apoE protein there is an increase in intracellular free calcium, with apoE4 protein resulting in higher levels of intracellular calcium compared to apoE3 protein.<sup>33</sup> This elevation in intracellular calcium is due to calcium influx, and not due to intracellular calcium storage release.<sup>34</sup> The apoE4 protein may also modulate the NMDA receptor directly as NMDA-dependent LTP induction in hippocampal *APOE4* slices is lower than slices from *APOE3* mice.<sup>35</sup> These changes in intracellular calcium may be responsible for the basal increase in calcineurin activity in the *APOE4* mice. Other studies indicate that apoE-containing lipoproteins can protect neurons against oxidative stress by inhibiting the activation of calcineurin.<sup>36</sup> ApoE4-containing lipoproteins are less effective than apoE3-containing lipoproteins at inhibiting this calcineurin-dependent neuroprotection.<sup>36</sup>

To determine if the observed elevation in calcineurin was responsible for the reduced spine levels in *APOE4* mice, we administered the calcineurin inhibitor FK506 and found that we could completely reverse spine loss in *APOE4* mice. Indeed, *APOE4* mice appeared more sensitive to FK506 treatment compared to either C57Bl/6 or *APOE3* mice, given that FK506 resulted in stronger effects on both calcineurin activity and spine density in *APOE4* mice. There is a strong correlation between the size of the spine and the strength of the synapse, whereas spines that are short and stubby are considered to be more mature than those that are long and thin.<sup>4</sup> The increase in spines in FK506-treated *APOE4* mice was associated with a higher proportion of immature spines, as indicated by the reduced average width of the synaptic protrusion. These data suggest that the turnover of dendritic spines is higher in *APOE4* mice compared to *APOE3* mice, but the newly formed immature spines caused by the suppression of calcineurin activity are not degraded as quickly as they are in vehicle-

treated mice. Further studies with longer timepoints post-treatment, or longer treatments, are needed to determine if these immature spines can mature or reverse cognitive deficits in *APOE4* mice.

In conclusion we find that the *APOE4* gene in mice is associated with elevated calcineurin activity and fewer dendritic spine numbers compared to *APOE3* mice. Importantly, calcineurin in *APOE4* remains sensitive to pharmacological inhibition and spine density can be rescued by treatment with FK506.

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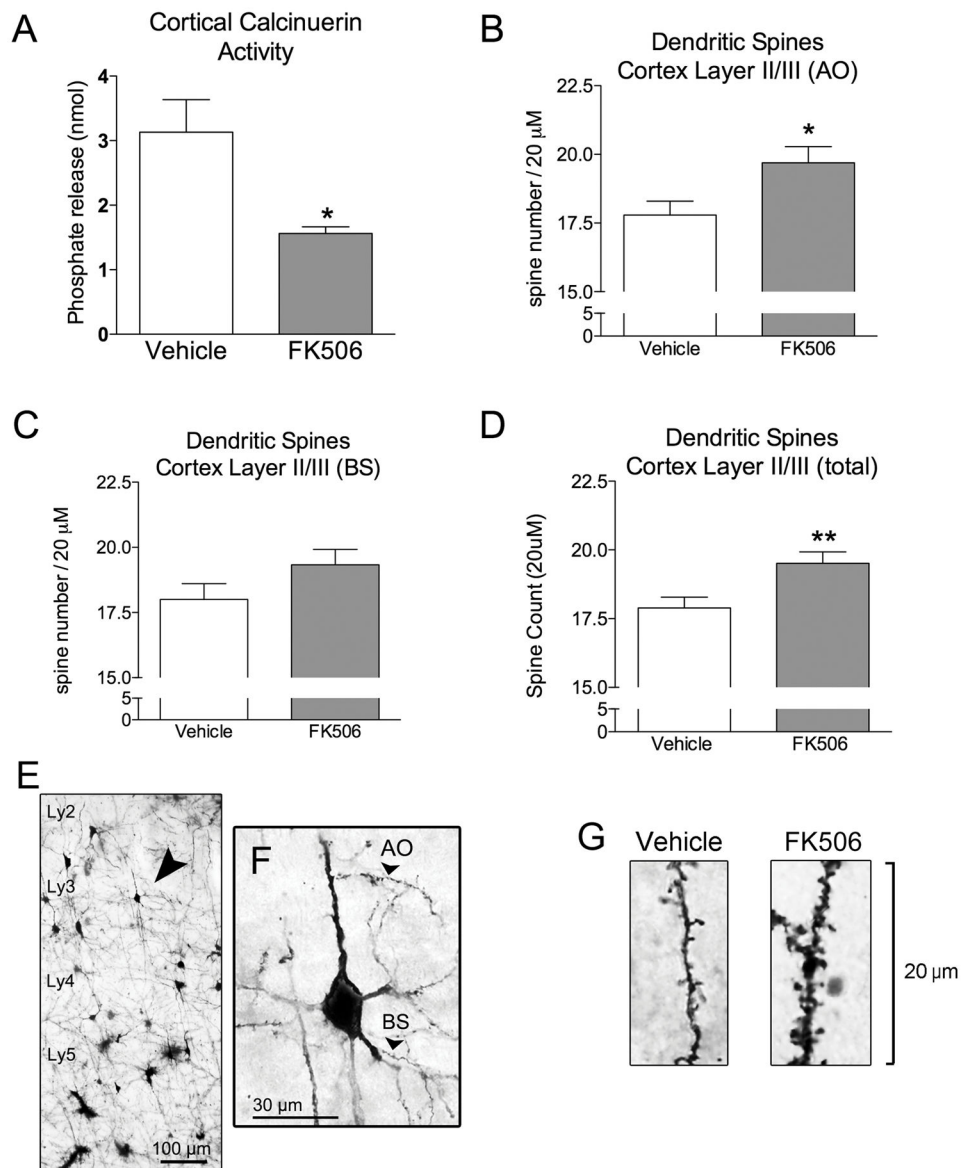
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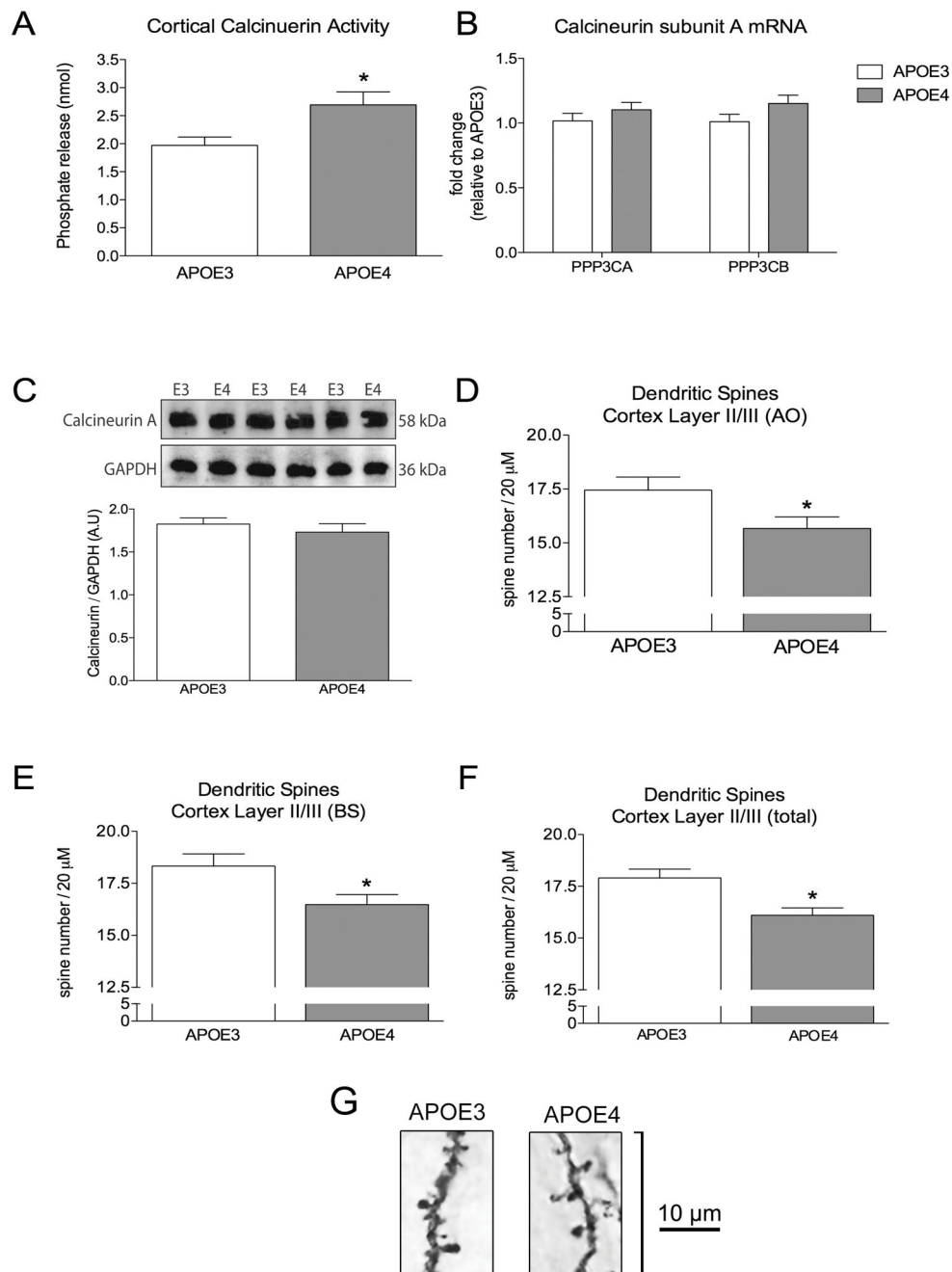
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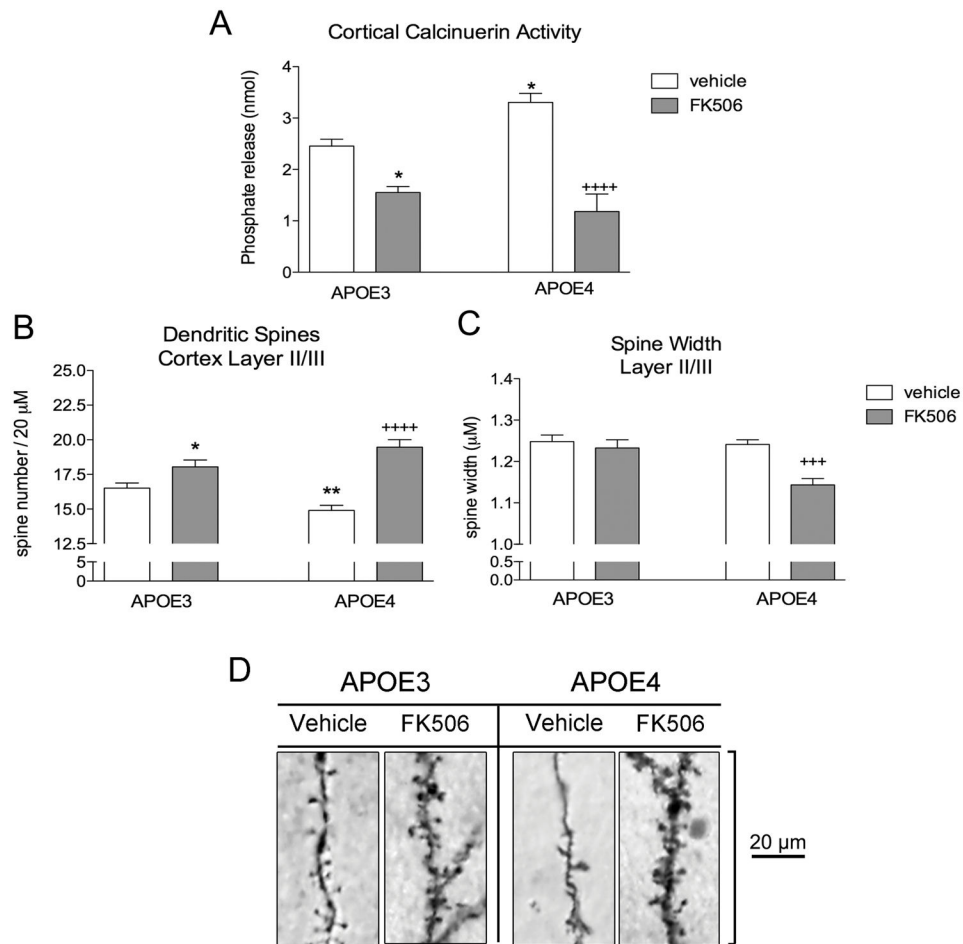


**Figure 1. Calcineurin inhibition by FK506 causes dendritic spine growth in C57Bl/6 mice**  
**A)** Four day peripheral administration of 100 mg/kg FK506 results in a decrease in calcineurin activity in the mouse cortex. **B)** Spine density quantification in the apical oblique (AO) dendrites, **C)** basal shaft (BS) dendrites, and **D)** total spine counts following four day vehicle or FK506 administration in mice. **E–F)** Golgi stained cortical section showing the layers of the cortex, and the areas of the dendrites where spines were quantified. **G)** Representative images of the Golgi stained AO dendrites spines following vehicle or FK506 treatment. Unpaired t-test. \* =  $P < 0.05$ ; \*\* =  $P < 0.01$ .



**Figure 2. *APOE4* targeted replacement mice have elevated calcineurin activity and reduced cortical dendritic spine density**

**A)** Calcineurin activity is increased in the cortex of naïve *APOE4* mice compared to *APOE3* mice. **B)** QT-PCR determined the relative levels of PPP3CA and PPP3CB, two of the genes responsible for producing isoenzymes of the catalytic calcineurin-A subunit. **C)** Protein levels of the catalytic calcineurin-A subunit were quantified by western blot. **D)** Spine density quantification of apical oblique (AO) dendrites, **E)** basal shaft (BS) dendrites, and **F)** total spine counts. **G)** Representative images of the Golgi stained AO dendrites in *APOE3* and *APOE4* mice. Unpaired t-test. \* =  $P < 0.05$ .



**Figure 3. Calcineurin inhibition by FK506 reverses spine loss in *APOE4* targeted replacement mice**

**A)** Four day peripheral administration of 100 mg/kg FK506 results in a decreased calcineurin activity in both *APOE3* and *APOE4* mouse cortex. **B)** Spine density quantification of apical oblique dendrites. **C)** Spine head width of apical oblique dendritic spines. **D)** Representative images of the Golgi stained AO dendrites following vehicle or FK506 treatment. Two way ANOVA with Bonferroni multiple comparisons. \* =  $P < 0.05$  vs *APOE3* vehicle; \*\* =  $P < 0.01$  vs *APOE3* vehicle; +++ =  $P < 0.001$  vs *APOE4* vehicle; ++++ =  $P < 0.0001$  vs *APOE4* vehicle.