Loss of Endothelial Nitric Oxide Synthase Promotes p25 Generation and Tau Phosphorylation in a Murine Model of Alzheimer’s Disease

Short Communication

Susan A. Austin, Zvonimir S. Katusic

Rationale: Alzheimer’s disease has an unknown pathogenesis; however, cardiovascular risk factors are associated with a higher incidence of Alzheimer’s disease. A defining feature of endothelial dysfunction induced by cardiovascular risk factors is reduced bioavailable endothelial nitric oxide (NO). We previously demonstrated that endothelial NO acts as an important signaling molecule in neuronal tissue.

Objective: We sought to determine the relationship between the loss of endothelial NO synthase (eNOS) and tau phosphorylation in neuronal tissue.

Methods and Results: We used eNOS knockout (−/−) mice as well as an Alzheimer’s disease mouse model, amyloid precursor protein (APP)/PSEN1dE9+/− (PS1) that lacked eNOS (APP/PS1/eNOS−/−) to examine expression of tau kinases and tau phosphorylation. Brain tissue from eNOS−/− mice had statistically higher ratios of p25/p35, indicative of increased cyclin-dependent kinase 5 activity as compared with wild-type (n=8; P<0.05). However, tau phosphorylation was unchanged in eNOS−/− mice (P>0.05). Next, we determined the role of NO in tau pathology in APP/PS1/eNOS−/−. These mice had significantly higher levels of p25, a higher p25/p35 ratio (n=12–14; P<0.05), and significantly higher cyclin-dependent kinase 5 activity (n=4; P<0.001). Importantly, APP/PS1/eNOS−/− mice also had significantly increased tau phosphorylation (n=4–6; P<0.05). No other changes in amyloid pathology, antioxidant pathways, or neuroinflammation were observed in APP/PS1/eNOS−/− mice as compared with APP/PS1 mice.

Conclusions: Our data suggests that loss of endothelial NO plays an important role in the generation of p25 and resulting tau phosphorylation in neuronal tissue. These findings provide important new insights into the molecular mechanisms linking endothelial dysfunction with the pathogenesis of Alzheimer’s disease.

Key Words: Alzheimer’s disease ■ Cdk5 ■ cerebrovascular disease ■ endothelial nitric oxide synthase ■ tau
Methods

Animals

Nos3<sup>−/−</sup>/J (eNOS<sup>−/−</sup>), stock no 002684, and C57BL/6 (wild-type) mice, stock no 000664, were purchased from Jackson Laboratory (Bar Harbor, ME). APPsw, PSEN1DE9<sup>−/−</sup> (APP/PS1) mice on the C57BL/6 genetic background were originally purchased from Jackson laboratory as stock no 005864. Subsequently, APP/PS1 mice were transferred from Jackson Laboratory to Mutant Mouse Resource & Research Centers (MMRRC), and mice were purchased from MMRRC as stock no 034832-JAX. eNOS<sup>−/−</sup> mice were bred from MMRRC as stock no 005864. Subsequently, APP/PS1 and APP/PS1/eNOS<sup>−/−</sup> mice were bred in house. The average breeding pair has 2 to 3 litters with viable pups, and these litters show a balance of both genotypes (Table 1). Importantly, the p25/p35 ratio was not different between the groups. 

A detailed methods section is provided in the Online Data Supplement and includes detailed methods regarding genotyping/polymerase chain reaction, tissue collection, confocal microscopy, Western blotting, ELISA for beta amyloid (Aβ), and statistical analysis, which are as previously described.6,7

Results

Ratio of p25/p35 Is Significantly Higher in the Brains of eNOS<sup>−/−</sup> Mice

We sought to determine whether chronic loss of endothelial NO affected protein kinases known to be involved in the phosphorylation of tau. First, we examined the levels of Cdk5 and its activators p35 and p25. Although there was no difference between protein levels of Cdk5 or p35, p25 tended to be increased in the brain tissue from eNOS<sup>−/−</sup> mice as compared with wild-type mice (Figure 1A–1E). Importantly, the increased ratio of p25/p35, an established index of increased Cdk5 activity, was significantly higher in the eNOS<sup>−/−</sup> brain tissue as compared with wild-type (Figure 1D; <i>P</i> &lt; 0.05).

We also examined the levels of GSK3β and Akt, 2 other kinases involved in aberrant tau phosphorylation. There were no differences observed in their expression or phosphorylation, suggesting that their expression and phosphorylation in the brain are unaffected by loss of eNOS (Online Figure I).

No Alteration in Tau Phosphorylation in eNOS<sup>−/−</sup> Brain Tissue

To determine whether the increased p25/p35 ratio led to increased tau phosphorylation, we measured protein levels of tau and phosphorylated tau (pTau) in the brains of wild-type and eNOS<sup>−/−</sup> mice. No differences were observed in tau or pTau levels in brain tissue (Figure 1F–1I; <i>P</i> &gt; 0.05).

Characterization of APP/PS1/eNOS<sup>−/−</sup> Mice

There was no difference in body weight between wild-type, APP/PS1, or APP/PS1/eNOS<sup>−/−</sup> mice (Online Table I). Systolic blood pressure was significantly elevated in APP/PS1/eNOS<sup>−/−</sup> mice as compared with both wild-type and APP/PS1 mice (Online Table I; <i>P</i> &lt; 0.001 from wild-type and <i>P</i> &lt; 0.01 from APP/PS1). Next, we examined circulating levels of glucose, total cholesterol, high-density lipoprotein cholesterol, and triglycerides (Online Table I). Of these, only triglycerides were significantly different between the groups. Triglycerides were significantly higher in APP/PS1/eNOS<sup>−/−</sup> mice as compared with both wild-type and APP/PS1 mice (Online Table I; <i>P</i> &lt; 0.01 from wild-type and <i>P</i> &lt; 0.05 from APP/PS1).

p25 and Tau Phosphorylation in APP/PS1/eNOS<sup>−/−</sup> Brain Tissue

We examined protein expression of p35, p25, and Cdk5 by Western blot. p25 protein levels and p25/p35 ratio were significantly higher in the brains of APP/PS1/eNOS<sup>−/−</sup> mice as compared with both wild-type and APP/PS1 mice (Figure 2A–2D; *<i>P</i> &lt; 0.05 from wild-type and **<i>P</i> &lt; 0.05 from APP/PS1). Importantly, p25 and the p25/p35 ratio were not significantly different in the APP/PS1 brain tissue as compared to wild-type (Figure 2). Notably, Cdk5 enzyme activity was significantly higher in Cdk5 isolated from APP/PS1/eNOS<sup>−/−</sup> brain tissue as compared with wild-type and APP/PS1 mice. Furthermore, expression of p25/p35 (Figure 3) and Cdk5 (Online Figure II) was mainly observed in neuronal tissue as demonstrated by the colocalization with NeuN, a neuronal marker.

Levels of total tau were unchanged between wild-type, APP/PS1, and APP/PS1/eNOS<sup>−/−</sup> mice (Figure 4A and 4C), whereas protein levels of pTau and the ratio of pTau/Tau were significantly increased in the brains of the APP/PS1/eNOS<sup>−/−</sup> mice as compared with the other mice (Figure 4A, 4B, and 4D; ***<i>P</i> &lt; 0.001, **<i>P</i> &lt; 0.01 from wild-type and *<i>P</i> &lt; 0.05 from APP/PS1). However, although pTau tended to be higher in APP/PS1 mice (<i>P</i> &lt; 0.05), the pTau/Tau ratio was not different between wild-type and APP/PS1 mice (Figure 4A–4D). Immunohistochemical analysis of pTau showed cellular localization within neurons as indicated by colocalization with the neuronal marker. Increased pTau immunoreactivity was observed in the cortex (Figure 4E) and hippocampus (data not shown) of APP/PS1/eNOS<sup>−/−</sup> mice as compared with wild-type and APP/PS1 mice. We did perform immunohistological examinations of brain tissue using an antibody for neurofibrillary tangles but did not observe any evidence of these tangles in the brain sections from APP/PS1 or APP/PS1/eNOS<sup>−/−</sup> mice at the 3 to 4 months of age we examined (data not shown). Although phosphorylated GSK3β and the ratio of phosphorylated GSK3β/GSK3β tended to be higher in the brains of APP/PS1/eNOS<sup>−/−</sup> mice, they did not reach statistical significance (Online Figure III; <i>P</i> &gt; 0.05).

Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
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<tr>
<td>Aβ</td>
<td>beta amyloid</td>
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<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
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<tr>
<td>APP</td>
<td>amyloid precursor protein</td>
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<tr>
<td>Cdk5</td>
<td>cyclin-dependent kinase 5</td>
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<tr>
<td>eNOS</td>
<td>endothelial nitric oxide synthase</td>
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<tr>
<td>eNOS&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>eNOS knockout/deficient</td>
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<td>GSK3β</td>
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<td>neuronal nitric oxide synthase</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>pTau</td>
<td>phosphorylated tau</td>
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Expression and Amyloidogenic Processing of APP in APP/PS1/eNOS−/− Mice

We examined protein levels of APP and β-site APP cleaving enzyme 1 in the brain tissue of wild type, APP/PS1, and APP/PS1/eNOS−/− mice. There was a significant increase in APP protein expression in APP/PS1 and APP/PS1/eNOS−/− mice as compared with wild-type mice (Online Figure IV A and IVB; *P<0.05 as compared with wild-type) and an increase in β-site APP cleaving enzyme 1 protein levels in APP/PS1/eNOS−/− as compared with wild-type mice (Online Figure IVC; *P<0.05); however, there was no difference between APP or β-site APP cleaving enzyme 1 protein levels between APP/PS1 and APP/PS1/eNOS−/− mice (Online Figure IV).

We measured circulating levels of Aβ40 and Aβ42 in these mice and found no significant differences between APP/PS1 and APP/PS1/eNOS−/− mice (data not shown, n=4–6 animals per background; P>0.05). Furthermore, when we examined brain tissue levels of soluble Aβ40 and Aβ42, we found no differences (data not shown, n=4–6 animals per background; P>0.05).

NOS Isoforms, Cyclooxygenase Pathway, Antioxidant Systems, and Microglia Activation

To determine whether there were compensatory changes in other important endothelial pathways, we examined protein levels of inducible NOS (iNOS), neuronal NOS (nNOS), cyclooxygenase-1, cyclooxygenase-2, and prostacyclin synthase. Importantly, iNOS and nNOS protein levels were unchanged by the loss of eNOS (Online Figure V). Furthermore, levels of cyclooxygenase enzymes and prostacyclin synthase were not altered in APP/PS1/eNOS−/− mice (data not shown, n=6–8 animals per background; P>0.05). Levels of the major antioxidant enzymes were unchanged between wild-type, APP/PS1, and APP/PS1/eNOS−/− mice (Online Figure V). Furthermore, levels of 2-hydroxyethidium, a measure of superoxide anion, were not different in APP/PS1/eNOS−/− mice (Online Figure VIF).

Figure 1. p25/35 ratio is significantly higher in the brain tissue of eNOS−/− mice as compared with wild-type, although tau phosphorylation is unchanged. A, Brain tissue from 4-month-old wild-type and eNOS−/− mice was analyzed by Western blot. A representative image is shown. Densitometric analysis for (B) p35, (C) p25, (D) p25/p35 ratio, and (E) Cdk is shown. Data are presented as relative mean optical density (OD)±SEM (n=8 animals per background, *P<0.05 from wild-type). B, Brain tissue from 4-month-old wild-type and eNOS−/− mice was analyzed by Western blot. A representative image is shown. Densitometric analysis of (G) pTau, (H) tau, and (I) pTau/tau ratio is shown. Data are presented as relative mean OD±SEM (n=8 animals per background). Cdk5 indicates cyclin-dependent kinase 5; eNOS, endothelial nitric oxide synthase; eNOS−/−, eNOS deficient; and pTau, phosphorylated tau.
Neuronal and astrocyte markers, NeuN, and glial fibrillary acidic protein, respectively, were unchanged (data not shown, n=4–6 animals per background, P>0.05). Importantly, microglial markers, cluster of differentiation 68, major histocompatibility complex II, and ionized calcium-binding adaptor molecule-1 were not different between the 3 groups of mice (Online Figure VII). Levels of interleukin-1α, as measured by ELISA, were not altered in the brains of APP/PS1/eNOS−/− mice (Online Figure VII). Finally, no differences were seen between wild-type, APP/PS1, or APP/PS1/eNOS−/− brain tissue in a proinflammatory array that examined 40 cytokines (data not shown).

**Discussion**

Our results are significant because they continue to demonstrate the importance of endothelial dysfunction as a plausible factor in the development of AD pathology. We demonstrate for the first time that loss of endothelial NO leads to alterations in neuronal p25, an aberrant activator of the tau kinase, Cdk5. Indeed, in our APP/PS1/eNOS−/− mice, increased p25 is accompanied by statistically higher enzymatic activity of Cdk5 and increased levels of pTau. It is important to note that this effect seems specific to loss of endothelial NO in the AD mouse model. Although we cannot completely rule out that alterations in NO produced by iNOS and nNOS may contribute to the upregulation of Cdk activity and increased pTau levels, these seem unlikely. First, in our previous studies, we reported that the loss of endothelial NO in eNOS−/− mice resulted in decreased microvascular levels of NOx, while overall brain NOx levels were unchanged, which suggests that loss of eNOS did not lead to compensatory changes in nNOS- or iNOS-produced NO within the brain tissue. Importantly, loss of eNOS alone, as seen in the eNOS−/− mice, was sufficient to increase generation of p25. Second, a search of the literature did not return any results suggesting that iNOS or nNOS protein levels or enzyme activity were increased in young APP/PS1 mice. In addition, we report that expression of iNOS and nNOS is not elevated in APP/PS1/eNOS−/− mice, thereby, reinforcing our conclusion that loss of eNOS function is primarily responsible for elevated phosphorylation of pTau. Finally, we did report increased systolic blood pressure in APP/PS1/eNOS−/− mice and, therefore, cannot completely rule out hypertension as a contributing factor in the changes we report.

It is established that tau is predominantly expressed in neurons, and we were not able to detect tau protein in cerebral microvessels or in cultured brain microvascular endothelial cells.
In addition, our immunohistochemical analysis demonstrated colocalization of pTau with the neuronal marker, NeuN. Cdk5 and GSK3β are the most relevant kinases involved in tau phosphorylation. Increased Cdk5 activity is associated with hyperphosphorylation of tau, paired helical fragments, and neurite death. The increased pTau we observed in APP/PS1/eNOS−/− mice seems to be mediated by increased activity of Cdk5 because loss of endothelial NO did not lead to changes in other tau kinases, namely GSK3β and Akt. Although phosphorylated GSK3β tended to be higher in APP/PS1/eNOS−/− mice, this phosphorylation site is an inhibitory site that would lead to decreased GSK3β activity and, thus, is not likely to be responsible for the increased pTau we see. Indeed, we report here for the first time that loss of eNOS led to an increased p25/p35 ratio. It is reported that increased p25/p35 ratio, caused by increased generation of p25 by calpain, leads to an aberrant activation of Cdk5, promoting tau phosphorylation and neurodegeneration. Importantly, our observed increased in p25 and p25/p35 ratio was accompanied by increased Cdk5 enzymatic activity in APP/PS1/eNOS−/− mice. Notably, in the present study, increased tau phosphorylation was seen in APP/PS1/eNOS−/− mice and not in eNOS−/− mice or APP/PS1 mice as compared with age-matched wild-type mice at 4 months of age. This suggests that there may be a synergistic effect between the loss of eNOS and amyloid alterations present in the APP/PS1 mouse, resulting in early appearance of tau pathology. Indeed, prior studies established that pTau was detected in adult APP/PS1 mice, only at 7 to 10 months of age. Tau acts to stabilize microtubules within neurons. Hyperphosphorylation of tau can cause tau to dissociate from the microtubules, thereby, leading to neurofibrillary tangle formation and eventually neuronal dysfunction and death. We did not observe neurofibrillary tangles in the 4-month-old APP/PS1/eNOS−/− mice; however, the earlier appearance of increased pTau in APP/PS1/eNOS−/− as compared with what is reported in the literature for APP/PS1 mice suggests that the loss of endothelial NO may accelerate the pathology timeline in these mice. Future studies will need to be performed to document the time course of pathological and cognitive alterations in these mice as they age.

There are 2 mechanisms by which NO can mediate signaling changes: cyclic guanosine monophosphate and S-nitrosylation. Treatment of Tg2576 mice with sildenafil, a phosphodiesterase 5 inhibitor that increases levels of cyclic guanosine monophosphate, led to decreased Cdk5 activity, as well as GSK3β activity. Furthermore, it is reported that S-nitrosylation can inhibit calpain activity, an enzyme responsible for cleavage of p35 to p25. Won et al reported that S-nitrosoglutathione treatment of purified calpain protein inhibited its activity. Consistent with our findings, Annamalai et al demonstrated that treatment of APP/PS1 mice with S-nitrosoglutathione led to decreased calpain-mediated cleavage of p35 and decreased Cdk5 activity. Taken together, these data suggest that loss of endothelial NO may alter the activity of calpain, thus, leading to increased Cdk5 activity. Although it is reported that several other stimuli, such as oxidative stress, Aβ exposure, and neuroinflammation, can all lead to activation of calpain, we did not observe any changes in Aβ levels, the antioxidant enzyme system or superoxide anion production, or inflammatory markers between APP/PS1 and APP/PS1/eNOS−/− mice, making these unlikely sources of calpain activation.

The results in this study provide novel findings regarding yet another role for vascular dysfunction in AD-related pathology. We report that loss of endothelial NO, a primary feature of endothelial dysfunction, leads to increased p25 production. Importantly, increased p25, increased p25/p35 ratio, is an established mechanism responsible for elevated Cdk5 activity.
and indeed, Cdk5 enzyme activity was significantly higher in APP/PS1/eNOS<sup>−/−</sup> mice. Furthermore, loss of endothelial NO in APP/PS1, an AD mouse model, led to statistically higher phosphorylation of tau. Our data, thus, provide significant evidence supporting the role of endothelial NO in the preservation of brain health. These findings also have significant

![Figure 4](http://circres.ahajournals.org/)
implications for the development of therapies designed to treat and prevent mild cognitive impairment and AD.

Sources of Funding
This work was supported by National Institutes of Health grants HL-111062 and HL-131515, the Mayo Alzheimer’s Disease Research Center (Z.S. Katusic), American Heart Association (AHA) Postdoctoral Fellowship (AHA no 12POST855003; S.A. Austin) and AHA Scientist Development Award (AHA no 14SDG20410063; S.A. Austin), and the Mayo Foundation.

Disclosures
None.

References

What Is Known?
1. Cardiovascular risk factors are associated with a higher incidence of Alzheimer’s disease (AD).
2. A common feature of cardiovascular risk factors is endothelial dysfunction, specifically, a loss of bioavailable endothelial nitric oxide (NO).
3. Hyperphosphorylated tau is the primary component of neurofibrillary tangles, one of the hallmark pathologies of AD.

What New Information Does This Article Contribute?
1. Endothelial nitric oxide synthase (eNOS)-deficient (eNOS−/−) mice display increased levels of p25, an aberrant activator of cyclin-dependent kinase 5, which is one of the primary kinases responsible for tau hyperphosphorylation, and a statistically higher p25/p35 ratio.

Novelty and Significance

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- We generated a novel AD mouse model that also lacks eNOS, APP/PS1/eNOS−/− mice.
- APP/PS1/eNOS−/− mice displayed increased p25, p25/p35, cyclin-dependent kinase 5 enzyme activity, and hyperphosphorylated tau.

Cardiovascular risk factors are associated with a higher incidence of AD. Loss of bioavailable endothelial NO is a common feature of these risk factors. The results of this study provide evidence that loss of endothelial NO leads to increased tau phosphorylation, a major mechanism responsible for the development of neurodegeneration in AD. Our findings identify a new role for endothelial NO in the pathogenesis of AD. Presented results support the concept that preservation of the endothelial NO pathway is a therapeutic target in the prevention and treatment of AD.
Loss of Endothelial Nitric Oxide Synthase Promotes p25 Generation and Tau Phosphorylation in a Murine Model of Alzheimer's Disease

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Circ Res. 2016;119:1128-1134; originally published online September 6, 2016; doi: 10.1161/CIRCRESAHA.116.309686

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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Supplemental Material

Methods

Animals
Nos3tm1Unc/J (eNOS−/−), stock #002684, and C57BL/6 (wild-type) mice, stock #000664, were purchased from Jackson Laboratory (Bar Harbor, ME). APPswe,PSEN1dE9−/+ (APP/PS1) mice on the C57BL/6 background were originally purchased from Jackson laboratory as stock #005864. Subsequently, APP/PS1 mice were transferred from Jackson Laboratory to Mutant Mouse Resource & Research Centers (MMRRC) and mice were purchased from MMRRC as stock #034832-JAX. eNOS−/− mice were bred with C57BL/6 mice to generate eNOS+−/− mice. To generate wild type, APP/PS1, and APP/PS1/eNOS−/− mice used in experiments, eNOS+−/− (female) and APP/PS1/eNOS+/− (male) mice were bred in house. The average breeding pair has 2-3 litters with viable pups and these resultant litters average 4-5 pups. We wish to point out that APP/PS1/eNOS−/− mice are found in very small numbers in the resulting litters, approaching 1 mouse in every 22-25 mice born. Male mice were sacrificed at 4-5 months of age by a lethal dose of pentobarbital.

Genotyping
Genotyping was performed using APP, PS1, and eNOS primers according to Jackson Laboratories. Briefly, DNA was isolated from a 2-3 mm piece of mouse tail using the PureLink Genomic DNA mini kit (Invitrogen) following manufacturer’s instructions. APP transgene primer sequences were 5’- AGG ACT GAC CAC TCG ACC AG -3’ and 5’ CGG GGG TCT AGT TCT GCA T -3’. The positive control gene used was TCR alpha with the sequences 5’- CAA ATG TTG CTT GTC TGG TG -3’ and 5’- GTC AGT CGA GTG CAC AGT TT -3’. PS1 transgene primer sequences were 5’- AAT AGA GAA CGG CAG GAG CA -3’ and 5’- GCC ATG AGG GCA CTA ATC ATC TT -3’. I12 was used as the positive control gene and the sequences were 5’- CTA GGC CAC AGA ATT GAA AGA TCT -3’ and 5’- GTA GGT GGA AAT TCT AGC ATC C -3’. The sequence for mutant (the knock out) eNOS was 5’- AAT TCG CCA ATG ACA AGA CG -3’. The wild type eNOS sequence was 5’- AGG GGA ACA AGC CCA GTA GT -3’. And the common reverse primer for eNOS was 5’- CTT GTC CCC TAG GCA CCT CT -3’.

Tissue collection
Brains were carefully removed and immediately placed in ice cold modified Krebs-Ringer bicarbonate solution containing 118.6 mmol/L NaCl, 4.7 mmol/L KCl, 2.5 mmol/L CaCl2, 1.2 mmol/L KH2PO4, 25.1 mmol/L NaHCO3, 0.026 mmol/L EDTA, 10.1 mmol/L glucose plus protease inhibitors as previously described[1]. Large cerebral arteries, including basilar and cerebral arteries were carefully removed prior to homogenization.

Glucose, Cholesterol, and Triglyceride measurements
Blood was collected via right ventricular puncture. Glucose was measured in whole blood using Accu Check (Roche Diagnostics, Indianapolis, IN). Blood was centrifuged (2,000 rpm, 10 mins, 4°C) and stored at -80° until all samples were collected. Total triglyceride and cholesterol levels were measured using the Hitachi 912 chemistry analyzer (Roche Diagnostics).
**Blood pressure**
Mice were trained for blood pressure measurements. Systolic blood pressure was measured in non-anesthetized mice using the tail cuff method as previously described[2] (Harvard Apparatus Ltd, Kent, England).

**Confocal Microscopy**
Mice were killed by an overdose of pentobarbital and perfused with PBS followed by 4% paraformaldehyde. Brains were dissected and fixed in a 4% paraformaldehyde solution. Tissue was embedded in paraffin and longitudinal sections (5 µm) were cut. Tissue was deparaffinized and rehydrated prior to antigen retrieval using sodium citrate heat-induced epitope retrieval. Briefly, sections were heated at 98°C for 20 minutes in sodium citrate buffer (10 mM sodium citrate, 0.05% Tween-20, pH 6.0). Tissue was permeabilized using 0.1% Triton X-100 in 2% fetal bovine serum. Sections were incubated with anti-pTau (Santa Cruz, Dallas, TX), anti-p35/p25 (Cell Signaling, Danvers, MA), or anti-Cdk5 (Millipore, Temecula, CA) antibodies and anti-NeuN (neuronal marker) Alexa Fluor 647 conjugated antibody. Sections were incubated with FITC conjugated secondary antibodies for visualization of pTau, p25/p35, and Cdk antibodies. To examine for neurofibrillary tangles, sections were incubated with antineurofibrillary tangle antibodies (Chemicon, and Millipore, Temecula, CA). 4',6'-diamidino-2-phenylindole dilactate (DAPI) was used to visualize nuclei. Sections were visualized using a Zeiss LSM 780 laser scanning confocal microscope.

**Western blotting**
To perform Western blot analyses, tissue homogenates were lysed in ice cold Triton lysis buffer (10 mmol/L Hepes, 50 mmol/L NaF, 50 mmol/L NaCl, 5 mmol/L EDTA, 5 mmol/L EGTA, 100 µmol/L Na3VO4, 50 mmol/L Na pyrophosphate and 1% Triton X-100) as previously described[1]. Equal protein amounts were resolved by SDS-PAGE and transferred to nitrocellulose membranes for Western blotting. Blots were probed with primary antibodies. BACE1, Cdk5, p35/p25, nNOS, pTau (Ser 396), GSK3β and pGSK3β (Ser 9) were purchased from Cell Signaling (Danvers, MA). Iba-1 was obtained from Abcam, (Cambridge, MA) and CD68 and MHC II were purchased from Santa Cruz Biotechnology (Dallas, TX). APP was obtained from Upstate Cell Signaling (Temecula, CA) and COX-1 was purchased from Invitrogen (Camarillo, CA). COX-2, eNOS, and iNOS were purchased from BD Transduction Laboratories (San Jose, CA). The GFAP antibody was purchased from StemCell Technologies (Vancouver, BC, Canada). Mn SOD, EC SOD, and CuZn SOD were purchased from Enso Life Sciences (Farmingdale, NY). The NeuN antibody was purchased from Millipore (Billerica, MA) and the PGI2S antibody was obtained from Cayman Chemical (Ann Arbor, MI). Catalase and Actin (loading control) were purchased from Sigma-Aldrich (St. Louis, MO).

**Intracellular superoxide anion**
Brain tissue, cut into small pieces, was incubated in Kreb’s-Hepes with 50 µmol/L dihydroethidium (Molecular Probes, Eugene, OR) at 37° for 15 minutes. Brain tissue was homogenized in methanol and intracellular superoxide anions were quantified using HPLC-based fluorescence and normalized using mg protein[3].
**Mouse Cytokine Array**
Brain tissue was collected and homogenized as described above. Brain tissue lysates were analyzed using Mouse Cytokine Array Panel A (# ARY006, R&D Systems, Minneapolis, MN) per manufacturer’s instructions. This array contained nitrocellulose membranes with 40 different cytokine antibodies.

**IL-1α ELISA**
Brain tissue levels of IL-1α were measured using a commercially available colorimetric ELISA kit following manufacturer’s instructions (R&D Systems, Minneapolis, MN).

**Aβ ELISA**
Circulating plasma levels of Aβ1-40 and Aβ1-42 were measured using a commercially available colorimetric ELISA kit following manufacturer’s instructions (Invitrogen, Camarillo, CA).

**Cdk5 enzyme activity**
Cdk5/p35 was precipitated, using anti Cdk-5 (Santa Cruz Biotechnology, Dallas, TX), from wild type, APP/PS1, and APP/PS1/eNOS-/- mouse brain tissue lysate. The Cdk5 kinase assay was then performed in kinase buffer (40 mmol/L Tris [pH 7.5], 20 mmol/L MgCl2, 0.1 mg/mL BSA) with 150 µmol/L ATP and 5 µg Histone H1 protein in a final reaction volume of 25 µL for 30 mins. at room temperature. The ADP-Glo Kinase assay was used according to manufacturer’s instructions (Promega, Madison, WI). The ADP-Glo kinase assay is a luminescent assay that measures the amount of ADP formed from the kinase reaction.

**Statistical analysis**
Data are represented as mean ± SEM. Statistical analysis was performed using one-way ANOVA with Tukey-Kramer post hoc comparison or the unpaired Student’s t-test for parametric data. For non-parametric data statistical analysis was performed using Kruskal-Wallis with Dunn’s multiple comparison as the post hoc comparison.
References

Online Table I. Characteristics of wild type, APP/PS1, and APP/PS1/eNOS−/− mice. Body weight, systolic blood pressure, glucose, total cholesterol, HDL cholesterol, and triglycerides were measured. Data are presented as mean ± SEM (n=6-14 animals per background, **P<0.01, ***P<0.001 from wild type and &P<0.05, #P<0.01 from APP/PS1).
Online Figure I. Protein and phosphorylation levels of GSK3β and Akt are unaltered in the brains of eNOS<sup>−/−</sup> mice. **A**, Brain tissue from 4 month old wild type and eNOS<sup>−/−</sup> mice was analyzed by Western blot. A representative image is shown. Densitometric analysis for **B**, pGSK3β, **C**, GSK3β, and **D**, pGSK3β/GSK3β ratio, is shown. **E**, Brain tissue from 4 month old wild type and eNOS<sup>−/−</sup> mice was analyzed by Western blot. A representative image is shown. Densitometric analysis for **F**, pAkt, **G**, Akt, and **H**, pAkt/Akt ratio, is shown. Data is presented as relative mean O.D. ± SEM (n=8 animals per background).
Online Figure II. Cdk5 immunoreactivity was found primarily in neuronal cells in the cortex. Fixed tissue sections from the brains of wild type, APP/PS1, and APP/PS1/eNOS−/− animals were immunolabeled with anti-Cdk5 (with an anti-rabbit IgG FITC secondary) and anti-NeuN Alexa Fluor 647 conjugated primary. 4′,6′-diamidino-2-phenylindole dilactate (DAPI) to visualize nuclei. Representative images of the cortex are shown. Magnification 40x; bar is representative of 20 µm.
Online Figure III. Protein and phosphorylation levels of GSK3β are unaltered in the brains of APP/PS1/eNOS−/− mice. A, Brain tissue from 4 month old wild type, APP/PS1, and APP/PS1/eNOS−/− mice was analyzed by Western blot. A representative image is shown. Densitometric analysis for B, pGSK3β, C, GSK3β, and D, pGSK3β/GSK3β ratio, is shown. Data is presented as relative mean O.D. ± SEM (n=9-11 animals per background).
Online Figure IV. APP is increased in the brains of APP/PS1 and APP/PS1/eNOS<sup>−/−</sup> mice while BACE1 is higher in only APP/PS1/eNOS<sup>−/−</sup> mice as compared to wild type. **A**, Brain tissue from 4 month old wild type, APP/PS1, and APP/PS1/eNOS<sup>−/−</sup> mice was analyzed by Western blot. A representative image is shown. Densitometric analysis for **B**, APP, and **C**, BACE1 is shown. Data is presented as relative mean O.D. ± SEM (n=8 animals per background, *P<0.05).
Online Figure V. iNOS and nNOS are unchanged in APP/PS1/eNOS−/− mice. A, Brain tissue from 4 month old wild type, APP/PS1, and APP/PS1/ eNOS−/− mice was analyzed by Western blot. A representative image is shown. Densitometric analysis for B, eNOS, C, iNOS, and D, nNOS is shown. Data is presented as relative mean O.D. ± SEM (n=10-14 animals per background, ***P<0.001 from wild type, $P<0.001 from APP/PS1).
Online Figure VI. Antioxidant enzyme levels from brain tissue of wild type, APP/PS1, and APP/PS1/eNOS<sup>−/−</sup> are not different. A, Brain tissue from 4 month old wild type, APP/PS1, and APP/PS1/eNOS<sup>−/−</sup> mice was analyzed by Western blot. A representative image is shown. Densitometric analysis for B, CuZn SOD, C, catalase, D, EC SOD, and E, Mn SOD is shown. Data is presented as relative mean O.D. ± SEM (n=3-6 animals per background). F, Brain tissue, cut into small pieces, was incubated with 50 µmol/L dihydroethidium at 37° for 15 minutes. Brain tissue was homogenized in methanol and intracellular superoxide anions quantified by HPLC-based fluorescence. Intracellular superoxide anions were normalized to mg protein of each sample (n=6). Data are represented as mean ± SEM.
Online Figure VII. Several microglial markers are unaltered in brains of APP/PS1/eNOS\(^{-}\) mice. 
A, Brain tissue from 4 month old wild type, APP/PS1, and APP/PS1/eNOS\(^{-}\) mice was analyzed by Western blot. A representative image is shown. Densitometric analysis for B, CD68, C, MHC II, and D, Iba-1 is shown. Data is presented as relative mean O.D. ± SEM (n=8-10 animals per background). E, Levels of IL-1\(\alpha\) in brain tissue lysates from 5-6 mice per background were measured via a commercially available ELISA. Data is presented as mean ± SEM.