Supplemental Material

Methods

Animals

Nos3^{tm1Unc}/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 AT -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 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 $CaCl_2$, 1.2 mmol/L KH_2PO_4, 25.1 mmol/L $NaHCO_3$, 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 anti-neurofibrillary tangle antibodies (Chemicon, and Millipore, Temecula, CA). 4',6'-diamiddino-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 Na_3VO_4, 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 $MgCl_2$, 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

1. Austin SA, Santhanam AV, Katusic ZS (2010) *Endothelial nitric oxide modulates expression and processing of amyloid precursor protein.* Circulation research 107: 1498-1502.
2. d'Uscio LV, Smith LA, Katusic ZS (2011) *Differential effects of eNOS uncoupling on conduit and small arteries in GTP-cyclohydrolase I-deficient hph-1 mice.* American journal of physiology Heart and circulatory physiology 301: H2227-2234.
3. Santhanam AV, d'Uscio LV, Smith LA, Katusic ZS (2012) *Uncoupling of eNOS causes superoxide anion production and impairs NO signaling in the cerebral microvessels of hph-1 mice.* Journal of neurochemistry 122: 1211-1218.

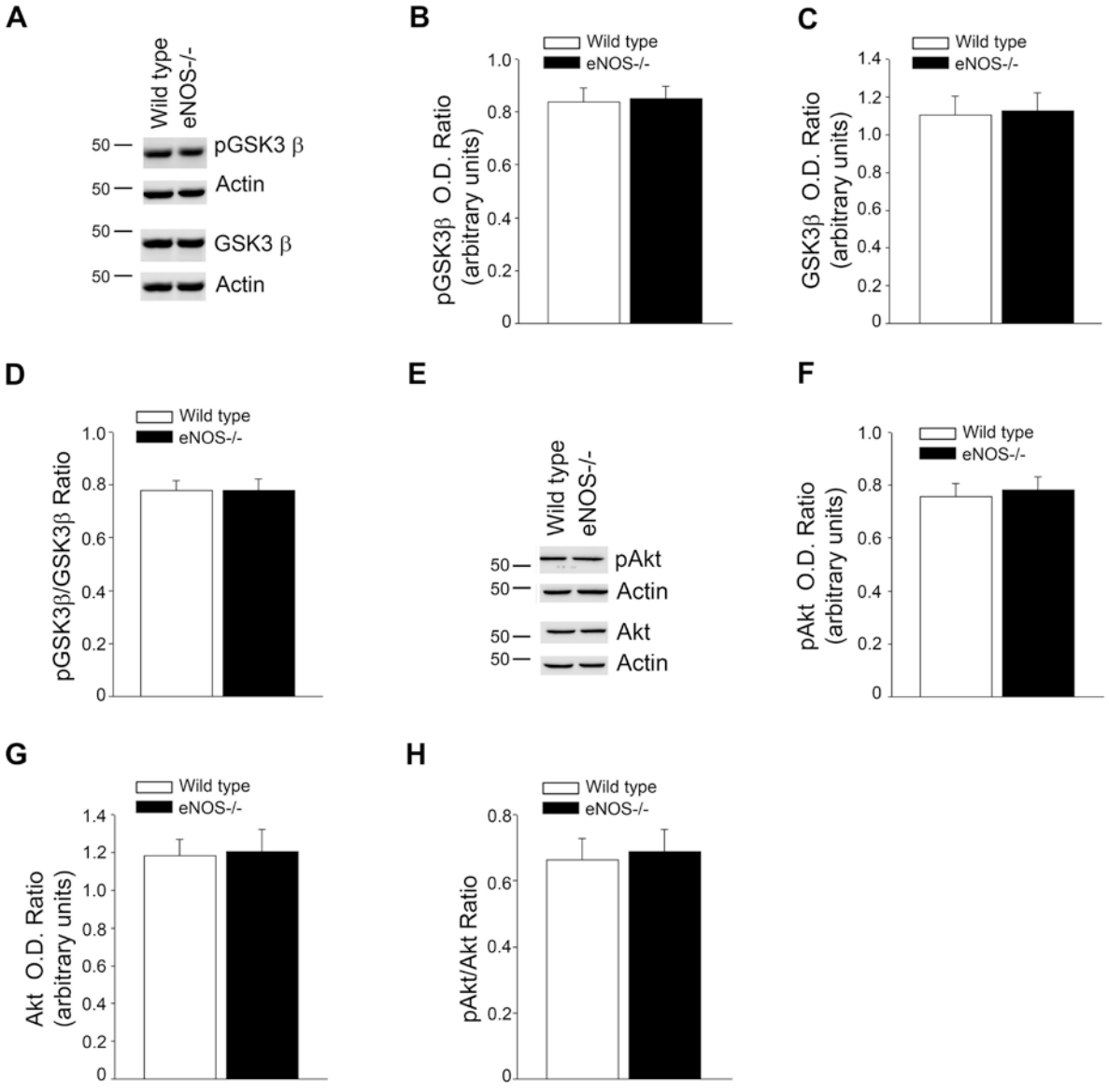
Supplemental Tables & Figures

	Wild type	APP/PS1 Tg	APP/PS1/eNOS-/-
Body weight (g)	29.73 ± 0.66	30.51 ± 1.00	29.28 ± 0.60
Systolic blood pressure	103.56 ± 2.73	117.95 ± 3.26	137.58 ± 7.28 ****, #
Glucose (mg/dL)	218.57 ± 12.71	218.91 ± 14.02	211.38 ± 13.09
Total cholesterol (mg/dL)	67.57 ± 4.16	61.17 ± 3.90	67.86 ± 2.69
HDL cholesterol (mg/dL)	50.71 ± 3.58	46.67 ± 3.25	51.08 ± 2.42
Triglycerides (mg/dL)	74.31 ± 6.78	68.83 ± 4.22	103.62 ± 6.37 **, &

**P<0.01 from wild type; ***P<0.001 from wild type
& P<0.05 from APP/PS1; # P<0.01 from APP/PS1

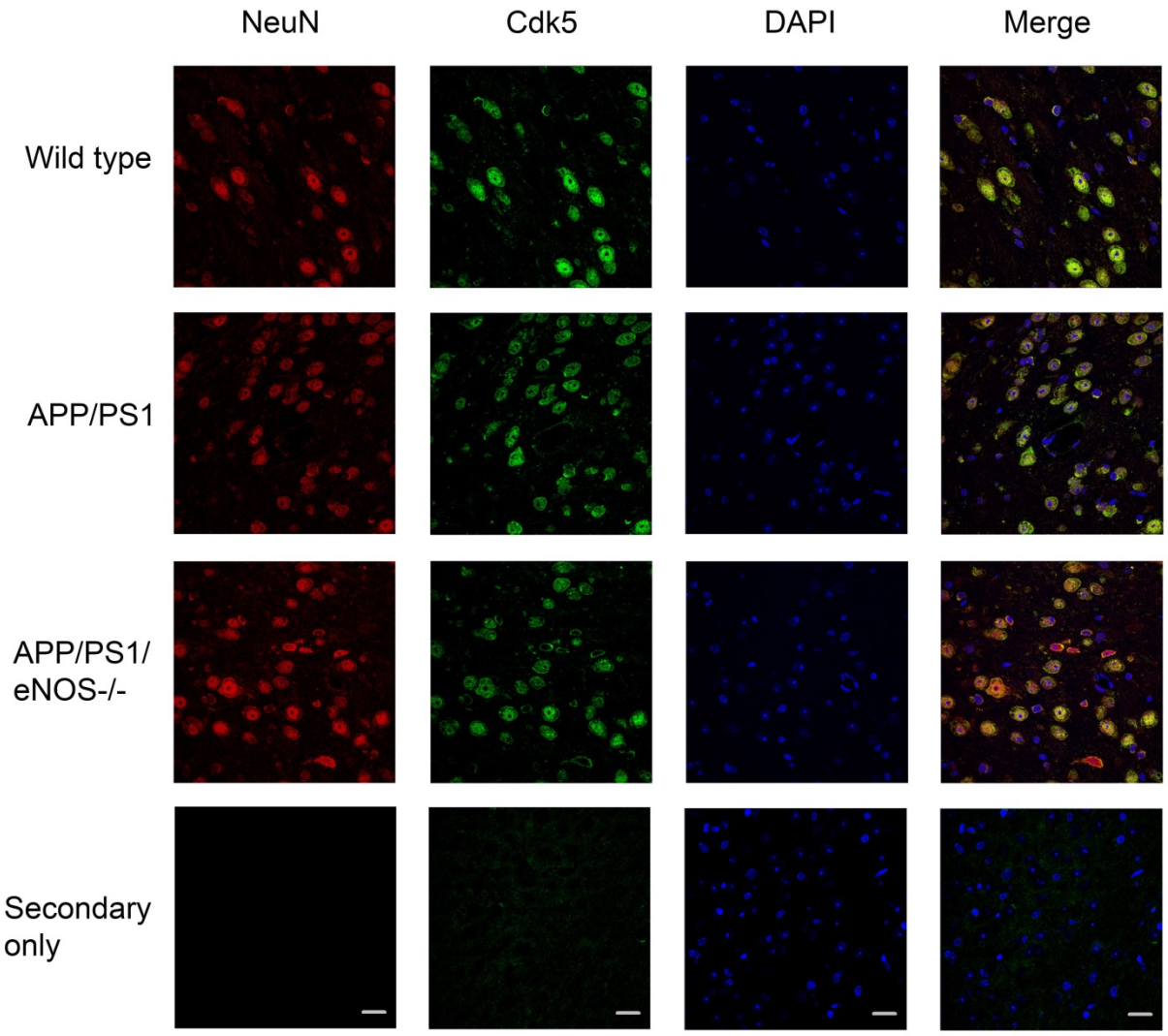
Online Table I. Characteristics of wild type, APP/PS1, and APP/PS1/eNOS$^{-/-}$ mice. Body weight, systolic blood pressure, glucose, total cholesterol, HDL cholesterol, and triglycerides were measured. Data are presented as mean ± SEM (n=6-14 animals per background, **P<0.01, ***P<0.001 from wild type and &P<0.05, #P<0.01 from APP/PS1).

Online Figure I



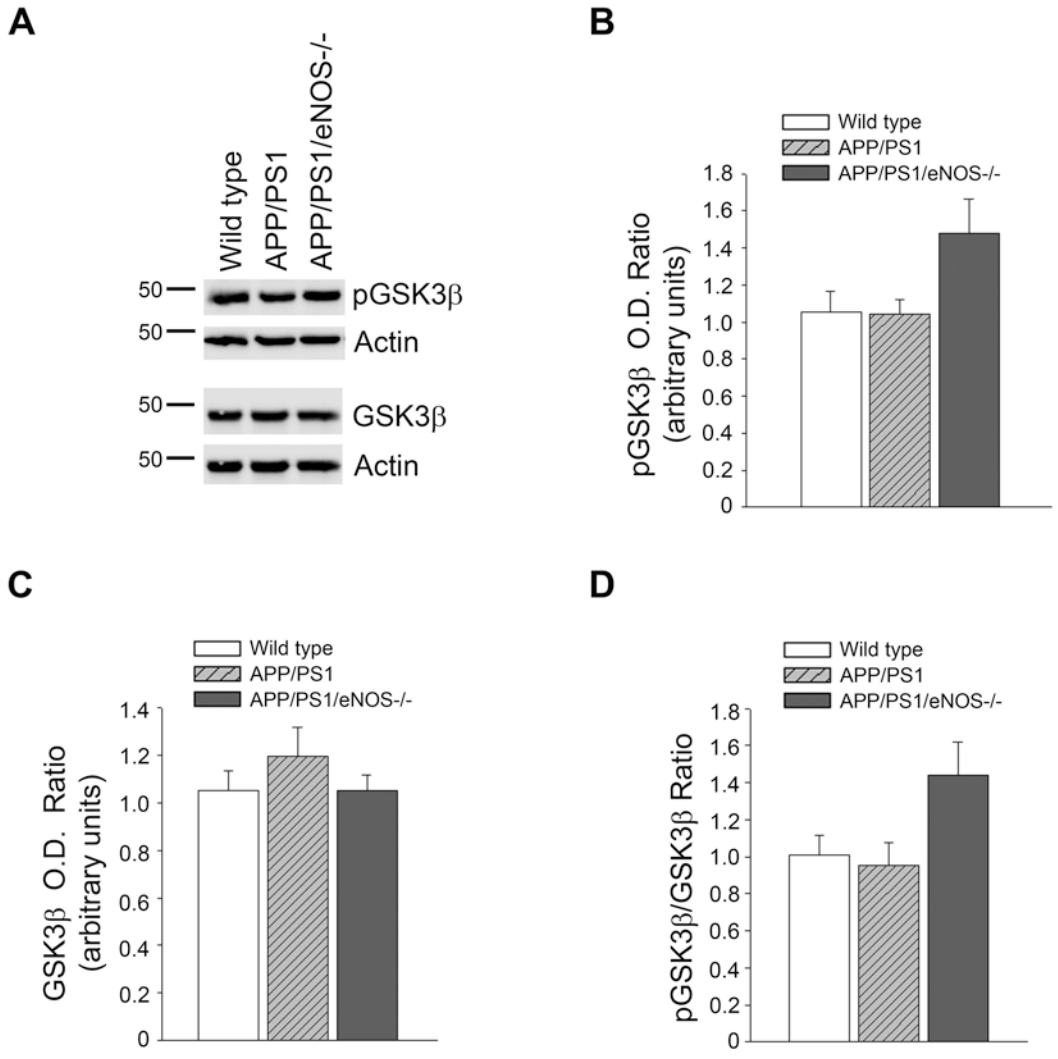
Online Figure I. Protein and phosphorylation levels of GSK3β and Akt are unaltered in the brains of eNOS$^{-/-}$ mice. **A,** Brain tissue from 4 month old wild type and eNOS$^{-/-}$ mice was analyzed by Western blot. A representative image is shown. Densitometric analysis for **B,** pGSK3β, **C,** GSK3β, and **D,** pGSK3β/GSK3β ratio, is shown. **E,** Brain tissue from 4 month old wild type and eNOS$^{-/-}$ 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



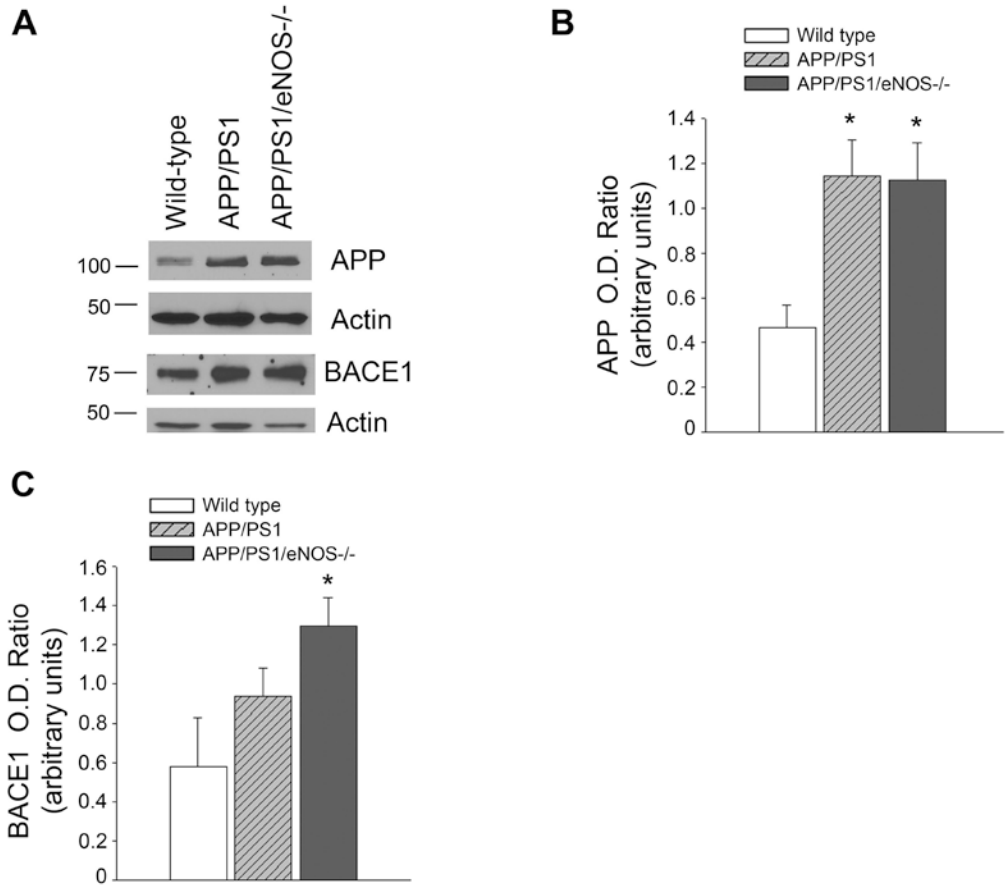
Online Figure II. Cdk5 immunoreactivity was found primarily in neuronal cells in the cortex. Fixed tissue sections from the brains of wild type, APP/PS1, and APP/PS1/eNOS$^{-/-}$ animals were immunolabeled with anti-Cdk5 (with an anti-rabbit IgG FITC secondary) and anti-NeuN Alexa Fluor 647 conjugated primary. 4',6'-diamiddino-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



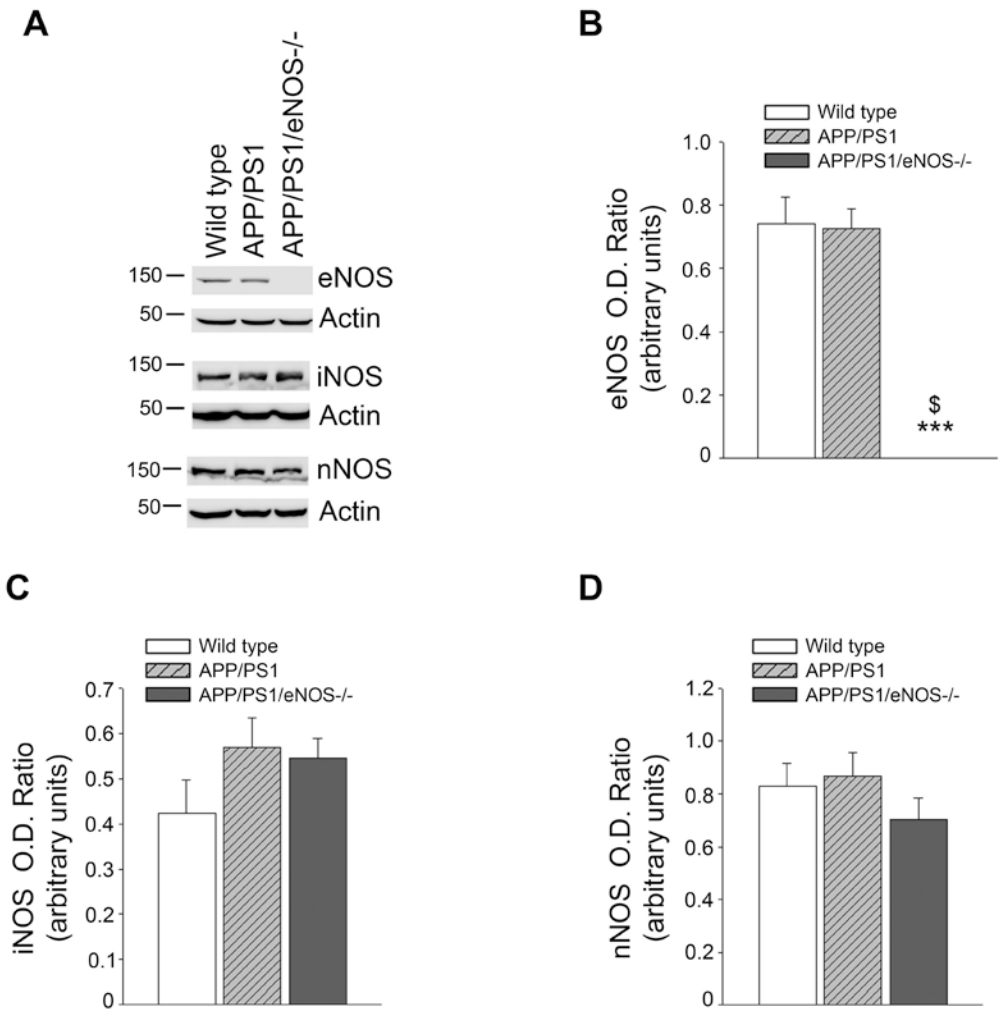
Online Figure III. Protein and phosphorylation levels of GSK3β are unaltered in the brains of APP/PS1/eNOS$^{-/-}$ mice. **A,** Brain tissue from 4 month old wild type, APP/PS1, and APP/PS1/eNOS$^{-/-}$ mice was analyzed by Western blot. A representative image is shown. Densitometric analysis for **B,** 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



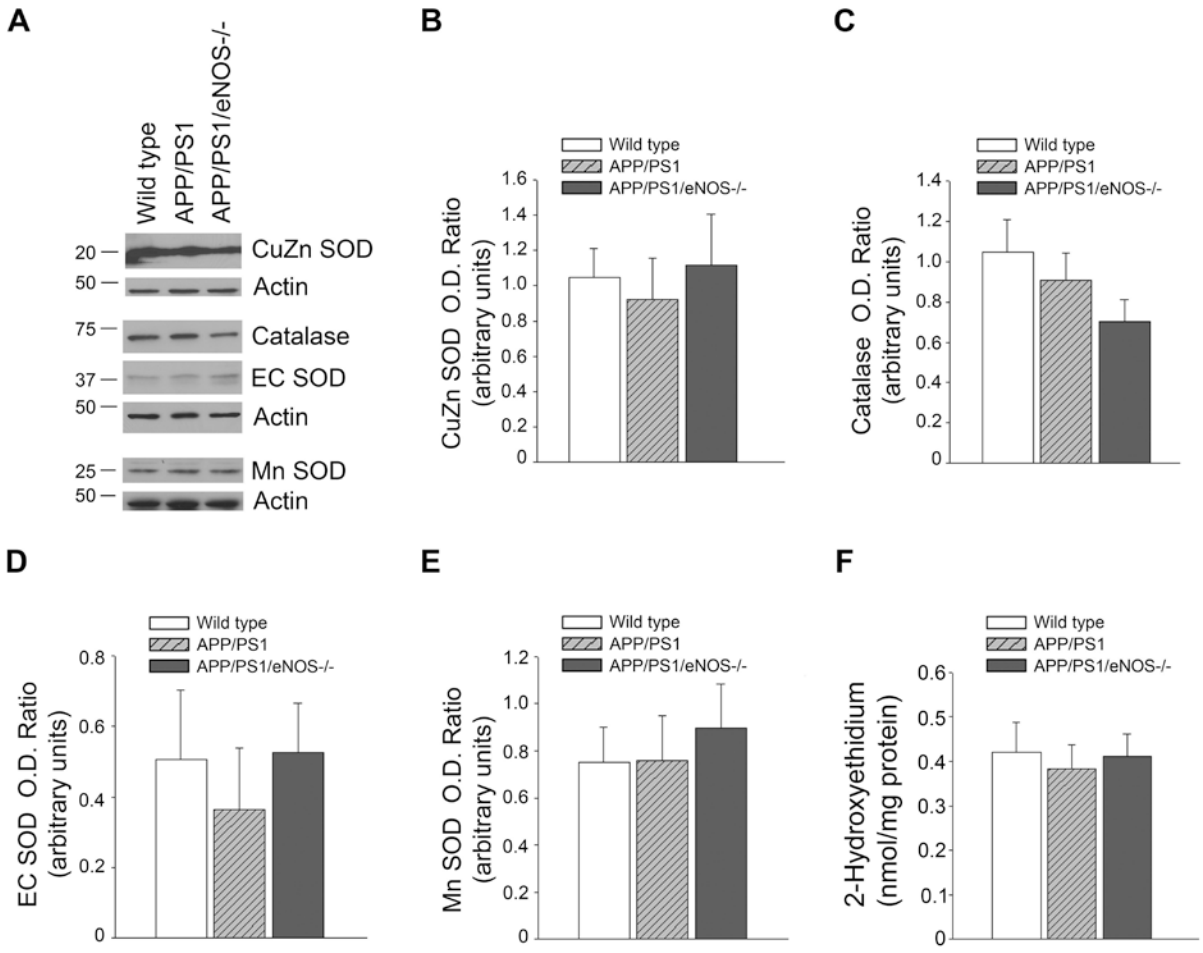
Online Figure IV. APP is increased in the brains of APP/PS1 and APP/PS1/eNOS$^{-/-}$ mice while BACE1 is higher in only APP/PS1/eNOS$^{-/-}$ mice as compared to wild type. **A,** Brain tissue from 4 month old wild type, APP/PS1, and APP/PS1/eNOS$^{-/-}$ mice was analyzed by Western blot. A representative image is shown. Densitometric analysis for **B,** 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



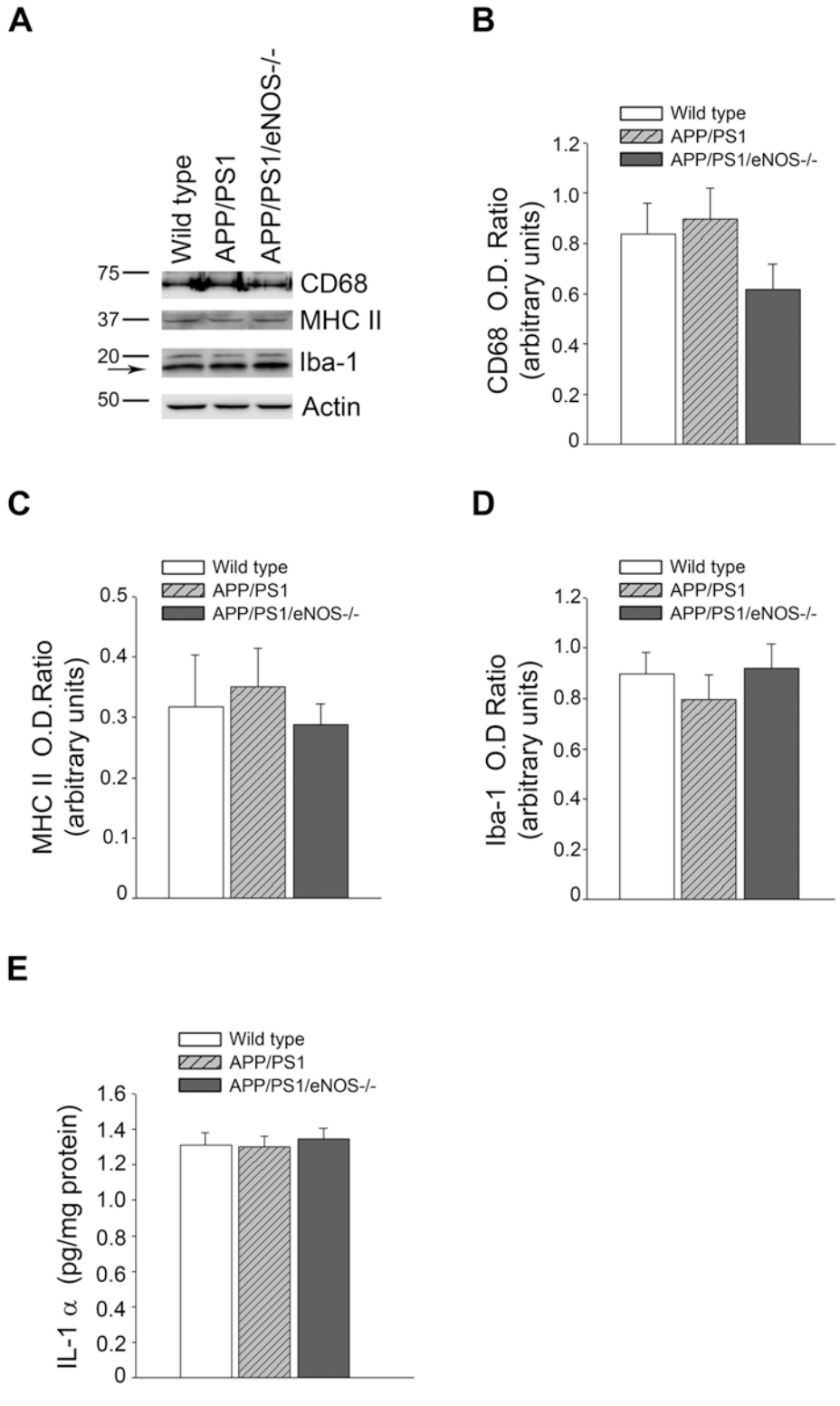
Online Figure V. iNOS and nNOS are unchanged in APP/PS1/eNOS$^{-/-}$ mice. **A,** Brain tissue from 4 month old wild type, APP/PS1, and APP/PS1/ eNOS$^{-/-}$ mice was analyzed by Western blot. A representative image is shown. Densitometric analysis for **B,** eNOS, **C,** iNOS, and **D,** nNOS is shown. 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



Online Figure VI. Antioxidant enzyme levels from brain tissue of wild type, APP/PS1, and APP/PS1/eNOS$^{-/-}$ are not different. **A,** Brain tissue from 4 month old wild type, APP/PS1, and APP/PS/ eNOS$^{-/-}$ 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



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-1α in brain tissue lysates from 5-6 mice per background were measured via a commercially available ELISA. Data is presented as mean ± SEM.