Supporting Information

Cirrito et al. 10.1073/pnas.1107411108

SI Materials and Methods

Animals. All experimental procedures involving animals were performed in accordance with guidelines established by the Animal Studies Committee at Washington University. We bred *PS1APP*$^{+/-}$ hemizygous mice (1) to wild-type C3H/B6 mice (Jackson Labs), then used the *PS1APP*$^{+/-}$ offspring at various ages for use in these experiments. These mice contain a PS1ΔE9 mutation and human amyloid precursor protein (APP) Swedish mutation, which were inserted into a single locus. Animals were screened for the presenilin-1 (PS1) and APP transgenes by PCR from tail DNA. At ∼5–6 mo of age, this mouse model begins to develop amyloid plaques.

Compounds. All pharmacological and chemical agents were purchased from Sigma-Aldrich unless otherwise noted. All compounds that were delivered to the hippocampus via reverse microdialysis were diluted in microdialysis perfusion buffer, which consisted of artificial cerebrospinal fluid (aCSF) with 4% BSA. FR180204 and PD98059 were purchased from Tocris Bioscience.

In Vivo Amyloid-β (Aβ) Microdialysis. In vivo microdialysis to assess brain interstitial fluid (ISF) Aβ in the hippocampus of awake, freely moving PS1APP$^{+/-}$ mice was performed similarly to previously described (2, 3). This technique samples soluble molecules within the extracellular fluid, which are smaller than 30 kDa, the molecular-weight cut off of the probe membrane. Aβ capable of entering the probe has been dubbed "exchangeable Aβ" (eAβ).

Under isoflurane volatile anesthetic, guide cannula (BR style; Bioanalytical Systems) were cemented into the left hippocampus (3.1 mm behind Bregma, 2.5 mm lateral to midline, and 1.2 mm below dura at a 12° angle). Two millimeter microdialysis probes were inserted through the guides so the membrane was contained entirely within the hippocampus (BR-2, 30-kDa molecular weight cut-off membrane; Bioanalytical Systems). Microdialysis perfusion buffer was aCSF (perfusion buffer in mM: 1.3 CaCl2, 1.2 $MgSO_4$, 3 KCl, 0.4 KH_2PO_4, 25 NaHCO3, and 122 NaCl, pH 7.35) containing 4% BSA (Sigma) that was filtered through a 0.1-μM membrane. Flow rate was a constant 1.0 μL/min. Samples were collected every 60–90 min with a refrigerated fraction collector into polypropylene tubes and assessed for $Aβ_{x-40}$ or $Aβ_{x-42}$ by ELISA at the completion of each experiment. Basal levels of ISF Aβ were defined as the mean concentration of Aβ over the 6-h preceding drug treatment. For each animal, all Aβ levels were normalized to the basal Aβ concentration. Once basal ISF Aβ levels were established, PS1APP mice were treated with either citalopram (10 mg/kg body weight, comparable to an adult human dose of 50 mg/day) (4), fluoxetine (10 mg/kg), desvenlafaxine (30 mg/kg), tianeptine (20 mg/kg), or vehicle (PBS) intraperitoneally or with serotonin (2 mM) by adding the drug to the microdialysis perfusion buffer to infuse the drug directly into the hippocampus (reverse microdialysis). Other agents were delivered at the doses and methods as noted.

For BDNF injection into the hippocampus, microdialysis probes with a separate injection port at the tip (IBR probes; Bioanalytical Systems) were used. This permitted us to perform microdialysis and still administer a large protein to the hippocampus, which would not readily diffuse out of the probe. BDNF was preloaded into a 10-μL Hamilton syringe and connected to the infusion port of the microdialysis probe with FEP tubing. BDNF, 0.5 μg in aCSF, was injected into the hippocampus using a syringe pump (Stoelting) at a flow rate of 0.05 μL/min for 20 min.

Aβ Elimination Half-Life. ISF Aβ half-life was determined as described (2) and microdialysis probes were inserted as described above. New steady-state ISF Aβ levels were established following treatment with either serotonin (2 mM) or vehicle (PBS) by reverse microdialysis for 8 h. At the end of 8 h, mice were coadministered a potent, blood-brain permeable γ-secretase inhibitor, Compound E, (synthesized by AsisChem) (5), (20 mg/kg) intraperitoneally to rapidly block Aβ production. Microdialysis samples were collected every 60 min for 6 h and then assayed for $Aβ_{x-40}$ by ELISA. The half-life of ISF Aβ was calculated on the basis of the slope of the semilog plot of % change in Aβ versus time (2). Only Aβ values that were continually decreasing were included in half-life analysis (the first 3 h following Compound E administration).

Aβ Sandwich ELISA. ISF Aβ levels were assessed using sandwich ELISAs as described (6). Briefly, a mouse anti-$Aβ_{40}$ antibody (mHJ2) or mouse anti-$Aβ_{42}$ antibody (mHJ7.4) was used to capture and a biotinylated central domain antibody (mHJ5.1) was used to detect, followed by streptavidin poly–HRP-40 (Fitzgerald Industries). All ELISAs were developed using Super Slow ELISA TMB (Sigma) and absorbance read on a Bio-Tek Epoch plate reader at 650 nm. The standard curves for each assay used synthetic human $Aβ_{1-40}$ or $Aβ_{1-42}$ peptide (American Peptide).

ERK Pathway Multiplex Assay. Hippocampal tissue was isolated from PS1APP hemizygous mice treated with citalopram (10 mg/kg i.p.) or vehicle (PBS) and killed 8 h later. Tissue was lysed in the EpiTag Cell Lysis kit (Novagen) by mechanical homogenization. ERK2 total protein and phosphorylated forms of MEK1/2 and ERK2 were measured together using the WideScreen EpiTag ERK Pathway panel I (Novagen) with concentrations calibrated against the EpiTag ERK Pathway Standards mix. Bead fluorescence was quantified using the xMAP Luminex platform.

Secretase Assays. Enzymatic activity of α-secretase and β-secretase was measured using FRET-based cleavage assays (R&D Systems). For studies in young mice (3–4 mo old), hippocampal tissue was isolated from PS1APP hemizygous mice treated with citalopram (10 mg/kg) or vehicle (PBS) and killed 8 h later. For 7-mo-old mice (plaque bearing), cortical tissue from mice in the chronic citalopram study was used. Tissue was lysed in Cell Extraction Buffer (Novagen) by mechanical homogenization. Cell extracts were incubated with secretase-specific peptides conjugated to the reporter molecules EDANS and DABCYL for 15–30 min. EDANS fluorescence was read on a Synergy 2 microtiter plate reader (BioTek).

Chronic Citalopram Administration. Beginning at 3 mo of age, PS1APP hemizygous female mice were administered normal drinking water (vehicle) or 8 mg/kg/day citalopram in drinking water for a total of 4 mo. Littermate mice were divided equally between the treatment groups. Mice were housed three to five animals per cage. Volume of water drunk per cage and animal body weight were tracked throughout the study and did not differ between cages or treatment groups. During the study, only one mouse from the vehicle-group died, which did not appear to be due to treatment. No adverse events were noted in either group. At 8 mo of age, mice were killed with CSF drawn from the cisterna magna (7) followed by transcardial perfusion of chilled PBS with 0.3% heparin. One hemisphere of the brain was postfixed overnight in 4% paraformaldehyde followed by processing for histological analysis of Aβ plaque burden. The other hemisphere had the hippocampus and cortex microdissected for biochemical analysis of brain Aβ levels.

Tissue Extraction of Aβ. To evaluate various pools of brain Aβ, we performed a sequential extraction of tissue with PBS, 1% Triton X-100 in PBS, and then 5 M guanidine to grossly assess the extracellular-enriched fraction, the membrane-bound and intracellular fraction, and the insoluble fraction, respectively. All lysis buffers were chilled to 4 °C and contained protease inhibitors without EDTA. Tissue was lysed at a 1:10 wet wt/vol ratio. PBS and Triton X-100 extractions were performed by mechanical dounce homogenization, whereas the guanidine extraction was performed with sonication to maximally solubilize remaining Aβ within the tissue. Tissue was spun in a microcentrifuge at 21,000 *g* for 15 min at 4 °C following each extraction.

Quantitative Analyses of Aβ Deposition. Brain hemispheres were placed in 30% sucrose before freezing and cutting on a freezing sliding microtome. Serial coronal sections of the brain at 50-mm intervals were collected from the rostral anterior commisure to caudal hippocampus as landmarks. Sections were stained with biotinylated anti-Aβ antibody, mHJ3.4 (hybridomas were a kind gift from Dr. David Holtzman, Washington University, St. Louis, MO). Stained brain sections were scanned with a NanoZoomer slide scanner (Hamamatsu Photonics). For quantitative analyses of mHJ3.4-biotin staining, scanned images were exported with NDP viewer software (Hamamatsu Photonics) and converted to 8 bit grayscale using ACDSee Pro-3 software (ACD Systems). Converted images were thresholded to highlight plaques and then analyzed by the "Analyze Particles" function in the ImageJ software (National Institutes of Health) (8). Identified objects after thresholding were individually inspected to confirm the object as a plaque or not. Three brain sections per mouse, each separated by 300 mm, were used for quantification. These sections correspond roughly to sections at Bregma −1.7, −2.0, and −2.3 mm in the mouse brain atlas. The average of three sections was used to represent a plaque load for each mouse. For analysis of Aβ plaque in the cortex, the cortex immediately dorsal to the hippocampus was assessed. All analyses were performed in a blinded manner by two independent researchers; their values for each mouse were averaged and then plotted for statistical analyses. The data that were statistically analyzed and plotted represent the average plaque load per section between the two researchers.

Quantitative Real-Time PCR (qPCR). Total RNA was extracted from mouse brain cortex using TRIzol Reagent (Invitrogen). Tissue was used from the PS1APP mice chronically treated with citalopram or vehicle for 4 mo. Tissue was reverse transcribed with Mir-X miRNA First-Strand Synthesis kit (Clontech). qPCR was performed with SYBR Advantage qPCR Premix (Clontech) in ABI 7900HT(Applied Biosystems) using the default thermal cycling program. To confirm the specificity of qPCR reactions, dissociation curves were analyzed at the end of qPCR assay. The sequences of primers are listed below. Mouse GAPDH endogenous control was used as a normalization reference. Relative mRNA levels were calculated by comparative Ct method using ABI 7900HT Sequence Detection Systems version 2.4 and GenEx 5.3.2 (MultiD analyses).

Gene name	Forward primer (5′->3′)	Reverse primer (5′->3′)
GAPDH	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA
ADAM10	ATGGTGTTGCCGACAGTGTTA	GTTTGGCACGCTGGTGTTTTT
APH1b	CCGCGCTCGCTCTTTATGT	TGTACTGGTCCATCTCTGTTGT
Memapsin-2	CAGTGGGACCACCAACCTTC	GCTGCCTTGATGGACTTGAC
LRP1	ACTATGGATGCCCCTAAAACTTG	GCAATCTCTTTCACCGTCACA
Neprilysin	CTCTCTGTGCTTGTCTTGCTC	GACGTTGCGTTTCAACCAGC
Nicastrin	TCCGTGGTACTGGCAGGATT	CCCCTGTATCCCCACTAATTGA
Presenilin-1	GGTGGCTGTTTTATGTCCCAA	CAACCACACCATTGTTGAGGA
Presenilin-2	TGCCTGTCACGCTGTGTATG	GTTAAGCACGGAGTTGAGGAG

Western Blots. Tissue from the PS1APP mice chronically treated with citalopram or vehicle for 4 mo was used for Western blotting; specifically tissue lysed in 1% Triton X-100 from the above sequential extracts used for Aβ ELISAs. Triton X-100 should solubilize primarily proteins within cell membranes or intracellular compartments, which is where APP and its intracellular fragments should be located. Western blotting for full-length APP was performed using 4–12% Bis-Tris NuPAGE gels (Invitrogen) under reducing conditions with 15 μg of protein loaded per lane. Nitrocellulose blots were probed with rabbit anti-APP directed against the C terminus of APP (Invitrogen), followed by donkey anti-rabbit antibody reabsorbed against mouse IgG and conjugated to peroxidase (Santa Cruz Biotechnology). Gels were stripped and reprobed with rabbit anti-tubulin (Sigma) as a loading control protein. Bands were detected with Lumigen-TMA6 (Amersham) and captured digitally using the Kodak ImageStation 440CF. Densitometry was performed using the Kodak 1D Image Analysis software and each band normalized to tubulin signal in each lane.

Human ApoE Genotyping. A blood sample was drawn from each individual. DNA was extracted from blood cells and ApoE genotyping was performed using a pyrosequencing protocol as described previously (9).

PET Imaging. Pittsburgh Compound B (PIB) is a derivative of an amyloid-binding dye, thioflavin T, that when radiolabeled with ^{11}C can enter the brain, bind amyloid plaques, and provide a signal to be detected by PET scanners (10). PIB mean cortical binding potential (MCBP) appears to be an accurate measure of amyloid plaques in the brains of living human participants (10). PET scans were acquired with 24 × 5 s frames, 9 × 20 s frames, 10 × 1 min frames, and 9 × 5 min frames). Dynamic PET images were reconstructed using standard processing, including attenuation correction, scatter correction, head motion correction (11), and alignment to an atlas target by registration of each individual participant's structural MRI image to an atlas target (12) that minimizes bias due to atrophy. Registration was visualized in each participant to verify proper alignment. Three dimensional regions of interest (ROIs) were created for each participant on the basis of their individual MRI using ANALYZE.

Amyloid-β PET imaging was carried out as has been described in detail (13), using i.v. injection of ∼12 mCi of [^{11}C]PIB and a 60-min dynamic PET scan in 3D mode (septa retracted). The MCBP was determined as previously described (13) from the average of the binding potential (BP) values in the precuneus, lateral temporal cortex, gyrus rectus, and prefrontal cortex regions. Participants with an abnormally elevated MCBP (MCBP ≥ 0.18) were defined as PIB^+; those with MCBP < 0.18 were defined as PIB^- (13, 14).

Statistical Analysis for Mouse Studies. Data in figures are presented as mean ± SEM. All statistical analysis was performed using

Prism version 6.0 Beta for Windows (GraphPad). Statistical analysis to compare the mean values for multiple groups was performed using a one-way ANOVA with correction for multiple comparisons. Comparison of only two groups was performed using a two-tailed unpaired *t* test. Analysis of effects of drug versus vehicle-treatment over time was done with a repeated measures two-way ANOVA with correction for multiple comparisons. Values were accepted as significant if $P \leq 0.05$.

Statistical Analysis for Human Studies. Two-tailed *t* tests or χ^2 analyses as appropriate were conducted to examine differences between participants treated with antidepressants within the past 5 y versus those not treated within the past 5 y. We examined the time treated within the past 5 y because we hypothesized that there would be a greater effect on Aβ deposition for recent treatment. Table 1 shows that antidepressant-treated versus -untreated participants did not differ in demographic variables, including sex, age, education, mini mental status examination (MMSE), and ApoE4 status ($E4^+$ versus $E4^-$). MCBP was not normally distributed as determined by the Shapiro–Wilk test for normality ($P < 0.0001$). Therefore, a Wilcoxon rank sum test was conducted to compare the MCBP between antidepressant-treated participants versus -untreated participants. A *t* test for the Spearman rank correlation examined the correlation between MCBP and the cumulative antidepressant treatment duration during the past 5 y. The frequency of each antidepressant drugs used by participants is indicated in Table S1.

1. Savonenko A, et al. (2005) Episodic-like memory deficits in the APPswe/PS1dE9 mouse model of Alzheimer's disease: Relationships to beta-amyloid deposition and neurotransmitter abnormalities. *Neurobiol Dis* 18:602–617.
2. Cirrito JR, et al. (2003) In vivo assessment of brain interstitial fluid with microdialysis reveals plaque-associated changes in amyloid-beta metabolism and half-life. *J Neurosci* 23:8844–8853.
3. Verges DK, Restivo JL, Goebel WD, Holtzman DM, Cirrito JR (2011) Opposing synaptic regulation of amyloid-β metabolism by NMDA receptors in vivo. *J Neurosci* 31: 11328–11337.
4. Reagan-Shaw S, Nihal M, Ahmad N (2008) Dose translation from animal to human studies revisited. *FASEB J* 22:659–661.
5. Grimwood S, et al. (2005) Determination of guinea-pig cortical gamma-secretase activity ex vivo following the systemic administration of a gamma-secretase inhibitor. *Neuropharmacology* 48:1002–1011.
6. Kim J, et al. (2009) Overexpression of low-density lipoprotein receptor in the brain markedly inhibits amyloid deposition and increases extracellular A beta clearance. *Neuron* 64:632–644.
7. DeMattos RB, et al. (2002) Plaque-associated disruption of CSF and plasma amyloid-beta (Abeta) equilibrium in a mouse model of Alzheimer's disease. *J Neurochem* 81: 229–236.
8. Kim J, et al. (2007) Abeta40 inhibits amyloid deposition in vivo. *J Neurosci* 27(3): 627–633.
9. Pastor P, et al. (2003) Apolipoprotein Eepsilon4 modifies Alzheimer's disease onset in an E280A PS1 kindred. *Ann Neurol* 54:163–169.
10. Klunk WE, et al. (2004) Imaging brain amyloid in Alzheimer's disease with Pittsburgh Compound-B. *Ann Neurol* 55:306–319.
11. Woods RP, Mazziotta JC, Cherry SR (1993) MRI-PET registration with automated algorithm. *J Comput Assist Tomogr* 17:536–546.
12. Talairach J, Tournoux P (1988) *Co-planar Stereotaxic Atlas of the Human Brain: 3-Dimensional Proportional System: An Approach to Cerebral Imaging* (Thieme Medical, Stuttgart, New York), p viii.
13. Mintun MA, et al. (2006) [11C]PIB in a nondemented population: Potential antecedent marker of Alzheimer disease. *Neurology* 67:446–452.
14. Roe CM, et al. (2008) Alzheimer disease and cognitive reserve: Variation of education effect with carbon 11-labeled Pittsburgh Compound B uptake. *Arch Neurol* 65: 1467–1471.

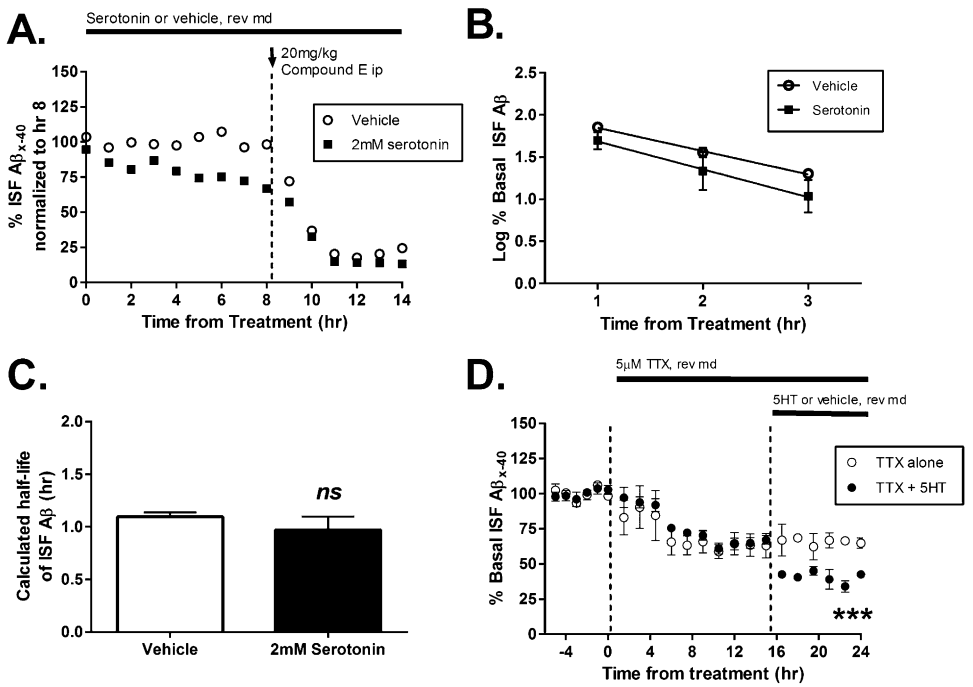

Fig. S1. Serotonin signaling reduces Aβ production. (*A*) PS1APP mice were treated with vehicle (aCSF) or 2 mM serotonin directly to the hippocampus by reverse microdialysis for 14 h (same study as Fig. 1*C*). By 8 h of administration, serotonin reduced ISF $A\beta_{x\text{-}40}$ levels to 66.7 ± 7.2% of baseline ($P = 0.003$, $n = 5$ per group). After 8 h, mice were administered a γ-secretase inhibitor, Compound E (20 mg/kg i.p.) to assess $A\beta_{x\text{-}40}$ half-life. (*B*) The elimination of ISF Aβ in vehicle and serotonin-treated mice followed first-order kinetics (*C*) with similar half-lives (1.1 ± 0.04 h and 0.97 ± 0.13 h). (*D*) TTX (5 μM) was infused into the hippocampus by reverse microdialysis. TTX alone reduced ISF Aβ levels by 65%. At hour 16 of TTX infusion, mice were coadministered either vehicle or 2 mM serotonin. Serotonin caused an additional 35.5 ± 0.68% decrease in ISF Aβ at hour 24 compared with TTX treatment alone ($P = 0.04$, $n = 5$–6 per group). Data are presented as mean ± SEM.

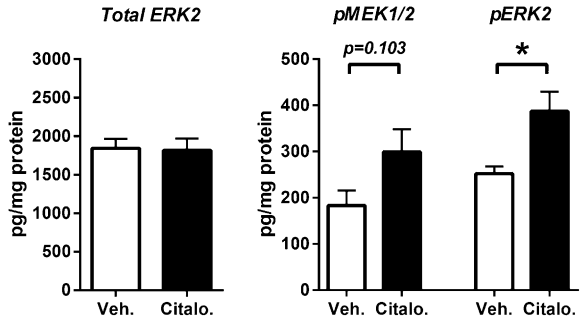

Fig. S2. Citalopram treatment increases ERK activation. PS1APP mice were treated with 10 mg/kg citalopram (i.p.) and brain tissue harvest 8 h later. Total ERK2 levels did not change in the hippocampus; however, pMEK and pERK levels were elevated by 45.2 ± 19.6% (P = 0.104) and 53.4 ± 16.6% (P = 0.02) compared with vehicle-treated mice (n = 8 per group). Data are presented as mean ± SEM.

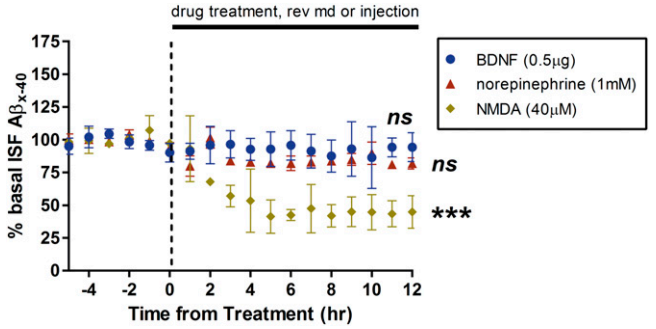

Fig. S3. Only select ERK activators alter ISF Aβ metabolism. Acute administration of ERK activators, BDNF (0.5 μg direct injection) and norepinephrine (1 mM by reverse microdialysis), did not significantly change ISF Aβ levels in vivo. Infusion of a high dose of NMDA (40 μM), however, significantly reduced ISF Aβ levels to 44.8 ± 12.4% of baseline levels (P < 0.0001) (n = 5 per group). Data are presented as mean ± SEM.

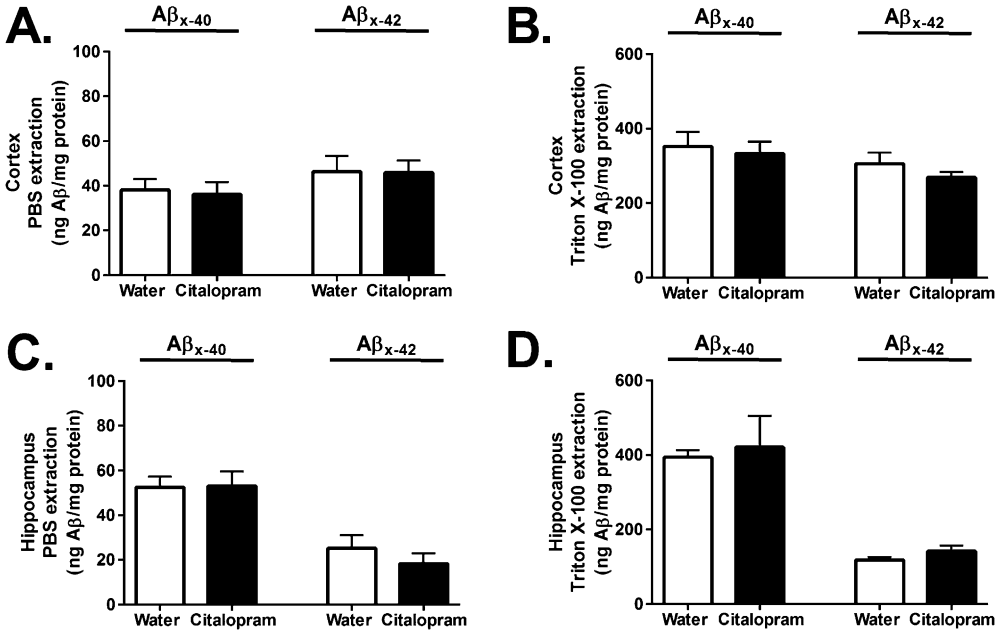

Fig. S4. Chronic citalopram administration did not alter soluble species of brain Aβ. Within the cortex and hippocampus, PBS-soluble Aβ (*A* and *C*) and Triton X-100–soluble Aβ (*B* and *D*) levels did not change significantly in citalopram-treated mice compared with littermate vehicle (water)-treated mice (n = 10 per group).

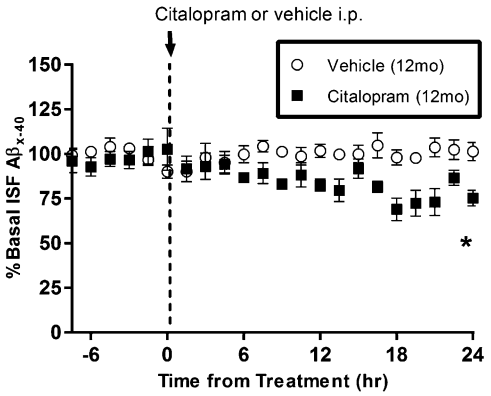

Fig. S5. Acute citalopram reduces ISF Aβ in plaque-bearing PS1APP mice. A cohort of naïve 12-mo-old PS1APP mice had ISF Aβ levels measured by microdialysis. Mice were treated with vehicle or citalopram (10 mg/kg i.p.). Citalopram reduced ISF $A\beta_{x\text{-}40}$ levels to 75.3 ± 4.4% compared with vehicle-treated mice ($P = 0.008$, $n = 5$ per group). This was not significantly different from the reduction seen from the same dose of citalopram in young, preplaque mice (Fig. 1*A*).

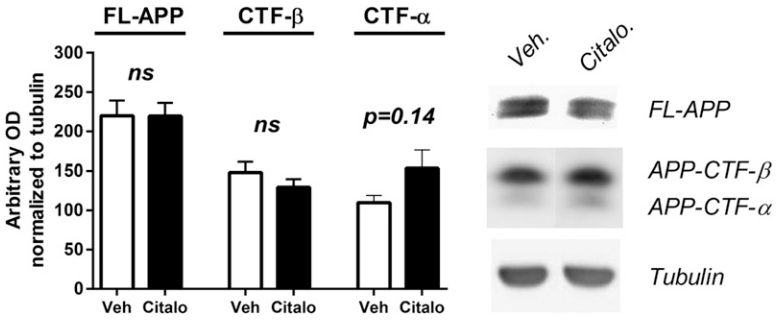

Fig. S6. Chronic citalopram administration did not significantly alter APP or APP fragments. Fragments of APP within Triton X-100 extractions of hippocampus were analyzed by Western blot. Neither full-length APP nor APP CTF-β changed significantly in mice treated with citalopram for 4 mo compared with littermate vehicle (water)-treated mice. APP CTF-α levels had a trend for an increase in citalopram-treated mice ($P = 0.14$; $n = 8$ per group). Data are presented as mean ± SEM.

Table S1. Antidepressant use history in human participants

Drug name	Number of participants
One antidepressant	
Sertraline	15
Citalopram	6
Fluoxetine	4
Bupropion	3
Escitalopram	3
Paroxetine	2
Trazadone	2
Venlafaxine	2
Duloxetine	1
Not certain*	3
Two or more sequential antidepressants	
Sertraline; Bupropion	1
Sertraline; Duloxetine	1
Sertraline; Fluoxetine	1
Sertraline; Nortriptyline	1
Venlafaxine; Fluoxetine	1
Escitalopram; Citalopram; Bupropion	1
Fluoxetine; Bupropion; Sertraline	1
Not certain*	1
Two or more simultaneous antidepressants	
Amitriptyline and trazadone	1
Paroxetine and duloxetine	1
Sertraline and nortriptyline and bupropion	1

*Participants were certain they had been treated with an antidepressant but were not sure exactly which one, e.g., paroxetine versus fluoxetine.