Supplementary Material for *Castellano et al.*, "Human apoE isoforms differentially regulate brain Amyloid-β peptide clearance"

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Supplementary Material includes the following items: Materials and Methods, Figures S1-S5, Table S1, Supplementary References.

Materials and Methods

Detailed materials and methods for in vivo stable isotopic labeling experiment

Mice were intraperitoneally injected with 200 mg/kg of stable isotope $^{13}[C_6]$-labeled leucine (Cambridge Isotope Laboratories, Inc., Andover MA). 20 or 40 minutes following injection, mice were transcardially perfused and tissue was immediately harvested and frozen at -80°C. Whole hippocampus from each mouse was lysed using 1% Triton X-100 lysis buffer, pH 7.6 (150mM NaCl, 50mM Tris-HCl, 1% Triton X-100 with complete protease inhibitor cocktail [Roche, Indianapolis, IN]). Aβ was immunoprecipitated from lysates using HJ5.2 antibody (anti-$A\beta_{13\text{-}28}$) that had been conjugated to Protein G-Sepharose 4 fast flow beads (GE Healthcare, Pittsburgh, PA). To prevent antibody elution from the beads, antibodies were crosslinked with freshly prepared 20 mM dimethyl pimelinidate (Sigma, St. Louis, MO). After three washes each of PBS and triethylammonium bicarbonate (Sigma, St. Louis, MO), Aβ was eluted twice with 100% formic acid. Formic acid was then dried, resuspending Aβ with 20% acetonitrile in 25mM triethylammonium bicarbonate prior to trypsin digestion (Promega, Madison, WI); samples were stored at 4°C prior to analysis.

Samples were then subjected to quantitative mass spectrometry to measure Aβ peptide containing $^{13}[C_6]$-leucine and $^{12}[C_6]$-leucine using a TSQ Vantage Triple Stage Quadrupole mass spectrometer, controlled by Xcalibur software (ThermoFisher Scientific, San Jose, CA) and equipped with a PST-MS nanospray source (Phoenix S&T, Chester, PA). Sample injection and liquid chromatography gradients were performed using a NanoLC-2D-Ultra (Eksigent Technologies, Dublin, CA). Prior to analysis, the TSQ Vantage was tuned to select the Aβ tryptic peptide LVFFAEDVGSNK (m/z = 663.340) and optimal conditions were set for a capillary temperature of 350°C, with a spray voltage of 1200 V. Peak widths for Q1 and Q3 were set at 2.0 Da, with a collision pressure in Q2 of 2.0 mTorr and a collision energy of 26 V. For the Aβ tryptic peptide LVFFAEDVGSNK (m/z = 663.340) and its $^{13}[C_6]$-leucine labeled form (m/z = 666.350), the MRM transition ions monitored were 819.384, 966.452 and 1113.521. These MRM transition ions were the three most intense ions and were validated using H4 APP695ΔNL cell-secreted Aβ standards. Standards were prepared by incubating H4 APP695ΔNL neuroglioma cells with known amounts of labeled and unlabeled leucine, followed by collection of media containing newly synthesized Aβ. 5-μL aliquots of samples containing Aβ immunoprecipitated from mouse hippocampal lysates were injected into a Zorbax SB300-C18 3μm particle-size nano-column (Agilent Technologies, Santa Clara, CA), packed in-house (0.15 x 150 mm). Peptide mixtures were separated at a flow rate of 500 nL/min using a gradient mixture of solvents A and B. Solvent A was 0.1% formic acid in water; solvent B was 0.1% formic acid in acetonitrile. The separation gradient program used for the nano-column was as follows: 15% to 65% B in 10 min, 65% to 95% B in 5 min, followed by a gradient back to 15% B in 5 min. The column was re-equilibrated for another 5 min to prepare for injection of the next sample.

Gas chromatography/mass spectrometry (GC/MS) was performed to measure free leucine tracer-to-tracee ratio (TTR) in plasma collected from PDAPP/TRE mice prior to harvesting brain tissue, as previously described (*1*). Plasma proteins were precipitated with ice-cold acetone, followed by

extraction of lipids with hexane solvent. The resulting aqueous fraction was then dried under vacuum (Savant Instruments, Farmingdale, NY). Free leucine TTR was measured by GC/MS by monitoring ions at m/z ratios of 200 and 203, corresponding to unlabeled and labeled leucine, respectively. Relative fractional synthesis rates of Aβ were calculated from the slope of the Aβ TTR over average leucine enrichment at the 20 min timepoint.

Quantitative measurements of ISF Urea concentration

ISF urea collected in microdialysis fractions was quantified using a commercially available colorimetric assay (Quanti-Chrom Urea Assay Kit, BioAssay Systems, Hayward, CA), as previously described (*2, 3*).

Western blot analysis of young PDAPP/TRE and TRE hippocampal homogenates

Hippocampal tissue from young PDAPP/TRE or TRE mice was manually dounce-homogenized with 75 strokes in radioimmunoprecipitation assay (RIPA) buffer (50mM Tris-HCl; pH 7.4, 150mM NaCl, 0.25% deoxycholic acid, 1% NP-40, 1mM EDTA) containing a cocktail of protease inhibitors (Roche, Indianapolis, IN). For assessment of APP levels in PDAPP/TRE mice, equivalent amounts of protein (7.5μg) were loaded on 4-12% Bis-Tris gels (Invitrogen, Carlsbad, CA) for SDS-PAGE before transferring protein to 0.2 μm nitrocellulose membranes. Full-length APP (FL-APP) was probed using 6E10 antibody (Covance, Princeton, NJ). For measurement of murine APP in TRE mice, equivalent amounts of protein (25μg) were loaded on 4-12% Bis-Tris gels (Invitrogen, Carlsbad, CA) for SDS-PAGE before transferring protein to 0.2 μm nitrocellulose membranes. Immediately following transfer, blots were boiled for 10 minutes prior to blocking and incubation with CT20 antibody (anti-APP C-terminal 20 amino acids; Calbiochem) to detect murine APP. Loading for 6E10 or CT20 westerns was normalized by stripping blots and re-probing with α-tubulin antibody (Sigma, St. Louis, MO). Normalized band intensities were quantified using Image J software (NIH).

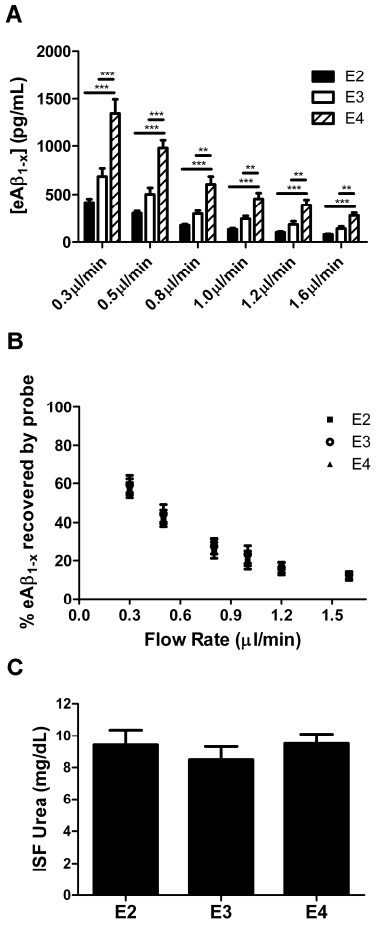
Sandwich ELISA for Aβ and apoE from CSF and sequentially extracted hippocampal tissue

Frozen hippocampi from young PDAPP/TRE mice were sequentially homogenized with PBS and 5M Guan-Tris buffer (pH 8.0) containing a cocktail of protease inhibitors (Roche), following by centrifugation at 4°C at 14,000rpm for 30 min. Levels of PBS-soluble (soluble) and Guan-soluble (insoluble) Aβ40 and Aβ42 were measured using sandwich ELISAs; HJ2 (anti-$A\beta_{35-40}$) or HJ7.4 (anti-$A\beta_{37-42}$) were used as capture antibodies, followed by detection with biotinylated HJ5.1 (anti-$A\beta_{13-28}$). ApoE levels in Triton X-100-soluble hippocampal extracts or CSF were quantified using an apoE sandwich ELISA (HJ6.2 for capture and HJ6.1B for detection) with recombinant human apoE as a standard.

Native-polyacrylamide gel electrophoresis/western blot analysis of apoE

Fresh CSF was isolated from the cisterna magna of young and old PDAPP/TRE mice before quantification using apoE sandwich ELISA. Equal amount of apoE (3ng), determined by ELISA, was loaded in each lane of 4-20% Tris-glycine gel for Native-PAGE (100V at 4°C for 14 hours) before transfer to 0.2 μm nitrocellulose membranes. Blots were probed with anti-apoE antibody (Calbiochem), and migration pattern of lipoproteins in the CSF was assessed using protein mixture of estimated hydrodynamic radii as a standard (GE/Amersham).

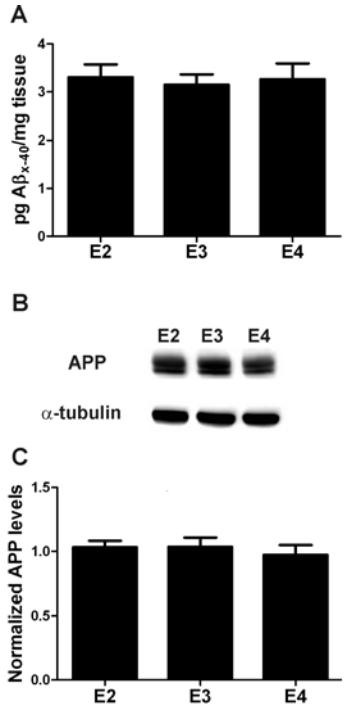
Fig. S1. eAβ_{1-x} concentration measured with equivalent microdialysis probe function differs according to apoE isoform.



(A) Mean concentrations of eAβ_{1-x} at each flow rate for zero flow extrapolation experiments performed in Fig. 4A. 2-way repeated-measures ANOVA was performed using genotype and flow rate as factors followed by pairwise comparisons of genotypes by flow rate using Tukey's post-hoc test (**p<0.01, ***p<0.001) **(B)** In vivo percent recovery by the microdialysis probe at each flow rate for each experiment in (A) was determined using the zero flow extrapolated method as previously described (*4-6*), revealing no significant difference in eAβ_{1-x} recovery by the probe among groups as assessed by 2-way repeated measures ANOVA with Huynh and Feldt adjustment. **(C)** Mean concentrations of urea

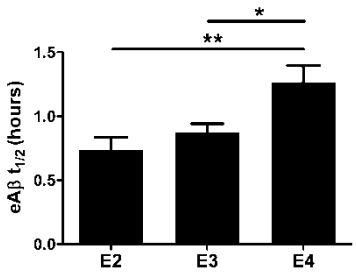
(mg/dL) collected from the hippocampal ISF of young, sex-matched PDAPP/TRE mice using a flow rate of 0.3µl/min (n=8 mice/group; 3-4 months old). Values represent mean ± SEM.

Fig. S2. PBS-soluble Aβ40 levels and APP levels do not vary according to apoE isoform in the context of murine APP/Aβ.



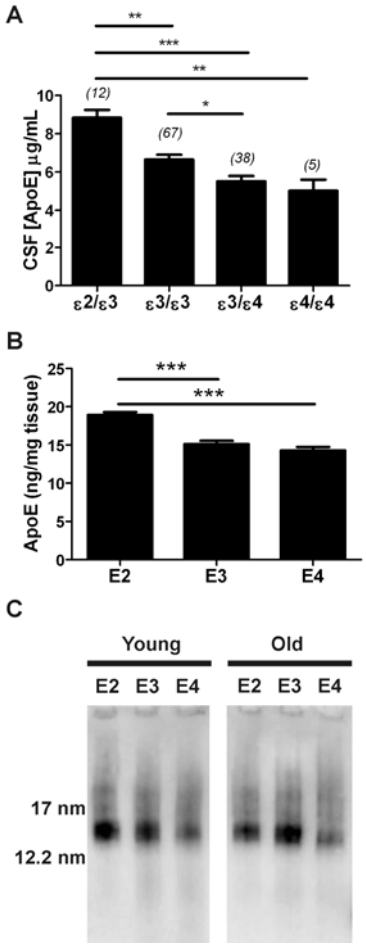
(A) Mean PBS-soluble $A\beta_{x\text{-}40}$ levels quantified by sandwich ELISA after homogenization of hippocampi from young, male TRE mice (n=5/group). 1-way ANOVA revealed no significant differences among groups. **(B)** Representative western blot probed with CT20 antibody (anti-APP) to detect murine APP from hippocampal homogenates from mice in (A). **(C)** Quantification of APP protein levels after normalization to α-tubulin band intensity (n=5/group). 1-way ANOVA revealed no significant differences among groups. Values represent mean ± SEM.

Fig. S3. Soluble Aβ clearance from the ISF is apoE isoform-dependent in young PSAPP/TRE mice



Clearance microdialysis experiments were performed in young PS1ΔE9/APPswe/human apoE knockin mice (PSAPP/TRE) on a BL6/SJL/C3 background (n=5-7 mice/group; 3 months old). After stable baseline measurement of $eA\beta_{1-x}$, mice were injected with 10mg/kg LY411,575 and $eA\beta t_{1/2}$ (hrs.) was calculated as in experiments in Fig. 3-4. 1-way ANOVA followed by Tukey's post-hoc test for multiple comparisons was performed (*$p<0.05$,**$p<0.01$). Values represent mean ± SEM.

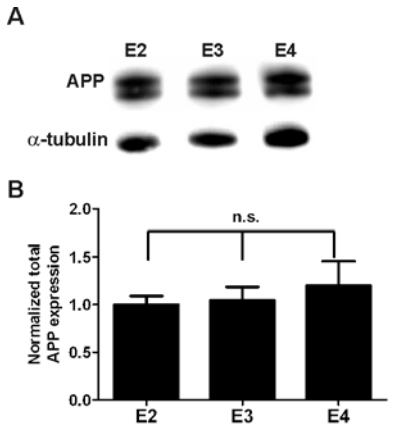
Fig. S4. ApoE concentration is higher in the context of apoE2 in both humans and in PDAPP/TRE mice.



(A) ApoE protein levels in the CSF of cognitively normal individuals were quantified using multi-analyte profiling. Number in parentheses indicates number of individuals in each group. Since 1-way ANOVA was significant, differences among groups were assessed using Tukey's post-hoc test for multiple comparisons (*p<0.05, **p<0.01, ***p<0.001). **(B)** ApoE protein levels measured by human apoE-specific sandwich ELISA after 1% Triton X-100 extraction of hippocampi dissected from young PDAPP/TRE mice (3.5 month-old; n=16-18 mice/group). 1-way ANOVA followed by Tukey's post-hoc test for multiple comparisons was performed (*p<0.05, **p<0.01, ***p<0.001). **(C)** Representative Native-PAGE/western blot probed with anti-apoE antibody (Calbiochem) after loading 3ng apoE/lane from CSF

of young (4 months) and old (17 months) PDAPP/TRE mice (n=4/genotype for each age). 12.2 nm and 17 nm sizes correspond to estimated hydrodynamic radii from protein marker.

Fig. S5. Full-length total APP levels do not differ according to apoE isoform.



(A) Representative western blot of RIPA-soluble total (mature and immature) full-length APP from hippocampal homogenates from young, sex-matched PDAPP/TRE mice. APP was detected using 6E10 antibody. All bands were normalized to α-tubulin band intensity (n=9 mice/group; 3-4 months old). **(B)** Quantification of total APP levels after normalizing each band's intensity to α-tubulin band intensity. All samples were represented relative to the PDAPP/E2 group mean. 1-way ANOVA revealed no significant differences among groups. Values represent mean ± SEM.

Table S1. Serial extraction of Aβ40 and Aβ42 from hippocampi of young PDAPP/TRE mice.

	***APOE* genotype**		
	PDAPP/E2	**PDAPP/E3**	**PDAPP/E4**
Soluble Aβ40	10.83***** ± 0.36	10.34****** ± 0.70	12.55 ± 0.21
Soluble Aβ42	2.83 ± 0.19	2.66***** ± 0.14	3.29 ± 0.18
Insoluble Aβ40	87.52 ± 3.09	79.55 ± 3.66	84.02 ± 3.29
Insoluble Aβ42	43.39 ± 1.74	41.09***** ± 2.79	53.07 ± 3.89

All values represent mean (pg/mg tissue) ± SEM. PBS-soluble (soluble) and Guan-soluble (insoluble) Aβ40 or Aβ42 was quantified using sandwich ELISAs following serial tissue extraction of hippocampi from young, sex-matched PDAPP/TRE mice with PBS, followed by 5M Guan-Tris buffer (n=8-9/group). When 1-way ANOVA was significant, pairwise comparisons of *APOE* genotypes were made using Tukey's post-hoc test; only significant differences were indicated (*$p<0.05$,**$p<0.01$). (* or **) denotes significant difference compared to PDAPP/E4 at the indicated levels of significance.

Supplementary References

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