

## Original Article

# MPEP reduces seizure severity in *Fmr-1* KO mice over expressing human A $\beta$

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**Abstract:** Metabotropic glutamate receptor 5 (mGluR<sub>5</sub>) regulates the translation of amyloid precursor protein (APP) mRNA. Under resting conditions, mRNA is bound to and translationally repressed by the fragile X mental retardation protein (FMRP). Upon group 1 mGluR activation, FMRP dissociates from the mRNA and translation ensues. APP levels are elevated in the dendrites of primary neuronal cultures as well as in synaptoneurosomes (SN) prepared from embryonic and juvenile *fmr-1* knockout (KO) mice, respectively. In order to study the effects of APP and its proteolytic product A $\beta$  on Fragile X syndrome (FXS) phenotypes, we created a novel mouse model (FRAXAD) that over-expresses human APP<sub>Swe</sub>/A $\beta$  in an *fmr-1* KO background. Herein, we assess (1) human APP<sub>Swe</sub> and A $\beta$  levels as a function of age in FRAXAD mice, and (2) seizure susceptibility to pentylenetetrazol (PTZ) after mGluR<sub>5</sub> blockade. PTZ-induced seizure severity is decreased in FRAXAD mice pre-treated with the mGluR<sub>5</sub> antagonist MPEP. These data suggest that A $\beta$  contributes to seizure incidence and may be an appropriate therapeutic target to lessen seizure pathology in FXS, Alzheimer's disease (AD) and Down syndrome (DS) patients.

**Key words:** APP,  $\beta$ -amyloid, FMRP, FRAXAD, PTZ, seizure, Tg2576

## Introduction

Amyloid precursor protein (APP) mRNA is translationally regulated by the fragile X mental retardation (FMRP) protein [1]. These data suggest a molecular link between Fragile X syndrome (FXS), a neurodevelopmental disorder, and Alzheimer's disease (AD), a neurodegenerative disease. Seizures are a prevalent phenotype in FXS, AD and Down syndrome (DS) [2-5] leading to the hypothesis that over-expression of APP/A $\beta$  in these disorders contributes to this pathology.

FXS mice have a neomycin cassette inserted into the *fmr-1* gene to block the synthesis of FMRP [6]. These mice mimic many of the pathological and behavioral phenotypes of FXS, over-express APP, A $\beta$ <sub>1-40</sub> and A $\beta$ <sub>1-42</sub> [1], and are highly susceptible to audiogenic-induced seizures (AGS) [7-9]. In order to study the effects of APP/A $\beta$  expression on FXS phenotypes, we created FRAXAD mice, a cross of *fmr-1* KO mice and Tg2576, an AD mouse model that over-expresses human APP<sub>Swe</sub>. FRAXAD mice (postnatal day 14-16) have 23%

more brain A $\beta$ <sub>1-40</sub> than Tg2576 and display significantly increased early mortality compared to Tg2576, WT and *fmr-1* KO mice, consistent with a developmental defect [10]. Both Tg2576 and FRAXAD have a significantly lower threshold to pentylenetetrazol (PTZ)-induced seizures compared to non-transgenic littermates, which suggests that APP/A $\beta$  contribute to juvenile mortality by decreasing seizure threshold [10].

How APP or its proteolytic products contribute to seizure propensity remains unknown. Others and we have shown that mGluR<sub>5</sub> inhibitors including MPEP can rapidly attenuate audiogenic seizures (AGS) in *fmr-1* KO mice [11]. mGluR<sub>5</sub> blockade also prevents DHPG-mediated up-regulation of APP synthesis in synaptoneurosomes (SN) [1]. Based on these data, we asked if mGluR<sub>5</sub> blockade would reduce seizure susceptibility in response to PTZ. Consequently, we assessed APP and A $\beta$  levels during development in Tg2576 and FRAXAD mice and the ability of mGluR<sub>5</sub> blockade to attenuate PTZ-induced seizures in these strains. PTZ-induced

seizure severity is decreased in FRAXAD mice pre-treated with the mGluR<sub>5</sub> antagonist MPEP. Thus, mGluR<sub>5</sub> blockade reduces both APP/A $\beta$  production as well as signaling.

## Methods

**Animal Husbandry:** WT, *fmr-1*<sup>-/-</sup>, Tg2576 and FRAXAD mice were generated, bred and housed as previously described [10]. All strains were in the C57BL/6 background (backcross n=5 or greater). APP<sub>Swe</sub> and *fmr-1* KO genotypes were determined by PCR analysis of DNA extracted from tail biopsies. Adequate measures were taken to minimize pain or discomfort to the mice, and all husbandry, seizure and euthanasia procedures were performed in accordance with NIH guidelines and an approved University of Wisconsin Madison animal care protocol administered through the Research Animal Resources Center.

**Molecular Analyses (westerns, ELISAs and secretase assays):** For the detection of mouse APP/A $\beta$ , brain regions (hippocampus, cortex and cerebellum) from WT and *fmr-1* KO brains of 3.5 month-old mice were homogenized in 0.5 mL protein extraction buffer (10 mM Tris [pH 7.6], 2 mM EDTA, 150 mM NaCl, 1% Triton X-100, 0.25% NP-40, and 1X protease inhibitor cocktail) and clarified at 12,000 rpm, 10 min, 4°C. Protein concentrations were determined by the Pierce BCA assay (<http://www.piercenet.com>). Lysates were analyzed by western blotting and ELISA. Western membranes were hybridized with anti-mouse APP antibody (Chemicon catalog #mAB348, dilution, 5  $\mu$ g/mL) and anti-mouse  $\beta$ -actin antibody (dilution, 1:10,000) followed by hybridization with anti-mouse HRP-conjugated secondary antibody (dilution, 1:2,000). Proteins were visualized by enhanced chemiluminescence on a STORM 860 phosphorimager. Sandwich ELISAs with the Signet A $\beta$ <sub>1-40</sub>/9131 and Signet A $\beta$ <sub>1-42</sub>/9134 capture antibodies and the rodent A $\beta$ /9154 reporter antibody to detect mouse A $\beta$  were performed as previously described [12].

For the detection of human APP/A $\beta$ , brain hemispheres were homogenized in 8M GnHCl buffer (8M GnHCl, 50 mM Tris pH 7.5), mixed at room temperature for 4 hr, aliquoted and frozen at -80°C. Protein concentrations were determined by the Pierce BCA Assay (<http://www.piercenet.com>). Lysates were diluted

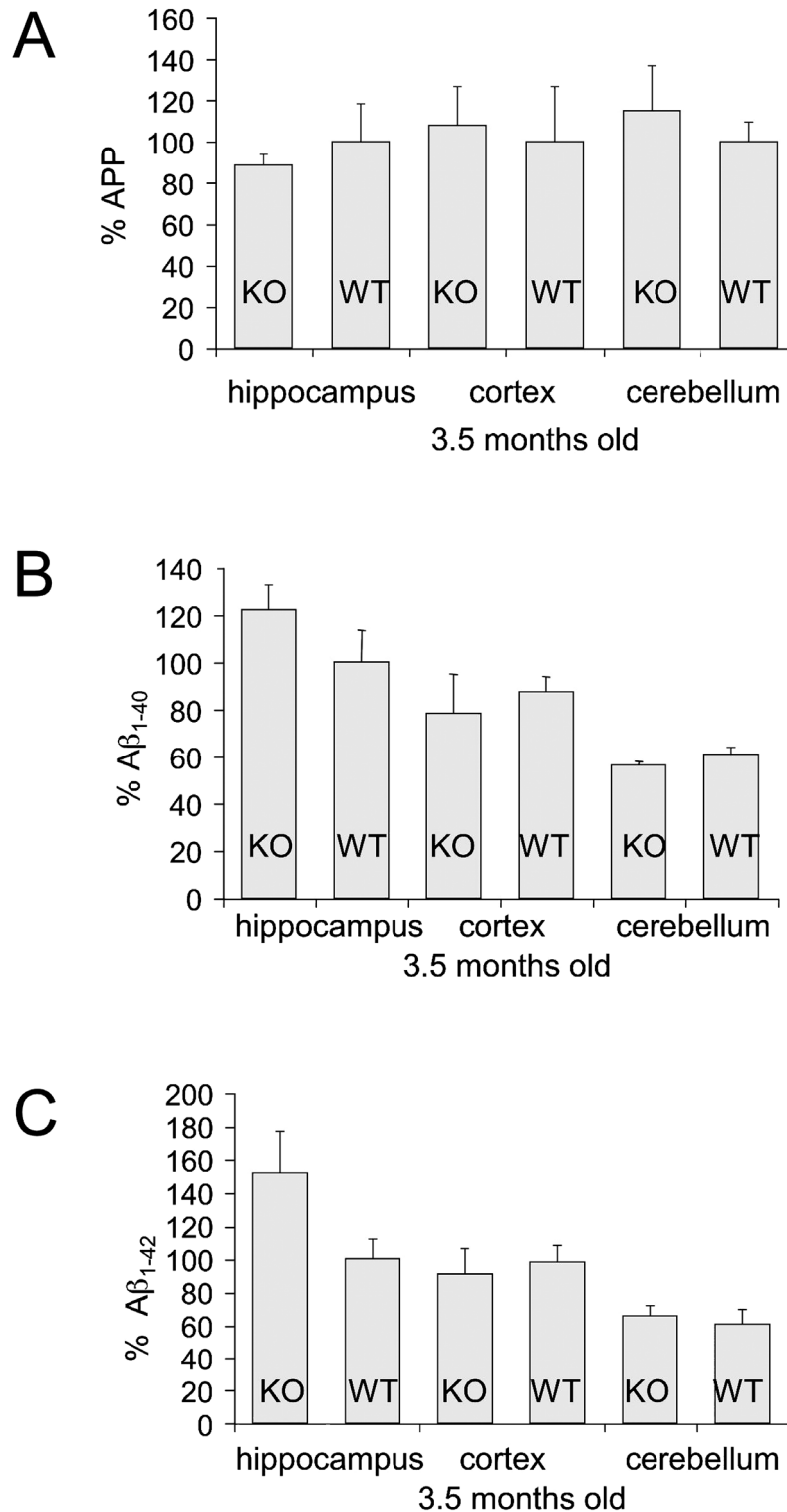
at least 80-100-fold to obtain GnHCl concentrations below 0.1 M and clarified at 16,000 rpm for 20 min at 4°C. Samples were further diluted as per the figure legends to obtain concentrations in the linear range of the standard curve. BioSource ELISA assays were employed to detect APP/APP $\alpha$  (catalog #KHB0051), A $\beta$ <sub>1-40</sub> (catalog #KHB3482) and A $\beta$ <sub>1-42</sub> (catalog #KHB3442) (<http://www.invitrogen.com>).

$\alpha$ - and  $\beta$ -secretase activities were assessed with R & D System kits #FP001 and #FP002 (<http://www.rndsystems.com>). Briefly, brain hemispheres were homogenized in 5 mL kit cell extraction buffer, set on ice for 30 min with occasional mixing and spun at 10,000 rpm for 1min at 4°C. The protein concentration of the cleared lysates was determined by the Pierce BCA assay (<http://www.piercenet.com>). Lysates (2 mg/mL stocks) were diluted 10-fold ( $\alpha$ -secretase assay) or 50-fold ( $\beta$ -secretase assay). Reactions contained 50  $\mu$ L diluted lysate, 50  $\mu$ L 2X reaction buffer and 5  $\mu$ L substrate and were incubated in the dark at 37°C for 1 hr (BACE) or 2 hr ( $\alpha$ -secretase) per the manufacturer's directions. Fluorescence was measured on a SpectraMax Gemini Fluorescence plate reader with Softmax Pro software from Molecular Devices (<http://www.moleculardevices.com>).

**PTZ-Induced Seizure Testing and Data Analyses:** MPEP was a kind gift from FRAXA Research Foundation (Newburyport, MA) and was dissolved at 1 mg/mL in DPBS before I.P. injection at 30 mg/kg body weight 30 min prior to PTZ treatment. PTZ (catalog #P6500) was purchased from Sigma Chemical Co. (<http://www.sigmaaldrich.com>), dissolved at 2 mg/mL in DPBS and administered by I.P. injection (50 mg/kg body weight). Seizure testing was conducted and scored as previously described [10].

## Results

We assessed APP and A $\beta$  levels in WT, *fmr-1*<sup>-/-</sup>, Tg2576 and FRAXAD mice as a function of age. There was no difference in brain APP levels in WT versus *fmr-1*<sup>-/-</sup> (3.5 months old) as assessed by western blotting of hippocampal, cortical and cerebellar lysates (**Figure 1A**). However, we have previously demonstrated increased APP in *fmr-1* KO synaptoneurosomes prepared from young mice (P14-17) as well as increased dendritic APP in *fmr-1* KO primary neuronal cells [1].



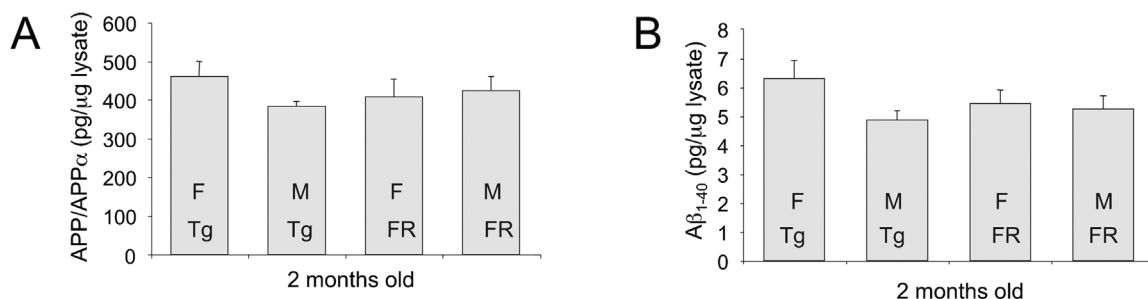
**Figure 1.** WT and *fmr-1* KO mice (3.5 months old) have equivalent levels of brain APP and A $\beta$ . (A) APP levels in lysates from hippocampus, cortex and cerebellum brain regions of WT and *fmr-1* KO mice (25  $\mu$ g per lane) were assessed by western blot analyses. Phosphorimager units for APP were normalized to  $\beta$ -Actin levels. All WT samples were set to 100%. There was no statistical difference between WT (n=3) and *fmr-1* KO mice (n=3 mice). (B) A $\beta_{1-40}$  and (C) A $\beta_{1-42}$  levels in hippocampus, cortex and cerebellum brain regions of WT (n=3) and *fmr-1* KO (n=3) mice were assessed by ELISA and presented as a percentage compared to levels in WT hippocampus. The 1.5X increase in A $\beta_{1-42}$  levels in *fmr-1* KO brain was not statistically significant (p=0.14).

Thus, APP expression is selectively elevated in embryonic and juvenile *fmr-1* KO mice compared to WT. FMRP levels decrease with aging in WT mice [13], suggesting APP translation and processing might gradually increase over time. Indeed, one-year old *fmr-1* KO mice have elevated A $\beta_{1-40}$  and A $\beta_{1-42}$  in whole brain lysates [1]. There was a trend towards similar data in hippocampal lysates from 3.5 month-old mice (**Figure 1B,C**), but the differences had not reached statistical significance at this age.

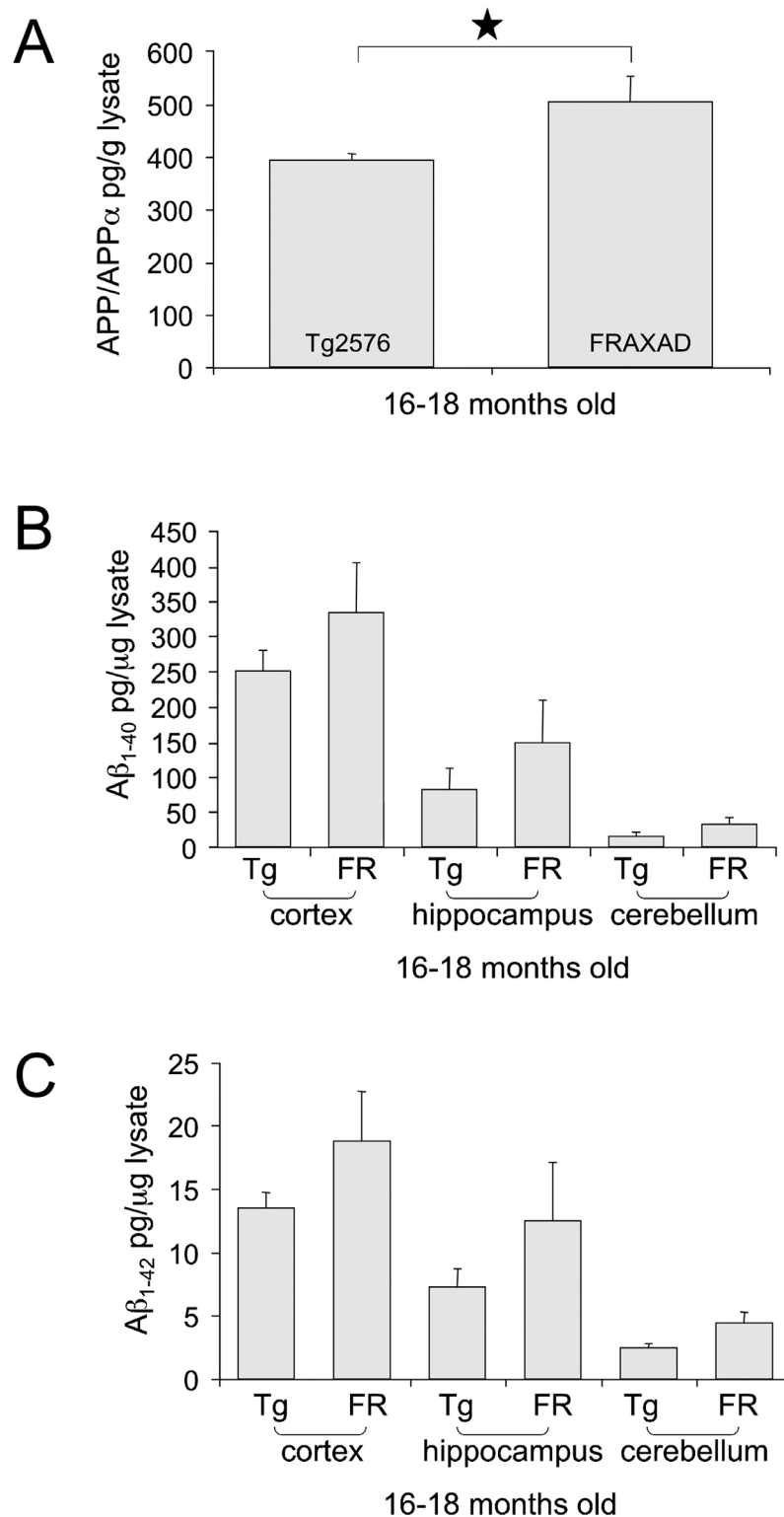
Next, we assessed APP and A $\beta$  levels in Tg2576 and FRAXAD mice from neonatal life to adulthood. FRAXAD mice produce significantly more A $\beta_{1-40}$  by 2 weeks of age than Tg2576 as assessed by ELISA of whole brain lysates [10]. Hence, we assessed APP and A $\beta$  levels in Tg2576 and FRAXAD mice at later stages of development. We hypothesized that the absence of FMRP in the FRAXAD mice would result in increased synthesis of APP and hence generation of A $\beta$ , which would accumulate with aging. We did not observe significant differences in APP or A $\beta_{1-40}$  between Tg2576 and FRAXAD by ELISA at 2 months of age (**Figure 2**). A $\beta_{1-42}$  was not detectable above background at this age. At 16-18 months of age, there was a statistically significant 28% increase in APP/APP $\alpha$  in whole brain lysates of FRAXAD versus Tg2576 (**Figure 3A**). There were trends for increased A $\beta_{1-40}$  and A $\beta_{1-42}$  in hippocampus, cortex and cerebellum (**Figures 3B,C**), which were not statistically significant. There were only n = 3-4 animals per cohort due to a 40% mortality rate by 60 days of age in both Tg2576 and FRAXAD [10]. The mice that survived 16-18 months were the healthiest and thus might be expected to have lower than typical levels of human APP<sub>Swe</sub> and A $\beta$ .

To test if the APP processing machinery was equally active in Tg2576 and FRAXAD, we assessed  $\alpha$  and  $\beta$ -secretase activity in mouse brain lysates. APP is cleaved by two proteases to produce A $\beta$ . The amino-terminal end of A $\beta$  is produced by APP cleavage between Met<sub>671</sub> and Asp<sub>672</sub> by  $\beta$ -secretase (BACE; aspartyl protease 2) and the carboxy-terminus by cleavage between Val<sub>711</sub> and Val<sub>712</sub> (A $\beta_{1-40}$ ) or between Ile<sub>713</sub> and Ala<sub>714</sub> (A $\beta_{1-42}$ ) by  $\gamma$ -secretase [APP<sub>770</sub> codon numbering system]. The Swedish familial mutation (Asn<sub>671</sub>/Leu<sub>672</sub>) of APP increases the propensity for cleavage by  $\beta$ -secretase. Cleavage of APP by  $\alpha$ -secretase within the A $\beta$  region precludes formation of A $\beta$ . There were equivalent  $\beta$ - and  $\alpha$ -secretase activities in lysates prepared from Tg2576 and FRAXAD brain lysates (**Figure 4A,B**) and equivalent levels of both the proform and mature form of BACE (**Figure 4C,D**). The anti-BACE antibody reacts with the carboxy-terminus of BACE and the calculated molecular weight of the protein based on amino acid content is 70 kDa. However, the observed molecular weight is in the range of 60-75 kDa due to glycosylation of mature and proform proteins. Equivalent secretase activity and levels of BACE in Tg2576 and FRAXAD indicate that the A $\beta$  processing machinery was intact in the FRAXAD mice.

The mGluR theory of FXS suggests that mGluR<sub>5</sub> antagonists are able to place a brake on protein translation and thus revert *fmr-1*<sup>-/-</sup> phenotypes [14]. MPEP is a specific and potent noncompetitive antagonist of mGluR<sub>5</sub> that is capable of crossing the blood brain barrier [15-16]. Hence, we tested the ability of MPEP to reduce PTZ-induced seizures in WT, *fmr-1*<sup>-/-</sup>, Tg2576 and FRAXAD mice (**Tables 1 & 2**). PTZ is a potent,

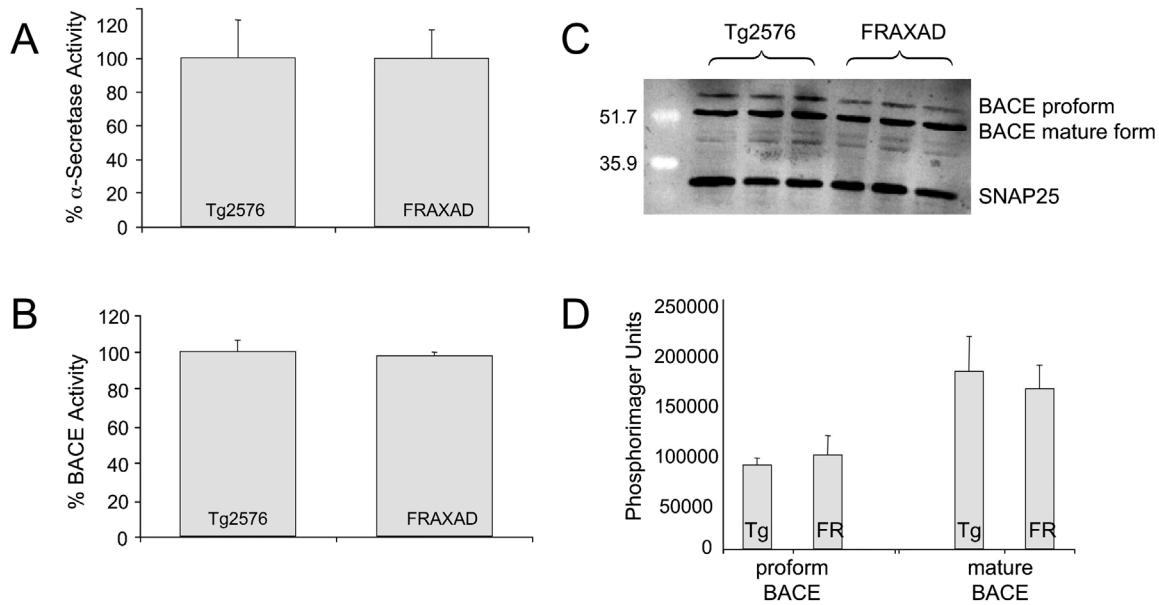


**Figure 2.** APP and A $\beta$  Levels in Young Adult Tg2576 and FRAXAD Mice. GnHCl-soluble lysates were prepared from brain hemispheres harvested from 2-month old mice and diluted 1:100 to reduce the GnHCl concentration to below 0.1 M. Lysates were further diluted 1:5 for APP/APP $\alpha$  analyses. (A) APP/APP $\alpha$  and (B) A $\beta_{1-40}$  ELISAs were performed as described in the Methods Section and plotted as pg/ $\mu$ g lysate. There were no statistically significant differences between Tg2576 (Tg) [females (F): n = 7, males (M): n = 8] and FRAXAD (FR) [F: n = 7, M: n = 7] mice.



**Figure 3.** FRAXAD Mice (16-18 months) Exhibit Elevated Brain APP/APP $\alpha$  Compared to Age-Matched Tg2576. GnHCl-soluble lysates were prepared from 16-18-month old Tg2576 (n=4) and FRAXAD (n=3) mice and diluted 1:800 (APP/APP $\alpha$ ), 1:25,000 (A $\beta_{1-40}$ ) or 1:75,000 (A $\beta_{1-42}$ ) for ELISA analyses of (A) APP/APP $\alpha$  in brain hemispheres and (B) A $\beta_{1-40}$  and (C) A $\beta_{1-42}$  in hippocampus, cortex and cerebellum brain regions. There was a statistically significant 28% increase in APP/APP $\alpha$  levels in FRAXAD brain (p < 0.05). There was a trend for increased A $\beta_{1-40}$  and A $\beta_{1-42}$  in cortex, hippocampus and cerebellum but the differences were not statistically significant between Tg2576 and FRAXAD.

## MPEP reduces seizure severity in *Fmr-1* KO mice over expressing human A $\beta$



**Figure 4.** Tg2576 and FRAXAD Mice Have Equivalent  $\alpha$ - and  $\beta$ -Secretase Activity. The enzymatic activities of  $\alpha$ -secretase (A) and  $\beta$ -secretase (B) were quantitated by R&D Systems fluorometric assays of whole brain lysates prepared from Tg2576 ( $n=3$ ) and FRAXAD ( $n=3$ ) mice (9 months old). The proform and mature forms of BACE were assessed by western blot analyses (C) and quantitated with ImageQuant software (Molecular Dynamics, Inc.) (D). Lysates (100  $\mu$ g per lane) prepared in cell extraction buffer were separated by 12% SDS-PAGE and transferred to 0.45  $\mu$ m nitrocellulose. Blocked membranes were hybridized with anti-BACE (dilution, 1  $\mu$ g/mL, Zymed catalog #34-4900) and anti-SNAP25 (dilution, 1:2000, Abcam catalog #ab5666) followed by anti-rabbit HRP-conjugated secondary antibody and visualization with ECL<sup>+</sup>,  $n=3$  each.

**Table 1.** Seizure Endpoint  $\pm$  MPEP

	n	Ave Age (days)	Avg Weight (g)	MPEP Seizure End-Point	n	untreated* Seizure End-Point	Difference	p
WT female	9	58.6 $\pm$ 0.84	21.6 $\pm$ 0.66	1.78 $\pm$ 0.15	9	1.89 $\pm$ 0.20	0.11	0.65
WT male	10	58.1 $\pm$ 0.78	28.6 $\pm$ 0.63	2.20 $\pm$ 0.25	12	2.42 $\pm$ 0.31	0.22	0.59
KO female	6	57.9 $\pm$ 0.95	20.8 $\pm$ 0.59	2.33 $\pm$ 0.42	20	2.55 $\pm$ 0.22	0.22	0.63
KO male	8	57.9 $\pm$ 0.74	27.0 $\pm$ 0.79	2.13 $\pm$ 0.29	13	2.85 $\pm$ 0.27	0.72	0.11
Tg2576 female	4	58.0 $\pm$ 1.1	20.1 $\pm$ 0.83	4.25 $\pm$ 0.75	6	4.67 $\pm$ 0.33	0.42	0.57
Tg2576 male	8	56.8 $\pm$ 0.90	26.3 $\pm$ 0.96	4.63 $\pm$ 0.26	7	4.57 $\pm$ 0.30	-0.06	0.89
FRAXAD female	5	58.2 $\pm$ 1.2	20.6 $\pm$ 0.73	4.4 $\pm$ 0.24	7	4.86 $\pm$ 0.14	0.46	0.12
FRAXAD male	5	58.0 $\pm$ 0.95	28.4 $\pm$ 0.85	4.0 $\pm$ 0.63	12	4.08 $\pm$ 0.31	0.08	0.89

\*Please refer to the reference [10].

competitive antagonist at inhibitory GABA<sub>A</sub> neurons [17] and *fmr-1* KO mice have a normal sensitivity to PTZ [7] whereas Tg2576 and FRAXAD are highly sensitive [10]. MPEP (30 mg/kg body weight via I.P. injection 30 min prior to PTZ) minimally reduced seizure end-point scores in WT and *fmr-1* mice from 1.89, 2.42, 2.55 and

2.85 to 1.78, 2.20, 2.33 and 2.13 for WT females and males and *fmr-1*<sup>-/-</sup> females and males, respectively (**Table 1**). These reductions were not statistically significant. However, MPEP did exhibit anti-convulsant activity by reducing the number of mice exhibiting grade 3 seizures. WT male mice reach a grade 3 seizure



Table 2. Latency to Seizure with MPEP

Mouse	Grade 1	Grade 2	Grade 3	Grade 4	Grade 5	Recovery Time (min)	Time of Death (sec)	% Mice Dead
WT Female	168 ± 25 n=3	842 ± 210 n=7	NA n=0	NA n=0	NA	39 ± 4.04 n=9	NA n=0	0
WT Male	140 ± 27 n=10	801 ± 151 n=9	522 ± 56 n=2	465 n=1	NA	39 ± 2.54 n=10	NA n=0	0
KO Female	90 ± 10 n=6	290 ± 68 n=5	387 ± 75 n=2	462 n=1	NA	43 ± 3.48 n=6	NA n=0	0*
KO Male	146 ± 13 n=6	393 ± 92 n=6	325 ± 80 n=2	244 n=1	NA	31 ± 3.47 n=8	NA n=0	0
Tg2576 Female	149 ± 33 n=2	198 ± 16 n=4	368 ± 44 n=3	595 ± 182 n=3	792 ± 179 n=3	60 n=1	1017 ± 5 n=2	50**
Tg2576 Male	121 ± 19 n=3	216 ± 41 n=8	344 ± 101 n=8	370 ± 114 n=7	576 ± 191 n=6	39 ± 11.00 n=2	725 ± 161 n=6	75
FRAXAD Female	155 ± 3 n=2	207 ± 25 n=5	322 ± 57 n=5	328 ± 55 n=5	223 ± 39 n=2	46 ± 5.05 n=3	267 ± 46 n=2	40
FRAXAD Male	ND n=5	261 ± 172 n=3	253 ± 33 n=3	220 ± 57 n=3	232 ± 56 n=3	52 ± 0 n=2	340 ± 139 n=3	60

\*2 *Fmr-1* KO female mice died from MPEP alone (no PTZ injection) and were not included.

\*\*1 Tg2576 female mouse died from MPEP alone (no PTZ injection) and was not included.

Of the 5 mice in the Tg2576 female group, 1 mouse had a stage 5 seizure and recovered within 1 hr, 1 mouse had a stage 2 seizure and did not recover within 1 hr, 2 mice died and 1 mouse died from MPEP alone.

[fully developed minimal seizure with clonus of the head muscles and forelimbs] at a rate of 50% (n=6/12) in response to 50 mg/kg PTZ [10], but only 20% achieve a grade 3 seizure after MPEP treatment (n=2/10) (**Table 2**) (statistically significant, Chi-square test,  $p < 0.05$ ). There was a reduction from 11% (n=1/9) to 0% (n=0/9), 38% (n=5/13) to 25% (n=2/8), and 40% (n=8/20) to 33% (n=2/6) in WT females, *fmr-1* KO males and *fmr-1* KO females, respectively. Thus, MPEP was more effective in reducing the rate of grade 3 seizures in WT than in *fmr-1* KO mice.

Tg2576 and FRAXAD mice have average seizure scores greater than 4 on a 5-point scale compared to  $2.42 \pm 0.31$  for WT male C57BL/6 mice in response to PTZ [10]. Tg2576 mice exhibited 83 and 100% rates of grade 3 seizures in response to PTZ as well as 83 and 71% death rates for females and males, respectively [10]. FRAXAD mice exhibited 100 and 75% rates of grade 3 seizures in response to PTZ as well as 86 and 42% death rates for females and males, respectively [10]. MPEP reduced the death rate from 83% to 50% in Tg2576 females and from 86% to 40% in FRAXAD females, but there was no reduction in the death rate for Tg2576 or FRAXAD male mice (**Table 2**). Of note, two *fmr-1* KO and one Tg2576 female died from the MPEP injection alone and were not included in the analyses. While the average seizure endpoint for the Tg2576 and FRAXAD mice was 4.0 or greater  $\pm$  MPEP, there were other measurable differences in seizure sensitivity. For example, with the Tg2576 males (n=8), three of the mice underwent two rounds of grade 3 seizures before progressing to grade 4 and then two rounds of grade 4 seizures before progressing to grade 5. One of these mice underwent two rounds of grade 5 seizures before dying. [Grade 5 is typically scored on the presence of hyperactive bouncing, tonic-clonic seizures and respiratory arrest, but for this analysis, the first round of grade 5 seizures includes only the hyperactive bouncing and tonic-clonic seizures.] An additional mouse in the cohort underwent one grade 3 seizure followed by two rounds of grade 4 seizures before progressing to two rounds of grade 5 seizures and death. Multiple rounds of seizure before progressing to the next seizure stage were not observed in the absence of MPEP. Of the four remaining mice in the Tg2576 male cohort, two mice had an increased latency time to grade 3

seizure after MPEP, 743 and 860 sec, versus  $337.3 \pm 62$  sec for PTZ alone [10]. The other two mice (25% of cohort) were indistinguishable from non-MPEP-treated Tg2576 males. This data suggests that MPEP partially attenuates PTZ-induced seizures by delaying progression to more severe seizure stages, but that blockade of mGluR<sub>5</sub> is not enough to prevent seizures.

In the case of the FRAXAD mice, we did not observe multiple rounds of seizures in any of the animals; however, MPEP reduced the odds of a FRAXAD mouse progressing to higher-grade seizures. An odds ratio [18] of seizure severity was calculated by dividing the number of mice displaying higher-grade (4+5) seizures by the number having the lower grade seizure (1+2+3) within each gender/genotype. For example, the odds that a Tg2576 mouse would exhibit a higher grade seizure score was  $(10+1)/(0+0+2)$ , which indicates that this genotype was 5.5 times more likely to exhibit a seizure scored at 4 or 5 than a lower grade seizure. The odds for a WT or *fmr-1* KO mouse to exhibit a grade 4 seizure were less than 0.2. The number of mice in each gender/genotype cohort as a function of seizure end-point is displayed in **Table 3**. An odds ratio for the probability of each strain (genders combined) undergoing high-grade (grades 4 & 5) seizures versus lower grade (grades 1, 2 and 3) is shown in **Table 4**. The odds ratio of a FRAXAD mouse undergoing a grade 4/5 seizure in response to PTZ drops from 8.5 to 4 after MPEP treatment. These data suggest that mGluR<sub>5</sub> antagonists can reduce seizure severity in transgenic mice over expressing human A $\beta$ .

## Discussion

We have demonstrated that fragile X mental retardation protein (FMRP) binds to and represses the translation of APP mRNA [1]. FMRP is a multi-functional mRNA binding protein that is ubiquitously expressed throughout the body with significantly higher levels in young animals [19]. FMRP is regulated in the neonatal brain where it peaks at the end of the first post-natal week and declines thereafter [20]. *Fmr-1* mRNA and protein are down regulated in mouse brain as a function of age with a 50% reduction between young (6 weeks) and middle-age (60 weeks) mice [13]. APP is also developmentally regulated with expression increasing during neuronal differentiation, maximal during



### Table 3. PTZ-Induced Seizure Endpoint Distribution

MPEP	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	no*	no*	no*	no*
gender	male	female	male	female	male	female	male	female	male	female	male	female	male	female
strain	WT	WT	fmr-1 KO	fmr-1 KO	Tg2576	Tg2576	FRAXAD	FRAXAD	FRAXAD	Tg2576	Tg2576	FRAXAD	FRAXAD	FRAXAD
n	10	9	8	6	8	4	5	5	5	7	6	12	7	7
grade 1 endpt	1	2	1	1	0	0	0	0	0	0	0	0	0	0
grade 2 endpt	7	7	6	3	0	1	1	0	0	0	0	2	0	0
grade 3 endpt	1	0	0	1	1	0	1	0	0	1	1	0	0	0
grade 4 endpt	1	0	1	1	1	0	0	3	0	1	0	5	1	1
grade 5 endpt	0	0	0	0	6	3	3	2	2	5	5	5	6	6
multiple grade 3	1	0	1	0	3	2	0	0	0					
multiple grade 4	0	0	0	0	4	1	0	0	0					
multiple grade 5	0	0	0	0	2**	0	0	0	0					

\*Please refer to reference [10].

\* Grade 5 seizures involve hyperactive bouncing followed by tonic-clonic seizures and death. These mice were scored as multiple grade 5 seizures due to two rounds of hyperactive bouncing and tonic-clonic seizures. The mice recovered from the first round and later entered a second round resulting in death.

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**Table 4.** Odds Ratio for Tonic Seizures and Death

MPEP	yes	yes	yes	yes	no	no
strain*	WT	<i>fmr-1</i> KO	Tg2576	FRAXAD	Tg2576	FRAXAD
n	19	14	12	10	13	19
grade 1 endpt	3	2	0	0	0	0
grade 2 endpt	14	9	1	1	0	2
grade 3 endpt	1	1	1	1	2	0
grade 4 endpt	1	2	1	3	1	6
grade 5 endpt	0	0	9	5	10	11
Odds Ratio**	0.06	0.17	5	4	5.5	8.5

\*genders combined.

\*\*odds that specified strain would exhibit grade 4 & 5 seizures.

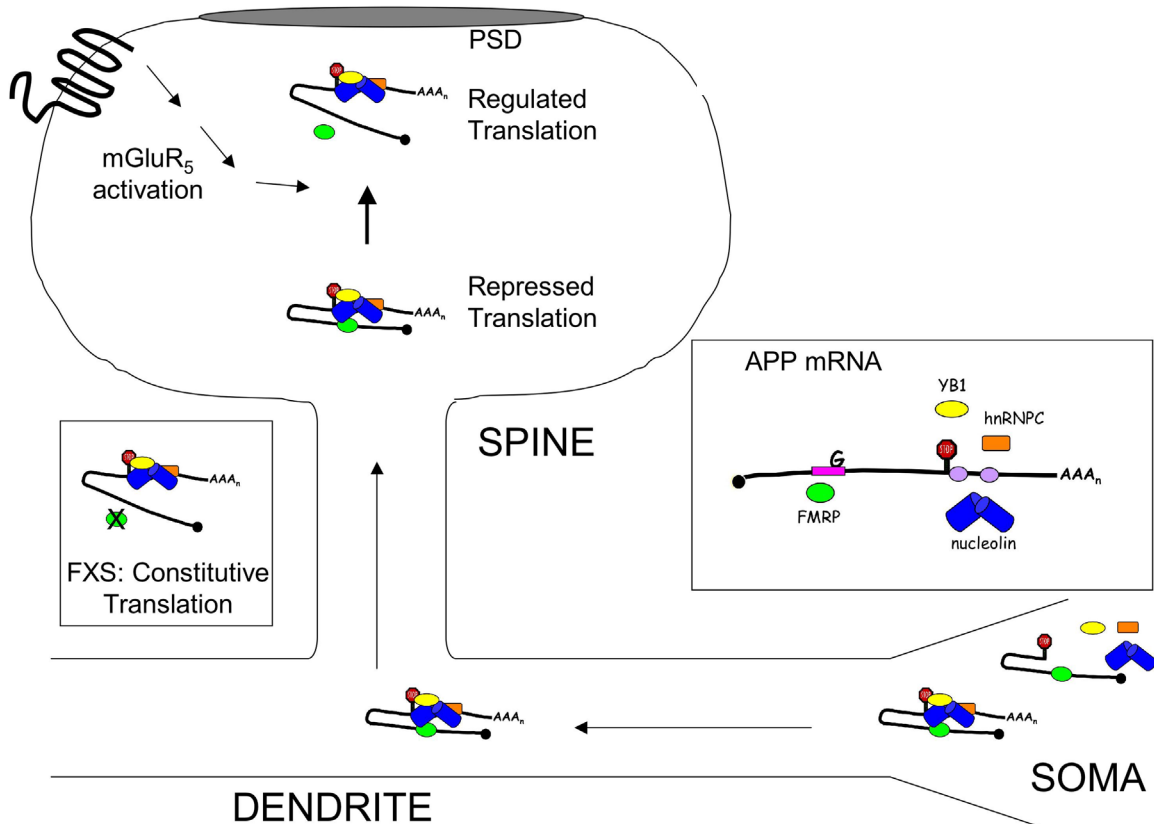
synaptogenesis, and subsequent decline when mature connections are completed [21-24].

APP plays a critical physiological role in synapse formation and maintenance. It promotes synapse differentiation at the neuromuscular junction in *Drosophila* [25] and increases the number of presynaptic terminals in transgenic mice [26]. siRNA targeted against APP decreases presynaptic APP/APLP2 levels and reduces synaptic activity in the rat superior colliculus [27]. APP/APLP2 double knockout mice exhibit defective NMJ, excessive nerve terminal sprouting and defective synaptic transmission [28]. Administration of anti-APP antibodies prevents memory formation in day-old chicks [29] and in rats [30]. Thus, misregulated expression and processing of APP during development likely play important roles in learning, memory and seizure induction/propagation.

APP levels are elevated in the dendrites of embryonic neurons (E18, cultured 11 days), SN (juvenile mice P14-17) and aged *fmr-1* KO brain (16-18 months old). Tg2576 over-express human APP and A $\beta$  in a WT C57BL/6 background hence they possess an intact FMRP/mGluR<sub>5</sub> signaling pathway whereas FRAXAD are lacking the FMRP translational brake. Thus, we expected to observe greatly exacerbated human APP and A $\beta$  expression in the FRAXAD mice. Surprisingly, we only observed a 28% increase in APP/APP levels in aged (16-18 months old) FRAXAD mice. We did not observe statistically significant differences in APP/APP $\alpha$  or A $\beta$ <sub>1-40</sub> levels between Tg2576 and FRAXAD at 2 months of age or in A $\beta$ <sub>1-40</sub> and A $\beta$ <sub>1-42</sub> at 16-18 months of age. We had previously observed a 23% increase in A $\beta$ <sub>1-40</sub> in

brain lysates, but not SN, prepared from 14-16 day-old FRAXAD mice compared to Tg2576 [10]. In addition, brain lysates from *fmr-1* KO mice showed 2.8-fold more A $\beta$ <sub>1-40</sub> compared to WT [1]. These data suggest differential regulation of murine versus human APP mRNA in these transgenic mice. The APP<sub>Swe</sub> cDNA expressed in Tg2576 lacks the 3'-UTR [31]. We have shown that FMRP binds to the 3'-UTR of APP mRNA suggesting that this fragment is required for appropriate translational regulation. In addition, the 3'-UTR may be required for dendritic targeting as shown for many other mRNAs [32-35]. Indeed, immunofluorescence analyses of cultured Tg2576 neurons reveals that APP<sub>Swe</sub> is predominantly in the soma rather than the dendrites (manuscript in preparation, Westmark et al.). Both of these events would diminish the effects of FMRP KO. We present a model for APP mRNA regulation in dendrites in **Figure 5**.

Tg2576 and FRAXAD both over-express human APP<sub>Swe</sub> and A $\beta$  and exhibit drastically reduced seizure thresholds to chemically induced seizures. The mGluR<sub>5</sub> inhibitor MPEP rescues prepulse inhibition of the startle response and dendritic spine protrusion morphology in cultured neurons [36], courtship behavior and mushroom body defects in *dfmr1* KO flies [37] and seizures induced by intracerebroventricular administration of CHPG or sound [11,38]. MPEP reduces the duration of synchronized charges in mouse hippocampal slices induced by the GABA<sub>A</sub> receptor antagonist bicuculline [39], the induction of burst prolongation of epileptiform bursts [40], seizures in rats induced by pilocarpine [41], and low dose PTZ-induced seizures in mice [42]. MPEP did not prevent PTZ-induced



**Figure 5.** Model of FMRP-Mediated Regulation of APP Translation. APP mRNA is a synaptic target for regulation by FMRP. Through UV crosslinking CLIP assays, we've shown that FMRP binds directly to the coding region of APP mRNA at a guanine-rich region. FMRP also protects a 29-base *cis*-element in the 3'-UTR from ribonuclease digestion of anti-FMRP immunoprecipitates [1]. RNA binding proteins such as nucleolin, hnRNP C and YB1 bind to *cis*-elements in the 3'-UTR [43-45]. Nucleolin and YB1 are protein binding factors of FMRP [46-47], which suggests that that protein/protein interactions bring multiple *cis*-elements in APP mRNA in close proximity to regulate translation (Repressed Translation State). Stimulation of cortical SN with DHPG, a group 1 mGluR agonist, releases FMRP from APP mRNA while increasing APP translation (Regulated Translation State). In the absence of FMRP (*fmr-1* KO SN and primary neuronal cells), basal APP levels are increased and nonresponsive to mGluR<sub>5</sub> signaling (FXS: Constitutive Translation State). mRNA/protein interactions are likewise important for the movement of APP mRNA from the soma to the dendrites as human APP<sub>Swe</sub>, which lacks the 3'-UTR, is localized in the soma.

seizures in Tg2576 or FRAXAD mice or significantly lower average seizure endpoints, however, MPEP did: (1) lower the death rate in Tg2576 and FRAXAD females, (2) produce multiple rounds of grade 3-5 seizures in Tg2576, and (3) decrease the odds ratio of a FRAXAD mouse undergoing a grade 4/5 seizure. Our data suggests that the mGluR<sub>5</sub> signaling pathway is involved in seizure initiation/propagation.

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# MPEP reduces seizure severity in *Fmr-1* KO mice over expressing human A $\beta$

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