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GENETIC CATHEPSIN B DEFICIENCY REDUCES β -AMYLOID IN TRANSGENIC MICE EXPRESSING HUMAN WILD-TYPE AMYLOID PRECURSOR PROTEIN

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Abstract

Neurotoxic β -amyloid ($A\beta$) peptides participate in Alzheimer's disease (AD); therefore, reduction of $A\beta$ generated from APP may provide a therapeutic approach for AD. Gene knockout studies in transgenic mice producing human $A\beta$ may identify targets for reducing $A\beta$. This study shows that knockout of the cathepsin B gene in mice expressing human wild-type APP (hAPPwt) results in substantial decrease of $A\beta_{40}$ and $A\beta_{42}$ by 67% in brain, and decreases levels of the C-terminal β -secretase fragment (CTF β) derived from APP. In contrast, knockout of cathepsin B in mice expressing hAPP with the rare Swedish (Swe) and Indiana (Ind) mutations had no effect on $A\beta$. The difference in reduction of $A\beta$ in hAPPwt mice, but not in hAPPSwe/Ind mice, shows that the transgenic model can affect cathepsin B gene knockout results. Since most AD patients express hAPPwt, these data validate cathepsin B as a target for development of inhibitors to lower $A\beta$ in AD.

Keywords

Beta-amyloid; cathepsin B; gene knockout; amyloid precursor protein; protease

Introduction

Alzheimer's disease (AD) is an age-related neurodegenerative disorder that results in progressive loss of memory and accumulation of neurotoxic β -amyloid ($A\beta$) peptides in brain [1–3]. The abnormal accumulation of $A\beta$ peptides has been demonstrated as a major factor in the disease, based largely on studies of transgenic mouse models which overproduce $A\beta$,

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resulting in memory deficit and amyloid plaque brain pathology similar to that observed in AD patients [4–6]. A β peptides are generated from the amyloid precursor protein (APP) by proteases known as β - and γ -secretases, which release the N- and C-termini of A β , respectively. Several forms of A β are produced, including A β 40 (1-40) and A β 42 (1-42), which have the same N-termini but differ in their C-terminal residues.

The accumulation of brain A β can be reduced by inhibiting A β production, via reduction of β -secretase processing of APP. The vast majority of AD patients express wild-type (wt) APP (hAPPwt) which contains the wt β -secretase cleavage site sequence [7–9]. The aspartyl protease BACE 1 is a β -secretase [10–14], but it is relatively inefficient at cleaving the wt β -secretase site [15–17]. Inhibitors of BACE 1 have not yet been shown to reduce brain A β or improve memory deficit in AD patients. Thus, there remains a need to identify other potential targets that may also reduce production of brain A β from APP [17,18].

The cysteine protease cathepsin B has been proposed as an alternative candidate β -secretase, which resides in the regulated secretory pathway of neurons, where it produces A β by efficient cleavage of the wt β -secretase site of APP [19–21]. In transgenic mice expressing human APP with the wt β -secretase site, inhibitors of cathepsin B significantly reduce brain levels of A β 40 and A β 42, and also reduce brain levels of C-terminal β -secretase fragment (CTF β) derived from APP by β -secretase [21]. Importantly, these inhibitors provide substantial improvement in memory deficit, and reduce brain amyloid plaque load [21]. Furthermore, in normal guinea pigs, which express APP containing the human wt β -secretase site sequence ([22,23], the cathepsin B inhibitors result in significant decreases in brain A β and CTF β [24, 25]. The conclusion drawn from these inhibitor data is that cathepsin B has potential as a target for developing inhibitors that lower brain levels of A β for possible therapeutic treatment of AD.

However, another study using a genetic knockout approach finds that deletion of the cathepsin B gene in transgenic mice expressing hAPP containing the rare Swedish (Swe) and Indiana (Ind) mutations at the β -secretase and γ -secretase sites, respectively, results in no change in brain A β or CTF β [26]. The conclusion drawn from the knockout data is that cathepsin B may not be a useful target for developing inhibitors to reduce brain A β as a therapeutic approach for AD.

To resolve the conflicting conclusions drawn from the cathepsin B inhibitor [21] and knockout data [26], this study directly compares the effects of cathepsin B gene knockout on A β in transgenic mice expressing hAPPwt, present in most Alzheimer's disease patients, and in mice expressing mutant hAPPSwe/Ind which was used in the previous cathepsin B knockout study [26]. Results from this study demonstrate that deletion of the cathepsin B gene in the hAPPwt mice significantly reduces brain A β 40 and A β 42 by ~67%, and reduces APP-derived CTF β by 41%. And, as previously reported [26], deletion of the cathepsin B gene in the hAPPSwe/Ind mice has no effect on brain levels of A β 40 or A β 42, and no effect on CTF β . The striking difference in the consequences of cathepsin B gene deletion on A β brain levels in hAPPwt transgenic mice, compared to mice expressing mutant hAPPSwe/Ind, highlight the importance of selecting a mouse model that most closely resembles the majority of Alzheimer's patients who express hAPPwt. These findings are consistent with the hypothesis that cathepsin B participates in A β production from APP containing the wild-type β -secretase site, but not from APP with the Swe mutant β -secretase site [21]. Significantly, because the majority of AD patients express hAPPwt, the knockout data of this study validates cathepsin B as a target for developing inhibitors to reduce brain A β generated from wt APP for potential therapeutic treatment of AD.

Materials and Methods

Transgenic cathepsin B knockout mice

The cathepsin B deficient mice (CatB^{-/-}) were generated as previously described [27] and backcrossed to the C57BL/6 background for more than 10 generations. Transgenic mice were obtained from the Jackson Laboratory in which either the hAPPwt, or hAPP containing the Swedish mutations (K670N/M671L) at the β -secretase site combined with the Indiana mutation (V717F) at the γ -secretase site (hAPPSwe/Ind), were expressed under the platelet-derived growth factor beta promoter [28]. All mice were maintained on a C57BL/6 background. The hAPPwtXCatB^{-/-} and hAPPSwe/IndXCatB^{-/-} mice were generated by crossing the indicated strains. PCR analysis was utilized to determine the genotype of the animals, performed as previously described [27,28]. Animal studies were conducted according to regulations by the NIH and as approved by the IACUC at the Medical University of South Carolina.

Preparation of Brain Extracts

Animals were sacrificed and brain extracts were prepared as previously described [21]. Briefly, brain extracts were homogenized (1:3 weight/volume of buffer) in buffer of 5 M guanidine HCl in 50 mM Tris-HCl, pH 7.6, 150 mM NaCl, plus protease inhibitors (Sigma). Homogenates were diluted to 0.5 M guanidine and centrifuged ($200,000 \times g$ for 20 min.), and supernatant and pellet fractions were collected. Protein content was determined by the Bradford method.

A β Analyses

Enzyme-linked immunosorbent assays (ELISAs) measured A β peptides by methods previously described [21]. The pellet from the brain extract procedure was sonicated in 6 M guanidine and centrifuged at $200,000 \times g$ for 20 min at 4 °C, and the supernatant was diluted to 0.5 M guanidine. The two supernatants were combined, and A β 40 and A β 42 were determined using ELISA kits specific for each peptide (KHB3481/KHB3441; Biosource International). The mean levels of A β peptides in CatB^{-/-} and CatB^{+/+} mice were compared

CTF β and sAPP α Analyses

Western blot assays measured CTF β and sAPP α as previously described with the same amounts of protein in each lane [21]. CTF β was determined in the pellet fraction from the brain extract (antibody 8717, Sigma). In addition, sAPP α was assessed in the supernatant fraction from the brain extract (antibody GE10, Signet Laboratories). Relative amounts of CTF β and sAPP α were measured by densitometry and results were expressed as percentage of the mean CTF β and sAPP α compared to the control group. As control, β -actin western blots (anti- β -actin from Cell Signaling Technology) monitored equal loading of the same amounts of samples (20 μ g protein) in gel lanes.

Statistical Evaluation

Experiments consisted of ten mice in each group. Each biochemical analysis consisted of two or three replicates. Statistical analyses utilized software designed for scientific data analysis by Student's t-tests (Prism 4 GraphPad).

Results

Deletion of the cathepsin B gene reduces brain A β and CTF β , and increases sAPP β , in transgenic mice expressing hAPPwt

Deletion of the cathepsin B gene (CatB^{-/-}) significantly reduced brain levels of A β 40 and A β 42 levels by 66% and 68%, respectively, in mice expressing hAPPwt, compared to control hAPPwt mice (CatB^{+/+}) (figure 1).

The effects of cathepsin B knockout on the C-terminal β -secretase fragment (CTF β) derived from APP by β -secretase processing was then examined. Deletion of the cathepsin B gene (CatB^{-/-}) resulted in significant reduction of brain levels of CTF β by 41% in hAPPwt transgenic mice compared to controls (CatB^{+/+}), illustrated by quantitation of CTF β western blots (figure 2A, B). These data are consistent with a role of cathepsin B in A β production from hAPPwt by β -secretase processing.

In addition, cathepsin B deficiency increased brain levels of sAPP α by 61% in cathepsin B knockout mice (CatB^{-/-}) relative to controls (CatB^{+/+}) (illustrated by western blots and quantitation in figure 2C, D). The sAPP α fragment is derived from APP by cleavage at the α -secretase site within the A β domain, which precludes A β production. The increase in sAPP α occurs during decreased production of A β in the cathepsin B knockout condition.

In transgenic mice expressing mutant hAPPSwe/Ind, deletion of the cathepsin B gene has no effect on brain A β , CTF β or sAPP α

Deletion of the cathepsin B gene (CatB^{-/-}) in mice expressing hAPPSwe/Ind did not change the mean A β 40 and A β 42 brain levels, compared to control hAPPSwe/Ind mice expressing cathepsin B (CatB^{+/+}) (Table 1) The cathepsin B deletion also had no effect on brain levels of CTF β or sAPP α in hAPPSwe/Ind transgenic mice compared to the hAPPSwe/Ind controls expressing cathepsin B (CatB^{+/+}) (Table 2).

All mice appeared healthy

Animals remained healthy and were observed to have normal eating, body weight, grooming, and general appearance. None of the mice prematurely died or became ill.

Discussion

The significant result of this study is that deletion of the cathepsin B gene results in substantial reduction of brain A β 40 and A β 42 by ~67% in mice expressing hAPPwt. This finding demonstrates that the cathepsin B gene has a major role in production of A β peptides from hAPPwt in brain. The key feature of this novel finding is that the decrease in A β occurs in transgenic mice expressing hAPPwt, which is present in the majority of AD patients. Thus, these data validate cathepsin B as a target for development of inhibitors to reduce A β for AD therapeutics.

CTF β is a proteolytic product derived from APP by β -secretase cleavage, and is a measure of β -secretase activity. The 41% reduction in CTF β resulting from eliminating the cathepsin B gene in mice expressing hAPPwt suggests that deletion of the cathepsin B gene decreases β -secretase activity, and that the reduced brain A β in these mice may be due to a reduction in β -secretase.

The sAPP α fragment is a proteolytic product of APP generated by α -secretase cleavage, which produces a non-amyloidogenic peptide product and competes with β -secretase for cleavage of APP. The 61% increase in sAPP α resulting from deletion of the cathepsin B gene in mice

expressing hAPPwt indicates that the deletion affects factors which influence α -secretase activity. The concomitant reduction in β -secretase activity in these animals may increase the availability of the APP substrate and thereby allow α -secretase to produce greater amounts of sAPP α .

These cathepsin B knockout data are consistent with results from *in vivo* cathepsin B inhibitor studies. Compounds which inhibit cathepsin B significantly reduce brain A β peptides (A β 40 and A β 42) and CTF β , and increase sAPP α in transgenic mice expressing hAPP containing the wt β -secretase site sequence [21]. Most importantly, the cathepsin B inhibitors improve memory deficit and reduce amyloid plaque load [21]. In normal guinea pigs, inhibitors of cathepsin B also reduce brain A β peptides (A β 40 and A β 42) and CTF β , and increase sAPP α [24,25]; guinea pigs are a model of human APP processing [29] and possess APP containing the human wt β -secretase site sequence [22,23,29].

The cathepsin B knockout data are further supported by cellular studies. Both siRNA or chemical inhibition of cathepsin B reduce the amount of A β secreted from rat hippocampal neurons in culture [30], which express APP containing the human wt β -secretase site sequence [22]. Also, an inhibitor of cathepsin B reduces levels of A β secreted from bovine chromaffin cells, accompanied by reduced CTF β [19]; these chromaffin cells express bovine APP containing the human wt β -secretase site sequence [22].

Thus, studies of genetic knockout, chemical inhibition, and siRNA silencing of cathepsin B in several animal species and cells expressing APP with the wt β -secretase site all result in a significant reduction of A β .

In contrast, in mice expressing mutant hAPPSwe/Ind, deletion of the cathepsin B gene does not reduce A β 40 or A β 42, and has no effect on CTF β or sAPP α , as shown in this study. These findings confirm the data previously reported for cathepsin B gene knockout mice expressing hAPPSwe/Ind [26].

The dramatically different results of cathepsin B knockout that occur in the hAPPwt compared to hAPPSwe/Ind mice are consistent with the ability of cathepsin B to cleave the wt β -secretase site, but not the Swe mutant β -secretase site of APP [20,21]. In hAPPwt mice, knockout of the cathepsin B gene reduces A β and CTF β , because cathepsin B has been shown to be highly efficient at cleaving the wt β -secretase site of hAPPwt [21]. Therefore, the absence of cathepsin B results in decreased amounts of A β . Similar to knockout of cathepsin B, inhibitors of cathepsin B reduce A β in mice expressing the wt β -secretase site of hAPP [21]. On the other hand, in hAPPSwe/Ind mice, deletion of the cathepsin B gene has no effect on A β or CTF β , because cathepsin B cannot cleave the Swe β -secretase site of hAPPSwe/Ind [21]; therefore, the absence of cathepsin B has no effect on the production of A β in hAPPSwe/Ind mice. Similarly, inhibitors of cathepsin B have no effect on A β in mice expressing mutant hAPP with the Swe β -secretase site of APP [21,31].

These data resolve the apparent conflicting conclusions drawn from the inhibitor [21] and previous knockout data [26] regarding cathepsin B as a useful target for developing AD treatments. Since deletion of the cathepsin B gene reduces A β in hAPPwt mice, cathepsin B is likely to be a useful drug target for development of inhibitors to reduce A β in the majority of AD patients expressing hAPPwt. However, inhibition of cathepsin B is not likely to be useful for reducing A β in the few AD patients expressing hAPPSwe [32], since deleting the cathepsin B gene does not effect A β in animals expressing Swe mutant hAPP.

In conclusion, deletion of the cathepsin B gene significantly reduces A β 40 and A β 42, as well as CTF β , in transgenic mice expressing hAPPwt, which is expressed in the majority of AD patients. Therefore, the key point of this study is that results of the cathepsin B gene knockout

in hAPPwt transgenic mice validates cathepsin B as a target for developing inhibitors to lower brain A β levels for the treatment of most AD patients.

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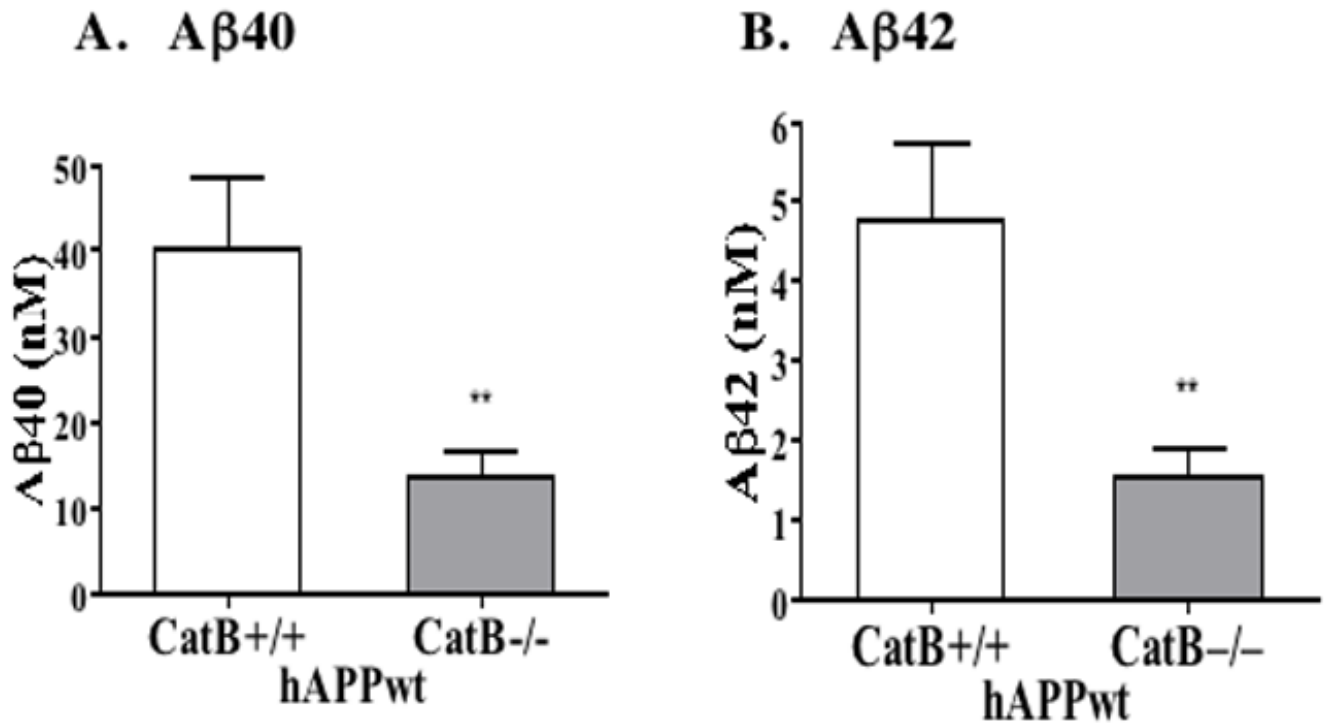


Figure 1. Deletion of the cathepsin B gene in transgenic mice expressing human wild-type APP (hAPPwt) reduces brain A β 40 and A β 42

A. A β 40 levels in brain. The Cat B^{+/+} and Cat B^{-/-} mice expressing hAPPwt contained mean brain A β 40 levels of $40.0 \pm 21\%$ and $13.6 \pm 22\%$ nM, respectively (significant, $**p < 0.007$). Knockout of the cathepsin B gene resulted in a 66% reduction in brain A β 40 in mice expressing hAPPwt.

B. A β 42 levels in brain. The Cat B^{+/+} and Cat B^{-/-} mice expressing hAPPwt contained mean brain A β 42 levels of $4.8 \pm 21\%$ and $1.5 \pm 24\%$ nM, respectively (significant, $**p < 0.007$). Knockout of the cathepsin B gene resulted in a 68% reduction in brain A β 42 in animals expressing hAPPwt.

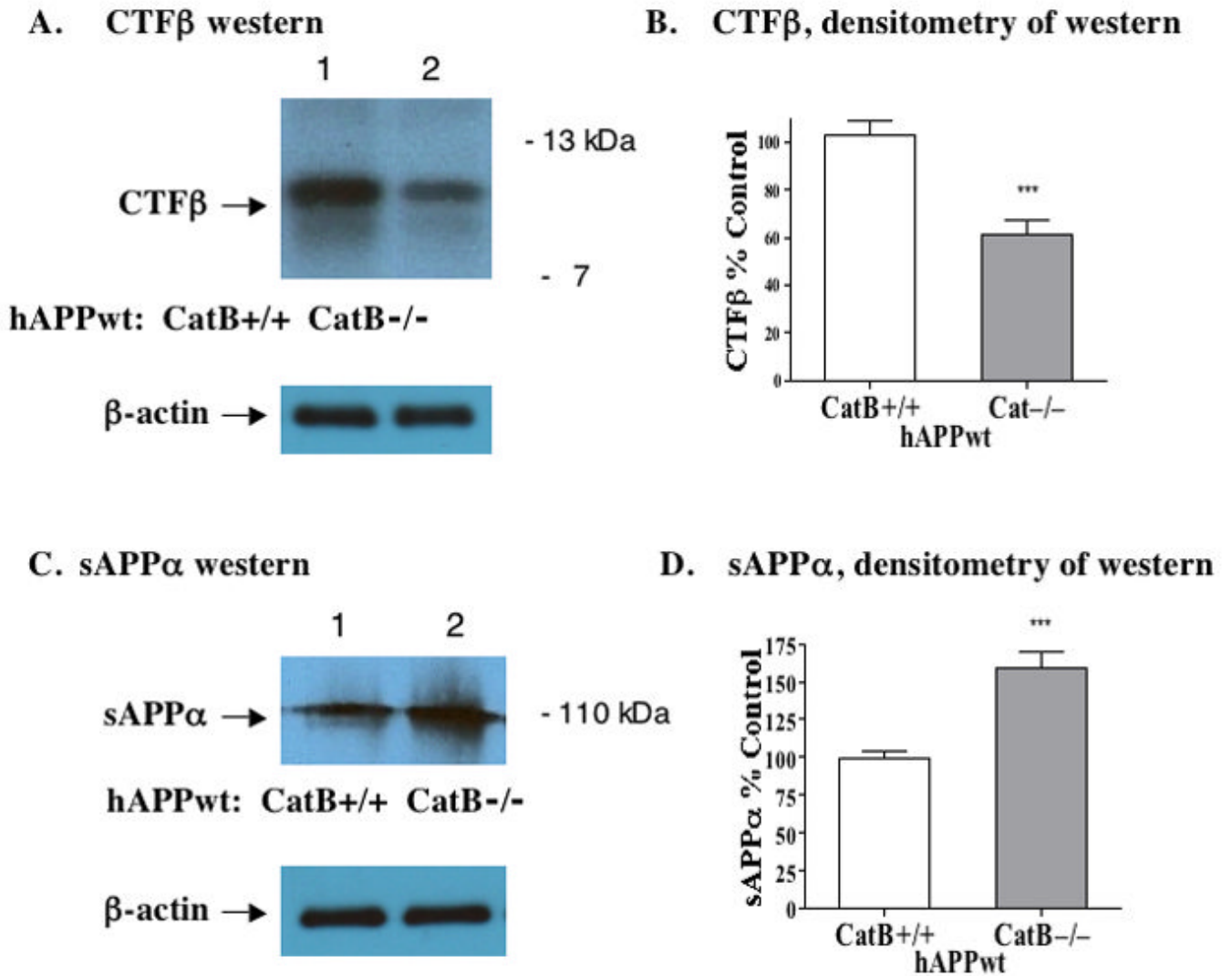


Figure 2. Deletion of the cathepsin B gene in transgenic mice expressing human wild-type APP (hAPPwt) reduces brain CTF β , and increases sAPP α , derived from APP

A. CTF β western blot images. Western blot of the CTF β band (~12kDa) shows its reduced levels in the Cat B^{-/-} mice compared to the Cat B^{+/+} mice. The control blot for β -actin illustrates equal loading of sample, 20 μ g protein, per gel lane.

B. CTF β levels quantitated by densitometry. Densitometry of CTF β western blots (from figure 2A) provided quantitation of CTF β . The Cat B^{+/+} and Cat B^{-/-} mice had mean brain CTF β levels of 103% \pm 6% and 61% \pm 10%, respectively (significant, *** p < 0.0002). Knockout of the cathepsin B gene reduced CTF β by about 41% in animals expressing hAPPwt.

C. sAPP α western blot images. Western blot shows higher levels of the sAPP α band (~110 kDa) in the CatB^{-/-} mice compared to the Cat B^{+/+} mice. The control blot for β -actin illustrates equal loading of sample, 20 μ g protein, per gel lane.

D. sAPP α levels quantitated by densitometry. Densitometry of sAPP α western blots (from figure 2C) provided quantitation. The Cat B^{+/+} and Cat B^{-/-} mice had mean brain sAPP α levels of 99% \pm 5% and 160% \pm 5%, respectively (significant, *** p < 0.0001). Deletion of cathepsin B increased sAPP α by about 61% in hAPPwt mice.

Table 1Deletion of the Cathepsin B Gene in hAPPSwe/Ind Mice has No Effect on A β 40 and A β 42 in Brain.

| A β peptide | A β peptide levels in brain extracts | |
|-------------------|--|--------------------|
| | Control, CatB +/+ | Knockout, CatB -/- |
| A β 40 | 104 \pm 14 nM | 115.5 \pm 16 nM |
| A β 42 | 16.6 \pm 12 nM | 19.8 \pm 9 nM |

A β 40 and A β 42 peptides in brain extracts from CatB+/+ and CatB-/- of hAPPSwe/Ind mice were measured as described in the methods.

Table 2Deletion of the Cathepsin B Gene in hAPPSwe/Ind Mice has No Effect on CTF β or sAPP α in Brain.

| APP-derived fragment | Relative levels in brain extracts of hAPPSwe/Ind Mice, % Control | |
|----------------------|--|--------------------|
| | Control, CatB +/+ | Knockout, CatB -/- |
| CTF β | 99 \pm 8 % | 103 \pm 7 % |
| sAPP α | 100 \pm 8 % | 98 \pm 6 % |

CTF β and sAPP α were evaluated in hAPPSwe/Ind mice with CatB+/+ or CatB-/- conditions, by western blots and densitometry, as described in the methods.