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Deletion of the Cathepsin B Gene Improves Memory Deficits in a Transgenic Alzheimer's Disease Mouse Model Expressing A β PP Containing the Wild-Type β -Secretase Site Sequence

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Abstract

Therapeutic agents that improve the memory loss of Alzheimer's disease (AD) may eventually be developed if drug targets are identified that improve memory deficits in appropriate AD animal models. One such target is β -secretase which, in most AD patients, cleaves the wild-type (WT) β -secretase site sequence of the amyloid- β protein precursor (A β PP) to produce neurotoxic amyloid- β (A β). Thus, an animal model representing most AD patients for evaluating β -secretase effects on memory deficits is one that expresses human A β PP containing the WT β -secretase site sequence. BACE1 and cathepsin B (CatB) proteases have β -secretase activity, but gene knockout studies have not yet validated that the absence of these proteases improves memory deficits in such an animal model. This study assessed the effects of deleting these protease genes on memory deficits in the AD mouse model expressing human A β PP containing the WT β -secretase site sequence and the London γ -secretase site (A β PPWT/Lon mice). Knockout of the CatB gene in the A β PPWT/Lon mice improved memory deficits and altered the pattern of A β -related biomarkers in a manner consistent with CatB having WT β -secretase activity. But deletion of the BACE1 gene had no effect on these parameters in the A β PPWT/Lon mice. These data are the first to show that knockout of a putative β -secretase gene results in improved memory in an AD animal model expressing the WT β -secretase site sequence of A β PP, present in the majority of AD patients. CatB may be an effective drug target for improving memory deficits in most AD patients.

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Keywords

Amyloid- β ; amyloid- β protein precursor; cathepsin B; gene knockout; protease

INTRODUCTION

The primary deficit in Alzheimer's disease (AD) is the severe loss of memory. There currently are no therapeutic agents that reverse or significantly retard the memory loss in AD. However, it may be possible to develop such therapeutics if new drug targets can be validated that improve memory deficits in appropriate AD animal models of the human disease.

The majority of AD patients (>90%) have sporadic AD and express normal, wild-type (WT) amyloid- β protein precursor (A β PP) [1–3]. Only a small percentage of patients develop familial AD involving inherited gene mutations including several mutant A β PP forms [1–3]. Expression of mutant human A β PP forms in transgenic mice results in development of memory deficits and brain neuropathology that resemble AD [4–8]. Such AD animal models may be considered for their representation of familial or sporadic forms of AD.

Proteases with β -secretase activity are viewed as potential targets for improving memory deficits in AD patients. The β -secretase in sporadic AD patients cleaves the WT β -secretase site sequence in A β PP, and, therefore, an appropriate animal model to evaluate potential β -secretase targets for improving memory deficits in most AD patients is one which expresses A β PP containing the WT β -secretase site sequence. Such a model is represented by transgenic mice expressing human A β PP containing the WT β -secretase site sequence combined with the London mutant (Lon) γ -secretase site sequence (A β PPWT/Lon mice), which results in memory deficits and neuropathology resembling AD [9]. Because the A β PPWT/Lon mouse model expresses the WT β -secretase site of A β PP expressed in most AD patients, it is an appropriate model for assessing candidate β -secretase genes as possible AD drug targets for improving memory loss.

Our recent studies show that treatment of A β PPWT/Lon mice with compounds that inhibit cathepsin B (CatB) results in improved memory deficits [9, 10]. These compounds reduce the biomarkers of brain amyloid- β peptides (A β) and C-terminal β -fragment (CTF β) derived from A β PP by β -secretase, suggesting that CatB may function as a WT β -secretase [9, 10]. But these data do not prove that CatB is a target for improving memory deficits because the compounds could have off-target effects. Therefore, to directly address the hypothesis that CatB participates in the memory deficits in an AD mouse model, this study investigated the effects of deleting the CatB gene on memory deficits and biomarkers in the A β PPWT/Lon mice.

The suggestion that CatB may have WT β -secretase activity is controversial because the aspartyl protease BACE1 has been thought to be the primary β -secretase [11, 12]. But deletion of the BACE1 gene in mice expressing A β PP containing the WT β -secretase site sequence results in worse memory [13], even though brain A β and amyloid plaque are reduced [14]. Therefore, further studies of BACE1 gene knockout effects on memory

deficits in transgenic mice expressing A β PP containing the WT β -secretase site sequence are needed to validate a β -secretase target suitable for improving memory deficits in most AD patients.

This study evaluated memory deficits in A β PPWT/Lon mice after deletion of the CatB or BACE1 genes. Knockout of the CatB gene resulted in substantial improvement in memory deficits, and reduced brain amyloid plaque, A β , and CTF β . However, knockout of the BACE1 gene in A β PPWT/Lon mice had no effect on memory deficits or the A β -related biomarkers. Since elimination of CatB reduced brain CTF β and A β , CatB may have WT β -secretase activity in A β PPWT/Lon mice. Significantly, these are the first results to show improved memory deficits after knockout of a protease gene, CatB, in an AD mouse model expressing the 'WT β -secretase site' of A β PP that is present in most AD patients. These findings validate CatB as a drug target to improve memory deficits in the majority of AD patients.

METHODS

Transgenic AD mice

Applied Neurotechnology, Inc. (Charleston, SC) generated the transgenic mice by methods previously described [9, 10, 15–17]. A β PPWT/Lon mice were generated by site-directed mutagenesis to insert the V717I London mutation [18] into human A β PP cDNA (A β PPWT/Lon mice, illustrated in Fig. 1), and the transgenic mouse was created in the C57BL/6 background using the platelet-derived growth factor beta (PDGF β) promoter containing an SV40 polyadenylation site and expressed the A β PP-695 gene. CatB deficient (CatB KO) mice were obtained from Christoph Peters (Albert Ludwig University, Freiburg, Germany), and BACE1 deficient (BACE1 KO) mice were obtained from Jackson Laboratory (Bar Harbor, ME). All mice were maintained on a C57BL/6 background. The transgenic A β PPWT/Lon mice were crossed with either the CatB KO mice or the BACE1 KO mice and the following transgenic mice were generated: A β PPWT/Lon, A β PPWT/Lon \times CatB KO, and A β PPWT/Lon \times BACE1 KO.

Transgenic mice expressing the rare Swedish mutation (Swe) at the β -secretase cleavage site (A β PPSwe) and the Lon mutation at the γ -secretase site were also generated (A β PPSwe/Lon mice, illustrated in Fig. 1). The A β PPSwe/Lon mice were a positive control because deletion of the BACE1 gene in transgenic A β PPSwe mice is known to improve memory deficits [19, 20]. The A β PPSwe/Lon mice were generated by site-directed mutagenesis to insert the K670 N/M671 L Swedish mutation into the human A β PP-695 cDNA containing the Lon mutation. Transgenic mice in the C57BL/6 background were crossed with the CatB and BACE1 gene knockout animals as described above, and the following transgenic mice were generated: A β PPSwe/Lon, A β PPSwe/Lon \times CatB KO, and A β PPSwe/Lon \times BACE1 KO.

PCR analysis was utilized to determine the genotype of the animals, as previously described [17]. All experimental mice were male. Mice were given free access to food and water before and during the experiment.

CatB and BACE1 protease activities in transgenic mice

CatB and BACE1 activities were determined in A β PPWT/Lon and A β PPSwe/Lon mice (at 3 months of age) with knockout of either the CatB or BACE1 genes, to confirm that the protease gene knockouts resulted in the absence of the respective protease activities.

CatB activity in brain was determined using a fluorometric assay kit from Abcam (ab65300) as described by the manufacturer. Briefly, tissues were washed twice in ice-cold PBS and then homogenized in extraction buffer. After 10 min incubation on ice, the extract was centrifuged at 10,000g for 5 min and 50 μ l of supernatant was mixed with an equal volume of 2 \times reaction buffer and 2 μ l substrate in a 96-well microplate. The plates were kept in the dark at 37°C for 1 h, and the fluorescence was recorded using FLUOstar Optima plate reader (BMG). Protein concentration was determined by the BCA method (Bio-Rad Laboratories). The CatB activity was expressed as fluorescent units/mg protein.

BACE1 activity in brain was determined using a fluorometric assay kit from Abcam (ab65357) as described by the manufacturer. Brain samples were prepared as described for CatB activity analysis.

Age of mice for analysis of memory deficits and biomarkers

Memory function, amyloid plaque and brain biomarkers were evaluated after significant memory deficits developed in the A β PPWT/Lon mice and the A β PPSwe/Lon mice. Because the purpose of this study was to compare the effects of deleting the CatB or BACE1 gene on memory deficits in the two different AD mouse models, it was, therefore, important to evaluate each of these mouse models at ages when they each show similar levels of memory deficits. Similar memory deficits for the A β PPWT/Lon and A β PPSwe/Lon mice occur at ages of 12 months and 9 months, respectively; the A β PPWT/Lon mouse acquires memory deficits at a later age than the A β PPSwe/Lon mouse. Thus, A β PPWT/Lon mice of 12 months and A β PPSwe/Lon mice of 9 months, with equivalent levels of memory deficits, were utilized to assess the effects of deleting either the CatB or BACE1 genes on memory deficits.

Spatial memory deficit

The memory deficit in the animals was measured by the Morris water maze test, as we have described previously [9, 10]. Briefly, the spatial memory capability of each animal was assessed with the Morris water maze test (700-0718-4W SD Instruments) which evaluates memory in a swimming test. Mice were individually trained in a 1.2 m open field water maze in a pool filled with water to a depth of 30 cm and maintained at 25°C. An escape platform (10 cm square) was placed 1 cm below the surface of the water. All animals underwent nonspatial pretraining for 4 consecutive days, which prepared the animals for the final behavioral test to determine the retention of memory to find the platform. Two days following the nonspatial pretraining, the hidden platform was placed in the center of one quadrant of the pool, the animal was released facing the pool wall in a random fashion, the time was recorded (latency period), and the distance traveled to reach the platform was measured using video recording (Smart Video Tracking System; SD Instruments).

On the day after the last training session, the platform was removed and a spatial probe test conducted. Each mouse was allowed to search for the platform for 60 seconds (memory retention) and the percent time spent in quadrant where the platform was located (NE quadrant) and in the outer annular area were determined.

Brain amyloid plaque

Amyloid plaque load was assessed in brain sections (10 from each mouse) as described previously [9, 10], achieved by immunohistochemical staining for A β (A β antibody 10D5, Elan Pharmaceuticals). Brain tissues were fixed in 4% paraformaldehyde and then in 4% parformaldehyde and 30% sucrose for 24 h each at 4°C. Tissues were washed in buffered saline and transferred to an optimum cutting temperature medium. Cryosections were cut and blocked with normal serum, incubated with anti-A β and stained with diaminobenzoic acid (Vector ABC Elite kit, Vector Laboratories). Bright field light microscopy imaged brain areas from which stained amyloid areas were quantitated using image analysis (NIH Image software).

Brain A β analysis

Brain A β analysis was conducted as previously described for transgenic AD mice [9, 10]. Briefly, animals were sacrificed and 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 g for 20 min), and supernatant and pellet fractions were collected. The pellet from the brain extract procedure was sonicated in 6 M guanidine and centrifuged at 200,000 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 (A β ₁₋₄₀ and A β ₁₋₄₂, respectively) were determined using ELISA kits specific for each peptide (IBL, JP27718 and JP27711). Enzyme-linked immunosorbent assays (ELISAs) measured A β peptides by methods previously described [9, 10]. Protein content was determined by the Bradford method.

CTF β and sA β PP α analyses

CTF β is generated from A β PP by β -secretase in the amyloidogenic pathway, and soluble A β PP (sA β PP α) is generated from A β PP by α -secretase in the non-amyloidogenic pathway. Western blots measured CTF β and sA β PP α in brains of transgenic mice, using the same amount of protein per gel lane, performed as previously described [9, 10]. CTF β was determined in the pellet fraction from the brain extract (antibody 8717, Sigma) and sA β PP α was assessed in the supernatant fraction from the brain extract (antibody 6E10, Signet Laboratories). Relative amounts of CTF β and sA β PP α were measured by densitometry and results were expressed as percentage of the mean levels of CTF β and sA β PP α of the control groups (without protease gene knockouts). Control β -actin western blots (anti- β -actin from Cell Signaling Technology) was conducted to monitor equal loading of the same amounts of samples (20 μ g protein) in each gel lane.

Animal treatment

Animal studies were conducted according to regulations by the National Institutes of Health and as approved by the IACUC at the Medical University of South Carolina and Ralph H. Johnson VA Medical Center.

Statistical evaluation

Experiments consisted of 10 mice in each group. Each biochemical analysis consisted of two or three replicates. Statistical analyses and data display were conducted utilizing computer software designed for scientific data analysis (Prism 4 GraphPad). Quantitative data are displayed as the mean and standard error of the mean (SEM). Differences between groups were determined by ANOVA analysis and Dunnett's multiple comparison test used to determine differences between transgenic control mice (A β PPWT/Lon or A β PPSwe/Lon) and the protease gene knockout (KO) mice (A β PPWT/Lon \times CatB KO and A β PPWT/Lon \times BACE1 KO or A β PPSwe/Lon \times CatB KO and A β PPSwe/Lon \times BACE1 KO, respectively). These statistical analyses also compared normal control WT mice (non-transgenic mice) and protease deficient mice (CatB KO or BACE1 KO).

RESULTS

A β PPWT/Lon AD mice and protease gene knockouts

Deletion of the CatB gene, but not the BACE1 gene, improves memory deficits in transgenic A β PPWT/Lon mice—The A β PPWT/Lon mice express human A β PP with the WT β -secretase site sequence and the London mutation at the γ -secretase site sequence (Fig. 1) and deletion of the CatB or BACE1 genes resulted in elimination of the respective protease activities (supplementary Figure 1; available online: <http://www.j-alz.com/issues/29/vol29-4.html#supplementarydata03>). The effects of deleting the CatB or BACE1 genes on memory function in the A β PPWT/Lon mice were then assessed by the Morris water maze test.

All animals first underwent nonspatial pretraining for four consecutive days to learn the location of the hidden platform. Analyses by the Morris water maze test on each day of the training period (Fig. 2) showed that the mice do learn, indicated by the reduced latency time for the mice to reach the hidden platform during the training period. By the fourth day of training, the A β PPWT/Lon with deletion of CatB showed the shortest latency period, representing enhanced learning, compared to control A β PPWT/Lon mice. Mice with deletion of the BACE1 gene showed similar learning as the control A β PPWT/Lon mice.

Two days following training, mice were subjected to the final behavioral Morris water maze test to determine the memory deficits. Deletion of the CatB gene in the A β PPWT/Lon mice resulted in substantial improvement in memory deficits, assessed by the latency period and distance traveled, which is the time and distance, respectively, that it took the animal to swim to the submerged platform (Fig. 3). The shorter time and distance traveled indicates better memory. The CatB gene deletion resulted in a 57% and 41% reduction in the latency period and distance traveled, respectively (Fig. 3A, B). The 16 second latency period for the

A β PPWT/Lon \times CatB KO mice is near that of normal mice (non-transgenic, same strain as A β PPWT/Lon mice) which show a 14 second latency period (Fig. 3A, dotted line).

Deletion of the CatB gene also resulted in substantial memory retention in the A β PPWT/Lon mice as illustrated by the higher percent time spent in the northeast (NE) quadrant (from which the submerged platform was removed), and the lower percent time spent in the outer annulus, compared to control A β PPWT/Lon mice (Fig. 4). The CatB deletion resulted in a 230% increase in the percent time spent in the NE quadrant and a 60% decrease in the percent time spent in the annulus. The A β PPWT/Lon mice (control) and A β PPWT/Lon \times CatB KO (CatB knockout) mice did not have a different swimming speed (data not shown). Thus, by the four parameters measured in the Morris water maze test, the deletion of the CatB gene improved the memory deficits that develop in the transgenic A β PPWT/Lon mice.

However, deletion of the BACE1 gene in the A β PPWT/Lon mice had no effect on memory deficits in the A β PPWT/Lon mice, assessed by the Morris water maze measurements of latency period and distance traveled (Fig. 3A, B, respectively), as well as percent time spent in the NE quadrant or annulus (Fig. 4A, B, respectively). The A β PPWT/Lon mice (control) and A β PPWT/Lon \times BACE1 KO mice had the same swimming speed (data not shown). Thus, the BACE1 gene deletion had no effect on memory deficits in the A β PPWT/Lon mice.

Deletion of the CatB gene, but not the BACE1 gene, reduces brain amyloid plaque load in transgenic A β PPWT/Lon mice—A β immunohistochemistry of brain sections showed that deletion of the CatB gene reduced brain amyloid plaque in the A β PPWT/Lon mice (Fig. 5A, B). But deletion of the BACE1 gene had no effect on amyloid plaque load in A β PPWT/Lon mice (Fig. 5A, C). Quantitative image analysis of the A β immunohistochemistry showed that the CatB deletion resulted in a significant 78% reduction in brain amyloid plaque load relative to control A β PPWT/Lon animals (Fig. 5D). In contrast, the quantitative analysis showed that the BACE1 gene deletion had no effect on amyloid plaque load in A β PPWT/Lon animals (Fig. 5D). These data show that knockout of the CatB gene, but not the BACE1 gene, reduces brain amyloid plaque load in the A β PPWT/Lon mice.

Deletion of the CatB gene, but not the BACE1 gene, alters brain biomarkers in a manner characteristic of inhibiting WT β -secretase in transgenic A β PPWT/Lon mice—The pattern of A β PP-derived A β peptides and A β PP-derived cleavage products resulting from amyloidogenic and non-amyloidogenic processing of A β PP was evaluated. Amyloidogenic processing of A β PP by β -secretase produces the CTF β fragment and A β peptides, and non-amyloidogenic processing of A β PP by α -secretase results in the sA β PP α fragment (secretase sites of A β PP are illustrated in Fig. 1).

Knockout of the CatB gene in the transgenic A β PPWT/Lon mice reduced both brain A β ₄₀ and A β ₄₂ by 81% compared to control A β PPWT/Lon mice (Fig. 6A, B), but knockout of the BACE1 gene in these mice did not alter A β ₄₀ or A β ₄₂ levels relative to control A β PPWT/Lon mice (Fig. 6A, B). The CatB gene deletion caused a 50% reduction in brain CTF β relative to controls (Fig. 6C, D), but the BACE1 gene deletion had no effect (Fig. 6C, D) in A β PPWT/Lon mice. The CatB gene deletion increased sA β PP α by 49% relative to

controls (Fig. 6E, F), but the BACE1 gene knockout had no effect on sA β PP α in the A β PPWT/Lon mice (Fig. 6E, F).

Since CTF β is a β -secretase cleavage product, the reduction of CTF β resulting from deletion of the CatB gene in the A β PPWT/Lon mice suggests that CatB has WT β -secretase activity. Reduced production of CTF β from A β PP in the CatB knockout is likely to result in an increased A β PP available for α -secretase to generate increase in sA β PP α . In contrast, since deletion of the BACE1 gene had no effect on the A β PP-derived peptide patterns, these data illustrate that BACE1 does not have endogenous WT β -secretase activity in A β PPWT/Lon mice.

A β PPSwe/Lon mice and protease gene knockouts

Effects of CatB or BACE1 gene deletion on memory deficits and A β -related biomarkers in A β PPSwe/Lon mice—A β PPSwe/Lon mice express the rare Swedish (Swe) mutant β -secretase site and the London mutation near the γ -secretase site (Fig. 1) and deletion of the CatB or BACE1 genes in the A β PPSwe/Lon mice results in the absence of the respective protease activities (supplementary Figure 2). The effects of CatB or BACE1 gene deletion on memory deficits in the A β PPSwe/Lon mice were compared (Fig. 7). During the training period, mice show reduced latencies to find the hidden platform (Fig. 7A), indicating that they learned the location of the platform. After the training period, analyses of memory deficits in the mice showed that knockout of the CatB gene in the A β PPSwe/Lon mice had no effect on memory deficits illustrated by measuring the latency period and the distance traveled (Fig. 7B, C, respectively). Also, CatB gene knockout had no effect on memory retention, measured by the percent time in the northeast quadrant (from which the hidden platform was removed) and the percent time in the outer annulus (Fig. 7D, E, respectively).

However, knockout of the BACE1 gene in the A β PPSwe/Lon mice improved memory deficits, observed by the reduced latency period and distance traveled (Fig. 7B, C, respectively), consistent with previous studies [19, 20]. BACE1 gene knockout also improved retention of memory in the A β PPSwe/Lon mice, assessed by the percent time mice spent in the northeast quadrant (from which the hidden platform was removed) and percent time in the annulus area (Fig. 7D, E).

Analyses of biomarkers showed that deletion of the CatB gene in the A β PPSwe/Lon mice had no effect on amyloid plaque load, A β brain levels, or A β PP-derived CTF β and sA β PP α fragments (Figs. 8 and 9). However, deletion of the BACE1 gene in the A β PPSwe/Lon mice reduced brain amyloid plaque, A β , and CTF β , combined with increased sA β PP α (Figs. 8 and 9), consistent with earlier reports [19, 20].

Comparison of CatB gene deletion in A β PPWT/Lon, A β PPSwe/Lon mice, and normal non-transgenic mice—The data of this study illustrate that the CatB gene knockout specifically improves memory deficits in the A β PPWT/Lon mice, but not in the A β PPSwe/Lon mice. Deletion of the BACE1 gene has no effect in the A β PPWT/Lon mice, but improves memory deficits in the A β PPSwe/Lon mice. The effect of deleting these protease genes in normal mice was also assessed. In normal non-transgenic mice (age-and

strain-matched), deletion of either the CatB or BACE1 genes had no effect on normal memory (age and strain-matched, supplementary Figure 3). These data together show that CatB or BACE1 gene knockout improves memory deficits in A β PPWT/Lon or A β PPSwe/Lon mice, respectively; but deletion of these protease genes has no effect on normal memory of non-transgenic mice.

DISCUSSION

The major result of this study is that deletion of the CatB gene improves memory deficits in the A β PPWT/Lon AD mouse model expressing human A β PP containing the WT β -secretase site sequence that is present in most AD patients. This is the first study that we are aware of to demonstrate that deletion of any gene results in improved memory deficits in an AD mouse model expressing the WT β -secretase site sequence that is present in the majority of AD patients. Moreover, mice with deletion of the CatB gene remain healthy [17]. These significant findings validate CatB as a target for developing therapeutic inhibitor compounds to improve memory deficits of AD.

The significant finding of this study is the identification of a new protease target, CatB, which improves memory deficits upon gene deletion in a mouse model expressing the WT β -secretase site sequence of A β PP that is relevant to sporadic AD patients, representing more than 90% of the AD population. Protease activity that cleaves the β -secretase site of A β PP is viewed in the field as a potential target for developing inhibitors to improve memory deficits. Identification of such protease activity involved in memory deficits was accomplished in this study by using the A β PPWT/Lon mouse model of AD, which expresses human A β PP containing the WT β -secretase site sequence combined with the London mutation near the γ -secretase site and results in development of memory deficits [5, 9, 10]. The A β PPWT/Lon AD mouse model is an appropriate for defining proteases functioning as 'wild-type' β -secretase activity utilized by the majority of AD patients.

Deletion of the CatB gene in the A β PPWT/Lon mice resulted in an altered biomarker pattern consistent with CatB having WT β -secretase activity. Biomarker analyses showed that knockout of the CatB gene in the A β PPWT/Lon mice reduced brain A β and CTF β derived from A β PP by β -secretase, and increased sA β PP α ; these changes represent an altered A β -related pattern characteristic of inhibiting the processing of A β PP at the WT β -secretase site. In transgenic mice expressing human wild-type A β PP (no mutations), deletion of the CatB gene alters these biomarkers in the same manner [17] as in the A β PPWT/Lon mice of this study. These data demonstrate that CatB has β -secretase activity in two transgenic mouse models expressing A β PP containing the WT β -secretase site sequence of A β PP. The biomarker data supports the hypothesis that deletion of the CatB gene in the A β PPWT/Lon mice improves memory by reducing CatB WT β -secretase activity. Moreover, the data suggest that the improvement in memory deficits occurring with administration of CatB inhibitors [9, 10] is also likely due to inhibition of CatB WT β -secretase function.

Of interest is the finding from this study that improvement in memory deficits results from CatB gene knockout in the A β PPWT/Lon mice, but not in the A β PPSwe/Lon mice. These data illustrate the specificity of CatB gene deletion in transgenic mice expressing the WT β -

secretase site but not the Swedish mutant site of human A β PP. The difference in CatB gene knockout in the A β PPWT/Lon mice compared to the A β PPSwe/Lon mice can be explained by the preference of CatB to readily cleave the WT β -secretase site, rather than the Swe mutant β -secretase site of A β PP [9].

One report speculated that inhibiting CatB may worsen memory deficits based on the finding that CatB degrades A β *in vitro* and over-expression of CatB by lentiviral vector injection into brains of transgenic mice resulted in a reduction of amyloid plaque in the region receiving the injection [21]. However, that study did not evaluate the effects of inhibiting CatB on memory deficits, whereas the data here shows that CatB gene deletion in the A β PPWT/Lon mice resulted in improved memory deficits. Moreover, we previously showed that chemical inhibition of CatB in the A β PPWT/Lon mice also results in improved memory deficits [9, 10]. Chemical inhibition of CatB in the guinea pig, a natural model of normal human WT A β PP processing [22], also results in reduction of brain A β [9, 10, 23, 24]. These data show that elimination or inhibition of CatB improves, and does not worsen, memory deficits in these animal models expressing the WT β -secretase site that is present in the majority of AD patients.

As control, our studies of memory function after BACE1 gene knockout found that its deletion in A β PPSwe/Lon mice expressing the Swe mutant β -secretase site (with the London mutation) resulted in improved memory deficits, similar to previous reports [19, 20]. But since the majority of AD patients express the WT β -secretase site of A β PP, we investigated the representative A β PPWT/Lon AD mouse model in BACE1 gene knockout experiments; results showed no effect of BACE1 knockout on memory deficits in the A β PPWT/Lon mice. Two other studies showed that in a mouse model expressing the WT β -secretase site sequence of A β PP, BACE1 gene knockout resulted in worse memory [13, 25]. So far, there is no report of improving memory deficits by BACE1 gene knockout in a mouse model expressing the WT β -secretase site of A β PP, expressed in most AD patients.

Biomarker results of this study show that deletion of the BACE1 gene in the A β PPWT/Lon mice has no effect on brain A β , CTF β , and sA β PP α , which argues that BACE1 is not acting as an endogenous WT β -secretase in this model. Our previous work also showed that knockout of the BACE1 gene in transgenic mice expressing human A β PP (no mutations) had no effect on these biomarkers [17]. But other reports show reduced A β after BACE1 gene knockout in transgenic PDGF-A β PP [14, 26] and A β PP51/16 [27] mice expressing the WT β -secretase site. These differences may be explained by the expression of A β PP695 in the A β PPWT/Lon mice, whereas the PDGF-A β PP [14, 26] and A β PP51/16 [27] mice express primarily the A β PP751 and A β PP770 isoforms that contain the Kunitz protease inhibitor domain [28, 29]. Different protease effects of the Kunitz domain, and differences in the distribution of A β PP695 in neurons that secrete A β compared to the presence of A β PP751 and A β PP770 in astrocytes [30, 31], may explain the different effects of BACE1 gene knockout in the different transgenic mouse models. Over-expression of BACE1 in A β PPWT/Lon mice results in elevated brain A β [32], indicating that BACE1 may have exogenous effects, but does not demonstrate an endogenous role. In normal mice expressing mouse WT A β PP [33], knockout or over-expression of BACE1 results in contradictory results showing reduction [18, 34] or no effect [35] on brain A β levels, respectively.

Importantly, careful examination of BACE1 knockout studies [11, 12, 36–38] indicate that the results do not exclude CatB as having WT β -secretase activity [39–41].

In conclusion, this study shows that deletion of the CatB gene in A β PPWT/Lon mice results in improved memory deficits and altered brain biomarkers consistent with CatB having WT β -secretase activity in that model. This is the first gene deletion study showing improvement in memory deficits in a transgenic mouse model expressing A β PP containing the WT β -secretase site sequence found in most AD patients. Importantly, these data validate CatB as a target for developing inhibitors to improve memory loss in most AD patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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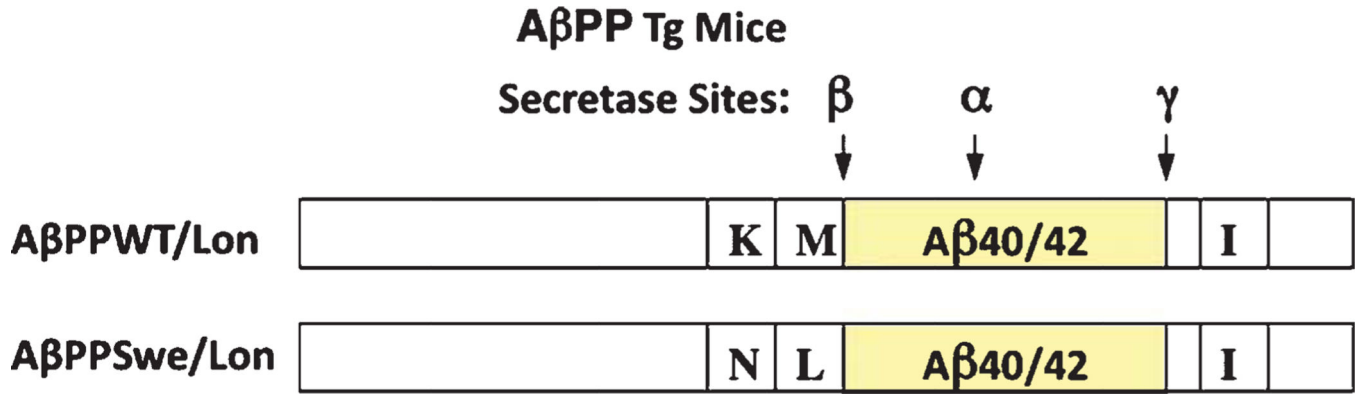


Fig. 1. A β PPWT/Lon and A β PPSwe/Lon transgenic (Tg) mice. This figure illustrates the human A β PP forms expressed in transgenic AD mouse models utilized in this study. The A β PPWT/Lon mice express human A β PP containing the WT β -secretase site (normal K–M residues at the N-terminal side of the β -cleavage site) and the London (Lon) mutation near the γ -secretase site sequence with isoleucine (I) substituting for a valine (V) at position 717. Because the A β PPWT/Lon mice express the WT β -secretase site of A β PP, it is a transgenic animal that has WT β -secretase activity. A β PPSwe/Lon mice express human A β PP containing the rare Swe mutation at the β -secretase site sequence consisting of asparagine (N) substituting for lysine (K) at position 670 and a leucine (L) for a methionine (M) at position 671. Cleavage of A β PP by β -secretase generates CTF β , which in turn is cleaved by γ -secretase to produce A β peptides. A β PP is also cleaved by α -secretase, which produces sA β PP α and precludes the production of A β .

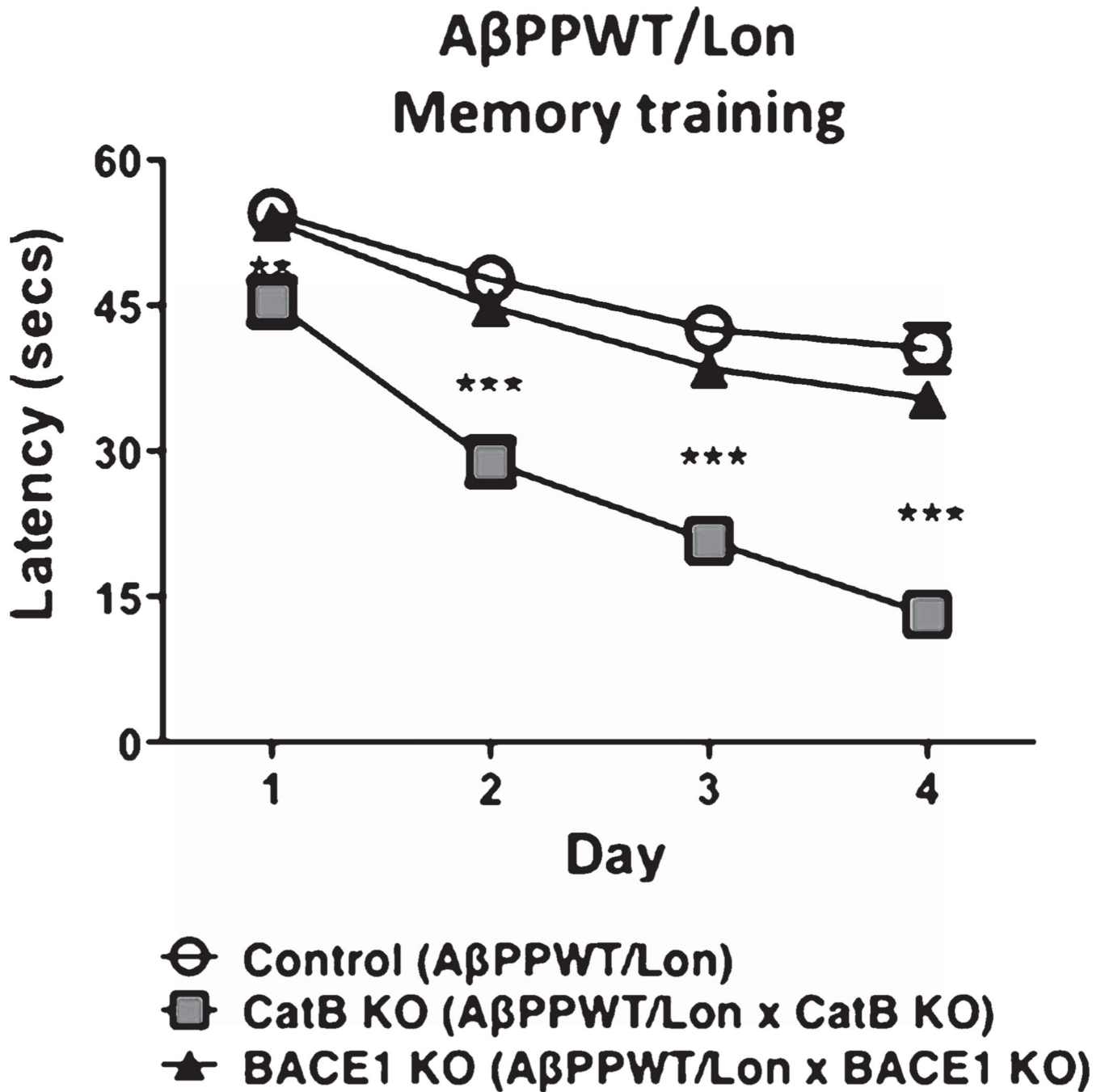
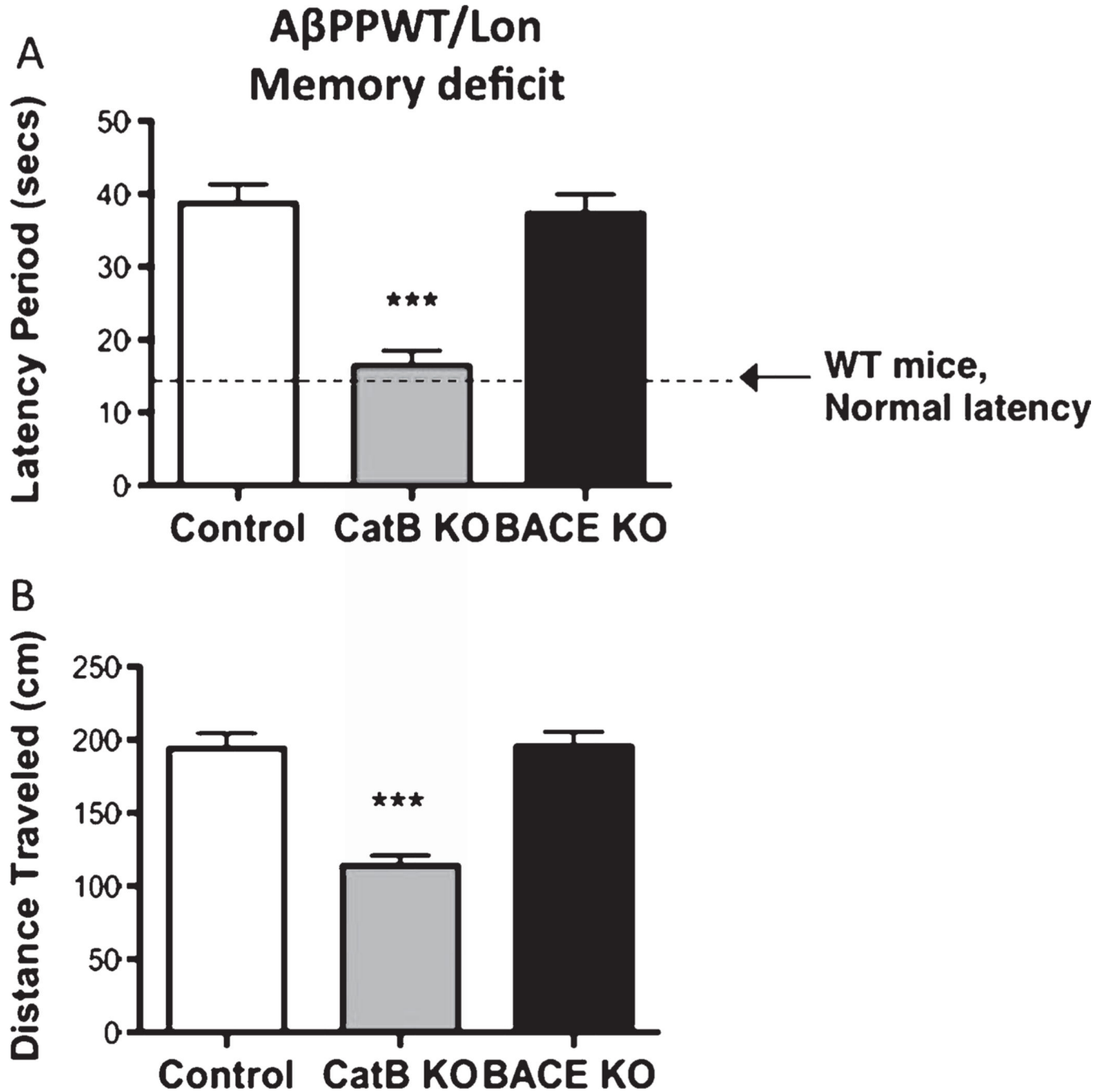


Fig. 2.

Knockout of the CatB gene, but not BACE1, in A β PPWT/Lon mice results in improved memory acquisition. A β PPWT/Lon mice (Control), A β PPWT/Lon \times CatB KO mice (CatB KO mice), and A β PPWT/Lon \times BACE1 KO mice (BACE1 KO mice) were trained in the Morris water maze test on each of 4 consecutive days to learn the location of a submerged, invisible platform in a pool of water. The time that it took the mice to swim to the platform was recorded each day, measured as the latency period (seconds), with shorter latency times

indicating better memory acquisition. Latency (secs) is shown as $x \pm \text{s.e.m.}$ (**, ***Statistically significant, $p < 0.05$, $n = 10$ per group).

**Fig. 3.**

Knockout of the CatB gene, but not BACE1, in A β PPWT/Lon mice improves memory deficits. Memory deficits of A β PPWT/Lon mice were assessed 2 days after completion of the training in the Morris water maze test by measuring the latency period (Panel A) and distance traveled (Panel B) for animals to swim to the submerged, invisible platform. The shorter latency periods and shorter distances traveled indicate improved memory. A β PPWT/Lon (control), A β PPWT/Lon \times CatB KO (CatB KO), and A β PPWT/Lon \times BACE1 KO (BACE1 KO) mice had mean latency periods of 39, 16, and 37 seconds,

respectively (Panel A), and mean distances traveled of 194, 114, and 195 cm, respectively (Panel B). Memory function of normal non-transgenic wild-type mice of the same strain and age is shown by the dotted line, of 14 seconds latency (Panel A) as reported previously [7]. Values are expressed as $x \pm \text{s.e.m.}$, and $n = 10$ per group. ***Statistically significant ($p < 0.05$).

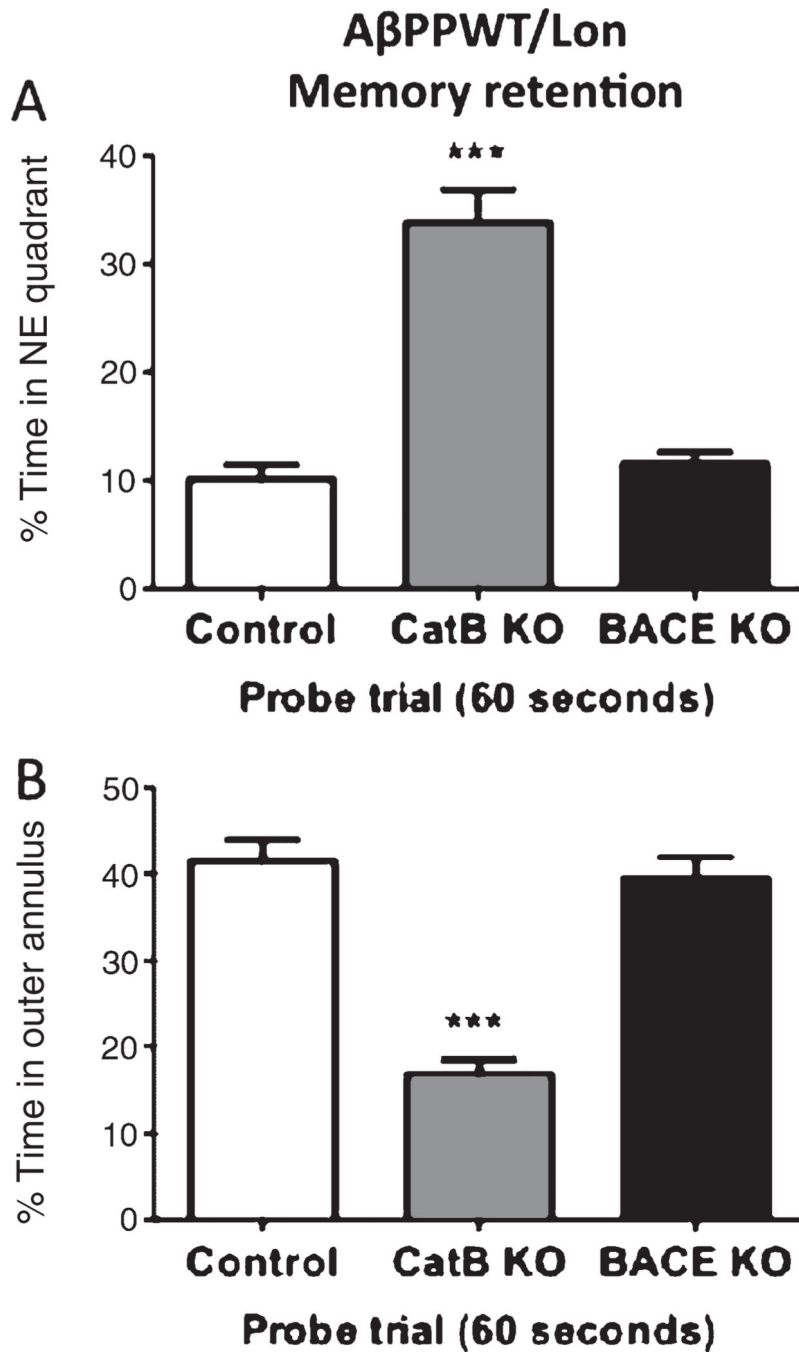


Fig. 4. Knockout of the CatB gene, but not BACE1, in A β PPWT/Lon mice improves memory retention. The day after the last training session, the submerged platform was removed and the mice were allowed to swim in the pool for 60 s. The percent time each animal swam in the quadrant from which the platform had been removed (Northeast (NE) quadrant, (Panel A) and the percent time an animal swam in the annulus of the pool were recorded (Panel B). Greater memory retention is reflected in a higher percent time in the NE quadrant and lower percent time in the annulus. A β PPWT/Lon (control), A β PPWT/Lon \times CatB KO (CatB KO),

and A β PPWT/Lon \times BACE1 KO (BACE1 KO) mice had percent times in the quadrant of 10%, 33%, and 11% (Panel A) and in the annulus of 42%, 17%, and 39% (Panel B). Values are expressed as the mean \pm s.e.m., and $n = 10$ per group. ***Statistically significant with $p < 0.05$.

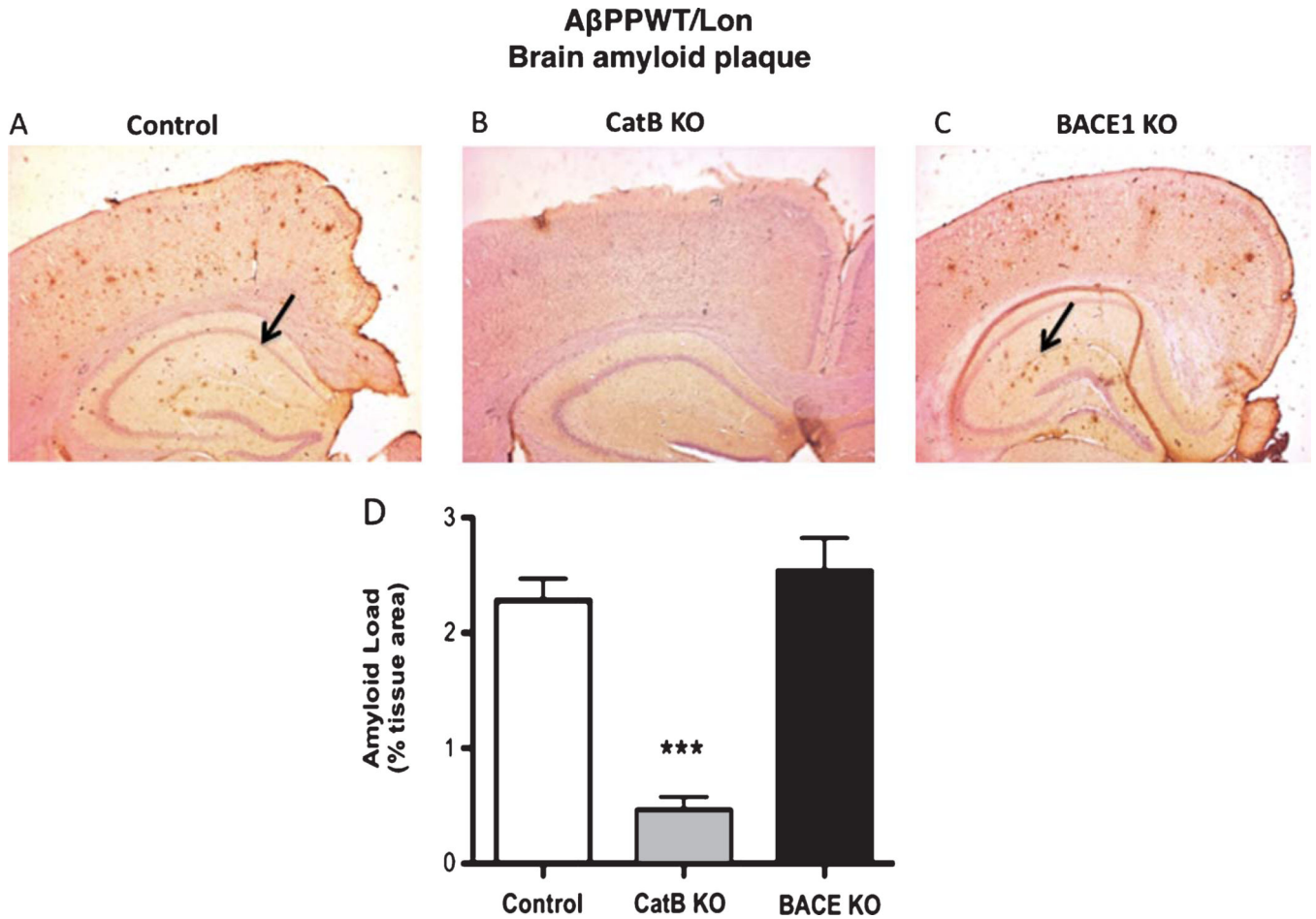


Fig. 5.

Knockout of the CatB gene, but not BACE1, in A β PPWT/Lon mice reduces brain amyloid plaque load. Amyloid plaque load was determined by immunohistochemistry and image analysis of brain sections from A β PPWT/Lon (control), A β PPWT/Lon \times CatB KO (CatB KO), and A β PPWT/Lon \times BACE1 KO (BACE1 KO) mice as shown in Panels A, B, and C, respectively. Arrows indicate amyloid plaque deposits (Panels A and C). Quantitation showed that A β PPWT/Lon, A β PPWT/Lon \times CatB KO, and A β PPWT/Lon \times BACE1 KO mice had mean percent amyloid plaque loads of 2.3%, 0.5%, and 2.5% of brain area, respectively (Panel D). ($n = 10$ per group, Values are expressed as $x \pm$ s.e.m., ***statistically significant with $p < 0.05$).

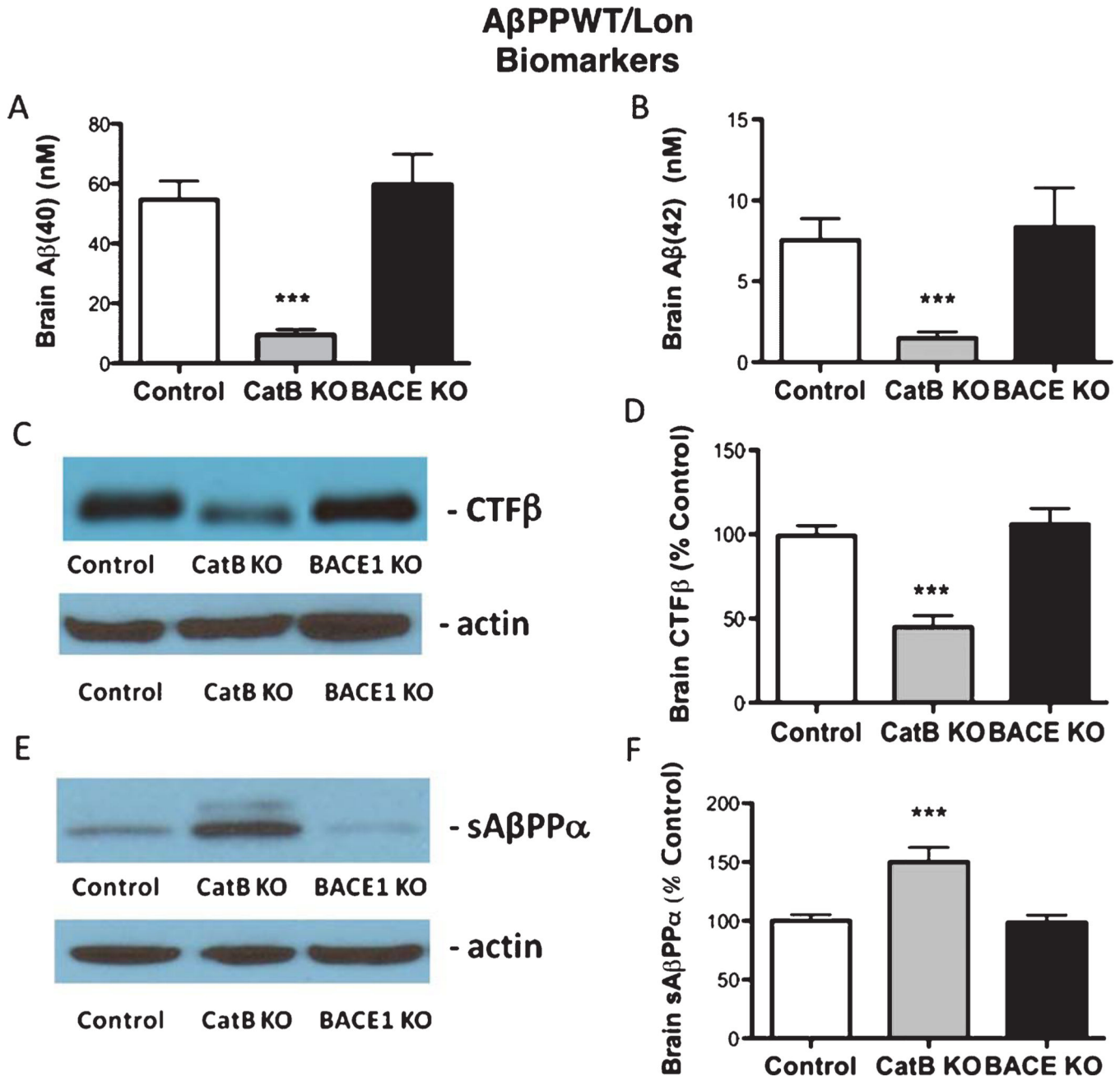


Fig. 6. Knockout of the CatB gene, but not BACE1, in A β PPWT/Lon mice results in changes in A β -related biomarkers. Brain A β ₄₀ and A β ₄₂ (A β ₁₋₄₀ and A β ₁₋₄₂, respectively) levels were determined by ELISA. The A β PPWT/Lon (control), A β PPWT/Lon \times CatB KO (CatB KO), and A β PPWT/Lon \times BACE1 KO (BACE1 KO) mice had mean brain A β ₄₀ levels of 54.6, 9.6, and 59.8 nM, respectively (A), and mean A β ₄₂ levels of 7.5, 1.5, and 8.4 nM, respectively (B). Brain A β PP-derived CTF β , generated by β -secretase, was assessed by western blot analysis (C, D). Relative quantitation by densitometry showed that the CatB KO and BACE1 KO mice had mean brain CTF β levels of 50% and 106% compared to

control A β PPWT/Lon mice (100%), respectively (D). A β PP-derived sA β PP α was evaluated by western blot analysis (E, F). Quantitation by densitometry showed that the A β PPWT/Lon, A β PPWT/Lon \times CatB KO, A β PPWT/Lon \times BACE1 KO mice had mean brain sA β PP α levels of 100%, 150%, and 99% of control A β PPWT/Lon mice, respectively (F). ($n = 10$ per group, values are expressed as $x \pm$ s.e.m., ***statistically significant with $p < 0.05$).

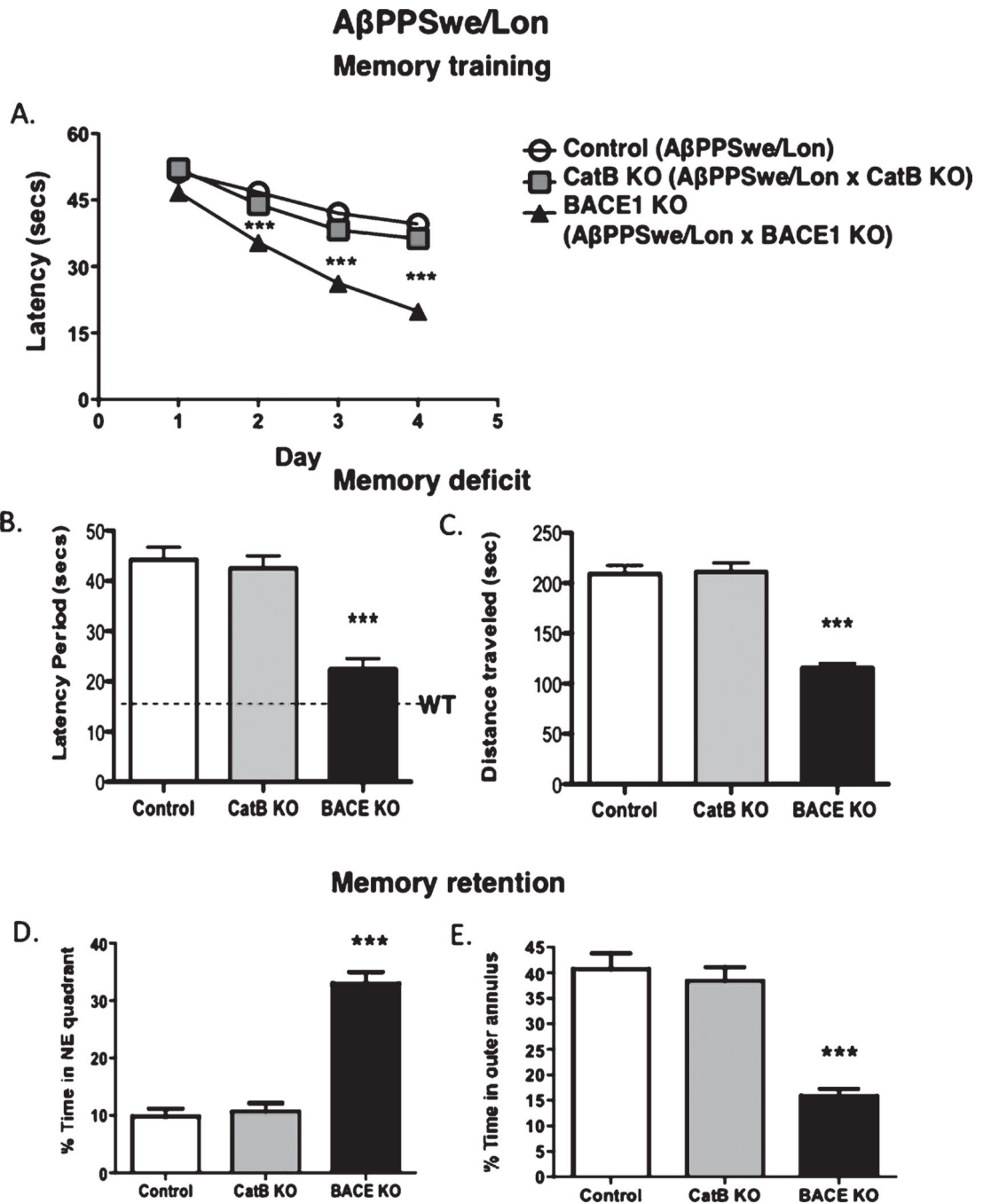


Fig. 7. Knockout of the BACE1 gene, but not CatB, in A β PPSwe/Lon mice results in improved memory acquisition, memory deficits, and memory retention. Control A β PPSwe/Lon mice, A β PPSwe/Lon \times CatB knockout (CatB KO), and A β PPSwe/Lon \times BACE1 KO (BACE1 KO) mice were evaluated in the Morris water maze test during each day of the training sessions by measuring the latency period (A). After the training sessions had concluded, memory deficits were determined by measuring the latency period (B) and the distance traveled to the platform (C). Memory retention was assessed by measuring the percent time

in the quadrant from which the platform had been removed (Northeast (NE) quadrant) (D) and the percent time in the annulus of the pool (E). Values are expressed as $x \pm \text{s.e.m.}$, $n = 10$ per group. ***Statistically significant ($p < 0.05$).

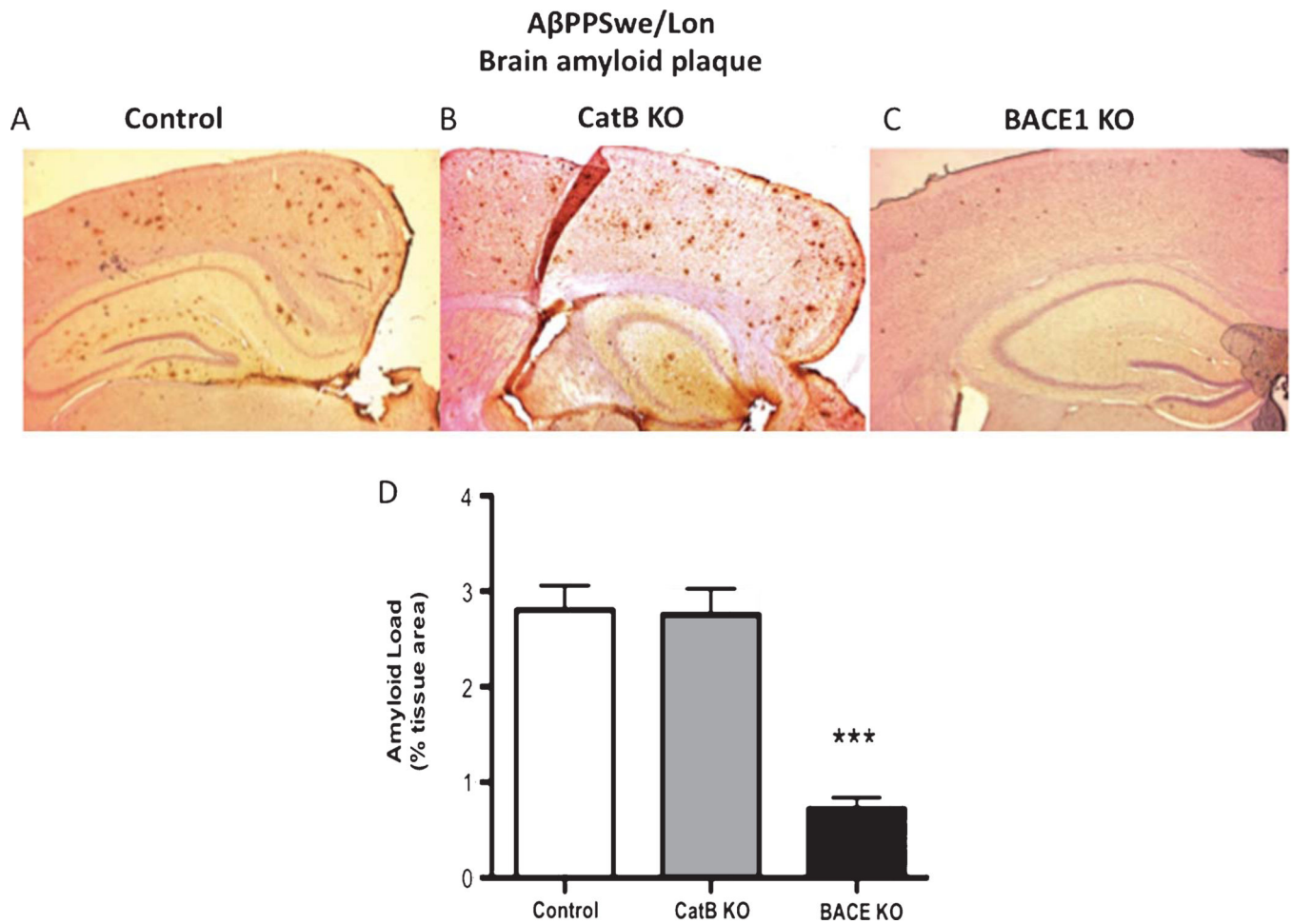


Fig. 8.

Knockout of the BACE1 gene, but not CatB, in A β PPSwe/Lon mice reduces brain amyloid plaque load. Amyloid plaque load was determined by immunohistochemistry and image analysis of brain sections from A β PPSwe/Lon (control), A β PPSwe/Lon \times CatB KO (CatB KO), and A β PPSwe/Lon \times BACE1 KO (BACE1 KO) as shown in A, B, and C, respectively. Arrows indicate amyloid plaque deposits (A, C). Qualitative analyses of the areas of plaque load were conducted (D). A β PPSwe/Lon, A β PPSwe/Lon \times CatB KO, and A β PPSwe/Lon \times BACE1 KO mice had mean percent amyloid plaque loads of 2.8%, 2.8%, and 0.7% of brain plaque area, respectively (D). Values in D are expressed as $x \pm$ s.e.m., and $n = 10$.

***Statistically significant ($p < 0.05$).

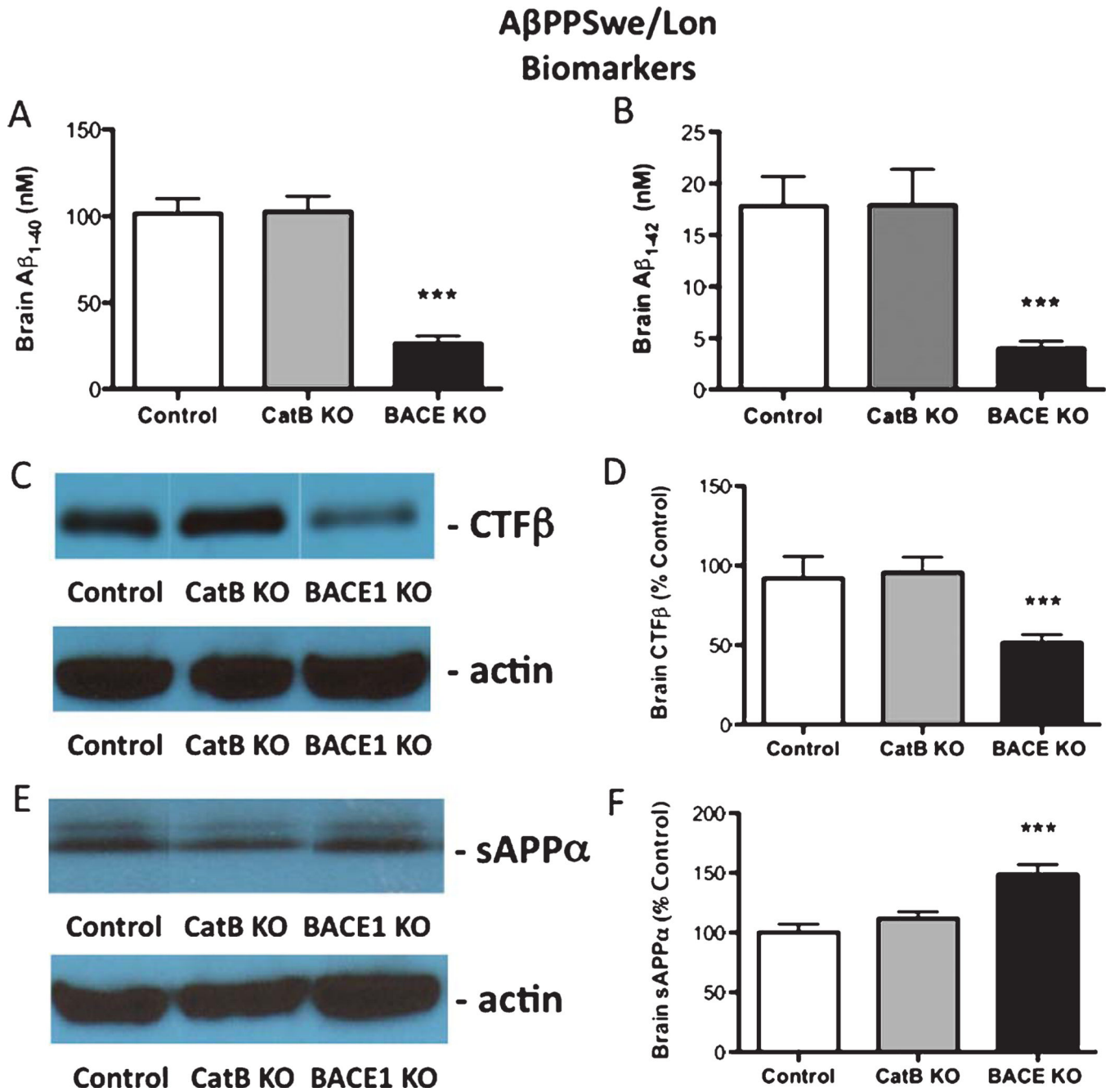


Fig. 9. Knockout of the BACE1 gene, but not CatB, in A β PPSwe/Lon mice alters A β -related biomarkers. Brain A β_{40} and A β_{42} brain levels (A and B, respectively) were determined by ELISA in A β PPSwe/Lon (control), A β PPSwe/Lon \times CatB KO (CatB KO), and A β PPSwe/Lon \times BACE1 KO (BACE1 KO) mice. Relative brain levels of CTF β were assessed by western blots (C) with actin in control westerns; quantitation of CTF β western blots for the three groups of mice is shown (D). Relative brain levels of sAPP α were assessed by western blots (E) with actin in control westerns; quantitation of sAPP α western

blots is shown (F). Values are shown as the $\bar{x} \pm \text{s.e.m.}$, and $n = 10$. ***Statistically significant ($p < 0.05$).