Cathepsin B Degrades Amyloid-β in Mice Expressing Wild-type Human Amyloid Precursor Protein*

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Background: The CysC-CatB axis affects levels of Aβ from hAPP with familial mutations. How it affects Aβ from wild-type hAPP remains unknown.

Results: Enhancing CatB reduces and deleting CatB elevates levels of Aβ derived from wild-type hAPP.

Conclusion: The CysC-CatB axis regulates Aβ degradation similarly regardless of familial mutations.

Significance: Enhancing CatB activity as an Aβ-lowering strategy might be applicable in familial and sporadic AD.

Accumulation of amyloid-β (Aβ), believed to be a key trigger of Alzheimer disease (AD), could result from impaired clearance mechanisms. Previously, we showed that the cysteine protease cathepsin B (CatB) degrades Aβ, most likely by C-terminal truncation, in mice expressing human amyloid precursor protein with familial AD-linked mutations (hAPPFAD). In addition, the Aβ-degrading activity of CatB is inhibited by its endogenous inhibitor, cystatin C (CysC). Reducing CysC expression markedly lowers Aβ levels by enhancing CatB-mediated Aβ degradation in hAPPFAD mice. However, because a vast majority of AD patients do not carry familial mutations, we investigated how the CysC-CatB axis affects Aβ levels in mice expressing wild-type hAPP (hAPPPWT). Enhancing CatB activity by CysC deletion significantly lowered total Aβ and Aβ42 levels in hAPPPWT mice, whereas CatB deletion increased Aβ levels. To determine whether neuron-derived CatB degrades Aβ in vivo, we generated transgenic mice overexpressing CatB under the control of a neuron-specific enolase promoter. Enhancing neuronal CatB activity in hAPPPWT mice significantly lowered Aβ42 levels. The processing of hAPPPWT was unaffected by increasing or ablating CatB activity. Thus, the CysC-CatB axis affects degradation of Aβ42 derived from hAPP lacking familial mutations. These findings support the notion that enhancing CatB activity could lower Aβ, especially Aβ42, in AD patients with or without familial mutations.

Amyloid-β (Aβ), a key pathogenic factor in Alzheimer disease (AD), accumulates and forms toxic oligomers as a result of overproduction or inefficient clearance in the brain (1). Given the importance of catabolic mechanism in controlling Aβ levels, enhancing Aβ degradation and clearance could lead to Aβ-lowering strategies (2). Several Aβ-degrading enzymes have been identified; most are metalloproteases, including nephrilysin, insulin-degrading enzymes, endothelin-converting enzymes, and matrix metalloproteinase-9 (3–6). Previously, we showed that cathepsin B (CatB), a cysteine protease, degrades Aβ by truncating Aβ42 at the C terminus (7). In transgenic mice overexpressing human amyloid precursor protein (hAPP) with familial AD (FAD)-linked Swedish and Indiana mutations (hAPPFAD/J20), plaque load was reduced by overexpression of CatB and increased by ablation of CatB. Consistent with the inhibition of CatB activity by its endogenous inhibitor cystatin C (CysC), genetic deletion of CysC enhanced CatB activity in the brain (8) and significantly reduced Aβ levels and Aβ-dependent behavioral and synaptic deficits in hAPP mice carrying FAD-linked mutations (8).

In contrast to our findings in hAPPFAD/J20 mice, deleting CatB was reported to cleave human wild-type APP (referred to as hAPPWT) at the β-secretase site, leading to production of Aβ and β C-terminal fragments (CTFs) (9). Because most AD patients do not carry FAD mutations, it is critical to determine how the CysC-CatB axis affects the production of Aβ and the processing of hAPPWT. To address this question, we deleted CatB or CysC in hAPPPWT/163 mice, which express hAPPPWT at levels similar to those produced by hAPPFAD/J20 mice but produce much lower levels of Aβ. Because CatB is expressed in neurons and glia in the brain (10, 11), we also established a transgenic line that overexpresses CatB under the control of a neuron-specific enolase (NSE) promoter to further assess the Aβ-degrading activity of neuronal CatB. To determine how enhanced CatB activity in neurons affects the catabolism of Aβ and the processing of hAPPPWT, we crossed NSE-catB mice with hAPPPWT mice.

CysC, cystatin C; CTF, C-terminal fragment; NSE, neuron-specific enolase; s-APPβ, soluble amyloid precursor protein-β; ANOVA, analysis of variance; FL-hAPP, full-length hAPP.

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§ The abbreviations used are: Aβ, amyloid-β; AD, Alzheimer disease; CatB, cathepsin B; hAPP, human amyloid precursor protein; FAD, familial AD; hAPPPWT, full-length hAPP.
**EXPERIMENTAL PROCEDURES**

*Mice—catB*−/− mice (12) and *cst3*−/− mice (13) were back-crossed to the C57BL/6 background for more than 10 (for *catB*−/− mice) or 8 (for *cst3*−/− mice) generations. *catB*−/− or *cst3*−/− mice were then crossed with hAPP<sub>WT</sub> mice (C57BL/6) from line I63 (14). The first cross resulted in hAPP<sub>WT</sub>/catB<sup>+/+</sup> or hAPP<sub>WT</sub>/cst3<sup>+/−</sup> mice, respectively. hAPP<sub>WT</sub>/catB<sup>+/+</sup> mice were crossed with catB<sup>−/−</sup> mice to generate hAPP<sub>WT</sub>/catB<sup>−/−</sup>, hAPP<sub>WT</sub>/catB<sup>+/−</sup>, hAPP<sub>WT</sub>/catB<sup>−/−</sup>, catB<sup>+/−</sup>, and catB<sup>−/−</sup> mice. Similarly, hAPP<sub>WT</sub>/cst3<sup>+/−</sup> mice were crossed with cst3<sup>−/−</sup> mice to generate hAPP<sub>WT</sub>/cst3<sup>−/−</sup>, hAPP<sub>WT</sub>/cst3<sup>+/−</sup>, hAPP<sub>WT</sub>/cst3<sup>−/−</sup>, cst3<sup>−/−</sup>, and cst3<sup>−/−</sup> mice.

**PCR-based genotyping for the catB and cst3 alleles and the hAPP transgene was performed as described (7).** All mouse studies were approved by the Animal Care and Use Committee of the University of California (San Francisco, CA).

**Generation of NSE-catB Transgenic Mice**—The cDNA encoding full-length mouse CatB was cloned from the pCMVSPORT6 vector containing mouse CatB (Addgene) by high fidelity PCR using the following primers (containing a HindIII site): 5′-TCT AAG CTG GGA TGT GGT GGT CCT TGA TCC-3′ and 5′-CTC TAA GCT TTT AGA ATC TTC CCC AGT ACT-3′. The PCR fragment was inserted into the NSE vector (15) with blunted HindIII linkers to generate the NSE-CatB plasmid. The orientation and sequence of mouse CatB were confirmed by sequencing. NSE-CatB was linearized by Sall digestion and purified by passing over an Elutip column (Schleicher & Schuell). The concentration of the linearized NSE-CatB construct was adjusted to 2 μg/ml in injection buffer (5 mM Tris, pH 7.5 and 0.2 mM EDTA) and microinjected into the pronuclei of single-cell embryos harvested from C57BL/6 mice. Transgenic offspring were identified by PCR analysis of tail lysates. Two founder lines of NSE-catB transgenic mice were generated. The line with the higher expression level was crossed with the hAPP-I63 line to generate NSE-catB transgenic mice (14). The first cross resulted in CST-3 (15) with blunted HindIII linkers to generate the NSE-CatB plasmid. The orientation and sequence of mouse CatB were confirmed by sequencing. NSE-CatB was linearized by Sall digestion and purified by passing over an Elutip column (Schleicher & Schuell). The concentration of the linearized NSE-CatB construct was adjusted to 2 μg/ml in injection buffer (5 mM Tris, pH 7.5 and 0.2 mM EDTA) and microinjected into the pronuclei of single-cell embryos harvested from C57BL/6 mice. Transgenic offspring were identified by PCR analysis of tail lysates. Two founder lines of NSE-catB transgenic mice were generated. The line with the higher expression level was crossed with the hAPP-I63 line to generate hAPP<sub>WT</sub>/NSE-catB mice.

**Aβ and Soluble Amyloid Precursor Protein-β ELISA**—Mice were perfused with 0.9% saline, and their hemi-brains were snap-frozen on dry ice and stored at −80 °C. Hippocampal and cortical Aβ levels were measured by ELISA as described (16). The capture antibodies were 266 (for Aβ<sub>1–40</sub> or Aβ<sub>1–42</sub>) and the detection antibody was biotin-conjugated donkey anti-rabbit IgG (1:500; Vector Laboratories, Burlingame, CA). Secondary antibodies, including fluorescein-labeled goat anti-mouse IgG (1:500; Invitrogen). For double staining, the sections were then incubated with primary antibodies, the sections were incubated with secondary antibodies, including Cy3-labeled donkey anti-rabbit IgG (1:100; Jackson ImmunoResearch Laboratories, West Grove, PA) and Alex Fluor 488-labeled donkey anti-sheep IgG (1:500; Invitrogen). For double staining, the sections were then incubated with mouse anti-NeuN antibody (1:500; Millipore), mouse anti-glia fibrillary acidic protein antibody (1:500; Millipore), or rabbit anti-iba1 antibody (1:500; Wako Chemicals USA, Richmond, VA), followed by incubation with secondary antibodies, including fluorescein-labeled goat anti-mouse IgG (1:500; Vector Laboratories, Burlingame, CA) and Cy3-labeled donkey anti-rabbit IgG (1:500). Images were acquired using a Eclipse Ti confocal microscope (Nikon, Melville, NY) and analyzed by Micro-Manager software (University of California, San Francisco).

**Western Blot Analysis**—Cortical samples were homogenized and sonicated at 4 °C in radioimmune precipitation assay buffer containing 10 mM HEPES (pH 7.4), 150 mM NaCl, 50 mM NaF, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na<sub>2</sub>VO<sub>4</sub>, 10 μg/ml leupeptin, 10 μg/ml aprotonin, and 1% SDS. Equal amounts of protein (by BCA assay) were resolved by SDS-PAGE and transferred to nitrocellulose membranes. After blocking, membranes were labeled with rabbit anti-CT15 antibody (1:1000; a kind gift of E. H. Koo, University of California, San Diego, La Jolla, CA) or mouse anti-GAPDH antibody (1:100,000; Millipore, Billerica, MA) and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:2000) or goat anti-mouse IgG (1:2000) antibody (Millipore). Bands were visualized by enhanced chemiluminescence, and the densitometry measurements of the bands were acquired from scanned images with Quantity One software (Bio-Rad).

**Immunohistochemistry**—Sliding microtome sections (30 μm) of NSE-catB transgenic mice were used for immunohistochemistry as described previously with the following modifications (7). Briefly, antigen retrieval was performed by heating the sections in 10 mM citric acid at 90 °C for 30 min. Sections were then incubated with rabbit anti-CatB antibody (1:100; Upstate Biotechnology, Lake Placid, NY) or sheep anti-CatB antibody (1:10; MP Biomedicals, Solon, OH). After overnight incubation with primary antibodies, the sections were incubated with secondary antibodies, including Cy3-labeled donkey anti-rabbit IgG (1:500; Jackson ImmunoResearch Laboratories, West Grove, PA) and Alex Fluor 488-labeled donkey anti-sheep IgG (1:500; Invitrogen). For double staining, the sections were then incubated with mouse anti-NeuN antibody (1:500; Millipore), mouse anti-glial fibrillary acidic protein antibody (1:500; Millipore), or rabbit anti-iba1 antibody (1:500; Wako Chemicals USA, Richmond, VA), followed by incubation with secondary antibodies, including fluorescein-labeled goat anti-mouse IgG (1:500; Vector Laboratories, Burlingame, CA) and Cy3-labeled donkey anti-rabbit IgG (1:500). Images were acquired using a Eclipse Ti confocal microscope (Nikon, Melville, NY) and analyzed by Micro-Manager software (University of California, San Francisco).

**Statistical Analysis**—Statistical analyses were conducted with GraphPad Prism 5. Values are expressed as means ± S.E. Differences among multiple means with one variable (catB or cst3 genotype) were evaluated by one-way analysis of variance (ANOVA) and Tukey-Kramer post hoc tests. Differences between two means were evaluated using unpaired Student’s t tests. *p < 0.05* was considered significant.

**RESULTS**

CatB Ablation Elevates Aβ Levels without Affecting hAPP Processing in Transgenic Mice overexpressing hAPP<sub>WT</sub>—CatB degrades Aβ in hAPP<sub>FAD</sub>-120 mice without affecting the processing of hAPP<sub>FAD</sub> (7). To directly determine the effects of CatB
CatB Degrades Aβ Derived from Wild-type hAPP

![Image](image)

**FIGURE 1.** CatB ablation increases levels of Aβ but does not affect hAPP processing in hAPP<sub>WT</sub> mice. A and B, ELISA measurements of hippocampal Aβ1–42 (A) and Aβ1–42 (B) in 3.5-month-old hAPP<sub>WT</sub>/catB<sup>+/+</sup>, hAPP<sub>WT</sub>/catB<sup>−/−</sup>, and hAPP<sub>WT</sub>/catB<sup>−/−</sup> mice. The levels of Aβ1–x were elevated significantly by CatB deletion (n = 9–13 mice/genotype). **, p < 0.01 (one-way ANOVA with Tukey’s post hoc test). C and D, ELISA measurements of cortical Aβ1–x (C) and Aβ1–42 (D) in 3.5-month-old hAPP<sub>WT</sub>/catB<sup>+/+</sup>, hAPP<sub>WT</sub>/catB<sup>−/−</sup>, and hAPP<sub>WT</sub>/catB<sup>−/−</sup> mice. The levels of Aβ1–x and Aβ1–42 were elevated significantly by CatB deletion (n = 7–10 mice/genotype). *, p < 0.05; **, p < 0.01 (one-way ANOVA with Tukey’s post hoc test). E, ELISA measurement of cortical s-APPβ in 3.5-month-old hAPP<sub>WT</sub>/catB<sup>+/+</sup>, hAPP<sub>WT</sub>/catB<sup>−/−</sup>, and hAPP<sub>WT</sub>/catB<sup>−/−</sup> mice. CatB deletion did not affect s-APPβ levels (n = 6 mice/genotype). F, representative Western blot analyses of hAPP metabolic fragments in the cortices of 3.5-month-old hAPP<sub>WT</sub>/catB<sup>−/−</sup> and hAPP<sub>WT</sub>/catB<sup>−/−</sup> mice. G, quantification of the levels of FL-hAPP (normalized to those of GAPDH) and α- and β-CTFs (normalized to those of FL-hAPP). CatB deletion did not affect the levels of hAPP fragments (n = 9–12 mice/genotype). Values are means ± S.E. (A–E and G).

deletion on hAPP<sub>WT</sub>, we crossed catB<sup>−/−</sup> mice with hAPP<sub>WT</sub>-I63 mice, which express hAPP at a level similar to hAPP<sub>FAD</sub>-I20 mice but produce much less Aβ. The levels of total Aβ (Aβ1–x) in the hippocampi and cortices of hAPP<sub>WT</sub> mice were slightly increased by deleting one catB allele and significantly increased by complete deletion of catB (Fig. 1, A and C). The levels of Aβ1–42 were also significantly increased in the cortices of hAPP<sub>WT</sub>/catB<sup>−/−</sup> mice and were slightly increased in the hippocampi, although not statistically significantly in the latter (Fig. 1, B and D). Nevertheless, these results suggest that endogenous CatB lowers the level of Aβ derived from hAPP<sub>WT</sub>. Deleting CatB did not affect the levels of s-APP in the cortex, as determined by ELISA (Fig. 1E), indicating that CatB may not function as a β-secretase for hAPP<sub>WT</sub>. In addition, Western blot analyses with a C terminus-specific antibody revealed that the levels of full-length hAPP (FL-hAPP) and α- and β-CTFs were similar in hAPP<sub>WT</sub> mice with or without CatB (Fig. 1, F and G), further supporting that neither the β- nor α-cleavage of hAPP<sub>WT</sub> is affected by CatB deletion.

CysC Reduction Lowers Aβ Levels without Affecting hAPP<sub>WT</sub> Processing—CatB activity is blocked by its endogenous inhibitor, CysC (cat3) (17, 18). To further explore the effects of CatB activity on Aβ and hAPP<sub>WT</sub> processing, we crossed hAPP<sub>WT</sub> mice with cat3<sup>−/−</sup> mice. CysC ablation yielded a gene dose-dependent increase in cortical CatB activity (Fig. 2A). Reducing CysC expression lowered the levels of total Aβ and Aβ1–42 in the hippocampi of hAPP<sub>WT</sub> mice (Fig. 2, B and C), in agreement with our previous findings in mice expressing hAPP<sub>FAD</sub> (8). Thus, elevated CatB activity lowered Aβ levels derived from hAPP with or without FAD mutations. Consistent with our observations in hAPP<sub>FAD</sub> mice (8), removing CysC did not affect the levels of FL-hAPP, α-CTF, or β-CTF in hAPP<sub>WT</sub> mice.
CatB Degrades Aβ Derived from Wild-type hAPP

Neuronal Overexpression of CatB Reduces the Levels of Aβ Derived from hAPP<sub>WT</sub>—CysC reduction enhances CatB activity in the brain, where CatB is expressed in neurons and glia. To determine whether neuron-derived CatB degrades Aβ in vivo, we established a transgenic mouse line that overexpresses mouse CatB under the control of a neuronal enolase promoter (NSE-catB) (Fig. 3A). CatB activity in the cortex was significantly higher in NSE-catB mice than in non-transgenic controls (Fig. 3B). Moreover, CatB staining was enhanced in hippocampal neurons expressing NeuN (Fig. 3C) but not in glial fibrillary acidic protein-expressing astroglia (Fig. 3D) or Iba1-positive microglia (Fig. 3E). These results suggest that CatB is increased predominantly in the neurons of NSE-catB mice.

We next crossed NSE-catB mice with hAPP<sub>WT</sub> mice and determined the effects of elevated neuronal CatB on Aβ and hAPP processing. Enhancing neuronal CatB in hAPP<sub>WT</sub>/NSE-catB mice significantly lowered the levels of Aβ1–42 in both the hippocampus and cortex (Fig. 4, B and E). The levels of total Aβ were modestly lower in hAPP<sub>WT</sub>/NSE-catB mice compared with hAPP<sub>WT</sub> mice (Fig. 4, A and D). Increased CatB activity in neurons also significantly reduced the relative abundance of Aβ1–42 in both the hippocampus and cortex (Fig. 4, B and E). These results suggest that neuronal CatB reduces Aβ1–42 preferentially, likely by truncating at the C terminus as described previously (7). Neuronal overexpression of CatB did not affect the levels of FL-hAPP, α-CTF, or β-CTF in the hip-
pocampus (Fig. 4G) or in the cortex (Fig. 4H), confirming that neuronal CatB does not affect hAPP<sub>WT</sub> processing.

**DISCUSSION**

This study shows that CysC-CatB affects Aβ levels in hAPP<sub>WT</sub> mice in a similar fashion as in hAPP<sub>FAD</sub> mice as described in our previous studies (7, 8). CatB removal elevated the levels of Aβ in hAPP<sub>WT</sub> mice, as in hAPP<sub>FAD</sub> mice (7). In mice expressing hAPP<sub>WT</sub>, neuron-derived CatB effectively reduced the levels of Aβ1–42, the most pathogenic of the Aβ peptides in the brain (19). Removing CysC, the endogenous inhibitor of CatB, in hAPP<sub>WT</sub> mice lowered total Aβ and Aβ1–42 levels. However, hAPP<sub>WT</sub> processing was unaffected by removal of CatB or CysC. Enhancing neuronal CatB also had no effect on hAPP<sub>WT</sub> processing. Thus, targeting the CysC-CatB axis to enhance Aβ degradation might be applicable not only in AD patients with FAD mutations but also in sporadic AD cases.

Our results differ from a previous study that showed that deleting CatB reduces the levels of s-APPβ, Aβ1–40, and Aβ1–42 in hAPP<sub>WT</sub> mice (9). In contrast, we found that total Aβ (Aβ1–x) and Aβ1–42 levels were reduced, whereas the levels of s-APPβ were unaffected. Although the exact reason underlying this discrepancy remains unclear, one key difference between the two studies is the Aβ ELISA used to measure total Aβ. We used a well established ELISA that allowed us to detect all Aβ species starting at position 1 and ending at or after position 23, which would include Aβ1–40, Aβ1–38, and Aβ1–42 among others (16), whereas previous ELISA probing total Aβ levels measured Aβ1–40 specifically (9). Because CatB could convert Aβ42 to Aβ40 and Aβ38, it is conceivable that CatB deficiency could lead to a reduction in Aβ40 and/or Aβ38 because Aβ42 could not be cleaved to generate Aβ40 and/or Aβ38. In support of this notion, a recent study showed that enhancing CatB activity pharmacologically significantly reduced Aβx–42 but increased levels of Aβ1–38 (20).

To determine the effects of the CysC-CatB axis on Aβ derived from hAPP<sub>WT</sub>, we used three approaches to modulate CatB levels. Besides deleting CatB, we enhanced CatB activity either by deleting CysC or by overexpressing CatB in neurons.
In both cases, enhancing CatB activity significantly lowered Aβ1–42 levels. Moreover, the enhanced activity reduced the relative abundance of Aβ1–42, supporting the notion that enhanced CatB activity reduces Aβ1–42 preferentially, most likely by C-terminal truncation. Consistent with these findings, systemic injections of the CatB-enhancing drug benzoxycarboxybenzyl-Phe-Ala diazomethyl ketone significantly reduced Aβ1–42/Aβx–42 levels in both 4-month-old hAPPWT mice and hAPPWT/NSE-catB mice. F, the Aβ1–42/Aβx–42 ratio was significantly lower in hAPPWT/NSE-catB mice than in hAPPWT mice (n = 6–8 mice/genotype). *p < 0.05 (unpaired Student’s t-test). G and H, quantification of hippocampal (G) and cortical (H) levels of FL-hAPP (relative to those of GAPDH) and α- and β-CTFs (relative to those of FL-hAPP) in hAPPWT mice by Western blotting (n = 8 mice/genotype). Values are means ± S.E.

FIGURE 4. Neuron-derived CatB overexpression reduces levels of Aβ but does not affect hAPP processing in hAPPWT mice. A and B, ELISA measurements of hippocampal Aβ1–x (A) and Aβ1–42 (B) in 4-month-old hAPPWT and hAPPWT/NSE-catB mice. C, the Aβ1–42/Aβ1–x ratio was significantly lower in hAPPWT/NSE-catB mice than in hAPPWT mice (n = 8–12 mice/genotype). **, p < 0.01 (unpaired Student’s t-test). D and E, ELISA measurements of cortical Aβ1–x (D) and Aβ1–42 (E) in 4-month-old hAPPWT and hAPPWT/NSE-catB mice. F, the Aβ1–42/Aβx–42 ratio was significantly lower in hAPPWT/NSE-catB mice than in hAPPWT mice (n = 6–8 mice/genotype). *p < 0.05 (unpaired Student’s t-test). G and H, quantification of hippocampal (G) and cortical (H) levels of FL-hAPP (relative to those of GAPDH) and α- and β-CTFs (relative to those of FL-hAPP) in hAPPWT mice by Western blotting (n = 8 mice/genotype). Values are means ± S.E.
directly up-regulated by Aβ42 in cultured microglia (26, 27), which might help explain the readily detectable CatB in microglia surrounding the plaques but not those away from the plaques (7). Further study is needed to determine the importance of microglial CatB in Aβ degradation and clearance in vivo.

Our results support an important role for neuronal CatB in Aβ degradation. However, where the CysC-CatB axis acts to modulate Aβ degradation at the subcellular level remains unknown. In most pyramidal neurons, mature and proenzyme forms of CatB are present in early endosomes (11, 29). Consistent with this finding, CatB in endosomes catalyzes C-terminal truncations of epidermal growth factor and insulin-like growth factor and inhibits their signaling (30, 31). Although CysC is generated mainly in astroglia, CysC immunoreactivity is found in neurons with a punctate distribution, which co-localize with CatB in AD brains (32). Thus, CysC could be taken up by neurons or microglia into the endocytic pathway and inhibit the Aβ-degrading activity of CatB. Regardless of the specific sites at which the CysC-CatB axis regulates Aβ degradation, our findings support enhancing proteolytic activity of CatB as a potential new Aβ-lowering strategy in sporadic AD.

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Note Added in Proof—A recent paper by K. Viswanathan et al. (33) identified new cathepsin B-enhancing small molecules that protect against synaptic deficits in a transgenic model of AD.

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