High β-Secretase Activity Elicits Neurodegeneration in Transgenic Mice Despite Reductions in Amyloid-β Levels

**IMPLICATIONS FOR THE TREATMENT OF ALZHEIMER DISEASE**

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Amyloid-β peptides (Aβ) are widely presumed to play a causal role in Alzheimer disease. Release of Aβ from the amyloid precursor protein (APP) requires proteolysis by the β-site APP-cleaving enzyme (BACE1). Although increased BACE1 activity in Alzheimer disease brains and human (h) BACE1 transgenic (tg) mice results in altered APP cleavage, the contribution of these molecular alterations to neurodegeneration is unclear. We therefore used the murine Thy1 promoter to express high levels of hBACE1, with or without hAPP, in neurons of tg mice. Compared with hAPP mice, hBACE1/hAPP doubly tg mice had increased levels of APP C-terminal fragments (C89, C83) and decreased levels of full-length APP. In contrast to non-tg controls and hAPP mice, hBACE1 mice and hBACE1/hAPP mice showed degeneration of neurons in the neocortex and hippocampus and degradation of myelin. Neurological deficits were also more severe in hBACE1 and hBACE1/hAPP mice than in hAPP mice. These results demonstrate that high levels of BACE1 activity are sufficient to elicit neurodegeneration and neurological decline in vivo. This pathogenic pathway involves the accumulation of APP C-terminal fragments but does not depend on increased production of human Aβ. Thus, inhibiting BACE1 may block not only Aβ-dependent but also Aβ-independent pathogenic mechanisms.

The precise mechanisms leading to neurodegeneration in Alzheimer disease (AD) are not completely understood. However, diverse lines of evidence suggest that alterations in the processing of the amyloid precursor protein (APP), leading to the accumulation of amyloid-β peptides (Aβ), play a key role in the pathogenesis of AD (1–3). Various products are derived from APP through alternative proteolytic cleavage, and enormous progress has recently been made in identifying the enzymes involved (3–11).

Cleavage of APP by α-secretase results in the secretion of a large N-terminal ectodomain. In an alternative pathway, β-secretase generates a shorter secreted N-terminal fragment and APP C-terminal fragments (CTFs) C89 and C99, which remain membrane-bound. The latter fragments are then further cleaved by γ-secretase, resulting in the production of Aβ peptides. The β-site APP-cleaving enzyme (BACE1) accounts for most of the β-secretase activity in the brain (1, 5–7, 9). BACE1 is a typical aspartic protease; cleavage of the prodomain to generate the mature enzyme occurs at the C-terminal site resulting in the generation of a mature protein starting at Gln-46 (12).

Recent evidence suggests that the pathogenesis of AD involves alterations in the activity of BACE1. A polymorphism in the BACE1 gene has been reported to influence AD risk (13). Compared with nondemented controls, BACE1 immunoreactivity was increased around amyloid plaques in AD brains; levels of BACE1 were elevated in AD brain homogenates (14–17) and correlated with the levels of APP CTFs and with Aβ1–42 (18). The potential pathogenic role of increased BACE1 activity has been investigated in vivo by analyzing the metabolism of APP in human (h) BACE transgenic (tg) mice (19–22). These studies have shown that expression of hBACE1 at moderate levels in hAPP tg models results in increased generation of hAPP CTFs and Aβ, which in some cases was associated with enhanced amyloid deposition. These studies have confirmed the importance of hBACE1 in APP processing in vivo. However, the relationship between high levels of hBACE1 activity, APP processing, and neurodegeneration remains to be established. To address this issue, we used the strong Thy1 promoter to express hBACE1 in neurons of tg mice, either alone or in combination with hAPP. High levels of BACE1 activity significantly increased the cerebral accumulation of hAPP CTFs, but not of Aβ, and caused prominent age-related neurodegeneration and neurological decline.

**EXPERIMENTAL PROCEDURES**

Generation of hBACE1 and hBACE1/hAPP tg Mice and Tissue Processing—A cDNA encoding wild-type hBACE1 was produced by reverse transcription-PCR from human brain mRNA as described previously (23). This cDNA includes a Kozak consensus sequence (GCC ACC ATG) at the 5′-end to enhance expression. The hBACE1 cDNA was inserted between exons 2 and 4 into the mThyl1 expression cassette (kindly provided by Dr. H. van der Putten, Ciba-Geigy, Basel, Switzerland), purified, and microinjected into one-cell embryos (C57BL/6 × DBA/2 F1). Transgenic founder mice were identified by PCR analysis of genomic DNA extracted from tail biopsies. Doubly tg mice were generated by crossing heterozygous hBACE1 mice from lines 1 or 39 with the hAPP tg line 41 we described previously (23). hAPP line 41 expresses human APP751 under the direction of the mThyl1 promoter and produces high levels of Aβ1–42 resulting in plaque formation by 3 months of age. All tg lines were maintained by crossing heterozygous tg mice with...
Neurodegeneration in hBACE1 tg Mice

**TABLE ONE**

| Antibody (clone)       | Region specificity                  | Antigen                   | Source                        | Dilution | Ref. |
|------------------------|-------------------------------------|---------------------------|-------------------------------|----------|------|
| hBACE (rabbit polyclonal) | Amino acids 485–501 of hBACE          | Peptide                   | ProSci, Inc., Poway, CA       | 1:500    | (6, 50) |
| APP C1T5 (rabbit polyclonal) | C99 and C89, terminal 15 residues of APP | Peptide                   | Dr. Edward Koo               | Immunohistochemistry, 1:100; Western blot, 1:1,000–1:20,000 | (51, 52) |
| APP A4 (22C11)         | Amino acids 66–81 of N terminus of FL APP molecule | Purified recombinant A4 fusion protein | Chemicon, Temecula, CA       | 1:500    | (53, 54) |
| Aβ (4G8)               | Amino acids 17–24 of hAβ (epitope is amino acids 18–22) | hAβ peptide               | Signet, Dedham, MA           | 1:2,000  | (55, 56) |
| Aβ (6E10)              | Amino acids 1–17 of hAβ (epitope is amino acids 3–8) | hAβ peptide               | Signet, Dedham, MA           | 1:1,000  | (55, 57) |
| Aβ (266)               | Amino acids 13–28 of hAβ (epitope is amino acids 16–23) | hAβ peptide               | Elan Pharmaceuticals, Inc., South San Francisco, CA | ELISA (capture), 10 µg/ml (final); Western blot, 5.7 µg/ml (final) | (25, 58) |
| Aβ (3D6)               | Amino acids 1–5 (epitope is likely the same) | hAβ peptide               | Elan Pharmaceuticals, Inc., South San Francisco, CA | ELISA (detection), 0.5 µg/ml | (58) |
| Aβ (21F12)             | Amino acids 33–42 (recognizes Aβ ending in ≥ amino acid 42) | hAβ peptide               | Elan Pharmaceuticals, Inc., South San Francisco, CA | ELISA (capture), 5 µg/ml | (58) |
| Phospho-APP Thr-668 (rabbit polyclonal) | Amino acids surrounding phosphorylated Thr-668 of APP | Phosphopeptide            | Cell Signaling Technology, Beverly, MA | 1:1,200 | (59) |

non-tg C57BL/6 × DBA/2 F1 breeders. All tg mice analyzed in this study were heterozygous with respect to individual transgenes, and non-tg littermates were used as controls.

At different ages, mice were anesthetized with chloral hydrate and flush-perfused transcardially with 0.9% saline. Brains and peripheral tissues were removed and brains divided sagittally. One hemibrain was postfixed in phosphate-buffered 4% paraformaldehyde, pH 7.4, at 4 °C for 48 h for neuropathological analysis. The other was snap frozen and stored at −70 °C for RNA and protein analyses.

**RNA Analysis**—Total RNA was extracted with TRI reagent (Molecular Research) at −20 °C. RNA was analyzed by solution hybridization ribonuclease protection assay (RPA), essentially as described previously (24). Samples were separated on 5% acrylamide, 8 M urea Tris borate, EDTA gels, and dried gels were exposed to Kodak XAR film (Eastman Kodak). mRNA levels were quantitated from PhosphorImager readings of probe-specific signals corrected for RNA content/loading errors by normalization to β-actin signals (24). The following 32P-labeled antisense riboprobes were used to identify specific mRNAs (protected nucleotides and GenBank accession numbers): hBACE1 (nucleotides 1305–1599, accession number AF190725); mouse (m) BACE1 (1280–1574, accession number AF190726); mAPP770 (811–1314, accession number XM_128362 of mAPP exon 6–9; and mβ-actin (480–565, accession number X03672).

**Western Blot Analysis of hBACE1, hAPP, APP CTFs, and Aβ**—After determination of the protein content by the Lowry method, frontal cortex homogenates were loaded (15 µg of protein/lane), separated on 10% SDS-polyacrylamide gels, and blotted onto nitrocellulose. Blots were labeled with a rabbit polyclonal antibody against hBACE1 (ProSci, Inc., Poway, CA; 1:1,000), a mouse monoclonal antibody against the N terminus of APP (22C11; Chemicon International, Temecula, CA; 1:1,000), a mouse monoclonal antibody against hAPP (8E5; Elan; 1:20,000), a rabbit polyclonal antibody against C99 and C89 of APP (CT15; courtesy of Dr. Edward Koo; 1:20,000), or a mouse monoclonal antibody against Aβ (4G8; Senetek PLC, Napa, CA; 1:1,000) followed by anti-mouse or anti-rabbit secondary antibodies. The blots were incubated with 125I-protein A (ICN Pharmaceuticals, Costa Mesa, CA) and exposed to PhosphorImager (Molecular Dynamics, Piscataway, NJ) screens, or incubated with Super Signal West Pico Chemiluminescent substrate (Pierce) and exposed to film. Further details regarding the antibodies utilized are described in **TABLE ONE**. To control for variations in loading, blots were stripped and incubated with a mouse monoclonal antibody against actin (Chemicon; 1:500). Signal intensities were quantitated with the ImageQuant software (Molecular Dynamics).

Additional Western blot analysis of Aβ was performed under highly denaturing conditions as described previously (25), with some modifications. For this, neocortical tissues were homogenized in 98% formic acid (80% final concentration, ICN) with a Dounce homogenizer and exposed to PhosphorImager (Molecular Dynamics, Piscataway, NJ) screens, or incubated with Super Signal West Pico Chemiluminescent substrate (Pierce) and exposed to film. Further details regarding the antibodies utilized are described in **TABLE ONE**. To control for variations in loading, blots were stripped and incubated with a mouse monoclonal antibody against actin (Chemicon; 1:500). Signal intensities were quantitated with the ImageQuant software (Molecular Dynamics).
out with Super Signal West Pico Chemiluminescent substrate (Pierce). Synthetic Aβ1-40 and Aβ1-42 peptides (750 pg each; Biopeptide, San Diego, CA) were run as standards.

Analysis of APP Metabolism and Related Pathways—Human Aβ peptides were quantitated by enzyme-linked immunosorbent assays (ELISAs) as described previously (26). The Aβ1-42 ELISA detects only Aβ1-42, whereas the Aβ1-40 and Aβ1-42 ELISAs detect various forms of Aβ containing amino acids 1–28. For analysis of APP CTFs, cortex homogenates were analyzed with the Lowry method to determine protein concentration, loaded at 75 μg of protein/lane (or 25 and 50 μg/lane for J20 and I5 mice, respectively) onto 14% Tricine/SDS-polyacrylamide gels, blotted onto nitrocellulose, and probed with antibodies against APP CTFs (CT15; 1:1,000), phospho (p) APP (Cell Signaling, Beverly, MA; 1:1,200), or tubulin (loading control; 1:500,000).

To evaluate the effects of hBACE1 expression on related pathways, Western blots were probed with antibodies against neprilysin (CD10, mouse monoclonal; Abcam, Cambridge, MA; 1:1000), insulin-degrading enzyme (IDE, rabbit polyclonal; Calbiochem, San Diego, CA; 1:1,000), or Notch4 (rabbit polyclonal; Santa Cruz Biotechnology, Santa Cruz, CA; 1:1,000).

Analysis of BACE1 Enzymatic Activity—BACE1 activity was determined by adapting the BACE1 fluorescence resonance energy transfer (FRET) assay kit (Panvera, Madison, WI), which uses a red FRET peptide derived from the Swedish (Sw) mutant APP as the substrate sequence as described previously (6). Briefly, brain homogenates from the tg mice were incubated with the BACE1 substrate, for a final concentration of 1 μM for each of the reagents. Then stop buffer (containing a 2.5M sodium acetate) was added, and the signal was determined at 545 nm with a spectrofluorometer. Control experiments were performed, and standard curves were generated using the baculovirus-expressed BACE1 and the BACE1 product standard (Rh-EVNL, a BACE1 inhibitor) provided with the kit.

To confirm BACE1 activity levels by an independent method, extracted supernatants from homogenized samples were neutralized with Tris and assayed for BACE1 activity utilizing as a substrate the bacterial maltose-binding protein (MBP) fused to the C-terminal 125
Neurodegeneration in hBACE1 tg Mice

FIGURE 2. Decreases in FL APP and increases in APP CTFs in hBACE1 tg mice. Homogenates of frontal cortex from 6-month-old hBACE1 tg mice and non-tg littermates were separated into cytosolic and particulate fractions and analyzed by Western blotting with antibodies against FL APP (22C11, mouse monoclonal), APP CTFs (CT15, rabbit polyclonal), and actin (mouse monoclonal), and expression levels were quantitated. A, representative Western blot of samples from different hBACE1 tg lines. Each lane contains a sample from a different mouse. B and C, quantification of Western blot bands (n = 4 mice/genotype). D–I, double immunolabeling and confocal microscopic analysis of hBACE1 (red) and APP CTFs (green) in the frontal cortex of 6-month-old non-tg (D–F) and hBACE1 tg (G–I) mice. Bar = 20 μm. *, p < 0.05 versus non-tg controls (Dunnett’s t test).

Behavioral Analysis—Mice had free access to food and water, and all experiments were carried out during the light cycle. Experimental and control groups contained similar proportions of males and females. The water maze test was carried out as described previously (28). Briefly, a pool (diameter 180 cm) was filled with opaque water (24 °C) and mice were first trained to locate a visible platform (days 1–3) and then a submerged hidden platform (days 4–7) in three daily trials 2–3 min apart. Mice that failed to find the hidden platform within 90 s were placed on it for 30 s. The same platform location was used for all sessions and all mice. The starting point at which each mouse was placed into the water was changed randomly between two alternative entry points located at a similar distance from the platform. On day 8, another visible platform trial was performed to exclude differences in motivation and fatigue. Time to reach the platform (latency), path length, and swim speed were recorded with a Noldus Instruments EthoVision video tracking system (San Diego Instruments, San Diego) set to analyze two samples/s.

Statistical Analysis—Analyses were carried out with the StatView 5.0 program (SAS Institute Inc., Cary, NC). Differences among means were assessed by student’s t test or by one-way ANOVA followed by Dunnett’s or Tukey-Kramer post hoc tests as indicated. Learning curves were performed essentially as described previously (28).

For ultrastructural analysis, blocks of neocortex and hippocampus were postfixed with 2% glutaraldehyde and 0.1% osmium tetroxide in 0.1 M sodium cacodylate buffer and embedded in epoxy. Blocks were sectioned with an Ultracut E ultramicrotome (Leica, Nussloch, Germany) (31).

Neuropathological Analysis—Vibratome sections were incubated overnight at 4 °C with anti-hBACE1 (1:500) and developed with the Tyramide Signal Amplification-Direct (Red) system (PerkinElmer Life Sciences; 1:100). Sections were then incubated overnight with an antibody against APP CTFs (CT15; 1:100), followed by incubation with FITC-tagged secondary goat anti-rabbit (Vector; 1:75) and imaging by laser scanning confocal microscopy (LSCM, MRC 1024, Bio-Rad).

For ultrastructural analysis, blocks of neocortex and hippocampus were postfixed with 2% glutaraldehyde and 0.1% osmium tetroxide in 0.1 M sodium cacodylate buffer and embedded in epoxy. Blocks were sectioned with an Ultracut E ultramicrotome (Leica, Nussloch, Germany) and analyzed with a Zeiss EM10 electron microscope (Carl Zeiss, Oberkochen, Germany) (31).

Statistical Analysis—Analyses were carried out with the StatView 5.0 program (SAS Institute Inc., Cary, NC). Differences among means were assessed by student’s t test or by one-way ANOVA followed by Dunnett’s or Tukey-Kramer post hoc tests as indicated.
Expression of BACE1 in Brains of mThy1-hBACE1 tg Mice—Microinjection of the mThy1-hBACE1 construct yielded four tg lines, three of which were selected for further analysis based on levels of hBACE1 mRNA determined by RPA. hBACE1 mRNA levels in whole hemibrains of these tg lines were 3–8-fold higher than in frontal cortex of humans without AD, with the highest levels detected in line 1 (Fig. 1, A and B). In all three lines, levels of endogenous mBACE1 were decreased by 30–50% compared with non-tg controls (Fig. 1C). Consistent with the mRNA analysis, hBACE1 protein levels (Fig. 1, D and E), hBACE activity (Fig. 1F), and neuronal hBACE1 immunoreactivity (Fig. 1, G–J) were higher in line 1 than in lines 2 and 39. In all three lines, high levels of hBACE1 immunoreactivity were observed in pyramidal neurons in layers 4–5 of the neocortex (Fig. 1, H–J). In the hippocampal formation, hBACE1 immunoreactivity was detected in pyramidal cells of the CA1 region and in granule cells of the dentate gyrus in line 1, and in pyramidal neurons of CA1–3, but not in granule cells, in lines 2 and 39 (not shown). Moderate hBACE1 immunoreactivity was also detected in subcortical nuclei and the cerebellum of all three lines (not shown). In brains of non-tg controls, no immunoreactivity above background levels was detected with the hBACE1-specific antibody (Fig. 1, D, E, and G).

High Levels of hBACE1 Expression Increase APP CTFs but Decrease Levels of Full-length APP and Aβ—Full-length (FL) APP was detected on Western blots of brain homogenates as a triple band (Fig. 2A). Compared with non-tg controls, high expresser hBACE1 tg mice had decreased levels of FL APP (Fig. 2A and B); this decrease was most marked in the two upper bands. In contrast, levels of APP CTFs were 2–3-fold higher in hBACE1 mice than in non-tg controls, as determined by Western blot analysis with the CT15 antibody, which recognizes the hBACE1-generated C89 and C99 fragments of APP (Fig. 2, A and C), as well as the α-secretase-generated C83 fragment (32). Double labeling analysis confirmed that intraneuronal accumulation of APP CTFs was higher in hBACE1 mice than in non-tg controls (Fig. 2, D–I). Moreover, neurons displaying the highest levels of hBACE1 expression also showed the highest levels of APP CTF immunoreactivity (Fig. 2, G–I). To further characterize the effects of high levels of hBACE1 expression on hAPP processing, homogenates from hAPP singly tg mice expressing a familial AD-mutant hAPP (line 41 (23)) and hBACE1 (line 1)/hAPP-P(line 41) doubly tg mice were analyzed by Western blot. Singly tg hAPP mice showed an increase in overall levels of cerebral APP expression compared with non-tg controls (Fig. 3, A–C). Compared with these mice, hBACE1/hAPP mice had lower levels of FL APP and higher levels of APP CTFs (Fig. 3, A–C). Immunoreactivities for hBACE1 and APP CTFs in hBACE1/hAPP mice were colocalized in the same neurons (Fig. 3, G–I), consistent with the notion that the increase in APP CTFs resulted from increased cleavage of hAPP by hBACE1.

To investigate APP metabolism in greater detail in these mice, APP CTFs were detected by immunoblot analysis after separation on Tricine/SDS-polyacrylamide gels. This analysis revealed that the increase in APP CTFs in hBACE1/hAPP mice represents primarily an accumulation of C89, which results from APP cleavage at the β' site (Fig. 4A). Whereas γ-secretase cleavage of C99 would result in the production of Aβ1–42, γ-secretase cleavage of C89 would result in the production of Aβ1–40. Surprisingly, hBACE1/hAPP mice had decreased levels of both Aβ1–42 and truncated Aβ species. Hippocampal levels of Aβ1–42 (Fig. 4B) and Aβ1–40 (Fig. 4C) were much lower in hBACE1/hAPP mice than in hAPP singly tg mice. Because the ELISAs used to
obtain these measurements specifically detect peptides containing the first five amino acids of \( \text{A}\beta \), we also analyzed \( \text{A}\beta \) levels with antibody 266, which was raised against the middle portion of the \( \text{A}\beta \) peptide (amino acids 13–28, TABLE ONE) (25). Extraction of hippocampal proteins with formic acid, followed by high resolution acid urea-PAGE and Western blot analysis with the 266 antibody, confirmed reductions in all...
Neurodegeneration in hBACE1 tg Mice

detectable Aβ species in hBACE1/hAPP mice compared with hAPP singly tg mice (Fig. 4D). Consistent with these biochemical data, amyloid plaques were detected at 3 and 6 months of age in the frontal cortex and hippocampus in hAPP mice but not in hBACE1/hAPP mice (Fig. 4, E and F, and data not shown).

These findings raise the possibilities that the decreased levels of Aβ1–40 and Aβ1–42 result from alterations in the activity of γ-secretase, in the availability of its substrate, or in the rate of Aβ turnover by enzymes such as neprilysin and insulin-degrading enzyme (IDE). Immunoblot analysis with antibodies against neprilysin or IDE showed roughly comparable levels of these enzymes in hBACE1 mice, hAPP mice, hBACE1/hAPP mice, and non-tg controls (Fig. 4, G and H). In addition, levels of Notch, another γ-secretase substrate, were comparable among the four groups of mice (Fig. 4, G and H). These results suggest that the decrease in Aβ levels in mice with high levels of hBACE1 activity does not result from increased degradation of Aβ or decreased γ-secretase activity.

Similar decreases in Aβ in an independent line of hBACE1 tg mice have recently been related to decreased availability of mature phosphorylated APP (APP-p) (19). Consistent with this possibility, Western blot analysis with an antibody against APP-p-threonine 668 revealed that the cerebral levels of FL APP-p and C99-p were lower in hBACE1/hAPP mice than in hAPP mice (Fig. 4, I and J). These results support the notion that high levels of hBACE1 activity increase hAPP cleavage in the early secretory pathway, depleting mature hAPP-p before it is transported to the axon terminals, where a large proportion of Aβ generation by the γ-secretase complex takes place (19).

Increased Activity of hBACE1 Elicits Learning Deficits and Neurodegenerative Alterations—At 3 months of age, hBACE1 tg mice from the highest expresser line 1 showed mild weakness and spasticity of the hind limbs. This phenotype was more apparent at 6 months and progressed to a prominent spastic paraparesis by 12 months of age. hBACE1 mice from the intermediate expresser lines 2 and 39 showed no obvious neurological deficits when inspected at 3 and 6 months of age, although at 12 months they had a mild tremor.

Because hBACE1 tg mice from line 1 developed age-dependent neurological deficits that precluded assessment in the water maze test, hBACE1 tg mice from line 39, hAPP mice, hBACE1/hAPP mice, and non-tg littermates were tested in the Morris water maze to examine their spatial learning and memory. By day 3 of the cued component of this test (platform visible), all four groups achieved similar escape latencies (Fig. 5). When the platform was hidden, all three groups of tg mice showed significant learning deficits compared with non-tg controls (Fig. 5). A trend toward more severe deficits was observed in the hBACE1/hAPP mice, but by repeated measures ANOVA the curves for the hidden platform were not different among the three groups of tg mice. However, on the last day of the test (day 7) the performance deficits of the hBACE1/hAPP group were significantly different (one way ANOVA, p < 0.05) compared with the hBACE1 and hAPP tg mice.

To assess the extent of neurodegenerative alterations in hBACE1 mice, immunolabeling was performed with antibodies against markers of neuronal and dendritic integrity. Compared with non-tg controls (Fig. 6A), mice expressing high levels of hBACE1 displayed shrinkage of pyramidal neurons in the CA3 region of the hippocampus (Fig. 6B), with the worst alterations seen in mice from the highest expresser line 1 (Fig. 6, B and C). Compared with non-tg controls (Fig. 6D), pyramidal neurons in layers 2–3 of the neocortex were also shrunk and condensed in all three hBACE1 tg lines (Fig. 6, E and F). In contrast to the normal labeling of neurites with antibodies against neurofilament (Flg) and MAP2 (Fig. 6F) in non-tg controls, axonal and dendritic processes of pyramidal neurons in hBACE1 mice were diminished, disrupted, and vacuolated (Fig. 6, H and K). These alterations were prominent at 12 months (Fig. 6, I and L) and detectable, albeit to lesser extent, at 3 and 6 months of age (not shown). At 12 months of age, the neurodegenerative alterations in hBACE1 tg mice were associated with a reactive astrocytosis (Fig. 6N) not observed in non-tg controls (Fig. 6, M and O). In contrast to non-tg littermate controls (Fig. 6P), hBACE1 mice from line 1 showed collapsed neuronal cytoplasm, accumulations of electron dense material, extensive vacuolization of dendritic arbors (Fig. 6Q), and widespread degeneration of axonal processes with splinting and disorganization of myelin laminations (Fig. 6R). Significant loss and alterations of neuritic structures were also detected in hAPP singly tg mice, but only hBACE1 mice and hBACE1/hAPP mice had considerable damage also in the CA3 region of the hippocampus (Fig. 7). Compared with non-tg controls (Fig. 7A), hAPP (Fig. 7B) and hBACE1 (Fig. 7C) mice had reduced levels of MAP2 immunoreactivity in the frontal cortex, an alteration that was even more prominent in hBACE1/hAPP mice (Fig. 7E). In the CA3 region of the hippocampus, all three tg groups showed a loss of dendritic complexity, which was most severe in hBACE1 and hBACE1/hAPP mice (Fig. 7, F–J).

DISCUSSION

The present study demonstrates that increased activity of hBACE1 in neurons of tg mice is sufficient to elicit profound alterations in APP metabolism, neurological deficits, and neurodegeneration. Although BACE1 is required for the generation of Aβ, high levels of neuronal hBACE1 activity decreased rather than increased Aβ levels in hBACE1/hAPP mice compared with hAPP singly tg mice, suggesting that Aβ was not the main mediator of the increased neuronal deficits identified in the doubly tg mice. Because APP-deficient mice (33, 34) do not show the neurological and neuropathological alterations we observed in several lines of hBACE1 tg mice, it is also unlikely that the decrease in FL or α-secretase-processed APP accounts for the neuronal deficits seen in hBACE1tg mice. A likelier pathogenic mechanism involves the accumulation of APP CTFs and/or the mismetabolism of alternative hBACE1 substrates.

hBACE1 cleaves APP at two sites (β and β′) generating the classical C-terminal stub (C99) and the alternative C89 fragment (15, 35).
FIGURE 6. Neuropathological and ultrastructural alterations in the brains of hBACE1 tg mice. Vibratome sections from 6-month-old mice were either stained with cresyl violet (A–F), immunostained with antibodies against neurofilaments (G–I), MAP2 (J–L) or GFAP (M–O), or processed for electron microscopy (P–R). For A and B, representative images are from the hippocampus, and for D–O, images are from the neocortex of non-tg and hBACE1 tg (line 1) mice. A and B, neuronal shrinkage and degeneration (arrows) in the CA3 region of an hBACE1 tg mouse. C, quantitative assessment of the cell density in CA3 revealing significant loss of neurons in mice from lines 1 and 2 (n = 6 mice/group). D and E, neuronal shrinkage and degeneration (arrows) in layers II–III of the fronto-parietal region in an hBACE1 tg mouse. F, quantitative assessment of the cell density in the neocortex revealing significant loss of neurons in mice from line 1 (n = 6 mice/group). G and H, loss of phosphorylated high and intermediate molecular mass neurofilaments in an hBACE1 tg mouse, as revealed by immunostaining with SMI312 (mouse monoclonal) and bright field microscopy. I, quantitation of SMI312 immunoreactivity showing a significant decrease in all three lines of hBACE1 tg mice (n = 6 mice/group). J and K, alterations of MAP2-positive neuronal and dendritic structures in an hBACE1 tg mouse, as demonstrated by LSCM. L, quantitative assessment of percent area of the neocortex occupied by MAP2-immunoreactive dendrites in the neocortex revealing significant losses in all lines of hBACE1 tg mice (n = 6 mice/group). M and N, reactive astrocytosis in an hBACE1 tg mouse, as revealed by GFAP immunostaining and bright field microscopy. O, quantitation of GFAP immunoreactivity in the neocortex showing a significant increase in hBACE1 tg mice from line 1 (n = 6 mice/group). P, ultrastructural analysis demonstrating well preserved neuronal (n), dendritic, and axonal structures in a non-tg mouse. Q, in contrast, an hBACE1 tg mouse from line 1 showed considerable neuronal shrinkage, condensation, and vacuolization of dendrites (d), R, axon (a) in an hBACE1 mouse showing thinning and degeneration of myelin sheaths. Bars = 25 μm (A, B, D, E, G, H, M, and N), 15 μm (J and K), 3 μm (P and Q), and 2 μm (R). *, p < 0.05 versus non-tg controls (Dunnett’s t test).
Although most of the current research in AD is focused on the potential neurotoxic effects of Aβ, APP CTFs have also been shown to have deleterious effects. For example, in primary neuronal cultures, accumulation of the C99 fragment promotes apoptosis (36); tg mice overexpressing CTFs in neurons develop hippocampal degeneration as well as deficits in memory and long term potentiation (36–39).

Increased activity of hBACE1 led to an increase in the levels of APP CTFs in both hBACE1 mice and hBACE1/hAPP mice, consistent with previous observations (19–21, 40, 41). Moreover, the levels of APP CTFs in different lines of hBACE1 mice were related to the extent of neurological and neuropathological alterations; the highest levels of APP CTFs were found in the most severely impaired line 1. Although previous studies (19–21, 40, 41) have shown an increase in C99 and C89 and a decrease in C83, in the present study we found that the most significant increase was in levels of C89, whereas C83 levels were decreased. This suggests that our model APP processing may occur preferentially at the β′ cleavage site (15, 35). Interestingly, despite an overall increase in C99 and C89 in previous studies, levels of C99-p were decreased significantly in hBACE1/hAPP mice, and this decrease in C-99 phosphorylation was more apparent in mice expressing higher levels of hBACE1 (19). Compared with hAPP tg mice, our hBACE1/hAPP mice showed lower levels of C99, and C99-p was decreased with hBACE1 overexpression. Because phosphorylation of APP is a post-translational modification necessary for maturation and axonal transport of C99 (42, 43), the decrease in C99-p indicates that APP cleavage in the hBACE1/hAPP mice might occur in early compartments (19).

High levels of hBACE1 and APP CTFs in our hBACE1/hAPP mice were associated with decreased levels of human Aβ1–40 and Aβ1–42 as determined by highly quantitative ELISA measurements. These results are consistent with a recent study (19) but differ from results obtained in other hBACE1/hAPP models (20, 21, 40, 41). Several factors might account for these differences, including the genetic background of the mice analyzed. However, the most critical factor in determining the effects of hBACE1 activity might be the levels of hBACE1 transgene expression (19). Although low and intermediate levels of hBACE1 expression enhanced amyloid production and deposition in doubly tg
mice, higher levels of hBACE1 expression reduced Aβ production, presumably because high levels of hBACE1 activity shifted the subcellular location of APP cleavage to the neuronal cell body and early secretory pathway, resulting in a depletion of mature APP in the trans-Golgi compartment (19). Thus, a lesser amount of APP might be targeted to the distal axon, which would preclude γ-secretase-mediated generation of Aβ at synaptic sites.

If not Aβ, what other factors may result in the degeneration of neurons with high levels of BACE1 activity? Recent evidence suggests that the toxicity of CTFs may be mediated by the caspase-generated C-terminal C31 fragment (44, 45). It will therefore be interesting to test whether preventing the generation of C31 prevents neurodegeneration in hBACE1/hAPP mice. In a similar vein, expression of hBACE1 in mAPP-deficient mice might reveal APP-independent mechanisms of hBACE1-induced neurotoxicity. Conceivably, increased BACE1 activity may trigger neurodegeneration by cleavage of substrates other than APP. For example, both in vitro (12) and in vivo (46) BACE1 cleaves St6Gal I, a sialyltransferase whose cleavage product is secreted. Recently, it has been shown that BACE1 also cleaves β-subunits of voltage-gated sodium channels (47). Increased levels of hBACE1 activity could further broaden the substrate specificity of this enzyme in both mouse and humans with AD.

Indeed, recent studies have identified increased levels of hBACE1 immunoreactivity and of APP CTIFs in AD brains (14–17), suggesting that increased BACE1 activity may be causally involved also in the pathogenesis of AD. The current study supports and extends this notion by demonstrating that increased neuronal hBACE1 activity can cause progressive neurodegenerative alterations in vivo. Whereas inhibiting γ-secretase, which does not alter α- or β-secretase processing, decreases Aβ production but increases the accumulation of APP CTIFs (48, 49), inhibiting BACE1 may block both Aβ-dependent and Aβ-independent pathogenic mechanisms in AD.

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