Human Molecular Genetics, 2001, Vol. 10, No. 12 **1317–1324**

BACE knockout mice are healthy despite lacking the primary β-secretase activity in brain: implications for Alzheimer's disease therapeutics

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Received 1 March 2001; Revised and Accepted 3 April 2001

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by accumulation of amyloid plaques and neurofibrillary tangles in the brain. The major components of plaque, β-amyloid peptides (Aβs), are produced from amyloid precursor protein (APP) by the activity of β- and γ-secretases. β-secretase activity cleaves APP to define the N-terminus of the Aβ1-x peptides and, therefore, has been a long-sought therapeutic target for treatment of AD. The gene encoding a β-secretase for beta-site APP cleaving enzyme (BACE) was identified recently. However, it was not known whether BACE was the primary β-secretase in mammalian brain nor whether inhibition of β-secretase might have effects in mammals that would preclude its utility as a therapeutic target. In the work described herein, we generated two lines of BACE knockout mice and characterized them for pathology, β-secretase activity and Aβ production. These mice appeared to develop normally and showed no consistent phenotypic differences from their wild-type littermates, including overall normal tissue morphology and brain histochemistry, normal blood and urine chemistries, normal blood-cell composition, and no overt behavioral and neuromuscular effects. Brain and primary cortical cultures from BACE knockout mice showed no detectable β-secretase activity, and primary cortical cultures from BACE knockout mice produced much less Aβ from APP. The findings that BACE is the primary β-secretase activity in brain and that loss of β-secretase activity produces no profound phenotypic defects with a concomitant reduction in β-amyloid peptide clearly indicate that BACE is an excellent therapeutic target for treatment of AD.

INTRODUCTION

Alzheimer's disease (AD) represents one of the great unsolved medical needs confronting society during this millennium. Despite considerable work during the past quarter century, no medicines exist that attack the underlying pathophysiology of the disease. One of the cardinal features of AD is deposition of plaques comprised of aggregated β-amyloid peptides (Aβs) in the brain, particularly in regions associated with cognition and memory (1). Overproduction of Aβ, which appears to be directly neurotoxic (2), can be detected at the earliest stages of AD and, in fact, before cognitive dysfunction is detectable (3). Aβ is produced from its precursor protein, amyloid precursor protein (APP), by proteolytic processing at its N- and C-termini by β- and γ-secretase enzymes, respectively. Mutations in APP (4), presenilin-1 (5–7) or presenilin-2 (7) genes result in overproduction of Aβ1–42 peptide and cause early onset, familial AD. The identity of the β- and γ-secretases have been studied since 1984 (8), and in 1999 the elusive N-terminal β-site APP cleaving enzyme (BACE) was reported virtually simultaneously by four laboratories (9–13). Although the same protein, BACE, was identified by all four groups, it remains possible that there are additional proteases with β-secretase activity. It

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is therefore critical to show that BACE comprises the major β-secretase activity in brain.

BACE mRNA is expressed widely at low levels, at moderate levels in the brain and at higher levels in the pancreas (9,11). However, β-secretase activity is low in pancreas and highest in brain (10). This discrepancy between mRNA and activity is probably due to a splice variant lacking two-thirds of exon 3 being the predominant BACE transcript in pancreas, resulting in a protein that is incompletely processed and retained in the endoplasmic reticulum (14). In the brain, BACE mRNA is widely expressed only in neurons, with most pronounced expression in the cerebellum, cortex and hippocampus observed by *in situ* hybridization (11). BACE is co-localized with its substrate, APP, in the trans-Golgi network of cells (11,12).

Because small-molecule BACE inhibitors are being studied as potential AD pharmacotherapeutics, it is necessary to prove that BACE is the primary β-secretase of brain and to understand what effects BACE inhibitors may have beyond inhibition of APP processing. Towards this end, we generated two independent lines of BACE knockout mice. The production of Aβ peptide was inhibited in primary cortical cultures of BACE knockout mice. These findings are consistent with two other strains of BACE knockout mice published while this manuscript was under review (15,16). Our data further demonstrated that disruption of the BACE gene ablated β-secretase activity in both primary cortical cultures and brain. Animals lacking BACE developed normally and displayed no profound physical, biochemical or behavioral abnormalities, suggesting that deletion of BACE activity results in no serious deleterious effects. Thus, small-molecule BACE inhibitors are excellent candidates for the treatment and prevention of AD.

RESULTS

We generated two lines of BACE knockout mice: (i) by replacing a part of exon 1 and (ii) by deleting exons 4–8 (Fig. 1). The exon 1 disruption contained an inserted expression cassette immediately downstream from the initiating methionine, and the exons 4–8 deletion removed one of the two aspartate residues at the active site. Deletion of one or both alleles of the BACE gene conveyed no developmental disadvantage, as the genotypes were transmitted in numbers approximating those expected for Mendelian inheritance. Specifically, genotyped offspring from heterozygote × heterozygote crosses to date of mice from the two lines are as follows: wild-type:heterozygous:homozygous = 29:77:29 (the offspring within each of the two lines also roughly followed Mendelian genetics although the number of offspring bred from the exons 4–8 line is still relatively small). Necropsy was performed on exon 1-disrupted and exons 4–8-deleted $BACE^{-/-}$, $BACE^{+/-}$ and $BACE^{+/+}$ mice to examine tissues for gross pathological changes (Table 1). No gross abnormalities clearly related to genotype were noted at any age. Terminal body weight and weights of brain, kidney, adrenals, thymus, liver, pancreas, testes and ovaries did not differ among the genotypes. In general, few gross or microscopic differences between $BACE^{-/-}$, $BACE^{+/-}$ and $BACE^{+/+}$ mice were observed. In particular, the morphology of the brain was normal. Representative photomicrographs of the hippocampus are shown in Figure 2, and thorough examination of sections throughout the brain failed to identify abnormalities in animals of any genotype. Detailed histopathological studies of other tissues at various ages are ongoing. Two other groups have, since the submission of this manuscript, reported viability in BACE knockout mice (15,16). One group performed a thorough histopathological analysis and found no abnormalities (15).

Table 1. BACE knockout animals examined by necropsy and histology. Exon 1-disrupted animals were also used for behavioral observations

Genotype	Age (weeks)	Males	Females
Exon 1			
–/–	7–17	8	2
+/–	7–13	10	0
+/+	8–17	3	7
Exons 4–8			
–/–	6	1	0
+/–	6	1	1
+/–	2	1	5
+/+	6	0	1
+/+	2	1	0

BACE-deficient animals weaned and thrived normally when compared with their wild-type and heterozygous littermates. Animals were repeatedly observed in their home cages for gross behavioral abnormalities, and none was noted. Urine was collected over a 24 h period on 4 consecutive days from 10 animals of each genotype, aged 7–17 weeks. No differences in standard urinalysis endpoints including urinary volume, pH, specific gravity, urinary sediment, urine glucose and protein levels were found among any of the genotypes. Also, no genotype-related differences in a standard battery of clinical chemistry parameters including blood urea nitrogen (BUN), creatinine, glucose, transaminases, bilirubin, electrolytes, creatine kinase and alkaline phosphatase were detected in these animals. Cellular composition of blood was indistinguishable between homozygous knockout, heterozygous and wild-type littermates.

Male and female mice lacking exon 1 of the BACE gene were tested on several measures of physiological function, as well as basal behavior and reactivity. Ten wild-type (+/+), heterozygous (+/–) and homozygous (–/–) mice ranging from 50–120 days of age (Table 1) were used for these tests. Age was balanced across sex and genotype, except female –/– mice (n = 2, both at the upper end of the range). Gross behavioral observations and scoring for physical and physiological function were done as per an amended version of the SHIRPA test (17), and open field activity was measured in photocell-equipped activity monitors. Although these results must be regarded as preliminary until larger numbers of mice are tested at various ages, we found no obvious differences observed between the genotypes.

All mice showed normal gait, with normal exploratory behavior including sniffing, rearing, urination and defecation. The exploratory phase was followed by habituation which was also similar for all groups. There were no differences in grip strength and all mice had a strong righting reflex with a normal reaction to being touched along their dorsal surface. The

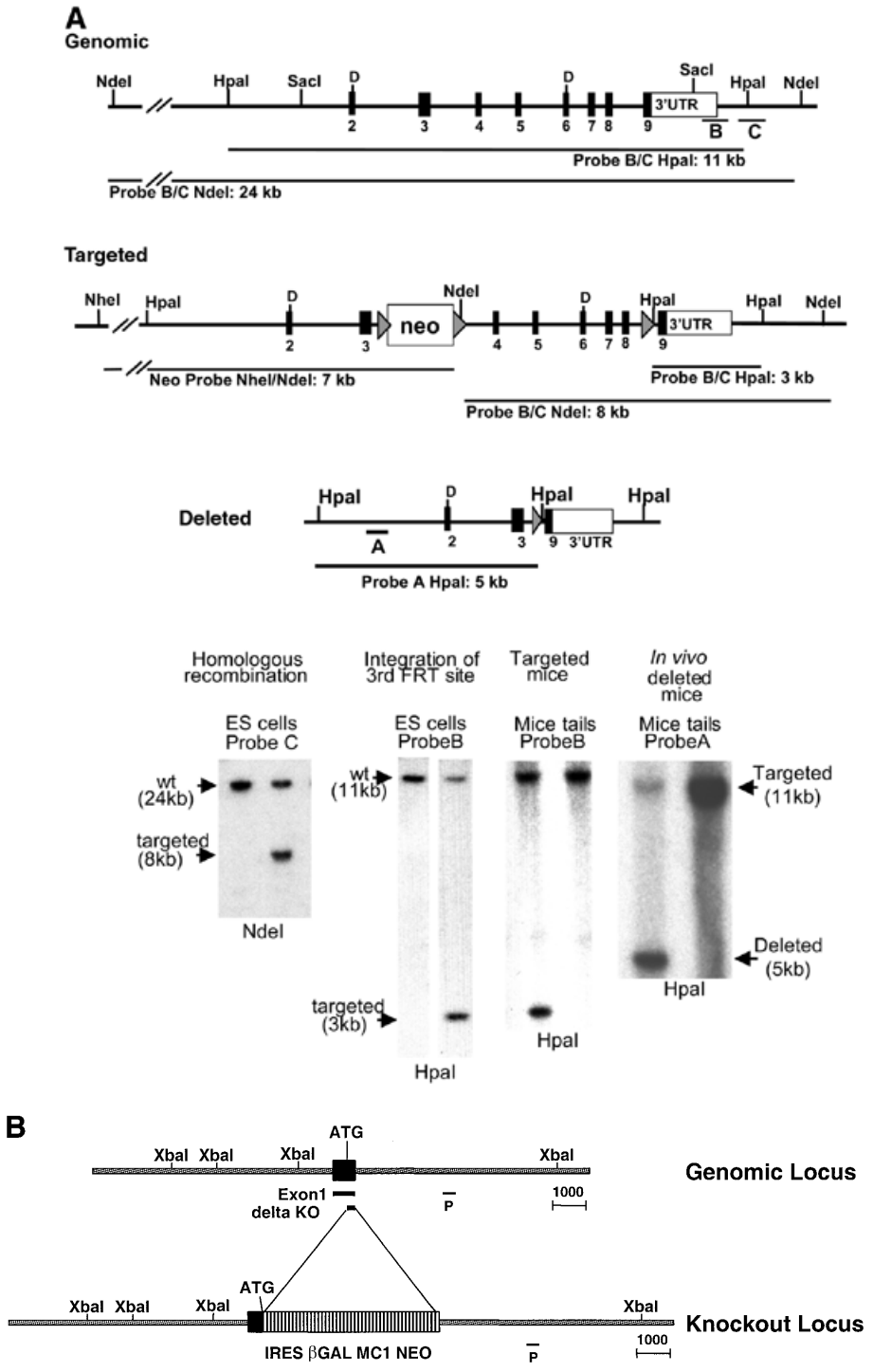

Figure 1. (**A**) Generation and genotyping of BACE knockout mice lacking exons 4–8. Three FRT sites (gray triangles) and a neomycin-resistance gene were appropriately integrated into ES cells as shown in the first two Southern blots. Mice produced using targeted ES cells also contained the targeted allele (third Southern blot). After crossing targeted BACE heterozygote mice with a transgenic mouse expressing FLP recombinase under the control of a CMV enhancer/chicken actin promoter, exons 4–8 had been deleted in heterozygous offspring as demonstrated by the fourth Southern blot. (**B**) Generation of BACE knockout mice with deletion and insertion in exon 1. The portion of the BACE genomic locus containing exon 1 is shown with the position of exon 1 (solid box) underlined and labeled and the position of the initiating methionine indicated by ATG. The region which is replaced by the expression cassette is underlined and labeled delta KO. The structure of the knockout locus is shown below with the inserted expression cassette shown by the striped box. IRES indicates the internal ribosomal entry site for polycistronic translation. βGAL, β-galactosidase gene; MC1 NEO, an expression cassette expressing the neomycin resistance gene driven by the polyoma enhancer/herpes simplex virus thymidine kinase promoter.

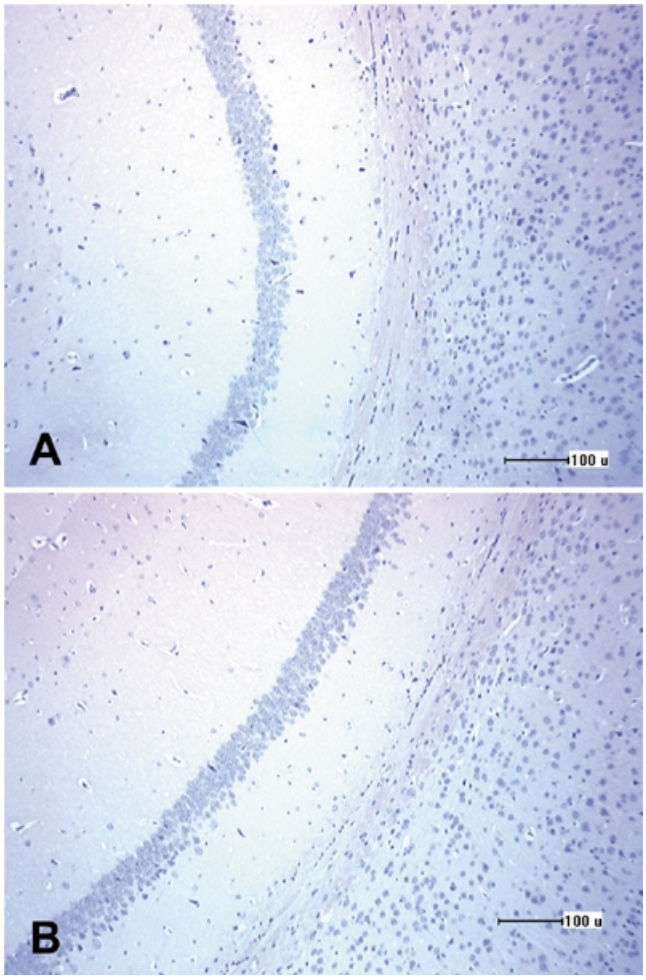

Figure 2. Representative histological sections of hemotoxylin and eosin-stained brain from exon 1-disrupted BACE knockout mice. No genotype-specific differences in the microscopic morphology of brain were observed, as exemplified by images of hippocampus from +/+ (**A**) and –/– (**B**) mice. Scale bar, 100 µm.

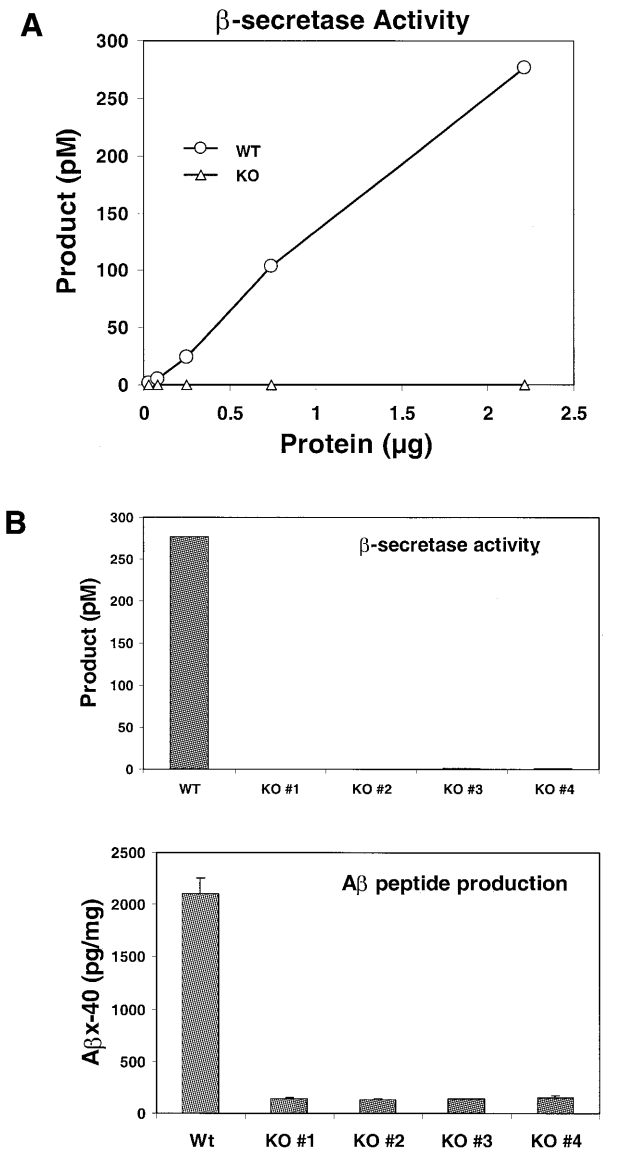

Figure 3. β-secretase activity and Aβ production in primary cortical cells from wild-type and homozygous BACE knockout mice. Primary cortical cultures were generated from individual –/– and +/+ fetuses generated from crossing male and female –/+ exon 1-disrupted BACE knockout mice. Five days after plating, the medium was exchanged and 2 days later cells were harvested. Medium was also harvested on days 1, 2 and 3 after the medium exchange. (**A**) β-secretase activity was measured in cell extracts from +/+ (WT, open circles) and –/– (KO, open triangles) primary cortical cultures and is shown as a function of cellular protein added to the reaction. (**B**) β-secretase activity in 2.3 µg of protein from cell extracts (top) and Aβx-40 released into the medium after 3 days of collection (bottom) are shown from individual +/+ (WT) and –/– (KO) fetuses. Aβx-40 measurements collected from multiple wells were normalized to mg of cellular protein in each well. Error bars indicate SD.

geotaxis response, measured as the time to turn 180° and begin to ascend a vertical screen, was very quick, and the same for all groups. All genotypes had eyes that were completely open on arousal, with a similar blink response to a light touch to the cornea. None of the animals showed body tremor or pilo-erection, and the resting respiration and body temperatures did not differ between the groups. Likewise, all genotypes showed a similar pinna reflex distribution in response to a light touch to the inside of the ear.

β-secretase was originally defined operationally as the activity generating a specific cleavage in APP to release the N-terminus of Aβ and an intact secreted fragment sAPPβ (18). Although BACE has been identified as a protease with such activity, it is not known whether BACE is the only, or even the primary, β-secretase in brain cells. To determine the degree to which BACE disruption decreased β-secretase activity, enzyme activity was measured in primary cortical cultures from –/– and +/+ fetuses. β-secretase activity from wild-type cultures was linear with the amount of cellular protein added (Fig. 3A). No β-secretase activity was detected in primary cortical cultures from homozygous BACE knockout animals (Fig. 3A and B). However, activity measured in wild-type cortical cultures showed activity above the background even when diluted 27-fold (Fig. 3A). Therefore BACE is the primary β-secretase in cortical cells.

If BACE is functioning in intact cells as β-secretase, one would predict that Aβ levels would be reduced in BACE

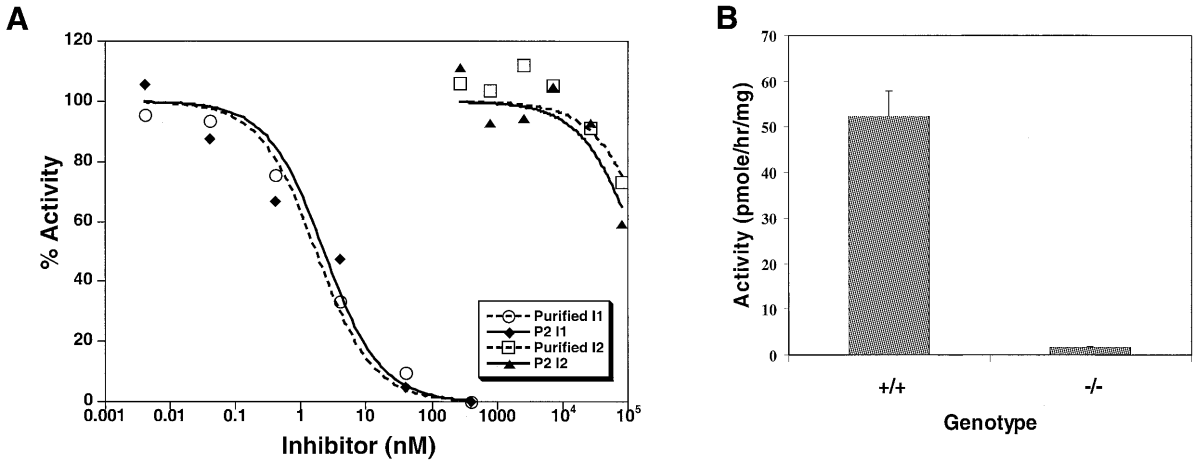

Figure 4. β-secretase activity and inhibition in brain from BACE knockout mice. (**A**) The statine-based substrate analog β-secretase inhibitors P10–P4′ statV (KTEEISEVNStaVAEF) (circle and diamond, PI1) and P10–P4′ stat (KTEEISEVNStaDAEF) (square and triangle, P12) previously described (10), were used to inhibit β-secretase activity from wild-type P2 pellets (closed diamond and triangle) and from BACE purified from human brain (open circle and square). I1 has an IC50 of 2 nM, and I2 has an IC50 of 200 μM for both purified BACE and P2 pellets. (**B**) β-secretase activity measured in brain homogenates from homozygous and wild-type exon 1-disrupted BACE knockout mice. Cortexes from four +/+ and four –/– mice were homogenized and β-secretase activity measured in P2 pellets as described in Materials and Methods. Activity per milligram of P2 membrane protein is shown. Activity from the –/– samples are below the limits of detection of the assay. Error bars indicate SD.

knockout cultures. To measure BACE effects on APP processing in brain cells, Aβx-40 was measured in primary cortical cultures used for β-secretase activity described above. Since the enzyme-linked immunosorbent assay (ELISA) for Aβx-40 readily detects rodent Aβ, it was used instead of other Aβ ELISAs. In knockout –/– cultures Aβx-40 production was reduced 15-fold relative to wild-type cultures. These decreases in Aβ peptide production are consistent with results from other BACE knockout mice recently reported (15,16). Furthermore, this finding is consistent with the amount of β-secretase activity we measured in these cultures. Therefore, BACE is the major β-secretase in cortical neurons and is required for Aβ production.

To confirm that BACE is the major β-secretase enzyme in brain tissue, measurements of enzyme activity were made in P2 pellets from cortex of exon 1-disrupted BACE knockout mice (Fig. 4). Enzyme activity from P2 pellets of cortex from wild-type mice was linear relative to the amount of protein added (data not shown) and fully inhibitable with a specific inhibitor of β-secretase activity (Fig. 4A, I1). The IC_{50} values for inhibition of enzymatic activity by two substrate analog inhibitors (Fig. 4A), differing by one amino acid and 100 000-fold in affinity, corresponded in the P2 pellet to that in enzyme purified from human brain (10). Thus the enzyme activity we are measuring in the P2 pellet behaves identically to purified BACE activity. Although robust activity was observed in wild-type mice, no activity was detectable in cortical extracts from knockout –/– mice (Fig. 4B). This confirms that BACE is the major β-secretase in intact mouse brain tissue.

DISCUSSION

We have demonstrated that BACE knockout mice are generally healthy and that they lack the major β-secretase in brain. To do so, we generated two independent lines of BACE knockout mice and studied these animals using biochemical, histological, clinical chemistry and behavioral techniques. Loss of BACE ablated β-secretase activity and reduced Aβ peptide production, yet these mice have no gross deficiencies in physiology or basic behaviors. Specifically, these mice develop normally *in utero*, as the knockout allele is inherited in a ratio expected by Mendelian genetics. No clearly genotypic abnormalities were noted upon assessment of fresh tissues nor upon histological examination of brain from young and adult animals. Taken together, these findings indicate that the BACE gene is dispensable for development and function through early adulthood.

Because brain contains higher levels of β-secretase activity than other tissues (10), performance in behavioral paradigms was used as a sensitive measure of brain function. Preliminary behavioral evaluation of homozygous and heterozygous BACE knockout mice revealed no obvious functional abnormalities when compared with wild-type littermates. Loss of BACE activity does not induce strong hyperactivity, behavioral sedation or other locomotor defects. In addition, gross behavioral observations and tests of neuromuscular activity showed no obvious deficits in basal neurological and physiological functions in these animals. These results are preliminary, and we are currently carrying out further detailed behavioral analyses on larger numbers of mice of both sexes and under different conditions to detect more subtle effects.

Importantly, β-secretase activity is undetectable in primary cortical cultures and in brains of homozygous BACE knockout mice, indicating that BACE is the major β-secretase enzyme in mouse brain. Moreover, BACE is also likely to be the major β-secretase in human brain, although this hypothesis must yet be tested. Because BACE2 is active in our β-secretase assay *in vitro* (data not shown), the lack of β-secretase activity in $BACE^{-/-}$ mice demonstrated in Figures 3 and 4 indicates that there is little or no active BACE2 in brain or cortical cultures.

Loss of BACE also results in a large decrease in Aβ production in primary cortical cultures. The small amount of detectable Aβ observed in the knockout cultures may represent forms initiating past the initial Asp of Aβ, since it was necessary to use an ELISA detecting all forms of Aβx-40 to detect Aβ production from endogenous murine APP. Nevertheless, these data further demonstrate that BACE is the major β-secretase required for production of Aβ.

Our data indicate that pharmacological inhibition of BACE activity will greatly diminish production of Aβ. It follows, then, that small-molecule BACE inhibitors should prevent the accumulation of amyloid plaque in the brain of individuals with AD and may prevent growth of pre-existing plaques. Notably, loss of BACE activity is well tolerated in mice, as no profound defects were found through 4 months of age. Although we have not yet evaluated aged mice, the overall healthy phenotype of BACE knockout animals suggests that BACE inhibition is unlikely to produce serious untoward effects.

Over the past 15 years, since the description of APP and elucidation of its relationship to Aβ, there has been an exhaustive search for the putative enzymes responsible for catabolism of APP to Aβ. Although the γ-secretase enzyme has not been conclusively identified, it appears to be associated with presenilins (19). Discovery of the BACE enzyme has provided a characterized molecular target that can be studied as a possible avenue to interdict Aβ-mediated neurotoxicity in AD. This report indicates that BACE is a key APP-processing enzyme which is mandatory for generation of Aβ. Moreover, our work demonstrates that knockout of BACE function does not lead to profound developmental abnormalities nor biochemical or behavioral dysfunction postnatally. This contrasts with perturbation of presenilin function, which leads to significant developmental abnormalities (20–22). At this point, the evidence suggests that BACE is an excellent therapeutic target for development of AD pharmacotherapeutics and that small-molecule inhibitors of this enzyme may be useful in halting the initiation and progression of AD.

MATERIALS AND METHODS

Generation of BACE targeted ES cells (exons 4–8)

The targeting vector was designed in a way that, upon homologous recombination, exons 4–8 of the BACE gene are flanked with FLP recombinase target sites (FRT sites). With respect to the genomic locus, the 5′ region of homology covered 4.5 kb and the 3′ region 4.3 kb until the third FRT site, and an additional 1.5 kb further 3′. Selection for homologous recombination was achieved by inserting an FRT-flanked neomycin resistance cassette between exons 3 and 4 of the BACE gene. The parental ES cell (23) line E14 (129/Ola) was employed for successful targeting of the BACE locus. Following electroporation, recombinant cells were positively selected with G418 (Geneticin, Gibco BRL). Successful targeting of the BACE gene and integration of the third FRT site were detected in resistant ES clones by *Hpa*I restriction enzyme digestion and Southern hybridization employing a 3′ external probe B. Probe B was generated by PCR from mouse genomic DNA using primers 5′-GACAGATGAATTC-CTATCTTG-3′ and 5′-GTCTCTTCCTCATCAACTGTC-3′. Single integration of one targeting vector was confirmed. ES cells with appropriate targeting were injected into blastocysts from C57BL/6 mice. Chimeric offspring were bred with C57BL/6 mice to produce animals heterozygous for the FLP-targeted BACE allele. These mice were bred with mice transgenic for FLP recombinase expressed under control of a CMV enhancer/chicken actin promoter on a C57BL/6 × CBA background for the *in vivo* deletion of the selection marker.

Generation of exon 1 BACE knockout mice

A lambda KOS genomic clone encoding BACE from murine strain 129/SvEvBrd covering 5.3 kb upstream and 2.5 kb downstream of exon 1 were used to generate the knockout targeting vector as described (24) (Fig. 1B). A 165 bp deletion of exon 1 starting from 2 bp past the initiating methionine and extending through the end of exon 1 was replaced with an expression cassette in the targeting vector. A neomycin resistance gene was included for positive selection of homologous recombinants and HSV-thymidine kinase gene for negative selection against random integrants. The targeting vector was electroporated into 129/SvEvBrd ES cells and clones were selected positively for neomycin resistance with G418 (Geneticin, Gibco BRL) and negatively against thymidine kinase with gancyclovir (Roche). Clones were screened for homologous recombination by Southern analysis using *Xba*I digestion and hybridized with probe P shown in Figure 1B. Probe P was generated by PCR from mouse genomic DNA using primers 5′-CGGAGCCCAACTGTCAA-AAAG-3′ and 5′-CCAACCGTGCCCTCCTGC-3′. Recombination was confirmed by digestion of genomic DNA with *Pvu*II and hybridization with a probe generated by PCR from mouse genomic DNA using primers 5′-GTGTCATGTAACTCAG-GCTG-3′ and 5′-GGTAATCTATGCAGAGCACTTG-3′ (data not shown). Single integration of one targeting vector was confirmed. ES cells with appropriate targeting were injected into blastocysts from C57Bl/6 albino mice. Chimeric offspring were bred with C57Bl/6 albino mice to produce animals heterozygous for the BACE knockout allele. Heterozygous mice were bred together to generate mice homozygous for the knockout allele. Genotyping on experimental mice was reconfirmed on tissue taken at the time of sacrifice.

Aβ ELISA

The Aβ ELISA for Aβx-40 was performed as described (25) except that antibody 2G3 was used as capture and coated at 10 μg/ml and antibody 266 was used as reporter at 1 μg/ml. Rodent Aβ was used for the standard and the same medium used for culturing of the primary cortical cells was added to the standard reaction in dilutions equivalent to that of the samples assayed.

Cortical brain cultures

Male and female heterozygous (+/–) BACE exon 1-disrupted mice were bred together and E16 mouse fetuses obtained from timed pregnancies. Primary cortical cultures were generated from cortexes of individual fetuses, and the hindquarters of each fetus were used for genotyping as described above. The cerebral cortex of E16 mouse fetuses was dissected from each pup individually and triturated and trypsinized to make a single

cell suspension. The cells were plated in polyethylenimine-coated 24-well plates at a density of 625 000 cells/well in neuronal medium (MEM, Gibco BRL) containing 5% fetal bovine serum (Gibco BRL) and 5% Chang's supplement (Irvine Scientific). After 4–5 days *in vitro* the medium was changed and the cells incubated for an additional 1–3 days before harvesting the conditioned medium for Aβ ELISA.

β-secretase activity assays on cultured cells

Primary cultured cortical cells were washed in PBS, lysed in extraction buffer (1 mM HEPES pH 7.5, 1 mM EDTA, 0.2% Triton X-100, 1 mM PMSF, 10 µg/ml E-64 and 10 µg/ml Pepstatin A) and centrifuged in a microfuge at 20 000 *g* for 5 min. Aliquots of the supernatant were assayed directly for β-secretase activity using as substrate a construct containing the bacterial maltose binding protein fused to the C-terminal 125 amino acids of APP, as described previously (10). Enzymatic assays incubated for 2 h at 37°C. Total cellular protein was <2.5 µg per reaction, and activity was linear with the amount of protein added.

β-secretase activity assays in brain homogenates

Cortexes were dissected, weighed and frozen from mice shortly after death. All subsequent operations were performed at 4°C or on ice. Mouse cortexes were homogenized in 4 ml of homogenization buffer (250 mM sucrose, 2 mM EDTA, 20 mM HEPES, pH 7.5) per gram of brain. The homogenates were centrifuged at 1000 *g* for 20 min. The supernatants were saved, and the pellets were resuspended in homogenization buffer and centrifuged as before. The supernatants were pooled with the respective first supernatants, and centrifuged at 16 000 *g* for 20 min. The resulting pellets (P2) were extracted with 1.5 ml of P2 extraction buffer (150 mM sodium chloride, 2 mM EDTA, 0.5% Triton X-100, 20 mM MES, pH 6.0, 5 µg/ml leupeptin, 2 µg/ml E64, 1.0 µg/ml pepstatin, 0.2 mM PMSF) for 1 h with agitation. The suspensions were centrifuged at 16 000 *g* for 20 min. The extracted supernatants were neutralized with Tris base and assayed for β-secretase activity using as substrate a construct containing the bacterial maltose binding protein fused to the C-terminal 125 amino acids of APP, as described previously (10) except that enzyme assay reactions were incubated for 2 h at 37°C and were diluted 1:5 prior to ELISA assay. Enzyme activity was normalized to the amount of protein from the P2 pellet assayed. Human BACE control was purified from brain as described previously (10).

Gross behavioral observations and measurement of open field activity

Visual observation and scoring for physical and physiological function were done as per an amended version of the SHIRPA test (17). Measurement of horizontal activity was performed in Digiscan activity boxes equipped with photocells (Accuscan) as described elsewhere (26).

Animal handling

All animal work used protocols approved by the institutional animal care and use committee. Prior to behavioral observations and necropsy, exon 1-disrupted animals were acclimated for 6 days in metabolism cages to collect urine. Urine was collected over a 24 h period on 4 consecutive days. Urinalysis parameters included volume, specific gravity, pH, protein, glucose, ketones, occult blood, urobilinogen, icto-test and urinary sediment. At necropsy, mice were anesthetized with isofluorane and exsanguinated through the posterior vena cava for complete blood counts and serum chemistries. Complete blood counts included standard quantitative cell count measures, and differential and morphological evaluation of the smear. Serum chemistry values included BUN, glucose, creatinine, cholesterol, alanine aminotransferase, aspartyl aminotransferase, alkaline phosphatase, total protein, albumin, phosphorus, calcium, creatine kinase, sodium, potassium, chloride, triglycerides, total bilirubin and direct bilirubin. A CBC was performed on exons 4–8-deleted animals in Table 1.

A detailed gross necropsy examination was performed. Terminal body weights and weights of brain, kidney, adrenals, thymus, liver, testes or ovaries were collected. Mice were perfused through the left ventricle with heparinized 0.9% saline to facilitate biochemical assays on brain tissue. The brain was removed, laterally bisected, and the right half dissected into cortex, hippocampus, cerebellum and midbrain and frozen for biochemical assays. The left half of the brain was immersion fixed in 4% paraformaldehyde. Multiple sections of brain (cerebellum, brain stem, cortex, hippocampus, thalamus and olfactory lobe) were examined.

ACKNOWLEDGEMENTS

We would like to thank Kenneth Platt and colleagues at Lexicon for work on generating the exon 1-disrupted mice, Riqiang Yan for cDNA cloning, Bruce Elder of Charles River Therion for genotyping, Mark Gurney for his many contributions to the BACE project and the veterinarians and animal care professionals who make this work possible.

NOTE ADDED IN PROOF

We know that the Aβ peptide measured in the medium collected from the knockout primary cortical cultures is due to an artifact of an overnight capture incubation used for the ELISA in these studies. When the capture incubation is reduced to 2 h, there is no measurable Aβ produced, even though the ELISA sensitivity in this format is equivalent to that with the longer incubation time. Therefore, no measurable Aβ is produced from primary cortical cultures of BACE1 knockout mice. These measurements concur with the lack of β-secretase activity observed in these animals.

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