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BACE1 gene deletion prevents neuron loss and memory deficits in 5XFAD APP/PS1 transgenic mic

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Abstract

Evidence suggests that β -amyloid (A β) peptide triggers a pathogenic cascade leading to neuronal loss in Alzheimer's disease (AD). However, the causal link between A β and neuron death *in vivo* remains unclear since most animal models fail to recapitulate the dramatic cell loss observed in AD. We have recently developed transgenic mice that overexpress human APP and PS1 with five familial AD mutations (5XFAD mice) and exhibit robust neuron death. Here, we demonstrate that genetic deletion of the β -secretase (BACE1) not only abrogates A β generation and blocks amyloid deposition but also prevents neuron loss found in the cerebral cortex and subiculum, brain regions manifesting the most severe amyloidosis in 5XFAD mice. Importantly, BACE1 gene deletion also rescues memory deficits in 5XFAD mice. Our findings provide strong evidence that A β ultimately is responsible for neuron death in AD and validate the therapeutic potential of BACE1-inhibiting approaches for the treatment of AD.

Keywords

Alzheimer's disease; β -amyloid (A β); β -secretase; BACE1 knockout; Tg6799; gliosis; cognitive impairment; spontaneous alternation; Y-maze

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder and the most common cause of dementia among the elderly population, but no treatments addressing the underlying cause of disease have been developed. One of the hallmarks of AD is extensive neuronal death in the brain, approaching 90% loss in certain regions such as entorhinal cortex and nucleus basalis (for review, see Morrison and Hof, 1997). Although the precise mechanisms of AD and

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related cell loss and memory deficits are not fully determined, data support the hypothesis that amyloid- β (A β) peptides trigger a pathologic cascade ultimately leading to neuron death and cognitive impairment in AD (Hardy and Selkoe, 2002; LaFerla and Oddo, 2005; Selkoe and Schenk, 2003; Sisodia and St George-Hyslop, 2002; Turner et al., 2003). Mutations in the genes for amyloid precursor protein (APP) and presenilins 1/2 (PS1/2) cause familial AD (FAD) and increase production of the 42-amino acid form of A β (A β 42). Furthermore, A β kills neurons in culture (for review, see Yankner, 1996), and A β also appears neurotoxic *in vivo*. For example, when directly injected into the brains of aged primates, fibrillar A β causes neuron death around the injection site (Geula et al., 1998). However, the case for the role of A β in neuron death has been challenged because many APP transgenic mice that overproduce A β form amyloid plaques and develop memory deficits but do not lose significant numbers of neurons (Irizarry et al., 1997a; Irizarry et al., 1997b; for review, see McGowan et al., 2006). Recently, new APP/PS1 transgenic mouse lines developed by our group and others show considerable neuron loss in the hippocampus (Casas et al., 2004; Schmitz et al., 2004) or the cortex and subiculum (Oakley et al., 2006). These studies suggest that A β does kill neurons *in vivo*, although they cannot formally exclude the possibility that overexpression of APP and PS1 with multiple FAD mutations is the cause of neuron death in these mouse models.

β -Site APP cleaving enzyme 1 (BACE1) has been identified as the β -secretase, the protease that initiates cleavage of APP to generate pathogenic A β peptides (Hanin et al., 2000; Hussain et al., 1999; Sinha et al., 1999; Vassar et al., 1999; Yan et al., 1999). As the rate-limiting enzyme for A β production, BACE1 is a prime therapeutic target for lowering cerebral A β levels in AD. Targeted deletion of the BACE1 gene (BACE1^{-/-}) in mice abrogates the production of A β (Cai et al., 2001; Luo et al., 2001; Roberds et al., 2001). Moreover, APP transgenic mice with the BACE1 null genotype do not form amyloid plaques (Laird et al., 2005; Luo et al., 2003) and are prevented from developing A β -dependent memory deficits (Laird et al., 2005; Ohno et al., 2006; Ohno et al., 2004; for review, see Ohno, 2006). In the present study, we have used BACE1^{-/-} mice to demonstrate that A β , rather than mutant APP or PS1 overexpression, is the cause of neuron loss in our APP/PS1 transgenic mouse line, 5XFAD (Oakley et al., 2006; Ohno et al., 2006). We determined that BACE1-deficient 5XFAD (BACE1^{-/-}.5XFAD) mice do not have the amyloid plaques, astrogliosis or memory deficits found in age-matched 5XFAD mice with wild-type BACE1 genes. Most importantly, neuron death in the cerebral cortex and subiculum was prevented in the BACE1^{-/-}.5XFAD mice. To our knowledge, this is the first demonstration of genetic rescue of neuronal loss via ablation of BACE1, and consequently of A β , in an Alzheimer's transgenic mouse model and provides strong evidence that A β ultimately kills neurons *in vivo*.

Materials and methods

Animals

We used APP/PS1 doubly transgenic mice that co-express and co-inherit both human APP and PS1 transgenes with a total of five FAD mutations under transcriptional control of the neuron-specific mouse Thy-1 promoter [5XFAD mice, Tg6799 line; (Oakley et al., 2006; Ohno et al., 2006)]. In 5XFAD mice, an APP transgene carrying triple FAD mutations [the Swedish mutation: K670N, M671L (Mullan et al., 1992); the Florida mutation: I716V (Eckman et al., 1997); the London mutation: V717I (Goate et al., 1991)] and a PS1 transgene carrying double FAD mutations [M146L and L286V (Citron et al., 1998)] were co-injected into the pronucleus of single cell C57BL/6XSJL hybrid mouse embryos. 5XFAD transgenic lines were maintained by crossing heterozygous transgenic mice with B6/SJL F1 breeders. In order to examine the impact of the BACE1 null mutation, hemizygous 5XFAD mice were crossbred to BACE1^{-/-} mice with the BlkSw/129 background (Luo et al., 2001). The resultant F1 progeny were further intercrossed, yielding age-matched littermates with the genotypes of interest in a subset of the

F2 progeny. Genotyping was performed by PCR analysis of tail DNA. The following four genotypes were analyzed: BACE1^{+/+}.5XFAD⁻, BACE1^{+/+}.5XFAD⁺, BACE1^{-/-}.5XFAD⁻, and BACE1^{-/-}.5XFAD⁺. All experiments were done at 15–18 months of age, blind with respect to the genotype of the mice, and were conducted with the approval of the Northwestern University Animal Care and Use Committee.

Spontaneous alternation Y-maze test

Spontaneous alternation performance was tested as described previously (Ohno et al., 2004). Each mouse was placed in the center of the symmetrical Y-maze and was allowed to explore freely through the maze during an 8-min session. The sequence and total number of arms entered were recorded. Arm entry was considered to be complete when the hind paws of the mouse had been completely placed in the arm. Percentage alternation is the number of triads containing entries into all three arms divided by the maximum possible alternations (the total number of arms entered minus 2) X 100.

Histology and immunostaining

After behavioral tests were completed, mice were decapitated under halothane anesthesia. One hemibrain of each mouse was fixed in 4% paraformaldehyde solution in phosphate buffered saline (PBS) and cryoprotected in 30% sucrose solution in PBS containing 0.01% sodium azide. Brains were sectioned sagittally on a freezing microtome at 30 μ m and successive sections were stored in azide-PBS solution at 4°C. For estimating neuronal loss, cresyl violet staining was performed to visualize neurons. For immunohistochemical analysis, the sections were stained by the avidin-biotin peroxidase complex method as described previously (Oakley et al., 2006). Briefly, the sections were incubated overnight at room temperature with the following primary antibodies: rabbit anti-A β 42 (1:5,000; Biosource, Camarillo, CA) and rabbit anti-glia fibrillary acidic protein (GFAP) (1:10,000; G9269, Sigma, St Louis, MO). After washes, the sections were incubated with secondary biotinylated goat anti-rabbit IgG at 1:2,000 for 2 hr at room temperature. The ABC kit (Vector Laboratories, Burlingame, CA) was utilized with 3,3'-diaminobenzidine tetrahydrochloride as a chromogen to visualize the reaction product. The sections were then mounted on charged slides, counterstained with hematoxylin, dehydrated in a series of alcohol, cleared in xylene, and covered with a coverslip. Light microscopy was conducted on a Nikon E800 microscope with a Spot Advanced digital camera for capturing images.

Immunoblotting

After behavioral tests were completed, hemibrain samples were taken from the mice under halothane anesthesia and were snap-frozen for immunoblot assays. Hemibrains were homogenized in 1% Triton X-100, PBS, protease inhibitor cocktail (Calbiochem, La Jolla, CA), and Halt Phosphatase Inhibitor Cocktail (Pierce, Rockford, IL). Following brief sonication, homogenates were diluted 1:1 with sample boiling buffer (60 mM Tris, 10% glycerol, 5% SDS), pH 6.8 and 5% loading dye, and boiled for 5 min. Equal amounts of protein (15 μ g) were resolved on 4–12% Bis-Tris NuPAGE gels (Invitrogen, Carlsbad, CA), transferred onto PVDF membranes, and blocked in 5% nonfat milk in Tris-buffered saline containing 0.05% Tween 20. For A β immunoblots, as a positive control 100 ng of synthetic A β 40 (Invitrogen, Carlsbad, CA) dissolved in distilled H₂O was electrophoresed along with brain samples. Membranes were probed with primary antibodies and incubated with HRP-conjugated secondary after washing. The following antibodies were used: anti-total A β (1:10,000; 6E10, Signet, Dedham, MA), anti-APP (1:5000; 22C11, Chemicon, Temecula, CA), anti-BACE1 (1:1000; BACE1-Cat, J. Zhao, L. Binder, R. Vassar, unpublished), and anti-actin (1:10,000; AC-15, Sigma, St. Louis, MO). Immunoblot signals were detected by

enhanced chemiluminescence plus (Amersham Biosciences, Arlington Heights, IL) and quantified using an Eastman Kodak Image Analyzer (Rochester, NY).

Statistical analysis

The significance of differences between the groups was determined by a one-way ANOVA, and *post-hoc* Fisher's PLSD tests were performed when appropriate.

Results

APP transgenic mouse models recapitulate several features of AD, such as amyloid pathology, synaptic dysfunction, and behavioral deficits, but there has been little demonstration of extensive neuronal loss in these models (Ashe, 2001; Dodart et al., 2002; German and Eisch, 2004; Irizarry et al., 1997a; Irizarry et al., 1997b; Janus and Westaway, 2001; Kobayashi and Chen, 2005; McGowan et al., 2006). We recently developed a novel APP/PS1 doubly transgenic mouse line [5XFAD mice, Tg6799 line; (Oakley et al., 2006; Ohno et al., 2006)] that harbors APP with three intramolecular FAD mutations (Swedish, Florida, and London mutations) and PS1 with two intramolecular FAD mutations (M146L and L286V). When expressed in combination, these five FAD mutations act together to additively increase A β 42 production. As a result, 5XFAD mice exhibit dramatically accelerated A β 42 accumulation, start to develop visible amyloid deposition in the deeper layer of the cerebral cortex (layer 5) and the subiculum at ~2 months of age, and have memory impairments in several behavioral paradigms beginning at ~4 months of age (Oakley et al., 2006; Ohno et al., 2006). In addition, at ~9 months of age, 5XFAD mice show pronounced loss of large pyramidal neurons in cortical layer 5 and subiculum, the same regions that have the greatest amyloid burden.

We hypothesized that neuron loss in 5XFAD brain was the result of A β 42-induced neurotoxicity. However, we could not exclude the possibility that abnormal interactions among the five FAD mutations in APP and PS1 or overexpression of the mutant transgenes, rather than A β 42 itself, led to the neuron death in 5XFAD mice. To address this problem, we crossed BACE1^{-/-} mice with 5XFAD transgenic mice to abrogate A β generation without blocking APP and PS1 transgene expression. Microscopic examination of cresyl violet-stained brain sections revealed that most of the large pyramidal neurons were lost in cortical layer 5 (Fig. 1C,D) and subiculum (Fig. 2C,D) of 5XFAD mice at 18 months of age, in agreement with our previous study (Oakley et al., 2006). The loss of large pyramidal neurons in 5XFAD cortex and subiculum was clearly obvious by qualitative visual inspection of micrographs, thus making it unnecessary to perform cell counting. Neurons in other cortical layers, hippocampus (including CA1–4 and dentate gyrus), and elsewhere in the brain of 5XFAD mice appeared intact by visual inspection, although we cannot exclude the possibility of less obvious cell loss in other brain regions. We also noted that 5XFAD brain sizes appeared normal.

In contrast to 5XFAD mice, BACE1^{-/-} mice appeared to have normal numbers of large pyramidal neurons both in the cortex (Fig. 1B) and in the subiculum (Fig. 2B), which were indistinguishable from those of wild-type controls (Figs. 1A and 2A), and no overt neural lesions were observed in other BACE1^{-/-} brain regions (Luo et al., 2001). Most importantly, large pyramidal neurons in cortical layer 5 (Fig. 1E,F) and subiculum (Fig. 2E,F) of BACE1-deficient 5XFAD (BACE1^{-/-}·5XFAD) mice were well preserved throughout successive sections and were comparable in number to those of wild-type controls. Furthermore, as noted in our previous study (Oakley et al., 2006), 5XFAD mice had a significantly thinner cortical layer 1 as compared with wild-type controls (data not shown), most likely reflecting the loss of layer 5 neuron dendrites that project to and ramify in layer 1. We also observed that this dendritic loss was prevented in BACE1^{-/-}·5XFAD mice, which showed layer 1 thickness comparable to controls (data not shown). Together, these findings provide compelling evidence

that elimination of A β via BACE1 deletion prevents neuronal loss found in the 5XFAD mouse model, even though FAD mutant transgene expression remains intact.

It has recently been reported that the expression and activity of β -secretase/BACE1 are significantly elevated in AD brain (Fukumoto et al., 2002; Li et al., 2004; Yang et al., 2003), raising the possibility that increased BACE1 levels may be causally involved in the pathogenesis of AD. Immunoblot analysis demonstrated that BACE1 levels were significantly increased in 5XFAD mouse brains as compared with wild-type controls [$F_{1,8} = 15.34$, $P < 0.01$] (Fig. 3A,B; J. Zhao, L. Binder, and R. Vassar, unpublished observations), validating 5XFAD transgenic mice as useful animal models that recapitulate the BACE1 elevation in AD. A very strong signal in 5XFAD brain homogenate was observed for A β (Fig. 3A), which is usually very difficult to detect by immunoblot, illustrating the extremely high cerebral A β 42 levels found in this mouse model (Oakley et al., 2006; Ohno et al., 2006). We confirmed that BACE1 null mutation completely abolished BACE1 protein and that A β peptides were not detectable in BACE1^{-/-}·5XFAD bigenic mouse brains by immunoblot (Fig. 3A). Interestingly, BACE1^{-/-}·5XFAD mice exhibited APP overexpression that was significantly higher than that of 5XFAD [$F_{3,16} = 95.28$, $P < 0.01$] (Fig. 3A,C), indicating that the full-length APP substrate accumulates in the brain if β -secretase cleavage of APP is ablated in 5XFAD mice.

Immunostaining with an A β 42 end-specific antibody revealed that 5XFAD mice had massive amyloid deposition in the cerebral cortex and subiculum at 18 months of age (Fig. 4C,G). Consistent with our previous study (Oakley et al., 2006), amyloid plaques spread to fill much of the cortex and other hippocampal regions by this age and were also observed in the thalamus, olfactory bulb, and brain stem, although deposition was less severe in these regions (data not shown). Importantly, A β deposition was completely abolished in all brain regions, including the cortex and subiculum, of BACE1^{-/-}·5XFAD mice (Fig. 4D,H), which were indistinguishable from wild-type control mice (Fig. 4A,E) and BACE1^{-/-} mice (Fig. 4B,F). Amyloid plaques in APP transgenic mouse brains are often accompanied by glial activation indicative of inflammatory processes reminiscent of AD (Akiyama et al., 2000; Dodart et al., 2002). Immunostaining for the astrocyte marker GFAP revealed that 5XFAD mice had robust astrogliosis in the cerebral cortex (Fig. 5C). In contrast, there was no evidence of astrogliosis in brains of BACE1^{-/-}·5XFAD mice (Fig. 5D) as well as in those of wild-type (Fig. 5A) or BACE1^{-/-} (Fig. 5B) mice, demonstrating that the accumulation of A β peptides causes glial activation in APP/PS1 transgenic mice. Taken together, these results show that BACE1 deletion completely prevents AD-related neuropathology, including amyloidosis, gliosis, and neuron death found in the 5XFAD transgenic mouse model.

To investigate the relationship between neuronal loss and memory deficits, we applied the spontaneous alternation Y-maze test at 15–18 months of age to the four genotypic groups of mice obtained by crossbreeding BACE1^{-/-} and 5XFAD mice (Fig. 6). This behavioral assay does not involve any training, reward, or punishment, and allows us to assess spatial working memory. ANOVA revealed significant differences in percent alternation between the groups [$F_{3,38} = 9.17$, $P < 0.01$]. 5XFAD transgenic mice showed dramatically reduced spontaneous alternation performance in the Y-maze as compared with wild-type controls ($P < 0.01$) (Fig. 6A). Consistent with our previous report (Ohno et al., 2004), BACE1 gene deletion by itself in mice resulted in decreased percent alternation in this task ($P < 0.01$). Importantly, the spatial working memory deficits were rescued to wild-type control levels in BACE1^{-/-}·5XFAD mice, which showed higher spontaneous alternation than 5XFAD mice ($P < 0.01$) and BACE1^{-/-} mice ($P < 0.05$). The total number of arm entries in the alternation Y-maze test was significantly increased in BACE1^{-/-} mice [$F_{3,38} = 5.76$, $P < 0.01$], suggesting that BACE1 null mutants exhibit increased levels of exploratory activity in the Y-maze (Fig. 6B).

Discussion

It is well established that A β kills neurons in culture (Pike et al., 1991; Roher et al., 1991; Yankner et al., 1990a; Yankner et al., 1989; Yankner et al., 1990b), but the link between A β and neuron loss *in vivo* has been equivocal. Cerebral injection of A β preparations into primates and rodents has produced variable effects, with some experiments demonstrating neurodegenerative changes (Frautschy et al., 1991; Geula et al., 1998; Kowall et al., 1991), while others yielding negative results (Podlisny et al., 1993). Moreover, most transgenic mice that express human APP with FAD mutations and overproduce A β lack significant cell loss (for review, see McGowan et al., 2006). For example, no significant neuron loss is reported in the hippocampus or cerebral cortex of Tg2576 and PDAPP mice (Irizarry et al., 1997a; Irizarry et al., 1997b). A small reduction in neuron number occurs in the hippocampal CA1 region of APP23 mice, but no loss is observed in the cortex in this model (Calhoun et al., 1998).

The lack of consistent results among the various earlier reports has cast some doubt upon the role of A β in neuron death in AD. However, recent work has begun to strengthen the connection between A β and neurodegeneration. For example, A β reduction by active or passive A β immunization protects against synaptic degeneration as determined by inhibition of progressive loss of synaptophysin in the hippocampus and cortex of PDAPP transgenic mice (Buttini et al., 2005). Moreover, potential toxic effects of intraneuronal A β or soluble A β oligomers have come to light and may be involved in neurodegeneration (Calhoun et al., 1998; Casas et al., 2004; Dickson, 2004; Schmitz et al., 2004; Urbanc et al., 2002). We have previously shown that 5XFAD transgenic mice have increased levels of the pro-neurodegeneration factor p25, exhibit decreased levels of the synaptic markers synaptophysin, syntaxin and PSD-95, and manifest a unique spatial pattern of large pyramidal neuron loss that occurs in cortical layer 5 and subiculum (Oakley et al., 2006). It is important to note that these same brain regions of 5XFAD have the highest levels of intraneuronal A β accumulation and extracellular amyloid plaques, both of which label with thioflavin S and therefore consist of β -pleated sheet aggregates. Our results are similar to those with another APP/PS1 transgenic mouse line having intraneuronal A β and marked death in the hippocampal CA1/2 pyramidal cell layer (Casas et al., 2004; Schmitz et al., 2004). 5XFAD brain also shows elevated levels of soluble A β oligomers (Ohno et al., 2006). Most importantly, the present study demonstrates that neuron loss as well as intraneuronal and extracellular A β deposition are prevented in brains of BACE1^{-/-} 5XFAD mice. Notably, APP or PS1 transgene expression was not decreased by BACE1^{-/-} genotype in this study as well as in our previous investigation (Ohno et al., 2006), suggesting that mere overexpression of FAD mutant APP and PS1 does not contribute to cell loss in 5XFAD mice. BACE1 deficiency also abolishes soluble oligomeric A β assemblies in 5XFAD mice (Ohno et al., 2006). Although it is difficult to unequivocally determine the form of A β that is responsible for neurodegeneration, our investigation provides direct evidence that A β , or a BACE1 cleavage fragment of APP, is ultimately responsible for neuronal loss in 5XFAD mice.

Since 5XFAD mice generate A β 42 almost exclusively (Oakley et al., 2006; Ohno et al., 2006), it is very likely that A β 42 was the trigger of the neuronal loss that we observed. However, we cannot completely exclude the possibility that neurotoxicity in 5XFAD mice may be caused by build-up of β -secretase-cleaved C-terminal fragment C99, which is also eliminated by BACE1^{-/-} genotype in APP and APP/PS1 transgenic mouse brains (Laird et al., 2005; Luo et al., 2001). Transgenic overexpression of the potentially amyloidogenic C99 results in progressive neurodegeneration in the cerebral cortex and hippocampus and causes memory deficits (Berger-Sweeney et al., 1999; Lee et al., 2006; Nalbantoglu et al., 1997). Furthermore, C99 has been shown to impair learning and memory when it is centrally administered (Choi et al., 2001; Song et al., 1998). Therefore, it is reasonable to speculate that both amyloidogenic proteins, A β and C99, may contribute to cell death and memory deficits in 5XFAD mice.

We have previously reported that BACE1 gene deletion and consequent reductions in A β prevent deficits in olfactory, temporal, and spatial (working and reference) memories in young plaque-free Tg2576 transgenic mice (Ohno et al., 2004) or plaque-bearing 5XFAD mice (Ohno et al., 2006). Consistent with these results, other groups have shown that lowering BACE1 levels *in vivo* by null mutation or lentiviral delivery of small interfering RNA ameliorates amyloid pathology and spatial memory impairments in APP or APP/PS1 transgenic mice (Laird et al., 2005; Singer et al., 2005; for review, see Ohno, 2006). Here, we show that BACE1 deletion also rescues memory deficits in 5XFAD mice at advanced age, when animals with BACE1^{+/+} genotype have developed dramatic loss of large pyramidal neurons in cortical layer 5 and subiculum. In this regard, it is interesting to note that neuron loss is also observed in both of these regions in AD brain (Bobinski et al., 1997; Giannakopoulos et al., 1996). Importantly, it is plausible that ablation of these neurons contributes to memory decline, since layer 5 pyramidal neurons receive the highest density of cholinergic innervation (Houser et al., 1985) and greater atrophy in the subiculum (which is part of the hippocampus) is associated with increased risk for conversion from mild cognitive impairment to AD (Apostolova et al., 2006). Our finding that BACE1 deletion prevents neuronal loss, amyloidosis, astrogliosis, and spatial working memory deficits in the Y-maze in 5XFAD mice of advanced age suggests a beneficial effect of BACE1 inhibition and A β reduction on later phases of cognitive impairments and AD-related pathologies. Inducible genetic and pharmacologic strategies to block BACE1 will be important for testing the reversibility of A β -dependent cognitive deficits by suppressing BACE1 at different stages of amyloid pathology and determining the extent and duration of ameliorative effects of BACE1 inhibition following treatment.

While cognitive rescue by BACE1 ablation is consistent across different transgenic mouse models of AD and various behavioral assays, BACE1^{-/-} genotype by itself affects memory performance in some learning paradigms. The present study as well as others (Laird et al., 2005; Ohno et al., 2004) demonstrates that BACE1^{-/-} mice are impaired in spatial working memory, as assessed by the spontaneous alternation Y-maze test. Spatial reference memory in a Morris water maze is also significantly impaired in BACE1^{-/-} mice (Laird et al., 2005; Ohno et al., 2006). In contrast, BACE1 deletion does not significantly impair olfactory memory assessed by the social recognition test (Ohno et al., 2004) or temporal associative memory assayed by trace fear conditioning (Ohno et al., 2006). It is therefore interesting to speculate that a BACE1-dependent mechanism may play a role in certain types of cognition, especially spatial components of learning and memory (for review, see Ohno, 2006). Since a variety of possible BACE1 physiological substrates have been reported in addition to APP [e.g., α -2,6-sialyltransferase (Kitazume et al., 2001), P-selectin glycoprotein ligand-1 (Lichtenthaler et al., 2003), β -subunits of voltage-gated sodium channels (Wong et al., 2005), low density lipoprotein receptor-related protein (von Arnim et al., 2005), and neuregulin-1 (Willem et al., 2006)], we cannot exclude the possibility that changes in the proteolytic products of these substrates may contribute to cognitive deficits in BACE1^{-/-} mice.

In the present study, we show that both 5XFAD and BACE1^{-/-} mice have spatial memory impairments, while BACE1^{-/-}·5XFAD bigenic mice are rescued from these cognitive deficits. Similar results were observed in our previous study analyzing Tg2576, BACE1^{-/-}, and BACE1^{-/-}·Tg2576 bigenic mice (Ohno et al., 2004) and in another investigation (Laird et al., 2005) (for review, see Ohno, 2006). Numerous studies suggest that the memory impairments of APP transgenic mice are caused by excessive levels of cerebral A β (for review, see McGowan et al., 2006), but the deficits of BACE1^{-/-} mice and the rescue of BACE1^{-/-}·APP bigenic mice are more difficult to explain. Genetic ablation of BACE1 in Tg2576 lowered cerebral A β level to only a few percent of that observed in BACE1^{+/+}·Tg2576 mice (Ohno et al., 2004), clearly demonstrating that BACE1 is the predominant β -secretase enzyme responsible for A β generation in the brain. However, a residual amount of brain A β was measured in BACE1^{-/-}·Tg2576 mice that was approximately equivalent to the level of

endogenous A β observed in non-transgenic wild-type brain. Endogenous levels of A β are normally quite low and difficult to detect in non-transgenic mouse brain, so only slight leakiness of A β generation in BACE1^{-/-}·Tg2576 brain would be required to produce the residual levels of A β that we measured. Importantly, A β levels in both BACE1^{-/-}·Tg2576 and wild-type brains were significantly higher than the background levels of A β found in BACE1^{-/-} monogenic brains. From this evidence, we hypothesized that an optimal level of A β was necessary to maintain normal memory function (Ohno et al., 2004). This notion is also consistent with the observation that spatial memory is impaired in APP-deficient mice (Dawson et al., 1999). Our results imply that both excessive (e.g., AD and APP transgenic) and deficient (e.g., BACE1^{-/-} and APP^{-/-}) A β levels may impair cognition. Although we have not quantified A β levels in our current study, we speculate that residual levels of A β in the brains of BACE1^{-/-}·5XFAD mice may be responsible for the behavioral rescue of these bigenic animals.

The enzyme responsible for generating residual A β in BACE1^{-/-}·APP bigenic mice is unknown. In our previous study (Ohno et al., 2004), we measured cerebral A β levels with an A β ELISA that detected endogenous mouse and transgenic human A β with equal sensitivity and that did not discriminate among A β peptides with heterogeneous N-termini. We have shown that BACE1 is responsible for generating the major A β peptides starting at Asp1 and Glu11 (Vassar et al., 1999). Work from Selkoe and colleagues identified minor A β forms beginning at Val-3 and Ile-6, demonstrated that these peptides are not made by β -secretase, and showed that inhibition of β -secretase activity in cells leads to elevated production of these minor A β species (Haass et al., 1995; Citron et al., 1996). Unlike Asp1 and Glu11 A β , the Val-3 and Ile-6 A β are not increased upon BACE1 overexpression (Vassar et al., 1999), indicating that they are indeed generated by a different enzyme(s). We speculate that the residual levels of A β measured in BACE1^{-/-}·Tg2576 brain (Ohno et al., 2004) represented A β peptides beginning at Val-3 and Ile-6. Genetic ablation of BACE1 would increase the generation of Val-3 and Ile-6 A β , similar to the effects of β -secretase inhibition (Haass et al., 1995; Citron et al., 1996). Moreover, transgenic overexpression of APP would provide greater levels of substrate for cleavage at Val-3 and Ile-6. Given these arguments, it appears likely that a small amount of residual A β beginning at Val-3 and Ile-6 would be generated in BACE1^{-/-}·5XFAD brains, thus providing an explanation for the rescue of memory deficits in these mice. Alternatively, BACE2, which also cleaves at Asp1 (but with lower efficiency than BACE1) and is expressed in the brain (but at much lower levels than BACE1), may play a role in producing residual A β in BACE1^{-/-}·5XFAD brain. Consistent with recent evidence suggesting potential physiological roles for A β peptides (Esteban, 2004; Kamenetz et al., 2003; Pearson and Peers, 2006; Plant et al., 2006; Yu et al., 2001), our findings favor the idea that normal levels of A β may be necessary for neuronal functions involved in learning and memory, although further investigation is required to elucidate potential underlying mechanisms.

In summary, the results presented here strongly support the hypothesis that A β is neurotoxic and initiates the pathogenic cascade leading to neuronal loss in AD. We conclude that inhibition of the β -secretase, BACE1, should prove beneficial as a disease-modifying therapeutic strategy to counter the cell death and cognitive impairments of this intractable neurodegenerative disorder.

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Fig 1.

BACE1 null mutation prevents neuronal loss in the cerebral cortex of 5XFAD transgenic mice. Sagittal brain sections from wild-type control (A), BACE1^{-/-} (B), 5XFAD (C,D), and BACE1^{-/-}·5XFAD (E,F) mice at 18 months of age were stained with cresyl violet. Two different mice are presented for each of the 5XFAD and BACE1^{-/-}·5XFAD genotypes. Shown are photomicrographs of cortical layer 5 (A₁–F₁: areas between the dashed lines) and higher magnification of pyramidal neurons in areas identified by respective rectangles (A₂–F₂). Note that large pyramidal neurons are nearly completely lost in layer 5 of 5XFAD mice as compared with the other three genotypes. In contrast, large pyramidal neurons are rescued to wild-type levels in BACE1^{-/-}·5XFAD brains. Scale bar = 200 μm in A₁–F₁; 50 μm in A₂–F₂.

Fig 2.

BACE1 null mutation prevents neuronal loss in the subiculum of 5XFAD transgenic mice. Sagittal brain sections from wild-type control (A), BACE1^{-/-} (B), 5XFAD (C,D), and BACE1^{-/-}·5XFAD (E,F) mice at 18 months of age (the same brains shown in Fig. 1) were stained with cresyl violet. Two different mice are presented for each of the 5XFAD and BACE1^{-/-}·5XFAD genotypes. Shown are photomicrographs of the subiculum (A₁–F₁: areas within dashed ovals) and respective higher magnification images (A₂–F₂). Note that large pyramidal neurons are significantly reduced in number in subiculum of 5XFAD mice as compared with the other three genotypes; numbers of glia are increased, as indicated by the high density of small cresyl violet-stained nuclei. In contrast, the quantity of large pyramidal

neurons in subiculum of $BACE1^{-/-}$.5XFAD brains appears similar to that of wild-type brains, demonstrating rescue of neuron loss by BACE1 gene deletion. Scale bar = 100 μm in A₁–F₁; 50 μm in A₂–F₂.

Fig 3.

Effects of BACE1 null mutation on cerebral Aβ, BACE1 and full-length APP levels in 5XFAD transgenic mice. (A) Immunoblot analyses of protein extracts (15 μg/lane) from hemibrain homogenates of wild-type control, 5XFAD, BACE1^{-/-}·5XFAD, and BACE1^{-/-} mice at 18 months of age. Shown are immunoblots for Aβ (top panels; anti-total Aβ antibody 6E10), APP (second from top panels; anti-mouse and human APP antibody 22C11), BACE1 (third from top panels; anti-BACE1 antibody BACE1-Cat), and actin (bottom panels; anti-actin antibody AC-15). 5XFAD brain homogenates show a strong Aβ band, while Aβ is below the level of detection by immunoblot analysis in brain extracts from the other three genotypes. The lane labeled “Aβ40” contains 100 ng of synthetic Aβ40 for use as a positive control; the two slower migrating bands (arrowheads) likely represent multimeric Aβ assemblies. (B) Quantification of BACE1 blots in which band intensities were measured by phosphorimaging and expressed as percentage of wild-type control levels. Note that BACE1 levels are significantly elevated in 5XFAD brains. (C) Quantification of APP blots in which band intensities were measured by phosphorimaging and expressed as percentage of wild-type control levels. Note that while 5XFAD mice overexpress human APP approximately fourfold relative to endogenous mouse APP protein, levels of APP are elevated even further in BACE1^{-/-}·5XFAD mice. Data are presented as the mean ± SEM of 5 animals. ***P* < 0.01 (vs. wild-type controls), ##*P* < 0.01 (vs. BACE1^{-/-}·5XFAD).

Fig 4.

BACE1 null mutation prevents amyloid deposition in 5XFAD transgenic mice. Sagittal brain sections from wild-type control (A,E), BACE1^{-/-} (B,F), 5XFAD (C,G) and BACE1^{-/-};5XFAD (D,H) mice at 18 months of age were immunostained with antibody specifically recognizing the Aβ42 C-terminus and counterstained with hematoxylin. Shown are photomicrographs of the layers of the cerebral cortex (A–D) and subiculum (E–H). Note that 5XFAD sections have robust Aβ42 staining in both brain regions, while BACE1^{-/-};5XFAD brain is protected from Aβ42 accumulation. Scale bar = 200 μm in A–D; 100 μm in E–H.

Fig 5.

BACE1 null mutation prevents gliosis in 5XFAD transgenic mice. Sagittal sections of cerebral cortex from wild-type control (A), BACE1^{-/-} (B), 5XFAD (C) and BACE1^{-/-}·5XFAD (D) mice at 18 months of age were immunostained for astrocyte marker GFAP and counterstained with hematoxylin. Note that 5XFAD cortex shows extensive astrogliosis, while BACE1^{-/-}·5XFAD brain has control levels of astrocytes. Scale bar = 200 μm.

Fig 6.

BACE1 null mutation prevents memory deficits in 5XFAD transgenic mice. (A) Spatial working memory of mice at 15–18 months of age was tested by spontaneous alternation performance in the Y-maze. BACE1^{-/-}·5XFAD mice are rescued completely back to wild-type levels of alternation in the Y-maze. Note also that both BACE1^{-/-} mice and 5XFAD mice show significantly lower levels of alternation performance as compared to wild-type control and BACE1^{-/-}·5XFAD mice, although the deficit for 5XFAD tends to be more severe. (B) Total number of arm entries indicates that BACE1^{-/-} mice show significantly higher levels of exploratory activity in the Y-maze as compared to wild-type control mice. Data are presented as the mean ± SEM of 7–14 animals. ** $P < 0.01$ (vs. wild-type controls), # $P < 0.05$, ## $P < 0.01$ (vs. BACE1^{-/-}·5XFAD).