Neuron, Vol. 41, 27–33, January 8, 2004, Copyright ©2004 by Cell Press

Report

BACE1 Deficiency Rescues Memory Deficits and Cholinergic Dysfunction in a Mouse Model of Alzheimer's Disease

Masuo Ohno,[1,*] Evgeny A. Sametsky,[1] Linda H. Younkin,[3] Holly Oakley,[2] Steven G. Younkin,[3] Martin Citron,[4] Robert Vassar,[2,*] and John F. Disterhoft[1,*]
[1]Department of Physiology
[2]Department of Cell and Molecular Biology
Northwestern University Feinberg School of Medicine
Chicago, Illinois 60611
[3]Mayo Clinic Jacksonville
Jacksonville, Florida 32224
[4]Department of Neuroscience
Amgen Inc.
Thousand Oaks, California 91320

Summary

β-site APP cleaving enzyme 1 (BACE1) is the β-secretase enzyme required for generating pathogenic β-amyloid (Aβ) peptides in Alzheimer's disease (AD). BACE1 knockout mice lack Aβ and are phenotypically normal, suggesting that therapeutic inhibition of BACE1 may be free of mechanism-based side effects. However, direct evidence that BACE1 inhibition would improve cognition is lacking. Here we show that BACE1 null mice engineered to overexpress human APP ($BACE1^{-/-}$·$Tg2576^{+}$) are rescued from Aβ-dependent hippocampal memory deficits. Moreover, impaired hippocampal cholinergic regulation of neuronal excitability found in the Tg2576 AD model is ameliorated in $BACE1^{-/-}$·$Tg2576^{+}$ bigenic mice. The behavioral and electrophysiological rescue of deficits in $BACE1^{-/-}$·$Tg2576^{+}$ mice is correlated with a dramatic reduction of cerebral Aβ40 and Aβ42 levels and occurs before amyloid deposition in Tg2576 mice. Our gene-based approach demonstrates that lower Aβ levels are beneficial for AD-associated memory impairments, validating BACE1 as a therapeutic target for AD.

Introduction

In its original conception, the amyloid cascade hypothesis postulates that amyloid plaques, composed of deposited aggregates of the β-amyloid (Aβ) peptide, are central to the pathogenesis of Alzheimer's disease (AD) (Hardy and Selkoe, 2002; Selkoe and Schenk, 2003; Turner et al., 2003). However, recent studies have begun to implicate small soluble assemblies of Aβ peptides as potential neurotoxic species in AD (Dodart et al., 2002; Hardy and Selkoe, 2002; Selkoe, 2002; Walsh et al., 2002), and thus the amyloid cascade hypothesis has been broadened to include so-called Aβ-derived diffusible ligands (ADDLs) and protofibrils of Aβ (Gong et al., 2003; Hartley et al., 1999; Lambert et al., 1998). Regardless of the form, size, and solubility of Aβ polymers, a large body of evidence suggests that Aβ plays a causal role in AD pathophysiology.

Amyloid precursor protein (APP) transgenic mice recapitulate many aspects of AD pathology including Aβ amyloidosis and related cognitive deficits and are thus used to evaluate novel therapeutic interventions for AD (Ashe, 2001; Janus and Westaway, 2001; Wong et al., 2002). Although these mouse models allow us to gather much evidence in support of the amyloid hypothesis, rigorous in vivo demonstration that Aβ is directly responsible for AD-associated cognitive impairment has been difficult to obtain. For example, in mice overexpressing mutant APP transgenes, it has not been possible to unequivocally prove whether transgene-dependent memory deficits are due to excessive cerebral Aβ generation or mutant APP overexpression. Some of the recent Aβ immunization studies showing behavioral improvement in APP transgenic mice suggest that Aβ may be deleterious to memory, but the mechanisms underlying the effects of Aβ immunization are not fully understood (Dodel et al., 2003; Schenk, 2002; Selkoe and Schenk, 2003).

Aβ is proteolytically generated from APP by the sequential action of two enzymes, the β- and γ-secretases (Hardy and Selkoe, 2002; Selkoe and Schenk, 2003; Turner et al., 2003; Vassar and Citron, 2000). BACE1 has been identified as a membrane bound aspartyl protease that fulfills all known criteria for β-secretase (Hussain et al., 1999; Lin et al., 2000; Sinha et al., 1999; Vassar et al., 1999; Yan et al., 1999) and thus is a prime therapeutic target (Citron, 2002; Selkoe and Schenk, 2003; Wolfe, 2002). Although the development of potent and specific small-molecule BACE1 inhibitors is being vigorously pursued (Citron, 2002; John et al., 2003; Tang et al., 2003), pharmacological strategies for inhibiting β-secretase in vivo are currently lacking.

We have taken a gene-targeting approach to determine the therapeutic potential of BACE1 inhibition for ameliorating AD-associated cognitive deficits and to rigorously test the amyloid cascade hypothesis in an in vivo model of AD. Knocking out the BACE1 gene in mice abrogates the formation of cerebral Aβ and amyloid deposits, and the mice remain healthy, fertile, and have no obvious neurological abnormalities (Cai et al., 2001; Luo et al., 2001, 2003; Roberds et al., 2001). These results suggest that BACE1 inhibition is a valid therapeutic strategy for AD and may not encounter severe mechanism-based toxicity. In this study, we crossed BACE1 null mutant ($BACE1^{-/-}$) mice (Luo et al., 2001) with transgenic mice that overexpress human APP-695 with the Swedish familial mutation ($Tg2576^{+}$) (Hsiao et al., 1996). We demonstrate that reduction of Aβ levels by BACE1 gene deletion can prevent learning and memory impairment and hippocampal cholinergic dysfunction in the Tg2576 model of AD. Since $BACE1^{-/-}$·$Tg2576^{+}$ bigenic mice still overexpress mutant APP, we conclude that excess Aβ, rather than APP overexpression, is directly responsible for the cognitive and electrophysiological abnormalities found in Tg2576 monogenic mice. These results strongly support the amyloid cascade hypothe-

*Correspondence: ohno@northwestern.edu (M.O.), r-vassar@northwestern.edu (R.V.), jdisterhoft@northwestern.edu (J.F.D.)

sis and show for the first time the beneficial effects of BACE1 (β-secretase) inhibition on AD-associated cognitive deficits.

Results

Rescue of Memory Deficits in BACE1$^{-/-}$·Tg2576^{+} Mice

Recent studies have focused on soluble Aβ assemblies, rather than insoluble Aβ fibrils, as potential pathogenic factors in AD (Dodart et al., 2002; Lambert et al., 1998; Selkoe, 2002; Walsh et al., 2002). Moreover, some aspects of the behavioral phenotypes of Tg2576 mice precede amyloid plaque formation (beginning at 9–12 months of age) (Dineley et al., 2002; Holcomb et al., 1998; Westerman et al., 2002). It has been unclear from these studies whether the memory deficits of APP transgenic mice result from excessive Aβ or APP overexpression in the brain. To address these questions, we tested Tg2576^{+}, BACE1$^{-/-}$·Tg2576^{+} bigenic, BACE1$^{-/-}$ monogenic, and wild-type littermates at 4–6 months of age, when Tg2576 mice lack histologically visible amyloid plaques but have high cerebral Aβ levels (Kawarabayashi et al., 2001).

First, we analyzed mice with a hippocampus-dependent social recognition task (Kogan et al., 2000), which tests the ability of mice to identify and remember conspecifics (specific details of an individual) and relies mostly on olfaction in rodents (Ferguson et al., 2002). In this task, a repeated exposure to the same juvenile results in decreased duration of investigation behavior, and thus the difference in investigation time between the first and second exposure can be used as an index of social recognition memory (Ferguson et al., 2002). The investigation times during the first exposure trial were not significantly different between the four groups tested [ANOVA $F(3,64) = 1.42$, $p = 0.25$; wild-type, 48.5 ± 3.3 s, mean ± SEM, n = 20; BACE1$^{-/-}$, 52.3 ± 4.7 s, n = 19; Tg2576^{+}, 43.5 ± 4.5 s, n = 19; BACE1$^{-/-}$·Tg2576^{+}, 58.4 ± 8.1 s, n = 10]. Percent investigation time during the second exposure trial relative to the first trial was significantly different between the groups [$F(3,64) = 4.07$, $p < 0.01$]. Post hoc Fisher's PLSD test revealed that social recognition memory, which was assessed by exposing the mice to the same juvenile 3 hr later, was significantly impaired in Tg2576^{+} mice ($p < 0.01$) (Figure 1A). Interestingly, BACE1$^{-/-}$·Tg2576^{+} bigenic mice performed significantly better than did Tg2576^{+} mice ($p < 0.05$) and were equivalent to wild-type littermates in this social recognition task.

Next, we tested memory with another hippocampus-dependent learning task, spontaneous alternation in the Y maze (Lalonde, 2002), which is relatively sensitive to early-onset cognitive deficits in Tg2576 mice (Holcomb et al., 1998, 1999). ANOVA revealed significant differences in percent alternation between the groups [$F(3,68) = 6.85$, $p < 0.01$]. Consistent with previous reports (Holcomb et al., 1998, 1999), Tg2576^{+} mice showed reduced spontaneous alternation performance in the Y maze at 4–6 months ($p < 0.01$) (Figure 1B), an age before substantial Aβ deposition is apparent (Kawarabayashi et al., 2001). Importantly, the spatial working memory deficit in the Y maze test was rescued to wild-type control level in the BACE1$^{-/-}$·Tg2576^{+} bigenic mice ($p < 0.01$). In contrast to social recognition, the BACE1 null mutation by itself moderately but significantly affected spontaneous alternation behavior ($p < 0.05$). The total number of arm entries in the alternation Y maze test, a measure of exploratory activity, was increased [$F(3,68) = 5.83$, $p < 0.01$] both in BACE1$^{-/-}$ monogenic ($p < 0.05$) and in BACE1$^{-/-}$·Tg2576^{+} bigenic mice ($p < 0.01$) (Figure 1C). This increased level of exploration is not likely to be responsible for the impaired alternation performance of BACE1$^{-/-}$ mice, since BACE1$^{-/-}$·Tg2576^{+} mice were normal in spontaneous alternation behavior even though they also exhibited increased exploration.

Reduction in Cerebral Aβ Improves Memory in BACE1$^{-/-}$·Tg2576^{+} Mice

After memory testing, sandwich ELISA assays were performed to correlate genotypes and behavioral phenotypes with Aβ levels in the brain. Consistent with our previous results (Luo et al., 2001), cerebral Aβ generation was nearly abolished in the BACE1$^{-/-}$·Tg2576^{+} bigenic mice as compared with Tg2576^{+} mice [Aβ40, $F(1,10) = 175.09$, $p < 0.01$; Aβ42, $F(1,10) = 89.50$, $p < 0.01$] (Table 1). Aβ40 and Aβ42 ELISA values of BACE1$^{-/-}$·Tg2576^{+} were only ~1% and ~5%, respectively, of Tg2576^{+} values, and were similar to Aβ values found in wild-type brain (~3% and ~5%, respectively). The origin of the Aβ signals in BACE1$^{-/-}$·Tg2576^{+} samples is unclear, although we suspect that the signals derive from Aβ species starting at positions other than Asp+1 generated by unidentified proteases. Alternatively, BACE1$^{-/-}$·Tg2576^{+} Aβ ELISA signals may result from antibody cross-reactivity to Aβ-containing APP fragments rather than to Aβ species. Nevertheless, our data indicates that reduction of cerebral Aβ to wild-type levels underlies the memory improvement in BACE1$^{-/-}$·Tg2576^{+} mice.

We also found that BACE1$^{-/-}$ monogenic brain had significantly lower levels of Aβ40 [$F(1,12) = 68.07$, $p < 0.01$] and Aβ42 [$F(1,12) = 85.44$, $p < 0.01$] as compared with wild-type brain (Table 1). Thus, BACE1$^{-/-}$ mutants have both below-normal cerebral Aβ levels and impaired spontaneous alternation performance in the Y maze (Figure 1B), although BACE1 null mutation does not affect social recognition memory (Figure 1A).

Rescue of Hippocampal Cholinergic Dysfunction in BACE1$^{-/-}$·Tg2576^{+} Mice

We next conducted hippocampal slice electrophysiology to elucidate the cellular mechanism by which the BACE1 null mutation rescues hippocampus-dependent learning and memory deficits in Tg2576 mice. Recent evidence suggests that low concentrations of exogenously applied Aβ can inhibit cholinergic signal transduction independent of apparent neurotoxicity (Auld et al., 1998; Huang et al., 2000; Kelly et al., 1996). This property of Aβ may contribute to the vulnerability of cholinergic neuronal populations in AD and to the early phases of cognitive impairment that we observe in 4- to 6-month-old Tg2576 mice. In fact, recent evidence demonstrates that cholinergic activation of protein kinase C and modulation of GABA transmission are impaired by 2 months of age in cortical neurons of Tg2576 mice (Zhong et al., 2003). These findings prompted us

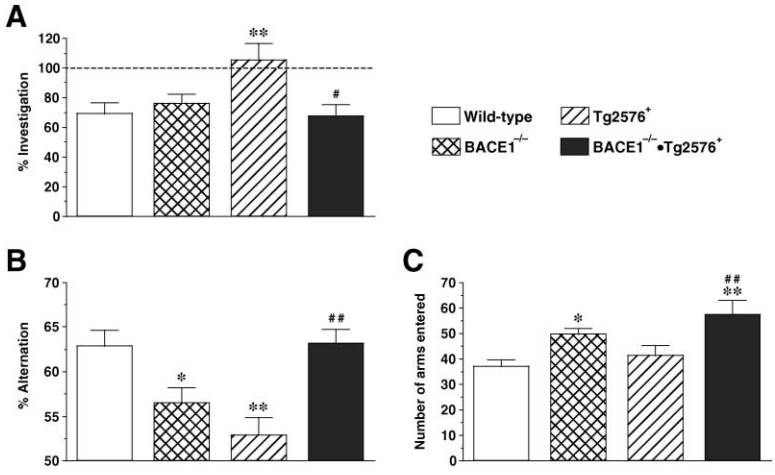

Figure 1. BACE1 Null Mutation Rescues Memory Deficits in the Tg2576 Alzheimer's Model

(A) Social recognition memory assessed with a 3 hr intertrial delay (n = 10–20). The amount of investigation time during the second exposure to the same juvenile mouse divided by that of the initial investigation time × 100 (% investigation) was used as an index of social recognition memory. Note that only the Tg2576$^+$ group does not show a reduction in spontaneous investigation to a familiar juvenile (approximately 100%) and thus is significantly impaired in this hippocampus-dependent test.

(B) Spontaneous alternation Y maze performance for the measurement of spatial working memory (n = 12–21). Tg2576$^+$ mice perform poorly (only slightly above 50% chance levels) in the Y maze test as compared to wild-type control and BACE1$^{-/-}$·Tg2576$^+$ mice. BACE1$^{-/-}$ mice are moderately but significantly impaired.

(C) Total number of arm entries reflecting exploratory activity in the Y maze (n = 12–21). Note that both BACE1$^{-/-}$ and BACE1$^{-/-}$·Tg2576$^+$ mice explore more than wild-type mice.

Each column represents the mean ± SEM. Significant differences from wild-type group (*$p < 0.05$, **$p < 0.01$) and Tg2576$^+$ group ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$), compared by ANOVA and post hoc Fisher's PLSD test.

to test the possibility that cholinergic dysfunction contributes to the memory deficits of Tg2576$^+$ mice and that improved cholinergic function may underlie the rescue of memory in BACE1$^{-/-}$·Tg2576$^+$ mice.

Increased hippocampal neuronal excitability, as assessed by a reduction in post-burst afterhyperpolarization (AHP), is known to be a cellular consequence of learning and is acetylcholine dependent (Saar et al., 2001; Wu et al., 2002). We measured the suppression of the AHP in CA1 pyramidal neurons following application of the cholinergic receptor agonist carbachol. While carbachol at 0.5 μM did not affect the peak amplitude of the AHP, it significantly increased neuronal excitability by inhibiting the slow component of the AHP (sAHP) (Figure 2A). Significant differences between groups were found in the effects of carbachol on sAHP as measured by decreases in amplitudes at 1 s after pulse offset [$F(3,31) = 3.34$, $p < 0.05$] (Figure 2B). We also assessed sAHP by calculating the integrated area from 0.3 s to 4.8 s (the end of recording) (Figure 2C), and reduction of the sAHP area following carbachol application was also significantly different between groups [$F(3,31) = 3.40$, $p < 0.05$]. For both sAHP measures, carbachol-induced increases in neuronal excitability, an in vitro model of learning and memory, was significantly impaired in hippocampal slices from Tg2576$^+$ mice ($p < 0.05$). Notably, cholinergic stimulation of neuronal excitability of CA1 pyramidal cells from BACE1$^{-/-}$·Tg2576$^+$ bigenic mice was restored to wild-type control level ($p < 0.05$) (Figures 2A–2C). In addition, the effects of carbachol on sAHP of BACE1$^{-/-}$ monogenic CA1 pyramidal cells were similar to those of wild-type cells, suggesting that the poor performance of BACE1$^{-/-}$ mice in the Y maze is not due to cholinergic dysfunction in the hippocampus. Overall, our findings demonstrate that BACE1 deficiency and reduced Aβ levels rescue hippocampal learning and memory deficits in the Tg2576 Alzheimer's model, at least in part, by correcting impaired cholinergic regulation of hippocampal neuronal excitability.

Table 1. BACE1 Null Mutation Blocks Cerebral Aβ Generation

Genotype	n	Aβ40 (%)	Aβ42 (%)
Wild-type	7	3.31 ± 0.35	4.80 ± 0.37
BACE1$^{-/-}$	7	0.42 ± 0.04	1.35 ± 0.03
Tg2576$^+$	7	100 ± 6.23	100 ± 8.36
BACE1$^{-/-}$·Tg2576$^+$	5	1.07 ± 0.11	5.13 ± 0.18

Levels of Aβ40 and Aβ42 were quantified by sandwich ELISA assays after behavioral tests were completed. Values are the mean (±SEM) expressed as percent, where 100% is Tg2576$^+$.

Discussion

Considerable advances have been made toward antiamyloid therapies. For example, recent studies demonstrate that Aβ immunotherapy reduces amyloid pathology and slows cognitive decline in some AD patients, although adverse symptoms consistent with meningoencephalitis were reported in a subset of patients (Hock et al., 2003; Nicoll et al., 2003). Clearly, much work remains for the identification, validation, and practical application of therapeutic approaches that are truly disease modifying and improve AD-associated cognitive deficits. Our combined use of behavioral, biochemical, and electrophysiological methods to analyze BACE1 knockout mice engineered to overexpress human APP (BACE1$^{-/-}$·Tg2576$^+$) has provided compelling evidence for the therapeutic potential of β-secretase inhibition for treating memory impairment in AD. We used social recognition and spontaneous alternation Y maze tasks, which are nonaversive paradigms that rely on natural social and exploratory behavior of mice (Ferguson et al., 2002; Lalonde, 2002). The present study clearly demonstrates that BACE1 deficiency rescues the memory deficits of the Tg2576 Alzheimer's model in the two different hippocampus-dependent learning tasks. The reduction of cerebral Aβ, especially the more neurotoxic

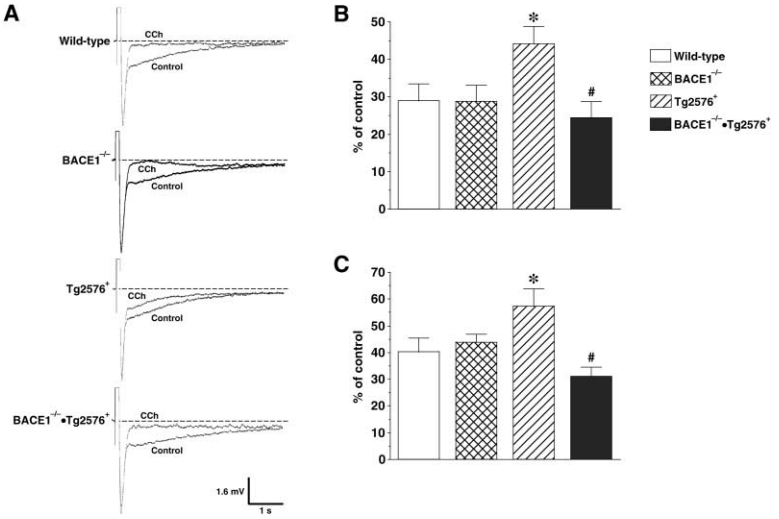

Figure 2. BACE1 Null Mutation Rescues Hippocampal Cholinergic Dysfunction in the Tg2576 Alzheimer's Model

(A) AHP in response to a 100 ms depolarizing current injection sufficient to elicit a burst of 7 action potentials was recorded from hippocampal CA1 pyramidal cells. Representative traces show the post-burst AHP before (control) and after the application of 0.5 μM carbachol (CCh). CCh at 0.5 μM selectively inhibits the slow component of AHP (sAHP) without affecting the peak amplitude of AHP. Note that the effect of CCh on sAHP in Tg2576$^+$ neurons is reduced as compared to hippocampal neurons from the other three groups. (B and C) Summary bar graphs showing CCh-induced reduction in sAHP measured by amplitudes at 1 s (B) and by integrated areas between 0.3 s and 4.8 s (C) after pulse offset. For both measures, the reduction of Tg2576$^+$ sAHP values following CCh application is less than that of wild-type and BACE1$^{-/-}$·Tg2576$^+$. Each column represents the mean ± SEM of post-CCh sAHP expressed as % of control (pre-CCh) levels (n = 5–10). Significant differences from wild-type group (*$p < 0.05$) and Tg2576$^+$ group ($^{\#}p < 0.05$), compared by ANOVA and post hoc Fisher's PLSD test.

Aβ42, from excessive (Tg2576$^+$) to near-normal (BACE1$^{-/-}$·Tg2576$^+$) levels is likely to underlie the memory rescue in BACE1$^{-/-}$·Tg2576$^+$ bigenic mice (Figure 3). Thus, our work suggests that lowering cerebral Aβ levels through the therapeutic inhibition of BACE1 should improve memory in AD patients.

Interestingly, abrogation of Aβ generation impairs spontaneous alternation performance of BACE1$^{-/-}$ single mutants in the Y maze (Figure 3), implying that Aβ may be required for normal memory in this assay. This result is consistent with recent reports on a potential physiological role of Aβ in normal neuronal function (Kamenetz et al., 2003; Yu et al., 2001). However, we cannot exclude the possibility that the absence of proteolytic products of BACE1 physiological substrates other than APP, such as sialyltransferase (Kitazume et al., 2001), may underlie the impaired performance of BACE1$^{-/-}$ mice in the Y maze. It should also be noted that BACE1$^{-/-}$ mice are unimpaired in the social recognition task (Figure 3), demonstrating that BACE1 deficiency does not affect all types of hippocampal learning. Although further studies will be required to fully characterize the behavioral phenotypes of BACE1-deficient mice, our findings raise the possibility that too much inhibition of Aβ generation may be deleterious to some forms of cognition. Nevertheless, this potential issue does not diminish BACE1 as a therapeutic target, since the dose of future BACE1 inhibitor drugs could be titrated to allow low-level Aβ generation to support normal memory function.

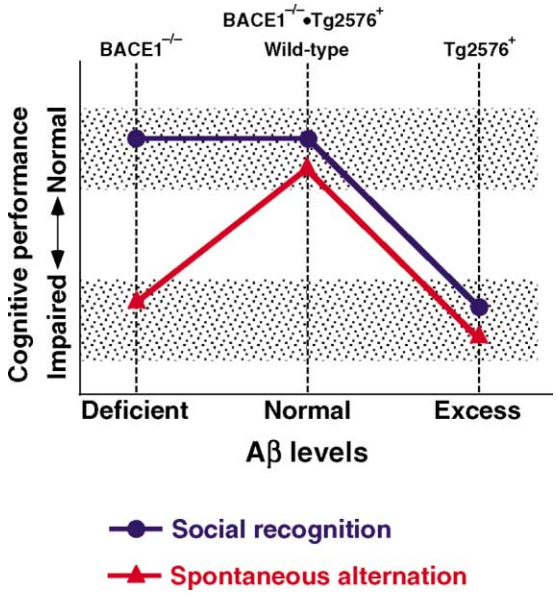

Figure 3. Relationship between Cerebral Aβ Levels and Cognitive Performance

Social recognition is impaired by excessive levels of Aβ in Tg2576$^+$ mice but is not affected by ablation of Aβ in BACE1$^{-/-}$ mice. In contrast, spontaneous alternation performance in the Y maze is sensitive to disruption by both excess (Tg2576$^+$) and deficient (BACE1$^{-/-}$) levels of cerebral Aβ. Importantly, BACE1$^{-/-}$·Tg2576$^+$ bigenic mice are unimpaired in both hippocampal learning tasks, most likely because brain Aβ levels have been restored to normal.

Our results demonstrate electrophysiological abnormalities in hippocampal cholinergic function in 4- to 6-month-old Tg2576 mice and are consistent with the marked cholinergic deficits observed in AD (Coyle et al., 1983) and the potential link between Aβ peptides and acetylcholine dysfunction (Auld et al., 1998; Tran et al., 2002). In particular, our data indicate that cholinergic stimulation of neuronal excitability following carbachol application, which is hypothesized to be an in vitro model of learning and memory (Saar et al., 2001; Wu et al., 2002), deteriorates significantly in hippocampal slices from Tg2576 mice. Social recognition and spontaneous alternation behaviors in mice are disrupted by pharmacological blockade of muscarinic neurotransmission with scopolamine (Lelong et al., 2003; Winslow and Camacho, 1995) and by reductions in hippocampal acetylcholine release during learning (Savage et al., 2003). Therefore, impaired cholinergic regulation of hippocampal neurons is likely to contribute to memory deficits in Tg2576 mice. Importantly, carbachol-induced in-

creases in excitability are restored to wild-type control level in hippocampal neurons of BACE1$^{-/-}$·Tg2576^{+} mice, suggesting that reduction of Aβ levels rescues cognitive impairment in the bigenic mice, at least in part, by ameliorating hippocampal cholinergic dysfunction.

Previous studies comparing APP transgenic versus nontransgenic mice could not unequivocally exclude the possibility that APP overexpression in the brain, rather than high cerebral Aβ levels, was the cause of learning and memory deficits in APP transgenics (Ashe, 2001). Here, we clearly show for the first time that reduction of cerebral Aβ levels by genetic ablation of BACE1 prevents memory deficits and hippocampal neuronal abnormalities in Tg2576 mice, despite massive APP overexpression in the brain. These results provide a rigorous test of the amyloid cascade hypothesis in vivo and indicate that Aβ is the primary cause of cognitive and hippocampal cholinergic impairments in the Tg2576 Alzheimer's model. Furthermore, the behavioral and electrophysiological abnormalities of the Tg2576, and the rescue of BACE1$^{-/-}$·Tg2576^{+}, were observed months before the start of amyloid deposition, indicating that soluble Aβ assemblies may be detrimental. It is relevant to note a recent study in which a single systemic injection of anti-Aβ antibody into PDAPP transgenic mice rapidly reversed cognitive deficits, as assessed by an object recognition task, presumably by sequestering soluble Aβ without affecting Aβ deposits (Dodart et al., 2002). Our findings are consistent with this conclusion and suggest that soluble, as opposed to deposited, Aβ assemblies are likely to be responsible for disrupting learning and memory in APP transgenic mice.

Since BACE1-deficient mice lack, in addition to Aβ, the β-secretase-cleaved C-terminal fragment (β-CTF) and secreted ectodomain (APPsβ) (Luo et al., 2001), we cannot exclude the possibility that some components of behavioral impairment in Tg2576 mice may involve these APP fragments. In particular, accumulation of the potentially amyloidogenic β-CTF may contribute to cognitive decline (Nalbantoglu et al., 1997; Suh, 1997). In this regard, it is important to note a recent finding that neuron-specific, postnatal deficiency of presenilin-1 (the active-site component of γ-secretase) and consequent decrease of cerebral Aβ levels fail to rescue cognitive defects, as assessed by an object recognition test, in APP[V717I] transgenic mice (Dewachter et al., 2002). This study suggests that β-CTF accumulation in the brain due to γ-secretase inactivation has detrimental effects that mask or interfere with any benefit that may have accrued by lowering Aβ levels. In our study, BACE1 deficiency is beneficial for memory in the Tg2576 Alzheimer's model possibly by preventing both Aβ and β-CTF generation (Luo et al., 2001), although further investigation is required.

In conclusion, our data provide the first demonstration that genetic inhibition of BACE1 can rescue memory deficits in an AD animal model by lowering brain Aβ levels and correcting hippocampal cholinergic dysfunction. Thus, our work validates the development of β-secretase inhibitors for the treatment of cognitive impairment in AD. Given that no mouse model fully recapitulates the entire neuropathological spectrum of AD (Wong et al., 2002), it will be important to test the impact of BACE1 deficiency with different Alzheimer's models, including a recently introduced triple-transgenic model that develops Aβ and tau-tangle pathology (Oddo et al., 2003). The present findings, nevertheless, shed significant new light on the relationship between cerebral Aβ levels and cognitive function and provide an experimental foundation supporting β-secretase inhibition for AD therapeutics.

Experimental Procedures

Animals

Mice lacking BACE1 (BACE1$^{-/-}$) with the BlkSw/129 background (Amgen Inc.) (Luo et al., 2001) were bred to mice transgenic for human APP-695 with the "Swedish" mutation (Tg2576^{+}) with the B6/SJL background (Taconic Farms Inc.) (Hsiao et al., 1996). The resultant F1 progeny were intercrossed, yielding animals with the genotypes of interest. Genotyping was performed by PCR analysis of tail DNA. All experiments were done with a subset of the F2 progeny and carried out blind with respect to the genotype of the mice. The mice from each genotype were age-matched littermates. The crossbreeding between BACE1 knockout mice and Tg2576 mice was done at Northwestern University, and the experiments were conducted with the approval of the Northwestern University Animal Care and Use Committee.

Social Recognition Task

The basic protocol for the social recognition experiments has been described previously (Kogan et al., 2000). Each mutant or wild-type littermate mouse to be tested was placed into a clean acrylic cage (the same plastic cage used for housing, 27 cm long × 16 cm wide × 12 cm high). Immediately, a male juvenile CD1 mouse (3- to 4-weeks-old) was placed into the cage with a test mouse for an initial interaction trial of 2 min. Following a 3 hr intertrial delay, the same juvenile was placed back into the test mouse's cage for a 2 min test trial. Social investigation of the juvenile by the test mouse was observed continuously and time-sampled by an experimenter. Social investigation behavior includes direct contact with the juvenile while inspecting any part of the body surface (grooming, licking, and pawing), sniffing of the mouth, ears, tail, and ano-genital area, and close following (<1 cm) of the juvenile. Since juvenile males provide relatively neutral stimulus value, they tend to provoke minimal amounts of fighting and sexual behavior from the resident adult subjects. Nevertheless, given any aggressive encounter between animals, the experiment was terminated immediately and the data excluded from analysis. The duration of investigation time during the second exposure divided by the initial investigation time × 100 (% investigation) was used to measure social recognition memory.

Spontaneous Alternation Y maze Task

Spontaneous alternation was tested as described previously (Holcomb et al., 1998). This learning task does not involve any training, reward, or punishment and allows us to assess spatial working memory that is dependent upon the hippocampus. The symmetrical Y maze made of acrylic consists of three arms separated by 120 degrees. Each arm is 40 cm long, 17 cm high, 4 cm wide at the bottom and 13 cm wide at the top. Each mouse was placed in the center of the Y maze and was allowed to explore freely through the maze during an 8 min session. The sequence and total number of arms entered was recorded. Arm entry was considered to be completed 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) × 100.

ELISA Quantitation of Brain Aβ Levels

After behavioral tests were completed, Aβ was measured essentially as described previously (Duff et al., 1996; Miller et al., 2003). Frozen hemibrains were extracted in 0.2% diethylamine in 50 mM NaCl and centrifuged at 20,000 × g for 1 hr at 4°C to remove insoluble material. Supernatant fractions were analyzed by sandwich ELISA using the well-characterized BNT77/BA27 and BNT77/BC05 antibody systems to detect Aβ40 and Aβ42, respectively. These sandwich ELISAs

recognize both human and mouse Aβ40 and Aβ42 with equivalent sensitivity.

Electrophysiology

Intracellular recordings were made from CA1 pyramidal neurons of mouse hippocampal slices as described previously (Colling et al., 1996; Mallucci et al., 2002). Transverse hippocampal slices (300 μm thick) were prepared and then maintained in an artificial cerebral spinal fluid (aCSF)-filled holding chamber at 36°C for at least 1 hr. The aCSF contained (in mM) 124 NaCl, 3 KCl, 2.4 $CaCl_2$, 2.0 $MgCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, and 10 D-glucose. Slices were transferred to the submerged glass-bottom recording chamber mounted onto the stage of an upright microscope (DM-LFS, Leica Microsystem). The chamber was constantly perfused with oxygenated aCSF (2 ml/min) at room temperature. Individual CA1 pyramidal cells were visualized using a long-distance water-immersion (×40) objective and infrared differential interference contrast (IR-DIC) illumination. Whole-cell current clamp recordings were made with an Axopatch 200B amplifier (Axon Instruments) at holding membrane potentials (−68 mV). Patch electrodes (4–5 MΩ) were filled with an intracellular solution containing (in mM) 130 potassium methylsulfate, 10 KCl, 10 HEPES, 2 K_2-ATP, 1 $MgCl_2$ (pH 7.3–7.4, osmolarity 290 ± 10 mOsm). Cells with input resistance <60 MΩ and resting membrane potential >−55 mV were excluded from recording. The AHP was evoked using a 100 ms depolarization current step that reliably elicited a burst of seven action potentials. The signal was digitized at 10 kHz and low-pass filtered at 5 kHz with a PCI-MIO-16E-4 board (National Instruments), and all data were stored on a PC computer with custom-made software and a NI DAQ 6.5 driver (National Instruments). A total of ten AHP measurements were made from each neuron at 20 s intervals. After the baseline measurements, the perfusate was changed to an aCSF containing 0.5 μM carbachol. The AHP was measured immediately before and 10 min after the start of carbachol application, by means of the amplitude at 1 s and the integrated area calculated from 0.3 s to 4.8 s after pulse offset. AHP measures following carbachol treatment divided by control AHP measures were used to compare the effects of carbachol between groups.

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.

Acknowledgments

This work was supported by grants from the NIH (R01 MH067251 to M.O., R37 AG08796 to J.F.D.) and from the Alzheimer's Association (IIRG-02-4282 to R.V., IIRG-02-4130 to J.F.D.).

Received: September 23, 2003
Revised: November 5, 2003
Accepted: November 24, 2003
Published: January 7, 2004

References

Ashe, K.H. (2001). Learning and memory in transgenic mice modeling Alzheimer's disease. Learn. Mem. *8*, 301–308.

Auld, D.S., Kar, S., and Quirion, R. (1998). β-amyloid peptides as direct cholinergic neuromodulators: a missing link? Trends Neurosci. *21*, 43–49.

Cai, H., Wang, Y., McCarthy, D., Wen, H., Borchelt, D.R., Price, D.L., and Wong, P.C. (2001). BACE1 is the major β-secretase for generation of Aβ peptides by neurons. Nat. Neurosci. *4*, 233–234.

Citron, M. (2002). Alzheimer's disease: treatments in discovery and development. Nat. Neurosci. *5*, 1055–1057.

Colling, S.B., Collinge, J., and Jefferys, J.G. (1996). Hippocampal slices from prion protein null mice: disrupted Ca^{2+}-activated K^+ currents. Neurosci. Lett. *209*, 49–52.

Coyle, J.T., Price, D.L., and DeLong, M.R. (1983). Alzheimer's disease: a disorder of cortical cholinergic innervation. Science *219*, 1184–1190.

Dewachter, I., Reverse, D., Caluwaerts, N., Ris, L., Kuiperi, C., Van Den Haute, C., Spittaels, K., Umans, L., Serneels, L., Thiry, E., et al. (2002). Neuronal deficiency of presenilin 1 Inhibits amyloid plaque formation and corrects hippocampal long-term potentiation but not a cognitive defect of amyloid precursor protein [V717I] transgenic mice. J. Neurosci. *22*, 3445–3453.

Dineley, K.T., Xia, X., Bui, D., Sweatt, J.D., and Zheng, H. (2002). Accelerated plaque accumulation, associative learning deficits, and up-regulation of α7 nicotinic receptor protein in transgenic mice co-expressing mutant human presenilin 1 and amyloid precursor proteins. J. Biol. Chem. *277*, 22768–22780.

Dodart, J.C., Bales, K.R., Gannon, K.S., Greene, S.J., DeMattos, R.B., Mathis, C., DeLong, C.A., Wu, S., Wu, X., Holtzman, D.M., and Paul, S.M. (2002). Immunization reverses memory deficits without reducing brain Aβ burden in Alzheimer's disease model. Nat. Neurosci. *5*, 452–457.

Dodel, R.C., Hampel, H., and Du, Y. (2003). Immunotherapy for Alzheimer's disease. Lancet Neurol. *2*, 215–220.

Duff, K., Eckman, C., Zehr, C., Yu, X., Prada, C.M., Perez-tur, J., Hutton, M., Buee, L., Harigaya, Y., Yager, D., et al. (1996). Increased amyloid-beta42(43) in brains of mice expressing mutant presenilin 1. Nature *383*, 710–713.

Ferguson, J.N., Young, L.J., and Insel, T.R. (2002). The neuroendocrine basis of social recognition. Front. Neuroendocrinol. *23*, 200–224.

Gong, Y., Chang, L., Viola, K.L., Lacor, P.N., Lambert, M.P., Finch, C.E., Krafft, G.A., and Klein, W.L. (2003). Alzheimer's disease-affected brain: presence of oligomeric Aβ ligands (ADDLs) suggests a molecular basis for reversible memory loss. Proc. Natl. Acad. Sci. USA *100*, 10417–10422.

Hardy, J., and Selkoe, D.J. (2002). The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. Science *297*, 353–356.

Hartley, D.M., Walsh, D.M., Ye, C.P., Diehl, T., Vasquez, S., Vassilev, P.M., Teplow, D.B., and Selkoe, D.J. (1999). Protofibrillar intermediates of amyloid β-protein induce acute electrophysiological changes and progressive neurotoxicity in cortical neurons. J. Neurosci. *19*, 8876–8884.

Hock, C., Konietzko, U., Streffer, J.R., Tracy, J., Signorell, A., Muller-Tillmanns, B., Lemke, U., Henke, K., Moritz, E., Garcia, E., et al. (2003). Antibodies against β-amyloid slow cognitive decline in Alzheimer's disease. Neuron *38*, 547–554.

Holcomb, L., Gordon, M.N., McGowan, E., Yu, X., Benkovic, S., Jantzen, P., Wright, K., Saad, I., Mueller, R., Morgan, D., et al. (1998). Accelerated Alzheimer-type phenotype in transgenic mice carrying both mutant amyloid precursor protein and presenilin 1 transgenes. Nat. Med. *4*, 97–100.

Holcomb, L.A., Gordon, M.N., Jantzen, P., Hsiao, K., Duff, K., and Morgan, D. (1999). Behavioral changes in transgenic mice expressing both amyloid precursor protein and presenilin-1 mutations: lack of association with amyloid deposits. Behav. Genet. *29*, 177–185.

Hsiao, K., Chapman, P., Nilsen, S., Eckman, C., Harigaya, Y., Younkin, S., Yang, F., and Cole, G. (1996). Correlative memory deficits, Aβ elevation, and amyloid plaques in transgenic mice. Science *274*, 99–102.

Huang, H.M., Ou, H.C., and Hsieh, S.J. (2000). Amyloid beta peptide impaired carbachol but not glutamate-mediated phosphoinositide pathways in cultured rat cortical neurons. Neurochem. Res. *25*, 303–312.

Hussain, I., Powell, D., Howlett, D.R., Tew, D.G., Meek, T.D., Chapman, C., Gloger, I.S., Murphy, K.E., Southan, C.D., Ryan, D.M., et al. (1999). Identification of a novel aspartic protease (Asp 2) as β-secretase. Mol. Cell. Neurosci. *14*, 419–427.

Janus, C., and Westaway, D. (2001). Transgenic mouse models of Alzheimer's disease. Physiol. Behav. *73*, 873–886.

John, V., Beck, J.P., Bienkowski, M.J., Sinha, S., and Heinrikson,

R.L. (2003). Human β-secretase (BACE) and BACE inhibitors. J. Med. Chem. *46*, 4625–4630.

Kamenetz, F., Tomita, T., Hsieh, H., Seabrook, G., Borchelt, D., Iwatsubo, T., Sisodia, S., and Malinow, R. (2003). APP processing and synaptic function. Neuron *37*, 925–937.

Kawarabayashi, T., Younkin, L.H., Saido, T.C., Shoji, M., Ashe, K.H., and Younkin, S.G. (2001). Age-dependent changes in brain, CSF, and plasma amyloid β protein in the Tg2576 transgenic mouse model of Alzheimer's disease. J. Neurosci. *21*, 372–381.

Kelly, J.F., Furukawa, K., Barger, S.W., Rengen, M.R., Mark, R.J., Blanc, E.M., Roth, G.S., and Mattson, M.P. (1996). Amyloid β-peptide disrupts carbachol-induced muscarinic cholinergic signal transduction in cortical neurons. Proc. Natl. Acad. Sci. USA *93*, 6753–6758.

Kitazume, S., Tachida, Y., Oka, R., Shirotani, K., Saido, T.C., and Hashimoto, Y. (2001). Alzheimer's β-secretase, β-site amyloid precursor protein-cleaving enzyme, is responsible for cleavage secretion of a Golgi-resident sialyltransferase. Proc. Natl. Acad. Sci. USA *98*, 13554–13559.

Kogan, J.H., Frankland, P.W., and Silva, A.J. (2000). Long-term memory underlying hippocampus-dependent social recognition in mice. Hippocampus *10*, 47–56.

Lalonde, R. (2002). The neurobiological basis of spontaneous alternation. Neurosci. Biobehav. Rev. *26*, 91–104.

Lambert, M.P., Barlow, A.K., Chromy, B.A., Edwards, C., Freed, R., Liosatos, M., Morgan, T.E., Rozovsky, I., Trommer, B., Viola, K.L., et al. (1998). Diffusible, nonfibrillar ligands derived from $A\beta_{1-42}$ are potent central nervous system neurotoxins. Proc. Natl. Acad. Sci. USA *95*, 6448–6453.

Lelong, V., Lhonneur, L., Dauphin, F., and Boulouard, M. (2003). BIMU 1 and RS 67333, two 5-HT_4 receptor agonists, modulate spontaneous alternation deficits induced by scopolamine in the mouse. Naunyn Schmiedebergs Arch. Pharmacol. *367*, 621–628.

Lin, X., Koelsch, G., Wu, S., Downs, D., Dashti, A., and Tang, J. (2000). Human aspartic protease memapsin 2 cleaves the β-secretase site of β-amyloid precursor protein. Proc. Natl. Acad. Sci. USA *97*, 1456–1460.

Luo, Y., Bolon, B., Kahn, S., Bennett, B.D., Babu-Khan, S., Denis, P., Fan, W., Kha, H., Zhang, J., Gong, Y., et al. (2001). Mice deficient in BACE1, the Alzheimer's β-secretase, have normal phenotype and abolished β-amyloid generation. Nat. Neurosci. *4*, 231–232.

Luo, Y., Bolon, B., Damore, M.A., Fitzpatrick, D., Liu, H., Zhang, J., Yan, Q., Vassar, R., and Citron, M. (2003). BACE1 (β-secretase) knockout mice do not acquire compensatory gene expression changes or develop neural lesions over time. Neurobiol. Dis. *14*, 81–88.

Mallucci, G.R., Ratte, S., Asante, E.A., Linehan, J., Gowland, I., Jefferys, J.G., and Collinge, J. (2002). Post-natal knockout of prion protein alters hippocampal CA1 properties, but does not result in neurodegeneration. EMBO J. *21*, 202–210.

Miller, B.C., Eckman, E.A., Sambamurti, K., Dobbs, N., Chow, K.M., Eckman, C.B., Hersh, L.B., and Thiele, D.L. (2003). Amyloid-β peptide levels in brain are inversely correlated with insulysin activity levels *in vivo*. Proc. Natl. Acad. Sci. USA *100*, 6221–6226.

Nalbantoglu, J., Tirado-Santiago, G., Lahsaini, A., Poirier, J., Goncalves, O., Verge, G., Momoli, F., Welner, S.A., Massicotte, G., Julien, J.P., and Shapiro, M.L. (1997). Impaired learning and LTP in mice expressing the carboxy terminus of the Alzheimer amyloid precursor protein. Nature *387*, 500–505.

Nicoll, J.A., Wilkinson, D., Holmes, C., Steart, P., Markham, H., and Weller, R.O. (2003). Neuropathology of human Alzheimer disease after immunization with amyloid-β peptide: a case report. Nat. Med. *9*, 389–390.

Oddo, S., Caccamo, A., Shepherd, J.D., Murphy, M.P., Golde, T.E., Kayed, R., Metherate, R., Mattson, M.P., Akbari, Y., and LaFerla, F.M. (2003). Triple-transgenic model of Alzheimer's disease with plaques and tangles: intracellular Aβ and synaptic dysfunction. Neuron *39*, 409–421.

Roberds, S.L., Anderson, J., Basi, G., Bienkowski, M.J., Branstetter, D.G., Chen, K.S., Freedman, S.B., Frigon, N.L., Games, D., Hu, K., et al. (2001). BACE knockout mice are healthy despite lacking the primary β-secretase activity in brain: implications for Alzheimer's disease therapeutics. Hum. Mol. Genet. *10*, 1317–1324.

Saar, D., Grossman, Y., and Barkai, E. (2001). Long-lasting cholinergic modulation underlies rule learning in rats. J. Neurosci. *21*, 1385–1392.

Savage, L.M., Chang, Q., and Gold, P.E. (2003). Diencephalic damage decreases hippocampal acetylcholine release during spontaneous alternation testing. Learn. Mem. *10*, 242–246.

Schenk, D. (2002). Amyloid-β immunotherapy for Alzheimer's disease: the end of the beginning. Nat. Rev. Neurosci. *3*, 824–828.

Selkoe, D.J. (2002). Alzheimer's disease is a synaptic failure. Science *298*, 789–791.

Selkoe, D.J., and Schenk, D. (2003). Alzheimer's disease: molecular understanding predicts amyloid-based therapeutics. Annu. Rev. Pharmacol. Toxicol. *43*, 545–584.

Sinha, S., Anderson, J.P., Barbour, R., Basi, G.S., Caccavello, R., Davis, D., Doan, M., Dovey, H.F., Frigon, N., Hong, J., et al. (1999). Purification and cloning of amyloid precursor protein β-secretase from human brain. Nature *402*, 537–540.

Suh, Y.H. (1997). An etiological role of amyloidogenic carboxyl-terminal fragments of the β-amyloid precursor protein in Alzheimer's disease. J. Neurochem. *68*, 1781–1791.

Tang, J., Ghosh, A.K., Hong, L., Koelsch, G., Turner, R.T., and Chang, W. (2003). Study of memapsin 2 (β-secretase) and strategy of inhibitor design. J. Mol. Neurosci. *20*, 299–304.

Tran, M.H., Yamada, K., and Nabeshima, T. (2002). Amyloid β-peptide induces cholinergic dysfunction and cognitive deficits: a minireview. Peptides *23*, 1271–1283.

Turner, P.R., O'Connor, K., Tate, W.P., and Abraham, W.C. (2003). Roles of amyloid precursor protein and its fragments in regulating neural activity, plasticity and memory. Prog. Neurobiol. *70*, 1–32.

Vassar, R., and Citron, M. (2000). Aβ-generating enzymes: recent advances in β- and γ-secretase research. Neuron *27*, 419–422.

Vassar, R., Bennett, B.D., Babu-Khan, S., Kahn, S., Mendiaz, E.A., Denis, P., Teplow, D.B., Ross, S., Amarante, P., Loeloff, R., et al. (1999). β-secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE. Science *286*, 735–741.

Walsh, D.M., Klyubin, I., Fadeeva, J.V., Cullen, W.K., Anwyl, R., Wolfe, M.S., Rowan, M.J., and Selkoe, D.J. (2002). Naturally secreted oligomers of amyloid β protein potently inhibit hippocampal long-term potentiation in vivo. Nature *416*, 535–539.

Westerman, M.A., Cooper-Blacketer, D., Mariash, A., Kotilinek, L., Kawarabayashi, T., Younkin, L.H., Carlson, G.A., Younkin, S.G., and Ashe, K.H. (2002). The relationship between Aβ and memory in the Tg2576 mouse model of Alzheimer's disease. J. Neurosci. *22*, 1858–1867.

Winslow, J.T., and Camacho, F. (1995). Cholinergic modulation of a decrement in social investigation following repeated contacts between mice. Psychopharmacology (Berl.) *121*, 164–172.

Wolfe, M.S. (2002). Therapeutic strategies for Alzheimer's disease. Nat. Rev. Drug Discov. *1*, 859–866.

Wong, P.C., Cai, H., Borchelt, D.R., and Price, D.L. (2002). Genetically engineered mouse models of neurodegenerative diseases. Nat. Neurosci. *5*, 633–639.

Wu, W.W., Oh, M.M., and Disterhoft, J.F. (2002). Age-related biophysical alterations of hippocampal pyramidal neurons: implications for learning and memory. Ageing Res. Rev. *1*, 181–207.

Yan, R., Bienkowski, M.J., Shuck, M.E., Miao, H., Tory, M.C., Pauley, A.M., Brashier, J.R., Stratman, N.C., Mathews, W.R., Buhl, A.E., et al. (1999). Membrane-anchored aspartyl protease with Alzheimer's disease β-secretase activity. Nature *402*, 533–537.

Yu, H., Saura, C.A., Choi, S.Y., Sun, L.D., Yang, X., Handler, M., Kawarabayashi, T., Younkin, L., Fedeles, B., Wilson, M.A., et al. (2001). APP processing and synaptic plasticity in presenilin-1 conditional knockout mice. Neuron *31*, 713–726.

Zhong, P., Gu, Z., Wang, X., Jiang, H., Feng, J., and Yan, Z. (2003). Impaired modulation of GABAergic transmission by muscarinic receptors in a mouse transgenic model of Alzheimer's disease. J. Biol. Chem. *278*, 26888–26896.