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Modeling an Anti-Amyloid Combination Therapy for Alzheimer's Disease

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Abstract

As only symptomatic treatments are now available for Alzheimer's disease (AD), safe and effective mechanism-based therapies remain a great unmet need for patients with this neurodegenerative disease. Although γ -secretase and BACE1 [β -site β -amyloid (A β) precursor protein (APP) cleaving enzyme 1] are well-recognized therapeutic targets for AD, untoward side effects associated with strong inhibition or reductions in amounts of these aspartyl proteases have raised concerns regarding their therapeutic potential. Although moderate decreases of either γ -secretase or BACE1 are not associated with mechanism-based toxicities, they provide only modest benefits in reducing A β in the brains of *APP^{swe}/PS1 Δ E9* mice. Because the processing of APP to generate A β requires both γ -secretase and BACE1, it is possible that moderate reductions of both enzymes would provide additive and significant protection against A β amyloidosis. Here, we test this hypothesis and assess the value of this novel anti-amyloid combination therapy in mutant mice. We demonstrate that genetic reductions of both *BACE1* and γ -secretase additively attenuate the amyloid burden and ameliorate cognitive deficits occurring in aged *APP^{swe}/PS1 Δ E9* animals. No evidence of mechanism-based toxicities was associated with such decreases in amounts of both enzymes. Thus, we propose that targeting both γ -secretase and BACE1 may be an effective and safe treatment strategy for AD.

INTRODUCTION

Alzheimer's disease (AD), the most common cause of dementia in the elderly, is characterized pathologically by the deposition of β -amyloid (A β) plaques and neurofibrillary tangles in the hippocampus and cerebral cortex. Because only symptomatic treatments are now available, safe and effective mechanism-based therapies are needed for this devastating neurodegenerative disease of later life. Experimental support of the "amyloid cascade hypothesis" has led investigators to identify and evaluate targets for anti-amyloid therapeutic

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strategies, which are designed to reduce the amyloid burden in the brains of individuals with AD (1,2). A β peptides are generated in the brain through sequential endoproteolytic cleavages of neuronal A β precursor protein (APP) by two membrane-bound enzyme activities, termed BACE1 (β -site APP cleaving enzyme 1) and γ -secretase. The latter is a complex consisting of four distinct membrane proteins, namely presenilin (PS1 and PS2), nicastrin, anterior pharynx defective-1 (Aph-1), and presenilin enhancer 2 (3,4).

Although both enzymes have been experimentally validated as targets for AD therapy (1), strong inhibition of either protease can be associated with mechanism-based toxicity because of the impact of these manipulations on other critical signaling pathways (for example, those using Notch and neuregulin-1) (5–9). Genetic ablation of *BACE1* prevents A β deposition and A β -associated cognitive deficits in a mouse model of A β amyloidosis (10,11), but the discovery that homozygous *BACE1* knockout (*BACE1*^{−/−}) mice exhibit cognitive deficits, impairment of synaptic function, hypomyelination of axons of sciatic and optic nerves, and schizophrenia-relevant endophenotypes (7,8,10–12) raised concerns about potential mechanism-based toxicities associated with strong inhibition of BACE1. Likewise, inhibition of γ -secretase causes untoward mechanism-based side effects, as pharmacological inhibition of γ -secretase by several potent inhibitors results in significant toxicity (13–15), likely because of influences on the Notch and epidermal growth factor receptor signaling pathway, leading to skin tumors such as squamous cell carcinoma (5,6).

Recent efforts have focused on modeling modest reductions of BACE1 (8,10,16) or γ -secretase (5,6,9,17) to minimize mechanism-based side effects. Although modest reduction of one of the secretases limits mechanism-based toxicity, it provides only mild protection against accumulation of A β in the brain. Because the processing of APP to generate A β requires both γ -secretase and BACE1, we hypothesize that simultaneous reductions of both enzymes could provide additive protection against A β amyloidosis while limiting side effects associated with reductions of these proteases. Here, we assess the value of this anti-amyloid combination therapy in a mouse model of A β amyloidosis (*APP*^{*swe*}/*PS1* Δ *E9* mice) lacking one allele each of *Aph-1a* and *BACE1* (10,18,19). We present findings that support the view that modest reductions of both γ -secretase and β -secretase provide greater attenuation of the amyloid burden and cognitive deficits than that conferred by the modest reduction of either enzyme alone.

RESULTS

Reductions of both γ -secretase and β -secretase additively attenuate A β deposition

Because strong inhibition of either BACE1 or γ -secretase was likely associated with mechanism-based toxicities, we previously demonstrated the value of moderate reduction of either BACE1 (10) or γ -secretase (6) as an effective strategy to reduce the amyloid burden while limiting mechanism-based side effects. Because the generation of A β is dependent on sequential cleavages of APP by the two secretases, greater attenuation of the amyloid burden may be achieved by reducing activities of both γ -secretase and BACE1. To test this hypothesis and to model such an anti-amyloid combination therapy, we took advantage of our previously characterized *Aph-1a*^{+/−} (6,19) and *BACE1*^{+/−} (10,18) mice harboring about 70% and 50% of γ -secretase and BACE1, respectively. These mice were crossbred to *APP*^{*swe*}/*PS1* Δ *E9* mice (20), a well-established model exhibiting accelerated A β amyloidosis attributable to coexpression of familial AD-linked mutations of APP (*APP*^{*swe*}) and PS1 (*PS1* Δ *E9*) (21). Using polymerase chain reaction (PCR) and protein blot analysis (fig. S1A), we confirmed the generation of a cohort of *APP*^{*swe*}/*PS1* Δ *E9* mice and *APP*^{*swe*}/*PS1* Δ *E9* mice lacking either one allele of *BACE1* (*APP*^{*swe*}/*PS1* Δ *E9*;*BACE1*^{+/−}), one allele of *Aph-1a* (*APP*^{*swe*}/*PS1* Δ *E9*;*Aph-1a*^{+/−}), or one allele each of *BACE1* and *Aph-1a* (*APP*^{*swe*}/*PS1* Δ *E9*;*BACE1*^{+/−};*Aph-1a*^{+/−}). To determine whether genetic reduction of both γ -secretase

and β -secretase confers greater amelioration of amyloidosis in the brain, we assessed the A β burden with complementary methods.

Biochemical analysis revealed a significant decrease in levels of insoluble (formic acid-extracted) A β 1–40 ($P < 0.01$, 99% reduction) and A β 1–42 ($P < 0.01$, 93% reduction) peptides from the brains of *APP^{swe}/PS1 Δ E9;Aph-1a^{+/-};BACE1^{+/-}* mice relative to those from *APP^{swe}/PS1 Δ E9* mice at 6 months of age (Fig. 1, A and B, and table S1). No significant differences were found in the amount of soluble [phosphate-buffered saline (PBS)-extracted] A β 1–40 and A β 1–42 among different genotypes (fig. S1, B and C). Moreover, whereas amounts of insoluble A β peptides in *APP^{swe}/PS1 Δ E9;Aph-1a^{+/-}* or *APP^{swe}/PS1 Δ E9;BACE1^{+/-}* mice were not significantly different from those observed in *APP^{swe}/PS1 Δ E9* mice, they were significantly different from those derived from *APP^{swe}/PS1 Δ E9;Aph-1a^{+/-};BACE1^{+/-}* mice (Fig. 1, A and B, and table S1). Together, these results indicate that modest decreases of activities of both BACE1 and γ -secretase confer greater attenuation of A β amyloidosis than modest reduction of either enzyme alone.

Immunohistochemical analyses with antisera specific for either ubiquitin (Fig. 1, E to H) or A β (fig. S1D) confirmed the biochemical data. Whereas a significant reduction (~50%) of amyloid plaque burden was observed in both the hippocampus and cerebral cortex of 6-month-old *APP^{swe}/PS1 Δ E9;BACE1^{+/-}* mice relative to that of *APP^{swe}/PS1 Δ E9* mice (Fig. 1, C and D; $P < 0.05$), as expected and in agreement with our previous findings (10), however, a greater decrement (~80%) in the hippocampus (Fig. 1C; $P < 0.01$, 86% reduction) and cerebral cortex (Fig. 1D; $P < 0.001$, 85% reduction) was seen in *APP^{swe}/PS1 Δ E9;Aph-1a^{+/-};BACE1^{+/-}* mice. Unbiased stereological analyses to assess the amyloid burden revealed similar results (fig. S1, E and F). Together, these data support the view that modest reductions in activities of both BACE1 and γ -secretases provide greater attenuation of A β amyloidosis in the brain than does a modest decrease of either enzymatic activity.

Reductions of both BACE1 and γ -secretase rescue memory deficits

We next determined whether such manipulations would provide functional rescue of A β -related cognitive deficits. Because we previously had shown that genetic ablation of one allele of *BACE1* failed to ameliorate cognitive deficits in aged *APP^{swe}/PS1 Δ E9* mice (10), we elected to test behavior in aged *APP^{swe}/PS1 Δ E9* mice (19 to 21 months of age). Aged *APP^{swe}/PS1 Δ E9* mice exhibiting high amyloid burden would also better mimic the clinical situation where such therapeutic strategy will be initially tested.

At the advanced stage of A β plaque deposition (23 months), unbiased stereological analyses revealed (Fig. 2, A to D) a greater decrement of A β deposition in the hippocampus (~70% reduction) (Fig. 2E and table S1) and in the cortex (~50% reduction) (Fig. 2F and table S1) of *APP^{swe}/PS1 Δ E9;Aph-1a^{+/-};BACE1^{+/-}* mice relative to those documented in either *APP^{swe}/PS1 Δ E9;BACE1^{+/-}* or *APP^{swe}/PS1 Δ E9;Aph-1a^{+/-}* mice. The effects of mild decreases in amounts of either of the secretases were less pronounced (ranging from a 25% decrease in the cortex of *APP^{swe}/PS1 Δ E9;Aph-1a^{+/-}* mice to a 50% decrease in hippocampus of *APP^{swe}/PS1 Δ E9;BACE1^{+/-}* mice) (Fig. 2, E and F, and table S1). Moreover, using an antibody that recognizes A β oligomers (A11), we showed a significant decrease in amounts of A β oligomers in the brains of *APP^{swe}/PS1 Δ E9;Aph-1a^{+/-};BACE1^{+/-}* mice relative to that of *APP^{swe}/PS1 Δ E9* mice (75% reduction; $P < 0.00001$), whereas no significant reductions were observed in *APP^{swe}/PS1 Δ E9;Aph-1a^{+/-}* or *APP^{swe}/PS1 Δ E9;BACE1^{+/-}* mice (Fig. 2, G to K, and table S1). Collectively, these results indicate that even in advanced stages of amyloid deposition, moderate reductions of both γ -secretase and β -secretase provide a greater protection against A β amyloidosis in the brain than do modest decreases of either enzyme alone.

To determine whether such protection correlates with functional recovery of cognition in aged mice, we used a classic version of the Morris water maze that is highly sensitive to the aging-related cognitive deficits in our *APP^{swe}/PS1 Δ E9* model (22). This task requires incremental learning of a constant platform location over multiple days of training and results in formation of long-lasting spatial reference memory. Learning of the constant platform location was analyzed in consecutive training trials in which the platform was submerged under the opaque water but accessible for the mouse (Fig. 3, A and B; see the Materials and Methods for details). *APP^{swe}/PS1 Δ E9* mice showed a significant decrease in distance to find the platform as training progressed [Fig. 3A; effect of blocks of trial $F(5,110) = 9.16$; $P < 0.0001$, two-way repeated-measures analysis of variance (ANOVA)]. Learning in the *APP^{swe}/PS1 Δ E9* group was not significantly different from that in the age-matched wild-type littermates [Fig. 3A and fig. S3A; effect of geno-type $F(1,22) = 3.33$, not significant (n.s.); genotype $\times$ block of trials interaction $F(5,110) = 1.68$, n.s.]. This improvement in performance was not significantly affected by the deletion of one allele of *Aph-1a* and/or *BACE1* [Fig. 3B; effect of genotype $F(3,25) = 0.67$, n.s.; genotype $\times$ block of trials interaction $F(5,125) = 1.03$, n.s.].

To confirm that learning of the platform location was based on the use of external environmental landmarks rather than acquisition of adaptive nonspatial strategies (23,24), we conducted probe trials in which the platform was lowered and inaccessible for the mouse (25). If the mouse learns the task by using spatial cues, it spends more time swimming around the platform location during the probe trial. Our previous studies indicated that a long-term delay (20 hours) increases sensitivity of the probe trial in detecting deficits in spatial memory (23). Thus, we focused on analyzing the accuracy of spatial memory by conducting the probe trial 20 hours after the last training trial. Bivariate histograms of the swimming patterns (Fig. 3C) indicated that wild-type mice as well as *APP^{swe}/PS1 Δ E9;Aph-1a^{+/-};BACE1^{+/-}* mice develop a clear spatial preference for the area around the platform. *APP^{swe}/PS1 Δ E9* mice lacking one allele of either *Aph-1a* or *BACE1* distribute their activities in other areas of the water maze, indicating impaired spatial memory. For quantitative analyses of outcomes (Fig. 3, D and F), we used measures of spatial preference with different levels of demand for accuracy of memory (22,23). The least demanding measure of spatial memory was the percentage of time spent in an area 40 cm in diameter around the center of the platform (area 40). Although it did not show significant genotype-related differences, this assessment revealed significant preferences for the correct quadrant only in wild-type mice ($P < 0.01$, t test) and *APP^{swe}/PS1 Δ E9;Aph-1a^{+/-};BACE1^{+/-}* mice ($P < 0.01$, t test) (Fig. 3D). Tests requiring higher levels of accuracy (area 20 and frequency of platform crossings) were highly sensitive in detecting A β -related deficits in *APP^{swe}/PS1 Δ E9* mice relative to wild-type age-matched controls [$F(1,22) = 4.95$ – 6.73 ; $P < 0.05$, one-way ANOVA; Fig. 3, E and F]. Both of these measures revealed a significant rescue of memory deficits in *APP^{swe}/PS1 Δ E9;Aph-1a^{+/-};BACE1^{+/-}* [$F(1,12) = 5.96$ – 6.07 ; $P < 0.03$, one-way ANOVA] but not in *APP^{swe}/PS1 Δ E9;Aph-1a^{+/-}* or *APP^{swe}/PS1 Δ E9;BACE1^{+/-}* mice (Fig. 3, E and F). Together, these results establish that modest reduction of both γ -secretase and BACE1 provides greater protection with regard to A β -induced memory impairments relative to reduction of either enzyme alone (10).

Behavioral performance is normal in mice with modest reductions of both γ -secretase and BACE1

We have shown that, relative to the decrease of either γ -secretase or BACE1, the reduction of both proteases provides greater protection for the brain. It is possible that pathways other than APP can be influenced by the modest decrements of both of these enzymes. Of particular interest in this respect is neuregulin 1 (NRG1), a ligand for members of ErbB family of receptor tyrosine kinases critical for brain development and function (26–28) and a substrate of both BACE1 (7,12) and γ -secretase (28). Moreover, because mice lacking two members of the *Aph-1* family (namely, *Aph-1b* and *Aph-1c*) (9) as well as *BACE1* knockout mice (18) show

behavioral abnormalities reminiscent of some aspects of schizophrenia (8,9), we determined whether mice lacking one allele each of *BACE1* and *Aph-1a* exhibit any schizophrenia-related endophenotypes.

For behavioral testing, we generated a young cohort of wild-type, *Aph-1a*^{+/-}, *BACE1*^{+/-}, and *Aph-1a*^{+/-};*BACE1*^{+/-} mice; confirmed their genotype by PCR and protein blot analysis (fig. S2); and used tasks that have been shown to be sensitive to behavioral abnormalities reported for *Aph-1b/c*^{-/-} (9) and/or *BACE1*^{-/-} mice (8)(Fig. 4 and table S2). First, we tested spontaneous alternation behavior with a Y-maze task that assesses working memory–dependent response to the environment driven by a natural tendency toward novel locations (29). The spatial working memory depends on the integrity of prefrontal and hippocampal systems (29) and is impaired in both *Aph-1b/c*^{-/-} (9) and *BACE1*^{-/-} (8) mice. Analysis of spontaneous alternations in *Aph-1a*^{+/-} or *BACE1*^{+/-} mice did not reveal any significant changes relative to wild-type littermates. Spatial working memory in this test was not affected even in *Aph-1a*^{+/-};*BACE1*^{+/-} mice (Fig. 4A and table S2). Testing for novelty-induced hyperactivity, a prominent trait in *BACE1*^{-/-} mice (8), did not show any significant changes in any of genotypes tested (Fig. 4, B and C, and table S2). The plus maze, a test that has been validated to measure anxiety levels (30), revealed no significant genotype-related modifications in the preference to the open arms of the maze (Fig. 4D and table S2). Total number of arms visited during the test (Fig. 4E) showed a mild increase in *BACE1*^{+/-} mice, but the magnitude was markedly lower than that seen in *BACE1*^{-/-} mice (8). This small change in activity detected in *BACE1*^{+/-} mice during plus maze testing was not influenced by the modest reduction of γ -secretase. Moreover, the difference between *Aph-1a*^{+/-};*BACE1*^{+/-} and wild-type mice in this task was not significant (table S2). Next, we tested whether ablation of *Aph-1a*^{+/-} and/or *BACE1*^{+/-} results in hypersensitivity to a psychostimulant, MK-801, a noncompetitive *N*-methyl-D-aspartate (NMDA) receptor blocker. Administration of this drug to rodents and primates is a well-established pharmacological model of features of schizophrenia (31). Locomotor hypersensitivity to MK-801 was observed in both *Aph-1b/c*^{-/-} and *BACE1*^{-/-} mice (8,9). MK-801 (0.3 mg/kg intraperitoneally; see Materials and Methods for dose response) elicited a strong locomotor response (Fig. 4F; $P < 0.0001$); however, no genotype-related differences were observed (table S2). Finally, we assessed sensorimotor gaiting by testing prepulse inhibition (PPI) of acoustic startle reaction (ASR). The deficit in PPI, one of the major endophenotypes related to schizophrenia (32), was observed in both *Aph-1b/c*^{-/-} and *BACE1*^{-/-} mice (8,9). In this paradigm, a brief low-intensity acoustic stimulus (the prepulse) inhibits the startle reaction caused by a loud stimulus. There were no significant effects of combined genetic ablation of one allele each of *Aph-1a* and *BACE1* on the amplitude of startle reaction (Fig. 4G) or the levels of inhibition of this reaction by prepulses (Fig. 4I and table S2). Likewise, no significant differences were observed between wild-type mice and either *BACE1*^{+/-} or *Aph-1a*^{+/-} mice, although *BACE1*^{+/-} mice showed some tendency for less efficient PPI (Fig. 4, G to I), which is evocative of more marked impairments reported for *BACE1*^{-/-} mice (8). Then, we examined the latency of the startle reaction that is altered in *BACE1*^{-/-} mice (8) possibly due to hypomyelination of long axons (7,12). No significant changes in the latency of startle reaction were observed between wild-type mice and *Aph-1a*^{+/-}, *BACE1*^{+/-}, or *Aph-1a*^{+/-};*BACE1*^{+/-} mice (Fig. 4H and table S2). Finally, on the basis of our previous findings that *BACE1*^{-/-} mice have aging-related deficits in long-term spatial memory (10), we tested whether this type of memory can be affected by modest reductions in activities of both secretases. Testing of 19- to 21-month-old wild-type, *Aph-1a*^{+/-}, *BACE1*^{+/-}, and *Aph-1a*^{+/-};*BACE1*^{+/-} mice in the classic version of the Morris water maze revealed no between-group differences in spatial learning or long-term reference memory (fig. S3). Together, these results support the view that the novel anti-amyloid combination therapy that modestly targets both β -secretase and γ -secretase represents not only an effective but also a safe treatment strategy to attenuate A β amyloidosis in AD.

Mild hypomyelination of axons in *BACE1*^{+/-} and *Aph-1a*^{+/-};*BACE1*^{+/-} mice

Because hypomyelination is a prominent feature of *BACE1*^{-/-} mice (7,12), we examined the effect of reduction of both γ -secretase and BACE1 on myelination of central and peripheral axons (Fig. 5, A to C). Morphometric analysis revealed that the g ratios (axon diameter to fiber diameter) of sciatic nerve axons were similar between wild-type and *Aph-1a*^{+/-} mice (Fig. 5D), although there was a small but significant difference between optic nerve axons in these two groups (Fig. 5E; $P < 0.02$, one-way ANOVA, Tukey's multiple comparison). A small but significant increase in g ratios was observed in axons of both sciatic and optic nerves of *BACE1*^{+/-} mice relative to wild-type littermates (Fig. 5, D and E; $P < 0.0001$). The g ratios of peripheral and central axons in *Aph-1a*^{+/-};*BACE1*^{+/-} mice were not different from those in *BACE1*^{+/-} mice, indicating that there is no convergence on hypomyelination of central nervous system or peripheral nervous system axons by the modest decrease of both γ -secretase and BACE1. It is important to note that, although significant, the magnitude of changes observed in g ratios in *BACE1*^{+/-} mice is very small relative to those observed in the optic and sciatic nerves of *BACE1*^{-/-} mice (reduction by factors of 5.5 and 4.5, respectively) (7).

Aph-1a^{+/-};*BACE1*^{+/-} mice exhibit normal life span and no increased risk of tumorigenesis or splenomegaly

Because reduction of γ -secretase has been shown to be associated with untoward side effects, including decreased life span, gastro-intestinal abnormalities, tumorigenesis, and splenomegaly (5), we determined whether any of these phenotypes were observed in *Aph-1a*^{+/-};*BACE1*^{+/-} mice. The life expectancy (Fig. 6A) and body weight (Fig. 6B) of *Aph-1a*^{+/-};*BACE1*^{+/-} mice were similar to that in the wild-type control littermates. In addition, pathological analyses revealed no increases in the number of mice with tumors in *Aph-1a*^{+/-}, *BACE1*^{+/-}, or *Aph-1a*^{+/-};*BACE1*^{+/-} mice. Although our previous data (6) suggested that a small percentage of aged *Aph-1a*^{+/-} mice show an enlargement of spleen (60 weeks or older), our current data disclosed very few cases of splenomegaly (1 of 12; Fig. 6C) or hepatomegaly in aged *Aph-1a*^{+/-};*BACE1*^{+/-} (1 of 23; Fig. 6D) or in wild-type control littermate mice (1 of 23) (all above 75 weeks of age) (Fig. 6C and fig. S4). The percentages of splenomegaly and hepatomegaly were higher in mice carrying the *APP*^{swe}/*PS1* Δ E9 transgenes than that on the wild-type background (fig. S5, A and B), indicating that the presence of these mutant transgenes can increase the incidence of these pathologies. Deletion of one allele of *Aph-1a* and/or *BACE1* did not significantly affect the frequency of these abnormalities in *APP*^{swe}/*PS1* Δ E9 mice. Collectively, these results support the idea that modest inhibition of both γ -secretase and BACE1 provides a greater protection for the brain while limiting mechanism-based toxicity. Thus, our results support the view that such anti-amyloid combination therapy may be beneficial for the prevention and/or treatment of AD.

DISCUSSION

Despite significant advances in our understanding of the pathogenesis of AD, safe and effective mechanism-based therapies are lacking and only symptomatic treatments are now available. On the basis of the amyloid cascade hypothesis, intense efforts have been made to reduce the generation of A β peptides (especially A β 42) or to enhance their clearance as potential therapeutic strategies for the treatment of AD (1). Pharmacological inhibition of γ -secretase or β -secretase has been considered as an attractive anti-amyloid therapy to ameliorate A β amyloidosis in AD. However, enthusiasm has been dampened because strong inhibition of their activities can be associated with mechanism-based toxicities (5–9). Further, recent findings indicate that selective targeting of components of γ -secretase (that is, *Aph-1b/c*^{-/-} mice) can provide anti-amyloid protection for the brain while avoiding Notch-related phenotypes (17), but there are negative effects on other signaling pathways, such as NRG1, associated with schizophrenia-like endophenotypes (9). This highlights the challenges

associated with targeting γ -secretase. Nevertheless, some γ -secretase inhibitors may be useful in attenuating A β amyloidosis and in understanding the mechanism-based toxicity associated with chronic reduction of γ -secretase activity (6,17). Although modest pharmacological inhibition of these proteases is a reasonable therapeutic strategy in efforts to ameliorate A β amyloidosis and limit side effects in AD, this approach only modestly decreases the amyloid burden in the brain (6,10,16). Our approach reported here is based on the idea that modest reductions of both γ -secretase and β -secretase would provide greater protection against A β amyloidosis while limiting mechanism-based side effects associated with either protease because the processing of APP to generate A β requires both γ -secretase and BACE1.

Our biochemical analyses show that the combined moderate reduction of γ -secretase and BACE1 not only attenuates AD pathology by additively reducing A β deposition but also decreases levels of A β oligomers in the brain. Accumulation of A β oligomers may be linked to neuronal dysfunction, as these oligomers are associated with impaired synaptic plasticity and memory in mouse models (33–35). We observed that the improvement of spatial reference memory in our aged (19 to 21 months old) *APP^{swe}/PS1 Δ E9;Aph-1a^{+/-};BACE1^{+/-}* mice is correlated with lower amounts of soluble A β oligomers in the brain; these mice performed as well as wild-type littermates. However, *APP^{swe}/PS1 Δ E9*, *APP^{swe}/PS1 Δ E9;Aph-1a^{+/-}*, or *APP^{swe}/PS1 Δ E9;BACE1^{+/-}* mice, with higher levels of A β oligomers, showed significant memory impairments. Our data suggest that the combined reduction of both γ -secretase and BACE1 provides greater ability to reduce soluble A β oligomers to rescue the memory deficits occurring in *APP^{swe}/PS1 Δ E9* mice. Because studies indicated that BACE1 and γ -secretase could modulate oxidative stress, it is also plausible that reduction of oxidative stress may contribute to the rescue of cognitive impairment in our *APP^{swe}/PS1 Δ E9;Aph-1a^{+/-};BACE1^{+/-}* mice (36). Our efforts emphasize the greater value of this novel therapeutic strategy in overcoming the age-related limitations in efficacy associated with modest reduction of just one of these secretases (10). Moreover, our modeling of this anti-amyloid combination therapy under the setting of advanced age and A β amyloidosis provides clinical relevance, as it mimics the treatment situation where elderly individuals with mild cognitive impairment or early stages of AD carry a high amyloid burden.

The observations that *Aph-1a^{+/-};BACE1^{+/-}* mice exhibit normal life span and show no overt pathological or behavioral abnormalities further strengthen the view that modest targeting of both γ -secretase and BACE1 may be an effective and safe therapeutic strategy used for attenuating A β amyloidosis in AD. Because endogenous A β peptides may play a role in normal cognitive functions by modulating synaptic plasticity (37–39), reduction of both secretases may affect learning and memory. However, our observation that *Aph-1a^{+/-};BACE1^{+/-}* mice exhibit normal cognition indicates that the level of endogenous A β is kept within the physiological range for normal synaptic function. Future electrophysiological studies of synaptic plasticity in *Aph-1a^{+/-};BACE1^{+/-}* mice should confirm this notion. Finally, it is possible that the combination of immunotherapy (40) and modest inhibition of secretases may provide a more effective and safe therapeutic strategy to reduce amyloid, protect synaptic connectivity and neuronal circuits from degeneration, and improve cognition without untoward side effects.

MATERIALS AND METHODS

Mice

BACE1 knockout and *Aph-1a* knockout mice were generated in our laboratory as previously described (41–43). Littermates of *Aph-1a* knockout mice, *Aph-1a^{+/-}* (129/SvJ background), were first intercrossed with *BACE1* knockout mice (C57BL/6J background) to obtain a cohort of *Aph-1a^{+/-};BACE1^{+/-}* mice. Subsequently, F1 (*Aph-1a^{+/-};BACE1^{+/-}*) mice were intercrossed to generate cohorts with one or two alleles of *Aph-1a* and *BACE1*. Four groups of

wild-type control, *Aph-1a*^{+/-}, *BACE1*^{+/-}, and *Aph-1a*^{+/-};*BACE1*^{+/-} mice were used for studies of behavior (5 to 6 months old and 12 to 15 months old) and myelination effect (3 months old). *APP^{swe}/PS1ΔE9* mice (line 85, C57BL/6J background) generated as described (44,45) were used to intercross with *Aph-1a*^{+/-};*BACE1*^{+/-} mice (F2 C57BL/6J × 129/SvJ background) to obtain wild-type, *Aph-1a*^{+/-}, *BACE1*^{+/-}, *Aph-1a*^{+/-};*BACE1*^{+/-}, *APP^{swe}/PS1ΔE9*, *APP^{swe}/PS1ΔE9*;*Aph-1a*^{+/-}, *APP^{swe}/PS1ΔE9*;*BACE1*^{+/-}, and *APP^{swe}/PS1ΔE9*;*Aph-1a*^{+/-};*BACE1*^{+/-} mice for neuropathological and behavior studies.

Genotype analysis was performed by PCR from mouse tail genomic DNA with the following primer sets: HC69 (5'-AGGCAGCTTTGTGGAGATGGTG-3'), HC70 (5'-CGGAAATCGGAAAGGCTACTCC-3'), and HC77 (5'-TGGATGTGGAATGTGTGCGAG-3') for *BACE1*; 5'-GCTGTCTCTGTCCTTCTACAGGAG-3' and 5'-CGGAAGATCACCCATCTCCATCC-3' for endogenous allele *Aph-1a*; and 5'-GTACACCATCACCCGACTGTC-3' and 5'-CTACCCGCTTCCATTGCTCAG-3' for the targeted allele were used to determine the genotype of *Aph-1a*. Apart from the conventional genotyping, results were verified by protein blot analysis (figs. S1A and S2). Procedures involving mice were performed under the guidelines of the Johns Hopkins Medical Institutions Institutional Animal Care and Use Committee.

Tissue handling

Animals for transmission electron microscopy (TEM) analysis were transcardially perfused with ice-cold PBS and then 4% paraformaldehyde–2% glutaraldehyde. Brain, sciatic, and optic nerves were immediately isolated and postfixed in 4% paraformaldehyde–2% glutaraldehyde for 48 hours at 4°C before processing. Animals for light immunohistochemical microscopy analysis were dissected rapidly. Hemisphere of the brain, heart, lung, liver, kidney, and spleen were isolated, weighted, and postfixed in 4% paraformaldehyde for 48 hours at 4°C before paraffin embedding, sectioning, hematoxylin and eosin (H&E) staining, and immunohistochemical staining. Other hemisphere of the brain was snap-frozen and homogenized in 10 volumes of ice-cold PBS buffer containing protease inhibitor (Roche Products) by pestle motor. Tissue lysates were left on ice for 15 min after homogenization. Half of the 10% (w/v) homogenate for protein blot analysis was adjusted to 1% SDS and centrifuged at a maximum speed for 10 min at 4°C. The remaining half of 10% (w/v) homogenate (without SDS) for Aβ enzyme-linked immunosorbent assay (ELISA) assay was centrifuged at 100,000g for 30 min at 4°C without adding any detergent. Supernatants containing soluble Aβ were collected for assays. The pellets were homogenized in 500 μl of 70% formic acid solution and incubated on ice for 1 hour. The formic acid lysates were centrifuged at 100,000g for 1 hour at 4°C. Supernatant containing insoluble Aβ was collected and neutralized by 25 volumes of 1 M Tris-base solution and stored at –80°C before usage. Concentrations of protein extracts were determined by the bicinchoninic acid method (Pierce).

Immunohistochemistry, stereology, and electron microscopy

For histochemical and immunocytochemical analyses, we performed sagittal brain, heart, lung, liver, kidney, and spleen sections (spaced 80 μm apart) in paraffin-embedded blocks. All samples were analyzed with H&E stain. Antibodies against APP/Aβ (monoclonal 6E10; Covance) and ubiquitin (Dako North America) were used for immunohistochemistry. Quantitative analysis of Aβ plaque load was determined by the area fraction of ubiquitin immunoreactivity as described (46) with a light microscope interfaced with a Stereo Investigator (MicroBrightfield). For electron microscopy, samples were processed for TEM under the standard protocol. Sections (55 to 70 nm thick) of optic and sciatic nerves were used for the TEM analysis. TEM images were captured by Hitachi 7600 transmission electronic microscope. The g ratios of axons from the sciatic and optic nerves were determined from six

animals (three males and three females) of wild-type, *Aph-1a*^{+/-}, *BACE1*^{+/-}, and *Aph-1a*^{+/-};*BACE1*^{+/-} mice as described (47) with ImageJ 1.38f software from the National Institutes of Health. The myelinated axon, total fiber circumferences, and axonal diameters were measured by digitally tracing around the perimeters of the inner and outer layers of myelinated fiber. The g ratio was calculated by dividing the inner circumference of an axon by outer circumference of the total fiber including myelin. Large sample sizes used for statistical comparisons of g ratio were sufficient to show significance even for small and substantially unimportant differences. To estimate the strength of the effects on myelination, rather than a significance (chance level), we calculated an effect size that characterizes a magnitude of the effect in units of variability of the population (Δ mean/SD in wild-type group) (48). The effect sizes of deletion of one allele of *BACE1* (*BACE1*^{+/-} mice with or without *Aph-1a*^{+/-}) ranged from 0.26 to 0.21 SD in the optic nerve and from 0.39 to 0.35 SD in the sciatic nerve. Thus, the magnitude of changes observed in g ratios in *BACE1*^{+/-} mice was not significantly influenced by the absence of one allele of *Aph-1a* and was very small relative to those observed in the optic and sciatic nerves of *BACE1*^{-/-} mice (reduction by factors of 5.5 and 4.5, respectively) (47).

Behavioral analyses

Group design for behavioral and cognitive assessment of *Aph-1a*^{+/-};*BACE1*^{+/-} mice—Groups of wild-type control ($n = 84$, 39 males and 45 females), *Aph-1a*^{+/-} ($n = 26$, 11 males and 15 females), *BACE1*^{+/-} ($n = 38$, 11 males and 27 females), and *Aph-1a*^{+/-};*BACE1*^{+/-} mice ($n = 31$, 13 males and 18 females) were used for studies of several behavioral tests, including spontaneous alternation task in Y maze, plus maze, open-field task, and PPI, to analyze the deficits in working memory, alterations in levels of anxiety, novelty-induced motor activity, and sensorimotor gating, respectively. These tasks have been used previously in studies of *APP*^{swe}/*PS1* Δ E9 and *BACE1*^{-/-} mice by our group (9,10,41). These behavioral tasks were used to examine cohorts of mice at 5 to 6 months and 12 to 15 months of age. Only data from 5- to 6-month-old mice were presented in this article, including wild-type control ($n = 24$, 17 males and 7 females), *Aph-1a*^{+/-} ($n = 12$, 6 males and 6 females), *BACE1*^{+/-} ($n = 19$, 3 males and 16 females), and *Aph-1a*^{+/-};*BACE1*^{+/-} ($n = 22$, 11 males and 11 females) mice. Male and female groups were collapsed together because of a lack of significant differences.

An additional cohort of mice was derived from crosses of *Aph-1a*^{+/-};*BACE1*^{+/-} and *APP*^{swe}/*PS1* Δ E9 mice and tested in the classic Morris water maze at the age of 19 to 21 months. The sample size for this cohort was as follows: wild-type ($n = 6$), *Aph-1a*^{+/-} ($n = 5$), *BACE1*^{+/-} ($n = 5$), and *Aph-1a*^{+/-};*BACE1*^{+/-} ($n = 6$) mice (fig. S3, B and C).

Assessment of cognitive deficits in *APP*^{swe}/*PS1* Δ E9;*Aph-1a*^{+/-};*BACE1*^{+/-} mice—The effects of partial ablation of *Aph-1a* and/or *BACE1* on cognitive deficits were determined in 19- to 21-month-old animals on *APP*^{swe}/*PS1* Δ E9 background. The sample size for *APP*^{swe}/*PS1* Δ E9 background was as follows: *APP*^{swe}/*PS1* Δ E9 ($n = 8$), *APP*^{swe}/*PS1* Δ E9;*Aph-1a*^{+/-} ($n = 9$), *APP*^{swe}/*PS1* Δ E9;*BACE1*^{+/-} ($n = 6$), and *APP*^{swe}/*PS1* Δ E9;*Aph-1a*^{+/-};*BACE1*^{+/-} ($n = 6$) mice (Fig. 3). To characterize the magnitude of A β -related cognitive deficits in the *APP*^{swe}/*PS1* Δ E9 model at this particular age and strain background, we compared the group of *APP*^{swe}/*PS1* Δ E9 mice ($n = 14$) and wild-type controls ($n = 10$) that were derived from the same crosses and tested at the same age as the four aforementioned genotypes (Fig. 3). Male to female ratio was ~1:1. Male and female groups were collapsed together because of a lack of significant differences in this test.

Behavioral tasks

Before behavioral testing, all mice were intensively handled (5 days by ~3 min per mouse). All testing was performed in an isolated behavior room at 23° to 24°C. Mice were acclimated to the behavior room for an hour before the test began. All tests were performed in the light part of the light-dark cycle. Behaviors in the Y maze, plus maze, and open field were recorded by a computer-based video tracking system (2100 Plus; HVS Image) and scored by trained experimenters blinded to genotype with a computer-assisted data acquisition system (Stopwatch+; www.cbn-atl.org/research/stopwatch.shtml).

Spontaneous alternation task in Y maze

Testing was carried out on a Y-shaped maze as described (49). Mice were placed at the end of one of the arms and allowed to explore freely for 5 min. The sequence of arm entries was recorded. The spontaneous alternation behavior was calculated as the number of triads containing entries into all three arms divided by the maximum possible alternations. The percentage of alternation was corrected by position bias according to McFarland (50).

Plus maze

A plus maze was raised 70 cm above the ground, and testing was performed in low diffuse lighting as described (49). Each subject was placed in the center of the apparatus and allowed to explore freely for 5 min. An experimenter scored the number of arm entries made to the open and closed arms as well as the time spent in each area.

Open-field task

Open-field task was performed as described by Laird *et al.* (41). The round white open-field arena had a diameter of 100 cm and 55 cm high sidewalls. The same illumination as in other tasks was used, consisting of indirect diffuse room light (eight 40-W bulbs, 12 lux). Each animal was released near the wall and observed for 15 min. As in all other tasks, performance in the open field was recorded by a computer-based video tracking system (2100 Plus). Activity measures included distance traveled, percentage of time spent in active exploration (episodes of movement ≥ 5 cm/s), and speed of movement during active exploration. To analyze anxiety levels, we broke down the activity measures into two zones. On the basis of our previous studies, a 20-cm-wide wall zone constituted the most preferred peripheral zone, whereas the rest of the open field was defined as a central zone comprising ~67% of the arena surface and was most aversive for mice. The number of entries to the central zone of the open field was also recorded.

Sensitivity to MK-801

Dizocilpine (MK-801), a noncompetitive NMDA receptor antagonist, induces hyperactivity and stereotypic behaviors in rodents (51). Because at higher doses MK-801 causes a set of stereotypic behaviors (head weaving and ataxic gait) and interferes with locomotor activity (52), in our pilot studies we used a range of MK-801 doses (0.1 to 0.6 mg/kg). A dose of 0.3 mg/kg resulted in changes in motor activity but minimal stereotypy (52). This dose of MK-801 was used in further experiments. MK-801 (Sigma) was dissolved in PBS and injected intraperitoneally (5 ml/kg). Control groups of mice received an injection of equal volume of vehicle (PBS).

For testing of drug-induced locomotor activation, each mouse was placed in a clean open field (45 × 45 × 40 cm) and activity was recorded over a 105-min period using the activity chambers with infrared beams (San Diego Instruments). Horizontal and vertical activities, stereotypic activities, and time spent in the center or along the walls (thigmotaxis) of the chamber were automatically recorded. Behavior was also video-taped for offline analyses by trained

observers blinded to genotype and treatment. The duration and incidence of grooming, rearing, and stereotypic behaviors (head weaving or ataxic gait) were scored with a computer-assisted data acquisition system (Stopwatch+).

PPI of ASR

PPI was performed as described (8). Briefly, after a 6-min acclimation period, the mouse was exposed to three 25-ms startle pulses of 120-dB white noise to determine the initial level of ASR. Subjects then received six blocks of eight trials each to measure the PPI. Each block of trials consisted of six different types of trial presented pseudo randomly across blocks: two types of trial were with startle pulse only (120 or 110 dB conducted twice in each block of trials) and four different types of trials in which prepulses were followed by the startle stimuli (one trial for each of the prepulse and startle intensities). The prepulses were 25-ms weak stimuli of white noise with intensities of 8 or 16 dB above the 63-dB background noises. The time interval between the prepulse offset and the startle pulse onset was 75 ms. The maximal amplitude of the reaction and its latency was recorded for each prepulse (during the interval 0 to 100 ms) and pulse stimulus (during the interval 100 to 200 ms). Trials were presented at a variable-interval schedule of 20 to 40 s.

The average value for every type of trial across six blocks was used for the statistical analysis. Startle reactivity (Fig. 4, G and H) was analyzed with maximal amplitudes of reactions to prepulse (71 and 79 dB) and pulse stimuli (110 and 120 dB). PPI (Fig. 4I) was measured as a percentage of ASR inhibition induced by each prepulse and was calculated as $[100 \times (\text{startle amplitude in the startle alone trial} - \text{startle amplitude in the prepulse trial}) / \text{startle amplitude in the startle alone trial}]$.

Classic Morris water maze task

In the classic Morris water maze task, testing was conducted as described (9,41). Animals were pretrained to climb and stay on a submerged platform (10×10 cm) placed in the center of a small pool (diameter, 45 cm). During the pretraining (five trials), a mouse was placed in the water (face to the platform) and allowed to swim, climb on the platform, and stay there for 10 s. Then, it was picked up by the tail, dried with a paper towel, and returned to a warm dry waiting cage for 7 to 8 min, so all mice were familiarized with procedural aspects of the task. The actual Morris water maze task was then conducted in a pool (diameter, 100 cm) filled with opaque water ($22 \pm 2^\circ\text{C}$) and surrounded by a set of distal and proximal spatial cues. Four daily sessions included 10 platform trials, in which the platform was submerged but accessible to the mouse, as well as two probe trials (before and after the platform trials). During the probe trials conducted with either a short (~15 min) or a long (20 hours) delay after the platform trials, the platform was collapsed for variable intervals (35 to 45 s) to test the subject's spatial preference. At the end of the probe trial, the collapsed platform was returned to its raised position, and the mouse was allowed to escape onto the platform. This probe trial protocol ensured the same response reinforcement contingency as in the platform trials and allowed to use probe trials repeatedly without the effect of extinction (53). If the mouse failed to locate the platform in 60 s, the experimenter directed the mouse to the platform by hand and the mouse remained on the platform for 10 s. On the day after the 4-day training, all mice were given a single probe trial with a 20-hour delay to assess the final strength and accuracy of memory. Distance (path from the start location to the platform, in centimeters) and swim speed (average speed during a trial, in centimeters per second) were measured during the platform trials. In the probe trials, the measures recorded were swim speed, percentage of time spent in the areas 20 and 40 cm in diameter around the location of platform (area 20 and area 40, respectively), and frequency of platform crossings (expressed as a number of crossings per 10 s).

All measures of performance in the Morris water maze were automatically calculated by 2100 Plus tracking system and then exported to STATISTICA 8.0 for analyses and data presentation. The measures of probe trials were presented for the correct and opposite quadrants to establish levels of spatial preferences in the control groups (Fig. 3, D to F, and fig. S3C). This is an important “quality” control for the experiment to ensure that a particular protocol used for a particular set of mice (strain background, age, etc.) is sufficient to result in good learning and memory. Presentation of measures for the correct and opposite quadrants was particularly important for the platform crossings. In contrast to assessment of spatial preferences by area 40 or area 20, the measure of platform crossings does not permit a simple calculation of the chance level of performance. It is critical to compare platform crossings between “baited” and “nonbaited” quadrant(s) to overcome this problem. Here, we have compared the frequency of platform crossings between correct and opposite quadrants, an assessment that based on our previous experience is sufficient to verify whether platform crossings reflect a significant spatial preference or chance level.

Protein blot analysis

Equal amounts of protein were subjected to SDS-polyacrylamide gel electrophoresis tris-glycine gel (Invitrogen) and transferred onto a polyvinylidene difluoride 0.2- μ m pore size membranes (Invitrogen). After the protein transfer, the blots were incubated with 4% bovine serum albumin blocking buffer and the specific antibodies as follows: antibody to rabbit BACE1 (1:1000) (42), antibody to human A β 1-42 (1:1000) (Covance), antibody to mouse β 3-tubulin (1:1000) (Sigma), and antibody to mouse β -actin (1:5000) (Sigma). Protein extracts were analyzed by immunoblotting with enhanced chemiluminescence (Millipore).

Determination of A β levels

Brain extracts (soluble protein from PBS and insoluble protein from formic acid) from 6-month-old *APP^{swe}/PS1 Δ E9* mice were used for two-site ELISAs that specifically detect the C terminus of A β to measure A β 1–42 and A β 1–40 levels as suggested by the manufacturer (Biosource International).

Statistical analysis

All data from biochemical studies were analyzed for statistical significance by GraphPad Prism statistical software with two-tailed *t* test or one-way ANOVA. For behavioral analyses, the data were analyzed with repeated-measures ANOVA with the statistical package STATISTICA 8.0 and a minimal level of significance of $P < 0.05$. Fisher's least significant difference post hoc tests were applied to significant main effects or interactions. In the figures, the data are presented as the mean $\pm$ SEM and significant P values are denoted with asterisks (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Reduction of γ -secretase and BACE1 additively attenuates A β 1–40 and A β 1–42 peptides and A β deposition. (**A** and **B**) ELISA assay of insoluble (formic acid extraction, pg/ μ l) A β 1–40 and A β 1–42 extracted from brain hemisphere of *APP^{swe}/PS1 Δ E9* (AP), *APP^{swe}/PS1 Δ E9;*Aph-1a*^{+/-}* (APA), *APP^{swe}/PS1 Δ E9;*BACE1*^{+/-}* (APB), and *APP^{swe}/PS1 Δ E9;*Aph-1a*^{+/-};*BACE1*^{+/-}* (APAB) mice at 6 months of age. (**C** and **D**) Average amyloid plaque number per section in the hippocampus and cerebral cortex of 6-month-old *APP^{swe}/PS1 Δ E9*, *APP^{swe}/PS1 Δ E9;*Aph-1a*^{+/-}*, *APP^{swe}/PS1 Δ E9;*BACE1*^{+/-}*, and *APP^{swe}/PS1 Δ E9;*Aph-1a*^{+/-};*BACE1*^{+/-}* mice. Sagittal serial brain sections were selected at eight-section intervals for the analysis. * P < 0.05, ** P < 0.01, *** P < 0.001. (**E** to **H**) Representative

sagittal hippocampal sections immunostained with ubiquitin-specific antisera from 6-month-old *APP^{swe}/PS1 Δ E9* (E), *APP^{swe}/PS1 Δ E9;Aph-1a^{+/-}* (F), *APP^{swe}/PS1 Δ E9;BACE1^{+/-}* (G), and *APP^{swe}/PS1 Δ E9;Aph-1a^{+/-};BACE1^{+/-}* (H) mice. Scale bars, 200 μ m.

Fig. 2.

Reductions of both γ -secretase and BACE1 protect against A β amyloidosis in aged *APP^{swe}/PS1 Δ E9* mice. (**A** to **D**) Representative sagittal hippocampal sections immunostained with ubiquitin-specific antisera from 23-month-old *APP^{swe}/PS1 Δ E9* (**A**), *APP^{swe}/PS1 Δ E9;Aph-1a^{+/-}* (**B**), *APP^{swe}/PS1 Δ E9;BACE1^{+/-}* (**C**), and *APP^{swe}/PS1 Δ E9;Aph-1a^{+/-};BACE1^{+/-}* (**D**) mice. (**E** and **F**) Unbiased stereology analysis of area fraction covered by amyloid plaques in the hippocampus (**E**) and cerebral cortex (**F**). (**G**) Average number of A11 immunoreactive clusters in hippocampus and cortex per brain section. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. (**H** to **K**) Immunohistochemical staining with A11, an oligomer-specific antisera, of the hippocampus in *APP^{swe}/PS1 Δ E9* (**H** and **J**) and *APP^{swe}/PS1 Δ E9;Aph-1a^{+/-};BACE1^{+/-}* (**I** and **K**) mice. Magnifications, $\times 4$ [(**H**) and (**I**)] and $\times 20$ of dentate gyrus from hippocampal section from (**H**) and (**I**) defined by the rectangular box. Scale bars, 200 μ m [(**H**) and (**I**)], 50 μ m [(**J**) and (**K**)].

Fig. 3.

Reductions of both γ -secretase and BACE1 rescue memory deficits in aged *APP^{swe}/PS1 Δ E9* mice. (A to F) Testing of spatial learning [(A) and (B)] and reference memory [(C) to (F)] in the classic Morris water maze. (A) Distances to the platform are shown as averages for consecutive blocks of five training trials for wild-type control (WT; $n = 10$) and *APP^{swe}/PS1 Δ E9* mice ($n = 14$). (B) Distance to the platform for groups of *APP^{swe}/PS1 Δ E9* ($n = 8$), *APP^{swe}/PS1 Δ E9;Aph-1a^{+/-}* ($n = 9$), *APP^{swe}/PS1 Δ E9;BACE1^{+/-}* ($n = 6$), and *APP^{swe}/PS1 Δ E9;Aph-1a^{+/-};BACE1^{+/-}* ($n = 6$) mice. These four groups of mice were tested at the same age and in the same strain background as mice shown in (A). (C) Representative examples of swimming patterns for each of the genotypes ($n = 6$ mice per genotype) are shown as bivariate histograms of coordinates of the animal location recorded every 0.1 s during the probe trial. The most frequently visited areas are indicated by orange and red. An insert shows a scheme of the water maze with a position of start location, platform location, and areas of 20 and 40 cm around the platform. [(D) to (F)] Accuracy of spatial reference memory assessed in the probe trial conducted 20 hours after the last training trial. Measures of spatial memory are presented in accordance with increasing demands for accuracy: Spatial preferences for the area of 40 cm (D) and 20 cm (E) in diameter around the platform are followed by the frequency of crossings (number of crossings per 10 s) through the exact platform location (10 cm in diameter) (F). The higher scores in the indices of probe trials reflect a better performance. * $P < 0.05$, ** $P < 0.01$, significant between-group differences in the measures for the correct quadrant; # $P < 0.05$, significant differences between the measures taken for the correct and opposite quadrants within the same genotype.

Fig. 4.

Aph-1a^{+/-};*BACE1*^{+/-} mice show no emotional or cognitive deficits. (A) Spontaneous arm alternation (%) in the working memory Y-maze task is shown for wildtype control, *Aph-1a*^{+/-} (A), *BACE1*^{+/-} (B), and *Aph-1a*^{+/-};*BACE1*^{+/-} (AB) mice. (B and C) Novelty-induced exploration tested in the open-field task. (B) Dynamic of motor activity in the open field during consecutive 3-min blocks of testing. (C) Total activity accumulated throughout a whole period of testing. (D and E) Testing the anxiety levels in the plus maze. (D) Percentage of visits to the open arms of the plus maze. (E) Total number of arms visited during the test was used as a measure of motor activity in this task. **P* < 0.05, significant difference relative to the wild-type control mice. (F) Psychostimulant-induced hyperactivity was tested after the intraperitoneal injection of MK-801 (0.3 mg/kg). Mice were first habituated for 45 min to a novel cage. After the MK-801 injection, activity was monitored for 60 min more. (G to I) PPI of ASR. (G) Amplitude of startle reaction to startle stimuli of different intensities. (H) Latency of startle reaction averaged for all trials with 110 dB. (I) PPI of ASR in four types of trials: with two levels of prepulses (8 or 16 dB above background) and two levels of startle stimuli (110 or 120 dB). The data shown are for mice tested at 5 to 6 months of age and are expressed as the mean ± SEM. An additional cohort of 12- to 15-month-old mice was tested in the Y maze, open field, and plus maze with no significant effect of genotype (data not shown).

Fig. 5.

BACE1^{+/-} and *Aph-1a*^{+/-};*BACE1*^{+/-} mice exhibit mild hypomyelination. (A) Representative examples of electron micrographs of sections of optic and sciatic nerves from 3-month-old wild-type control mice, *Aph-1a*^{+/-}, *BACE1*^{+/-}, and *Aph-1a*^{+/-};*BACE1*^{+/-} mice. Scale bars, 1 μm (optic nerves), 4 μm (sciatic nerves). (B and C) Scatterplots of axonal diameters are shown against the g ratios of myelinated axons from the optic (B) and sciatic (C) nerves. (D and E) Quantitative analyses of genotype-related differences in the g ratio for the optic (D) and sciatic (E) nerves ($n = 6$ mice per genotype, at least 100 axons per nerve per mouse). For the optic nerve, significantly higher g ratios were observed in *BACE1*^{+/-} ($P < 0.001$) and *Aph-1a*^{+/-};*BACE1*^{+/-} ($P < 0.001$) mice relative to that of control mice (D). (E) For the sciatic nerve, g ratios of *Aph-1a*^{+/-} ($P < 0.01$), *BACE1*^{+/-} ($P < 0.001$), and *Aph-1a*^{+/-};*BACE1*^{+/-} ($P < 0.001$) were higher than that of control mice.

Fig. 6.

Aph-1a^{+/-};*BACE1*^{+/-} mice exhibit normal life span and organ histology. (A) Kaplan-Meier survival plots for *Aph-1a*^{+/-} [*n* = 52, median life expectancy (MLE) = 95 weeks], *BACE1*^{+/-} (*n* = 61, MLE = 105 weeks), *Aph-1a*^{+/-};*BACE1*^{+/-} mice (*n* = 66, MLE = 104 weeks), and wild-type control mice (*n* = 67, MLE = 103 weeks). No significant differences in life span were found between any of these genotypes. (B) Average body weight in *Aph-1a*^{+/-} (*n* = 12), *BACE1*^{+/-} (*n* = 27), and *Aph-1a*^{+/-};*BACE1*^{+/-} (*n* = 23) mice is shown as percentage of average body weight in wild-type control (*n* = 23) mice. No significant differences in body weight were observed. (C) Average spleen weight in grams. Splenomegaly (>3 SDs from the average spleen size) was rare and observed only in one animal in each of the wild-type control, *Aph-1a*^{+/-},

and *Aph-1a*^{+/-};*BACE1*^{+/-} groups. **(D)** Average liver weight in grams. The incidents of hepatomegaly (>3 SDs from the average liver size) increased as aging progressed; however, no significant differences in liver size were found between different genotypes. An individual case of 91-week-old wild-type control male [denoted in (C) and (D) with asterisks] represented with splenomegaly and hepatomegaly accompanied by metastases in multiple organs (fig. S4). **(E to L)** H&E staining of spleen [(E), (G), (I), and (K)] and liver [(F), (H), (J), and (L)]. No histopathology was found in wild-type control or *Aph-1a*^{+/-};*BACE1*^{+/-} mice. Scale bars, 100 μ m [(E) to (H)], 50 μ m [(I) to (L)].