Neurobiology of Disease

BACE1, a Major Determinant of Selective Vulnerability of the Brain to Amyloid-β Amyloidogenesis, is Essential for Cognitive, Emotional, and Synaptic Functions

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A transmembrane aspartyl protease termed β-site APP cleavage enzyme 1 (BACE1) that cleaves the amyloid-β precursor protein (APP), which is abundant in neurons, is required for the generation of amyloid-β (Aβ) peptides implicated in the pathogenesis of Alzheimer’s disease (AD). We now demonstrate that BACE1, enriched in neurons of the CNS, is a major determinant that predisposes the brain to Aβ amyloidogenesis. The physiologically high levels of BACE1 activity coupled with low levels of BACE2 and α-secretase anti-amyloidogenic activities in neurons is a major contributor to the accumulation of Aβ in the CNS, whereas other organs are spared. Significantly, deletion of BACE1 in APPswe/PS1E9 mice prevents both Aβ deposition and age-associated cognitive abnormalities that occur in this model of Aβ amyloidosis. Moreover, Aβ deposits are sensitive to BACE1 dosage and can be efficiently cleared from the CNS when BACE1 is silenced. However, BACE1 null mice manifest alterations in hippocampal synaptic plasticity as well as in performance on tests of cognition and emotion. Importantly, memory deficits but not emotional alterations in BACE1+/− mice are prevented by coexpressing APPswe;PS1E9 transgenes, indicating that other potential substrates of BACE1 may affect neural circuits related to emotion. Our results establish BACE1 and APP processing pathways as critical for cognitive, emotional, and synaptic functions, and future studies should be alert to potential mechanism-based side effects that may occur with BACE1 inhibitors designed to ameliorate Aβ amyloidosis in AD.

Key words: BACE1 null mice; selective vulnerability; Aβ amyloidosis; Alzheimer’s; cognition; synaptic plasticity; RNAi

Introduction

Alzheimer’s disease (AD), the most common cause of dementia in the elderly (Morris and Price, 2001; Mayeux, 2003; Petersen, 2003), selectively damages the brain but does not involve other tissues/organs. Although the biological basis for the selective vulnerability of the brain to amyloid-β (Aβ) amyloidosis is not well understood, we postulate that high levels of amyloid-β precursor protein (APP) and levels and activities of β-site APP cleavage enzyme 1 (BACE1), a proamyloidogenic enzyme, play critical roles in the susceptibility of the CNS to this disease (Holsinger et al., 2002; Yang et al., 2003). In the CNS, Aβ peptides are generated by sequential endoproteolytic cleavages of neuronal APP by two membrane-bound enzyme activities: BACE1 cleaves APP to generate APP-β C-terminal fragments (APP-βCTFs) (Hussain et al., 1999; Sinha et al., 1999; Vassar et al., 1999; Yan et al., 1999; Lin et al., 2000), and the γ-secretase cleavage of APP-βCTFs leads to secretion of Aβ peptides (Francis et al., 2002; De Strooper, 2003; Selkoe and Kopan, 2003; Takasugi et al., 2003). APP can also be cleaved within the Aβ sequence through an alternative, non-amyloidogenic pathway involving α-secretase, thought to be tumor necrosis factor-α converting enzyme (TACE) (Allinson et al., 2003), or BACE2 (Farzan et al., 2000; Yan et al., 2001). Despite initial efforts to clarify the physiological roles of APP and its derivatives (Zheng et al., 1995; Dawson et al., 1999), their exact functions remain elusive. Emerging evidence indicates that γ-secretase cleavages of the APP-βCTF or αCTF leads to the release of cytosolic fragments termed the APP intracellular domain (AICD), which forms a multimeric complex with Fe65, a nuclear adapter protein (Cao and Sudhof, 2001). This complex or Fe65 alone enters the nucleus and binds to the histone acetyltransferase Tip60 to regulate gene transcription (Cao and Sudhof, 2001; Baek et al., 2002; Cao and Sudhof, 2004). An APP and
Fe65 complex has also been implicated in neurite growth and synapse modification (Sabo et al., 2003). Because BACE1 cleavage of APP is critical in Aβ amyloidosis, it has been suggested that inhibition of BACE1 would be an attractive strategy to ameliorate Aβ deposition in AD (Citron, 2002; Vassar, 2002). Supporting this notion are studies demonstrating that deletion of BACE1 prevents Aβ secretion in cultured neurons and in brain (Cai et al., 2001; Luo et al., 2001; Roberds et al., 2001; Wong et al., 2002; Luo et al., 2003), and that young (before Aβ deposits) mutant APP mice lacking BACE1 do not develop memory deficits (Ohno et al., 2004). However, it is unclear whether deletion of BACE1 will prevent age-associated memory impairments in aged mutant APP mice. Moreover, it is not known whether Aβ deposition or Aβ-associated cognitive deficits in these mice are sensitive to the dosage of BACE1. It will be important to assess the consequences of partial deletion of BACE1 because complete inhibition of this enzyme could potentially be associated with problems for several reasons. First, additional BACE1 substrates may exist (Kitazume et al., 2001; Gruninger-Leitch et al., 2002; Lichtenhaller et al., 2003; Li and Sudhof, 2004; Wong et al., 2005), and the consequences of inhibiting these processing events are unknown. Second, Aβ may normally play an important role in modulating activities of certain synapses (Kamenetz et al., 2003). Consequently, chronic inhibition of BACE1 could be associated with mechanism-based toxicities. Although it is not clear whether deletion of BACE1 impacts on cognition in older individuals, the recent findings that 4-month-old BACE1 knock-out mice have mild deficits in a task that assesses spatial memory (Ohno et al., 2004) suggests that chronic reduction in BACE1 activity could impact learning and memory.

Thus, despite recent advances, several key issues remain to be addressed. Here, we assessed whether the relative levels of BACE1 and BACE2 activities in neurons predispose the brain to amyloidosis. In addition, we determined whether age-associated memory deficits occurring at advanced age in mouse models of Aβ amyloidosis can be prevented in the absence of BACE1 and whether Aβ deposition is sensitive to the dosage of BACE1 and efficiently cleared from the brain by silencing BACE1. Furthermore, we evaluated the involvement of BACE1 and the APP processing pathway in cognitive, emotional, and synaptic functions. Results of these studies indicate that the balance of various BACE1-dependent processes appear to be important for both normal and abnormal behavioral performance. These discoveries are critical for the design and testing of novel therapeutic strategies for AD.

**Materials and Methods**

**Immunoblot analysis and ELISA**

Mouse tissues were dissected and homogenized in T-PER buffer (Pierce, Rockford, IL) in the presence of protease inhibitors (Roche Products, Welwyn Garden City, UK). After homogenization, the lysates were centrifuged at 100,000 × g, and the supernatants were saved for Western blot, Ciphergen (Palo Alto, CA) ProteinChip Array, and Aβ ELISA analyses. Equal amounts of lysates were subject to SDS-PAGE (Tris–glycine mini gel, Invitrogen, Carlsbad, CA) and Western blot analysis using antibodies specific for the following: BACE1 [1:2500 (Cai et al., 2001); BACE2 (1:1000; AnaSpec, San Jose, CA); APP (1:1000; Chemicon, Temecula, CA); presenilin-1 (PS1) (1:2000; Chemicon); GFAP (1:5000; Sigma, St. Louis, MO); β3-tubulin (1:5000; Covance Research Products, Berkeley, CA); and actin (1:5000; Chemicon). The quantitative sandwich ELISA was used to determine levels of both Aβ42 and Aβ40 in the conditional media of primary cultured cortical neurons and astrocytes and in brain lysates of mutant transgenic mice as suggested by the manufacturer (Biosource International, Camarillo, CA).

**Primary cultures, infection, and metabolic labeling**

Cortical neuronal cultures were established from brains of embryonic day 16.5 fetal mice following published protocols (Naruse et al., 1998). After 7 d of culture, the cells were infected with 5 × 106 plaque-forming units of adenovirus expressing APPswe for 4 d in serum-free medium. For metabolic labeling, neuronal cells were preincubated for 30 min in methionine-free DMEM with 1% dialyzed bovine serum and then labeled with 500 μCi/ml 35S-methionine in methionine-free medium for 5 h. After metabolic labeling, cell extracts were immunoprecipitated with an APP C-terminal antibody (Chemicon), and the immunoprecipitates were fractionated on 4–20% Tris–glycine SDS-PAGE. The gels were dried and exposed, and radioactive bands were quantified by phosphorimaging analyses. For cortical astrocyte cultures, the dissected brain cortices were suspended in HBSS supplemented with 0.25% trypsin and 0.01% DNasel and incubated at 37°C for 10 min. The tissues were then transferred to DMEM supplemented with 10% fetal bovine serum and dissociated by repeated trituration. The dispersed cells were collected by centrifugation and plated at ~1 × 106 cells per well on six-well cell culture plates (coated with poly-L-lysine) in DMEM supplemented with 10% fetal bovine serum. Astrocytes were allowed to mature for 3 d in culture before they were treated with 10 μM glutamate to kill the neurons and split once in DMEM supplemented with 10% fetal bovine serum on an uncoated culture dish, before they were infected with 5 × 106 plaque-forming units of adenovirus expressing human APPswe or BACE1 for 4 d. The conditional medium and cell lysates were saved for later analysis.

**Ciphergen ProteinChip array**

On each spot of PS1 series ProteinChip array, 1 μg of purified 6E10 monoclonal antibody (Signet Laboratories, Dedham, MA) was applied and incubated at room temperature for 1 h in a humidified chamber. After blocking the unbound chip surface with 0.1 g/ml glycerine, pH 8.0, for 30 min and washing the chip three times with PBS buffer containing 0.5% Triton X-100, samples were added onto antibody-coated spots and incubated for 2 h at room temperature. After extensive wash with PBS buffer in the presence of 0.5% Triton X-100 followed by 5 ml HEPES buffer, pH 7.5, chips were analyzed by surface-enhanced laser desorption/ionization time of flight mass spectrometry (Ciphergen) in the presence of CHCA matrix solution. Mass identification was made on each spot by 100 averaged shots. External standards were used for calibration.

**Animals**

BACE1 knock-out mice, mouse/human APPswe (line C3-3), and human PS1ΔE9 (line S-9) transgenic mice were described as generated previously (Borchelt et al., 1996; Lee et al., 1997; Cai et al., 2001). To generate a cohort of 16- to 18-month-old APPswe/PS1ΔE9 mice with zero, one, or two alleles of BACE1, BACE1+/–/– mice (C57BL6/J;129/Sv background) were first crossed with APPswe (C57BL6/129 background) or PS1ΔE9 (C57BL6/129 background) mice to create APPswe/BACE1+/–/– and PS1ΔE9; BACE1+/- mice (F1). Subsequently, F1 APPswe;BACE1+/– and F1 PS1ΔE9/BACE1+/– mice were intercrossed to obtain five groups of mice (F2) used for behavioral testing at 16–18 months of age: APPswe; PS1ΔE9, APPswe/PS1ΔE9;BACE1+/-, APPswe/PS1ΔE9;BACE1+/-, PS1ΔE9;BACE1+/-, and nontransgenic mice. This F2 cohort of mice therefore possesses the C57BL6 and 129sv hybrid background. We verified that the effect of the transgene(s) was not influenced by this hybrid genetic background by confirming that the C57BL6 and 129sv hybrid background used in our study did not significantly affect our ability to detect genotype-related differences (supplemental Figs. S1, S2, available at www.neurosci.org as supplemental material).

A second cohort of BACE1+/–, BACE1+/-, and nontransgenic mice of 3–6 months of age was generated from intercrosses of BACE1+/– mice on C57BL6/129 congenic background. All procedures involving animals were under the guidelines of Johns Hopkins University Institutional Animal Care and Use Committee.

**Histology and immunohistochemical analysis**

Mice were perfused with 4% paraformaldehyde in PBS. The brain was removed, embedded in paraffin, sectioned, and processed for both histological and immunohistochemical analyses. Hirano silver stain used Hirano’s modification of the Bielschowsky method (Yamamoto and
Hirano, 1986; Borchelt et al., 1996), and Cgo red staining was performed according to standard techniques. For immunohistochemical studies, the peroxidase–antiperoxidase method used antibodies specific for the following: BACE1; synaptophysin (Dako, High Wycombe, UK); syntaxin (Sigma); GFAP (Sigma); F4/80 (Serotec, Oxford, UK); and microtubule-associated protein 2 (MAP2) (Sigma). Sections were counterstained with hematoxylin and eosin. For BACE1 immunostaining, an IgG-purified anti-BACE1 fusion protein antibody was applied (1 µg/µl) as primary antibody in Tris-buffered saline buffer (10 mM Tris-HCl and 4°C). Before filtering, the samples were diluted with PBS (100 – 200 µl) and adjusted to a final concentration of 1% SDS. After filtering, using a 96-well dot-blot apparatus (Bio-Rad, Hercules, CA), the membranes were washed twice with 500 µl of PBS, pH 7.4, per well. Proteins trapped by the filter were detected by immunostaining following protocols used in immunoblotting.

Filter trap assay

For filter trap assay (Xu et al., 2002), we used one hemisphere from the brains of APPswe;PS1ΔE9 and APPswe;PS1ΔE9;BACE1+/− mice at 12 and 20 months of age, whereas the other hemispheres were used for stereological analyses. Cellulose acetate membranes with 0.2 µm pore size (OE66; Schleicher & Schuell, Keene, NH) were used to trap the Aβ aggregates from the brain lysate. Briefly, mouse brains were weighed and then homogenized in 10 vol of PBS, pH 7.4, containing a protease inhibitor mixture (Roche Products). Homogenates were centrifuged at 3000 rpm for 5 min at 4°C in a microcentrifuge. The protein concentrations in the supernatants were determined by the BCA method (Pierce). Before filtering, the samples were diluted with PBS (100 – 200 µl) and adjusted to a final concentration of 1% SDS. After filtering, using a 96-well dot-blot apparatus (Bio-Rad, Hercules, CA), the membranes were washed twice with 500 µl of PBS, pH 7.4, per well. Proteins trapped by the filter were detected by immunostaining following protocols used in immunoblotting.

Unbiased stereology

A stereological method (Jankowsky et al., 2003) was performed to quantitatively analyze the fraction of the hippocampus occupied by neuritic plaques. Neuritic plaques were defined with an anti-ubiquitin antibody (Sigma). The hippocampal region was outlined under a microscope equipped with stepping motors driven by Stereo Investigator Software (MicroBrightField, Colchester, VT). Starting from a random site, a counting frame with a superimposed counting grid composed of small equidistant “crosses” was generated to systematically sample the outlined field. We counted the total number of crosses (intersections) and the crosses that coincided with immunoreactivity “hits.” Finally, the fractional area (FA) was calculated as the sum of hits for all counted frames divided by the total number of crosses. According to the Delesse principle, the FA is equal to the fraction of the volume occupied by the neuritic plaques being quantified.

Behavioral testing

Groups in the first cohort, five groups of 16- to 18-month-old mice were behaviorally tested: APPswe;PS1ΔE9 and APPswe;PS1ΔE9;BACE1+/− mice (n = 14); APPswe;PS1ΔE9;BACE1−/− mice (n = 6); APPswe;PS1ΔE9;BACE1+/− mice (n = 22); BACE1−/− mice (n = 11); and nontransgenics (n = 15). Approximately equal numbers of female and male mice were used (females, 44%; males, 56%). Female to male ratio for each genotype was as follows: APPswe;PS1ΔE9 (57%); APPswe;PS1ΔE9;BACE1−/− (50%); APPswe;PS1ΔE9;BACE1+/− (41%); BACE1−/− (36%); and nontransgenic mice (40%). All mice were analyzed in three behavioral tasks: first, in the classic Morris water maze task; second, in the visual discrimination task; and finally, in the open field. A second cohort of mice was tested at 3 months of age: nontransgenics (n = 7), BACE1+/− mice (n = 20), and BACE1−/− mice (n = 7). In this cohort only, male mice were used and testing was performed in the classic Morris water maze task, radial water maze task, Y-maze, plus maze, and on an open-field task. Before behavioral testing, all mice were intensively handled (5 d by ~3 min per mouse) and weighed once a week.

Classic Morris water maze task. Testing was conducted as described previously (Savonenko et al., 2005). Before the task, mice 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 (2 d for 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 tail, dried with a paper towel, and returned to a warm dry waiting cage for 7–8 min (an average duration of intertrial interval). Thus, before starting the classic Morris water maze task, all mice were familiarized with procedural aspects of the task. The Morris water maze task was conducted in the pool (diameter, 100 cm) filled with opaque water (22 ± 2°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 block of platform trials). During the probe trials, the platform was collapsed at the bottom of the tank for variable intervals (30–40 s). 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 the use of probe trials repeatedly without the effect of extinction. 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. 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 different quadrants, as well as time spent in the area 40 cm in diameter around the location of the platform (annulus 40).

Visual discrimination task in the water maze. After the Morris water maze task, mice were pretrained to swim to and climb on a visible platform as described for pretraining with hidden platform. The platform was made visible by the attachment of a high-contrast extension (1.5 cm above water surface). Thus, before starting the visual discrimination task, mice were familiarized with a visible platform as a new escape platform to minimize possible confounding effects of neophobia and strategy of searching for hidden platform. Visual discrimination training was conducted in a 100 cm pool with spatial cues removed and consisted of two daily sessions of six trials each. For every trial, the visible platform was located in a different quadrant of the pool and at a different distance from the walls. The start position was changed randomly for each trial. Measures of performance were the same as those for the platform trials in the Morris water maze task.

Radial water maze. The radial water maze task was conducted as described by Savonenko et al. (2005). Briefly, clear plastic inserts (30 × 30 cm) were placed within the Morris water maze such that six arms were created within the pool. Training took place over 6 d with daily new position of the platform. Each training session consisted of six trials, in which the mouse was placed at different start positions from trial to trial and allowed 120 s to find the hidden platform located at the end of one of the maze arms. Two additional sessions were run with the same protocol, but the start position was constant across the session. Distance to the platform and the number of errors (entry of the mouse into an arm that did not hold the platform) were recorded for each trial.

Spontaneous alternation task in Y-maze. Testing was performed on a Y-shaped maze as described by Andreasson et al. (2001). Mice were placed into the end of one arm 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.

Plus mask task. A plus maze described by Andreasson et al. (2001) was used for testing anxiety in the young cohort of mice. For this test, the maze was raised 70 cm above the ground, and testing was performed in low diffuselighting. Each subject was placed in the center of the apparatus and allowed to explore freely for 5 min. An observer scored the number of arm entries made to the open and closed arms as well as time spent in each area.

Open-field task. 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 subject was released near the wall and observed for 5 min. As in all other tasks, performance in the open field was recorded by a computer-based video tracking system (Analysis VP-200; HSV Image, Hampton, UK). Activity measures included distance traveled, percentage of time spent in active exploration (episodes of movement ≥5 cm/s), and...
Electrophysiological studies in hippocampal slices

Hippocampal slices were prepared from adult (3–6 months old) male BACE1 knock-out or wild-type mice as described previously (Zhao and Lee, 2003). Briefly, under deep anesthesia by halothane, mice were killed by decapitation, and their brains were removed quickly and transferred to the ice-cold dissection buffer containing the following (in mM): 212.7 sucrose, 2.6 KCl, 1.23 NaH$_2$PO$_4$, 26 NaHCO$_3$, 10 dextrose, 3 MgCl$_2$, and 1 CaCl$_2$ (bubbled with a mixture of 5% CO$_2$ and 95% O$_2$). A block of hippocampus was removed and sectioned into 400-μm-thick slices using a vibratome. The slices were recovered for 1.5 h at room temperature in artificial CSF (ACSF) (in mM): 124 NaCl, 5 KCl, 1.25 NaH$_2$PO$_4$, 26 NaHCO$_3$, 10 dextrose, 1.3 MgCl$_2$, and 2.5 CaCl$_2$ (bubbled with a mixture of 5% CO$_2$ and 95% O$_2$). All recordings were done in a submersion recording chamber perfused with ACSF (29.5–30.5°C, 2 ml/min). Synaptic responses were evoked by stimulating the Schaffer collaterals with 0.2 ms pulses delivered through concentric bipolar stimulating electrodes (Frederick Haer Company, Bowdoinham, ME) and recorded extracellularly in CA1 stratum radiatum. Baseline responses were recorded using half-maximal stimulation intensity at 0.033 Hz. To induce long-term potentiation (LTP), four trains of theta-burst stimulation (TBS) were delivered at 0.1 Hz. Each train consists of 10 bursts (four pulses at 100 Hz per burst) repeated at 5 Hz frequency. Long-term depression (LTD) was induced by a paired-pulse 1 Hz protocol (PP-1 Hz) with interstimulus interval (ISI), 50 ms delivered at 1 Hz for 15 min. We used three episodes of paired-pulse, 1 Hz stimulus delivered with 15 min interepisode intervals to induce the saturated LTD. For measurement of paired-pulse facilitation (PPF), we used ISIs of 25, 50, 100, 200, 400, 1000, and 2000 ms. Pharmacologically isolated NMDA receptor-mediated synaptic responses were measured using 0 mM MgCl$_2$ ACSF with 10 μM NBQX. APV at 100 μM was added at the end of each experiment to confirm the NMDA receptor-mediated responses.

Generation and injection of lentiviral-small hairpin RNA for BACE1

Two short hairpin RNA (shRNA) (sequences shown in supplemental Fig. S4A, available at www.jneurosci.org as supplemental material) were designed according to guidelines described previously (Elbashir et al., 2002; Paddison and Hannon, 2002; Dykhooorn et al., 2003; Rubinson et al., 2005). The two shRNA specifically target BACE1 mRNA and are predicted not to anneal to any other mRNA in mice as determined by homology search of the mouse database. The shRNAs were subcloned into a lentiviral vector generously provided by Dr. Parijs (Massachusetts Institute of Technology, Cambridge, MA) (Rubinson et al., 2003). The shRNAs were packaged into lentiviruses (LV) (Intriviron) and subsequently concentrated by centrifugation, and its titer (≈5.2 × 10$^4$ IU/μl) was determined by the expression of green fluorescent protein (GFP) in infected HEK293 cells. HEK293 cells were cultured under standard conditions in DMEM media with 2 mM l-glutamine and 10% fetal calf serum (Intriviron). N2A cells were maintained in 45% DMEM, 45% Opti-DMEM, 10% fetal calf serum, and 2 mM l-glutamine. N2A cells were transfected with two different short hairpin RNAs, SH1-BACE1 and SH2-BACE1, and, 24 h later, cells were infected with adenoviral vector expressing APPsw. At 48 h after transfection, conditioned media were collected, and levels of β$\beta_1$-40 were determined by sandwich ELISA (Bio-source International).

Using a glass pipette attached to a Hamilton syringe, 3 μl of high-titer lentivirus expressing either SH1-BACE1 or GFP alone were stereotaxically injected into the right hippocampus of APPsw/PS1ΔE9 mice using a syringe pump (New Era Pump System, Farmingdale, NY) at a rate of 0.25 μl/min. Two injections were made using the following coordinates (in mm): injection 1, anteroposterior 2.5, mediolateral 2.5, dorsoventral 2.5; and injection 2, anteroposterior 2.5, mediolateral 2.5, dorsoventral 2.5. For unbiased stereological analysis, every 15th section of 10-μm-thick sections was sampled within ~1 mm surrounding the injection site and the respective region of the contralateral hippocampus. The amount of AB deposits as visualized by immunostaining with antisera specific to ubiquitin in the polymorph layer/CA3 (the path of mossy fibers and their terminals) was determined.

Statistical analyses

The data were analyzed using repeated measures or simple main effect ANOVAs and two-tailed t-test with the statistical package Statistica 6.0 (StatSoft, Tulsa, OK) and a minimal level of significance p < 0.05. The main factors were as follows: group, a comparison between groups of different genotypes (BACE1$	extsuperscript{-/-}$, APPsw/PS1ΔE9, APPsw/PS1ΔE9; BACE1$	extsuperscript{-/-}$, APPsw/PS1ΔE9; BACE1$	extsuperscript{-/-}$, and nontransgenics), and gender. However, comparisons between male and female mice are not reported because there were no significant effects of gender or gender × group interactions. The repeated measures were as follows: sessions, a comparison between means from trials during sessions, and trials, a comparison between trials. Newman–Keuls post hoc tests were applied to significant main effects or interactions.

Results

BACE1 selectively accumulates in the CNS

The relative differences between BACE1 and BACE2 mRNA expression in various tissues (Vassar et al., 1999; Bennett et al., 2000) led us to hypothesize that BACE1 is a major factor in selective predisposition of AB accumulation in the CNS (Wong et al., 2001). We determined levels and distributions of BACE1 in the CNS compared with these parameters in other organs. In contrast to the ubiquitous expression pattern of BACE1 mRNA in a variety of tissues (Vassar et al., 1999), BACE1 protein accumulates to high levels in the brain (Fig. 1A), although it is undetectable in non-neural tissues, including the pancreas, which had been shown to have the highest levels of expression of BACE1 transcripts (Vassar et al., 1999). In interpreting the protein blots, it is important to note there is a nonspecific cross-reacting band (migrating at slightly higher molecular weight than BACE1) (Fig. 1A). Without the BACE1$	extsuperscript{-/-}$ tissues as controls, this band could be mistakenly identified as BACE1. To examine the distribution of BACE1 within the brain, we determined the levels of BACE1 in different regions of the newborn (Fig. 1B) and adult (Fig. 1C) mouse brains. With the exception of the relatively higher levels of BACE1 in the olfactory bulb, the levels of BACE1 in most regions of the brain appear to be uniform when normalized to the level of β3-tubulin (Fig. 1B, C). Altogether, our results are consistent with the view that the selective accumulation of BACE1 in the brain predisposes the CNS to Aβ amyloidosis.

Although BACE1 protein is present at comparable levels in most brain regions (Fig. 1B, C), BACE1-specific immunoreactivities are particularly localized in the hippocampus, a region that is critical for learning and memory and one that is vulnerable in AD (Fig. 1D). Notably, strong signals were observed in the hilus of dentate gyrus and stratum lucidum of CA3 region (terminal field of mossy fiber pathway) (Fig. 1D, I). As expected, no specific signals were observed in brain sections prepared from BACE1 knock-out mice (Fig. 1E). To determine whether BACE1 has a presynaptic location, we compared the immunostaining patterns of BACE1 with that of several markers. The pattern of BACE1 immunoreactivity in the hippocampus was remarkably similar to two well characterized presynaptic terminal markers, synaptophysin (Fig. 1F) and syntaxin1 (Fig. 1H). In contrast, the BACE1 pattern was distinct from that of MAP2 (Fig. 1G), a postsynaptic marker. Moreover, under higher magnification, BACE1 immunoreactivities were localized to the giant boutons of the mossy
Levels of BACE1 activity in neurons is a major determinant of selective predisposition of CNS to amyloidosis

Because cleavages of APP by α-secretase and BACE2 (between positions 16–17, 19–20, and 21–22 of Aβ) result in truncated non-amyloidogenic peptides, we hypothesized that relative levels of BACE1-mediated activities versus BACE2 and α-secretase activities could significantly influence the ability of neurons (in contrast to glial cells) to secrete Aβ. To test this hypothesis, we determined the relative levels of protein and enzymatic activities of BACE1, BACE2, and α-secretase in primary cortical neurons compared with astrocytes. Whereas levels of BACE1 in neurons are significantly higher than that of astrocytes, levels of BACE2 are higher in glial cells compared with neuronal cultures (Fig. 2A). To determine the relative secretase activities between neuronal and glial cultures, we coated the SELDI protein chip array with a monoclonal antibody (6E10) that specifically recognizes the N-terminal 1–16 of Aβ, to monitor simultaneously relative amounts of Aβ1–40 and Aβ1–42 (derived from BACE1 and γ-secretase cleavages), Aβ fragments 1–19 and 1–20 (generated from BACE1 and BACE2 cleavages), and Aβ fragments 1–15 and 1–16 (derived from BACE1 and α-secretase cleavages) secreted from conditioned media of cells infected with recombinant adenovirus expressing APPswe (Fig. 2B–E). Consistent with our protein blot analysis, we observed that neurons exhibit lower levels of BACE2 and α-secretase generated peptides compared with levels of peptides produced by BACE1 activity. In contrast, astrocytes show higher levels of BACE2 and α-secretase activities compared with BACE1 activity (Fig. 2C). Thus, relative amounts of BACE2-dependent 1–19 and 1–20 or α-secretase-dependent 1–15 or 1–16 Aβ fragments when normalized to that of Aβ1–40 reveal that both α-secretase and BACE2 activities are significantly higher in astrocyte cultures compared with cultured neurons...
BACE1 is critical for cognitive and emotional functions

Although the deletion of BACE1 did not lead to overt developmental abnormalities, an observation that BACE1 null mice exhibit mild cognitive deficits in the Y-maze task (Ohno et al., 2004) raised the question as to whether BACE1 plays a critical role in learning and memory. We used three different behavioral tasks to assess hippocampus-dependent cognitive function in BACE1 null mice. First, using the Morris water maze task (Morris, 1984; Markowska et al., 1993; Andreasson et al., 2001) to assess spatial reference memory, we observed that 3-month-old BACE1−/− mice were able to learn and remember the hidden platform location as efficiently as BACE1+/− or nontransgenic littermates (F(2,31) = 0.22–0.32; p > 0.7) (Fig. 3A,B). However, 16-month-old BACE1−/− mice exhibited a significant impairment in spatial reference memory during probe trial measures compared with littermate controls (F(1,24) = 8.30; p < 0.01) (Fig. 3D). During platform trials, the performances of aged BACE1−/− mice were not significantly different from nontransgenic mice (F(1,24) = 3.64; p > 0.07) (Fig. 3C), indicating that they were able to adopt nonspatial strategies in finding the hidden platform. These results indicated that BACE1−/− mice exhibit an age-dependent deficit in spatial reference memory.

We next used the radial water maze tasks to analyze the impact of BACE1 on spatial working memory (Arendash et al., 2001; Dudchenko, 2004). BACE1−/− mice were significantly impaired in the radial water maze (Fig. 3E,F); BACE1−/− mice made significantly more errors before finding the platform compared with BACE1+/− mice (F(2,31) = 6.03; p < 0.006; Newman–Keuls post hoc test, p < 0.005) or BACE1+/+ mice (Newman–Keuls post hoc test, p < 0.006). This deficit cannot be attributed to differences in visual acuity because 3-month-old BACE1−/− mice were not impaired on the classical Morris water maze in which the visual demands are equivalent. Additionally, specific testing of vision in the visual discrimination task showed no differences between BACE1−/− mice and littermate controls (data not shown). One critical aspect of the radial water maze task is a variable start position randomly assigned to a different arm for each trial. This variability in start position ensures that the task is solved using allocentric, hippocampus-dependent, rather than egocentric, hippocampus-independent, strategies (Eichenbaum et al., 1990; King et al., 2002). Interestingly, when a start position was made invariant across the trials, performances of the BACE1−/− mice in the radial water maze were normal (Fig. 3F). Therefore, by reducing the hippocampal demands of the task, the BACE1−/− mice were able to perform as well as littermate controls, possibly by using hippocampus-independent strategies to complete the task.

In addition, the Y-maze task was used as an independent method to assess spatial working memory in BACE1−/− mice. Similar to the radial water maze, we also observed a deficit in cognitive function in BACE1−/− mice as judged by the Y-maze task. Although BACE1−/− mice visited a similar number of arms as the BACE1+/− or BACE1+/+ mice (F(2,31) = 0.87; p > 0.5), BACE1−/− mice showed significant deficits in arm alternation (F(2,31) = 3.67; p < 0.5; Newman–Keuls post hoc test, p > 0.05) (Fig. 3G), indicating that the ablation of BACE1 resulted in the
early cognitive deficits in spatial working memory. Together, these findings support the view that BACE1 plays a critical role in both spatial reference memory and working memory.

BACE1−/− mice exhibited a reduced speed of swimming compared with littermate controls (Fig. 3A, C) in the Morris water maze task, suggesting that anxiety levels in these mice might be reduced because swim speed in rodents may reflect a stress/anxiety reaction to the placement in cold water (Winocur and Hasher, 2004). To test this hypothesis, an open-field task, a popular model of anxiety-like behaviors (Crawley, 1999; Prut and Belzung, 2003), was used to confront animals with an unknown environment. In such a situation, mice spontaneously prefer the periphery of the apparatus (thigmotaxis) as a result of an anxiety-induced inhibition of exploration in the aversive central parts of the open field. An increase in time spent, distance traveled, and number of entries to the central part, as well as a decreased latency to enter the central part, would indicate anxiolysis. In the open-field task, BACE1−/− mice traveled a significantly longer distance (F(2,31) = 3.45; p < 0.5; Newman–Keuls post hoc test, p < 0.05) and showed a significantly higher proportion of activity (F(2,31) = 3.85; p < 0.5; Newman–Keuls post hoc test, p < 0.05) (Fig. 3F) and number of visits (F(2,31) = 3.68; p < 0.5; Newman–Keuls post hoc test, p < 0.05) (Fig. 3J) in the central parts of the open field compared with BACE1+/− or BACE1+/+ mice. This “low anxiety” phenotype was also confirmed in the plus maze in which 3-month-old BACE1−/− mice visited open arms of the maze more often compared with BACE1+/− mice or littermate controls (F(2,31) = 4.07; p < 0.5; Newman–Keuls post hoc test, p < 0.03) (Fig. 3J). Collectively, our findings are consistent with the view that BACE1 null mice exhibit a lower level of anxiety compared with control littermates, implicating an important role for BACE1 in emotion.

BACE1−/− mice exhibit specific deficit in synaptic plasticity

To investigate the role of BACE1 in synaptic transmission and plasticity, we used young BACE1−/− mice (3–6 months old) and focused on area CA1, one of the main integral outputs from the hippocampus. Parameters of basal synaptic transmission, which predominantly measure the AMPA receptor-mediated component, were not different between wild-type and BACE1−/− mice (Fig. 4A). Pharmacologically isolated NMDA receptor-mediated responses were similar between wild-type and BACE1−/− mice (Fig. 4B). We then examined whether presynaptic function is altered in BACE1−/− mice by measuring the PPF ratio. Interestingly, we found that there is a significant increase in the PPF ratio in BACE1−/− mice compared with littermate controls (Fig. 4C) at 50 ms interstimulus interval (PPF ratio at 50 ms IS: BACE1+/−,
1.59 ± 0.03, n = 40 slices, 12 mice; BACE1−/−, 1.75 ± 0.06, n = 43 slices, 12 mice; Fisher’s PLSD post hoc analysis, p < 0.02). Because changes in PPF ratio have been attributed to alterations in presynaptic release probability (Manabe et al., 1993; Saura et al., 2004), the increased PPF ratio observed in BACE1−/− mice may indicate a deficit in presynaptic release.

To determine whether synaptic plasticity is altered in the BACE1−/− mice, we examined LTP using four trains of TBS. We found that there is no difference in the magnitude of LTP up to 2 h after the TBS protocol (BACE1+/+, 165 ± 11% at 2 h after TBS, n = 18 slices, 10 mice; BACE1−/−, 151 ± 10%, n = 7 slices, 5 mice) (Fig. 5A). Similarly, we did not find a deficit in LTD induced with one train of paired-pulse 1 Hz protocol (PP-1 Hz, 15 min, ISI = 50 ms) (BACE1+/+, 75 ± 4% at 1 h post-onset of PP-1 Hz, n = 12 slices, 5 mice; BACE1−/−: 78 ± 5%, n = 12 slices, 5 mice) (Fig. 5B). Because one episode of PP-1 Hz does not saturate LTD, we repeated the study with 3 trains of PP-1 Hz to determine whether the “lower ceiling” of synaptic plasticity has changed in BACE1−/− mice. As shown in Figure 5C, we did not find a difference between control and BACE1−/− mice (BACE1+/+; 58 ± 4% at 1 h post onset of last PP-1 Hz, n = 16, 7 mice; BACE1−/−; 63 ± 6%, n = 18, 8 mice). To determine whether LTD reversal (de repression) is affected in BACE1−/− mice, we delivered four trains of TBS after LTD saturation. Surprisingly, we found that BACE1−/− mice produced a significantly larger de-repression compared with control mice (BACE1+/+, 168 ± 16% at 30 min after TBS, n = 13 slices, 7 mice; BACE1−/−, 252 ± 27% n = 13 slices, 7 mice; Student’s t test, p < 0.01) (Fig. 5D). Both LTD and de-repression are induced by the same TBS protocol, but our data indicate that BACE1−/− mice show a selective increase in de-repression. To determine whether this is attributable to differential summation of responses during TBS, we compared the area under the field potentials during the TBS (Fig. 5E). We found a significant increase in responses during TBS in the BACE1−/− only during the de-repression (ANOVA, repeated measures, F(1,23) = 6.573; p < 0.02) (Fig. 5E). Collectively, our results indicate that BACE1−/− mice display specific deficits in paired-pulse facilitation and de-repression, implicating significant alterations in mechanisms of presynaptic release and synaptic plasticity.

Deletion of BACE1 prevents age-dependent cognitive deficits occurring in APPswE;PS1ΔE9 mice

Although it has been shown that deletion of BACE1 prevents both Aβ deposition (Luo et al., 2003) and cognitive impairment in young APPswE mice (Ohno et al., 2004), an important question is whether, in aged animals, the absence of BACE1 prevents Aβ amyloidosis and age-dependent cognitive deficits. Another critical question that has not been addressed is whether partial reduction of BACE1 ameliorates Aβ amyloidosis and cognitive impairments in aged mutant APP mice. To address these questions, we used the Morris water maze task, which is sensitive to age-associate cognitive deficits in different mutant APP transgenic models (Ashe, 2001), including our APPswE;PS1ΔE9 mice (Savonenko et al., 2005). We initially crossedbred BACE1−/− mice with APPswE;PS1ΔE9 mice to generate APPswE;PS1ΔE9 mice with normal levels of BACE1 (APPswE;PS1ΔE9;BACE1+/+), with 50% of normal BACE1 levels (APPswE;PS1ΔE;BACE1+/+), and without BACE1 (APPswE;PS1ΔE;BACE1−/−). Immunocytochemical and biochemical analyses of the resulting offspring are presented in supplemental Figures S4 and S5 (available at www.jneurosci.org as supplemental material). Briefly, APP-βCT, Aβ1-40, and Aβ1-42 and Aβ deposits are abolished in brains of APPswE;PS1ΔE9;BACE1−/− mice.

We next analyzed the cognitive effects of BACE1 ablation in 16- to 18-month-old APPswE;PS1ΔE9 mice with 0.50, or 100% of BACE1 activity. As expected, APPswE;PS1ΔE9 mice swam a significantly longer distance to find the platform (Fig. 6A) (Newman–Keuls post hoc test, p < 0.0001) applied to significant effect of group, ANOVA, F(4,63) = 7.69, p < 0.0001) and spent less time in the vicinity of the platform (Fig. 6C,D) (probe trials, Newman–Keuls post hoc test, p < 0.0005; effect of group, ANOVA F(4,63) = 5.49, p < 0.001) than control nontransgenic mice. However, APPswE;PS1ΔE9;BACE1−/− mice performed as well as nontransgenic mice in both platform (Fig. 6A) and probe (Newman–Keuls post hoc test, p > 0.35) trials (Fig. 6C,D), indicating that deletion of BACE1 prevented age-dependent cognitive deficits observed in APPswE;PS1ΔE9 mice. However, partial decrease of BACE1 to 50% of normal levels was not sufficient to ameliorate cognitive deficits because APPswE;PS1ΔE9;BACE1−/− mice were significantly impaired compared with nontransgenic controls (Newman–Keuls post hoc test, p < 0.0001 for platform and probe trial measures) and indistinguishable from APPswE;PS1ΔE9 mice.
We have already shown that cognitive deficits occurring in a mouse model of Aβ amyloidosis, but a 50% decrease of BACE1 is not sufficient to significantly ameliorate cognitive deficits.

**Cognitive deficits occurring in BACE1<sup>−/−</sup> mice are prevented by APP and PS1 transgenes**

We have already shown that BACE1 null mice exhibited an age-dependent cognitive deficit (see above) (Fig. 3C,D) as assessed by the Morris water maze. Intriguingly, the memory impairments observed during probe trials in BACE1<sup>−/−</sup> mice did not occur in APP<sup>swe</sup>;PS1<sup>ΔE9</sup>;BACE1<sup>−/−</sup> mice (Fig. 6A,D), indicating that the BACE1-dependent cognitive deficits are attenuated by coexpression of APP and PS1 transgenes. Importantly, despite the rescue of cognitive impairments in BACE1<sup>−/−</sup> mice overexpressing APP and PS1, BACE1<sup>−/−</sup> mice showed a slower swim speed when compared with nontransgenic controls, APP<sup>swe</sup>;PS1<sup>ΔE9</sup>, or APP<sup>swe</sup>;PS1<sup>ΔE9</sup>;BACE1<sup>−/−</sup> mice (Fig. 6A,B). These results indicated that, in contrast to cognitive deficits, a "low swim speed" phenotype was associated with the deletion of BACE1 and appeared to be independent of coexpression of APP and PS1 transgenes. The low swim speed phenotype was observed in both hidden and visible platform trials (Fig. 6A,B), suggesting that this...
Figure 6. Analyses of cognitive and emotional behaviors in aged BACE1−/−, APPswe;PS1ΔE9, and APPswe;PS1ΔE9;BACE1−/− mice. A–F, Cognitive deficits present in aged (16 –18 months) BACE1−/− and APPswe;PS1ΔE9 mice but absent in APPswe;PS1ΔE9;BACE1−/− mice. Distance to find the platform is shown for consecutive blocks of "platform" trials in which the platform was available but hidden under the milky water (A). Inset shows an average swimming speed during platform trials. Note that BACE1−/− and APPswe;PS1ΔE9;BACE1−/− mice swim significantly slower than other groups. No between-group differences were observed in distance to find the platform when it was made visible by a high-contrast extension (B). Inset shows that BACE1−/− and APPswe;PS1ΔE9;BACE1−/− mice swim slower than other groups similar to that observed in hidden platform trials (see A). Spatial preferences for the hidden platform location tested in the "probe" trials (C–F) in which the platform was lowered and inaccessible for the mouse for a variable interval (30 – 40 s). Acquisition of spatial preference is shown as a percentage of time spent in annulus 40 in consecutive probe trials (C) conducted before and after each block of 10 platform trials (shown in A). Nontransgenic controls and APPswe;PS1ΔE9;BACE1−/− mice acquired significantly higher preference for the platform location compared with all other groups. Percentage of time spent in different quadrants of the water maze during the last probe trial (D). Inset in D shows the scheme of quadrants in the water maze. The small circle indicates the location of the hidden platform, and the larger circle shows a borderline of annulus 40, an area of 40 cm in diameter centered around the platform. Nontransgenic and APPswe;PS1ΔE9;BACE1−/− mice swim predominantly in the correct quadrant (white bars), whereas the spatial preferences in other groups were significantly lower and distributed between the correct and one of the adjacent (gray bars) quadrants. The triangles indicate that the preferences for the correct and one of the adjacent quadrants are not different (p > 0.2). Preference for the correct quadrant is shown in consecutive blocks of 10 s in the last probe trial (E). The spatial preferences were stable in nontransgenic and APPswe;PS1ΔE9;BACE1−/− mice but not in other groups. In all genotypes, swim speed increased in the course of the probe trial (F). BACE1−/− and APPswe;PS1ΔE9;BACE1−/− mice swam consistently slower than other groups (see also insets in A and B). The asterisks in A and C–F indicate a significant difference from other groups as determined by Newman–Keuls post hoc test applied to a significant effect of group (ANOVA, p < 0.05). Dotted lines in C–E show chance levels of performance for the variables used. G–I, Anxiolytic phenotypes present in BACE1−/− and APPswe;PS1ΔE9;BACE1−/− mice in the open-field test. Integral measures of activity (G) and anxiety (H) averaged for whole duration of testing (5 min) is shown. The asterisks and triangles indicate a significant difference from nontransgenic (NTG) and APPswe;PS1ΔE9 mice, respectively, as a result of Newman–Keuls post hoc test applied to significant effect of group (ANOVA, p < 0.01). Note that BACE1−/− and APPswe;PS1ΔE9 mice had contrasting deficits in distance, time, and number of visits to the central part of the open field, indicating low and high anxiety levels, respectively. Dynamics of visits to the central part of the open field (I) are shown for consecutive minutes of testing. Pound signs indicate significant differences from nontransgenic group as a result of Newman–Keuls post hoc test (p < 0.05) applied to significant group × minute interaction (ANOVA, F(16,252) = 1.75; p < 0.05).
behavioral trait of mice lacking BACE1 was independent of cognitive demands of the tasks.

To further delineate a possible interaction between spatial memory and swim speed in BACE1 knock-out mice, we evaluated in greater detail the last probe trial in which between-group differences were most robust (Fig. 6D–F). In the probe trial, the swimming speeds of BACE1−/− mice (with or without mutant APP;PS1 transgenes) were significantly lower than those found in other groups (Fig. 6F) (effect of group, F(4,63) = 4.50; p < 0.005). Importantly, the swim speed of BACE1−/− mice increased over the course of the probe trials, indicating that the low swim speed was not simply a result of fatigue (which would be observed as a reduced swim speed as the probe trials progressed) (effect of 10 s blocks, F(1,32) = 17.15; p < 0.0001; group × block interaction is not significant). Comparisons of swim speeds and spatial preferences between BACE1−/− mice with or without mutant APP;PS1 transgenes showed that, despite having similar swim speeds, these two groups were significantly different in their preferences for the correct quadrant (Fig. 6I). Together, our results indicated that, in the observed range of changes, the swim speeds did not interfere with the spatial performance during probe trials. Thus, spatial memory deficits observed in BACE1−/− mice appeared to be independent of alterations in swim speeds and could be prevented by coexpression of mutant APP;PS1 transgenes, implicating an important role for the APP processing pathway in cognition.

**Disassociation between cognitive and emotional deficits in BACE1−/− mice**

Although cognitive deficits occurring in BACE1−/− mice were prevented by coexpression of APP;PS1 transgenes, we next assessed the impact of these transgenes on the emotional alteration observed in BACE1−/− mice. We used the open-field task to assess the anxiety level in mutant APP;PS1 mice lacking BACE1. As observed previously for young BACE1−/− mice (Fig. 3), 16- to 18-month-old BACE1−/− mice traveled a significantly longer distance and showed a significantly higher proportion of activity and number of visits in the central parts of the open field compared with nontransgenic controls (ANOVAs, F(1,63) = 6.90–10.56, p < 0.005; Newman–Keuls post hoc tests, p < 0.03), indicating a low level of anxiety (Fig. 6G–I). In contrast to BACE1−/− mice, APPswE;PS1ΔE9 mice spent less time and traveled a shorter distance in the central parts of the open field than nontransgenic mice (Newman–Keuls post hoc test, p < 0.05) (Fig. 6G–I). Importantly, the differences between APPswE;PS1ΔE9 mice and nontransgenic mice were observed exclusively in the central but not peripheral (relatively safe) parts of the open field (Fig. 6G). These behavioral traits are typical of high anxiety rather than low motor activity. Thus, in the open-field task, BACE1−/− mice and APPswE;PS1ΔE9 mice showed contrasting emotional deficits with low and high anxiety levels, respectively.

Given that the cognitive deficits occurring in BACE1 null mice and APPswE;PS1ΔE9 mice were prevented in APPswE;PS1ΔE9 mice lacking BACE1, one may expect that a similar interaction be observed for emotional deficits. However, anxiety measures in APPswE;PS1ΔE9;BACE1−/− mice did not support this assumption because, in all anxiety measures, APPswE;PS1ΔE9;BACE1−/− mice were significantly different from APPswE;PS1ΔE9 mice (Newman–Keuls post hoc tests, p < 0.05) but not from BACE1−/− mice (Fig. 6G). Furthermore, APPswE;PS1ΔE9;BACE1−/− mice closely mimicked the low anxiety phenotype observed in BACE1−/− mice, particularly at the beginning of testing in a novel environment (Fig. 6I). Altogether, these results indicated that, whereas cognitive deficits occurring in BACE1−/− mice were prevented in APPswE;PS1ΔE9;BACE1−/− mice, the emotional phenotypes of BACE1−/− mice (low anxiety levels) were not ameliorated by coexpression of mutant APP;PS1 transgenes.

**Aβ deposition is sensitive to BACE1 dosage and efficiently cleared from brains of aged APPswE;PS1ΔE9 mice**

Because of the potential value of inhibiting BACE1 in efforts to ameliorate Aβ deposition in AD, we examined the following: (1) the relationships between the levels of BACE1 protein and amounts of Aβ production/deposition in our lines of mice, including BACE1−/− mice that express 50% of normal level of BACE1 protein; and (2) whether silencing BACE1 through RNA interference (RNAi) ameliorates Aβ deposition in the hippocampus of a mouse model of amyloidosis. To determine whether Aβ burden is sensitive to dosage of BACE1 in the CNS, we initially assessed Aβ production in young APPswE;PS1ΔE9;BACE1−/− and APPswE;PS1ΔE9;BACE1−/− mice before the onset of Aβ deposition. In 3-month-old APPswE;PS1ΔE9;BACE1−/− mice compared with age-matched APPswE;PS1ΔE9 mice, both APP–BACE1 and Aβ42 were significantly reduced (Aβ42 reduced 31%; n = 6, p < 0.005, Student’s t test; Aβ42 reduced 40%, n = 6, p < 0.05, Student’s t test) (Fig. 7A,B). To quantify the reduction in Aβ burden after the onset of Aβ deposition, we used 12- and 20-month-old APPswE;PS1ΔE9;BACE1−/− mice. Filter trap (Xu et al., 2002) and unbiased stereological approaches were used to measure the amount of aggregated Aβ and percentage of the brain occupied by Aβ deposits, respectively. Importantly, no Aβ aggregation was detected from the filter trap assay in 12- or 20-month-old APPswE;PS1ΔE9 transgenic mice in the BACE1 null background. The filter trap assay reveals a 27% reduction of Aβ aggregates in brains of 12-month-old APPswE;PS1ΔE9 transgenic mice compared with those in the BACE1+/- background (Fig. 7B) (n = 5; t test, p < 0.05). However, no significant differences were observed in 20-month-old animals (filter trap blot not shown; quantification shown in Fig. 7B). Unbiased stereological analysis of brain sections of the same sets of mice revealed a 37% (Fig. 7E) (n = 5; t test, p < 0.01) decrease in percentages of brain volume occupied by Aβ plaques in 12-month-old APPswE;PS1ΔE9;BACE1−/− (Fig. 7D) compared with APPswE;PS1ΔE9;BACE1−/− (Fig. 7C) mice. However, no significant reduction of Aβ deposition was found in brains of 20-month-old APPswE;PS1ΔE9;BACE1−/− mice, paralleling the result from filter trap assays (Fig. 7E). Together, our results demonstrate that Aβ burden is sensitive to BACE1 dosage in young but not aged mice, indicating that Aβ clearance mechanisms may be compromised in aged animals.

To determine whether Aβ deposits can be efficiently cleared from the brain by reducing the level of BACE1, we used an RNAi strategy to silence BACE1 in the hippocampus of aged (13- to 18-month-old) APPswE;PS1ΔE9 mice. First, we assessed the effectiveness of two shRNA (SH1 and SH2) to silence BACE1 in N2A cells. Transient transfections of either SH1 or SH2 into N2A cells led to a significant reduction in levels of BACE1 mRNA and BACE1 protein, as well as secretion of Aβ42 (supplemental Fig. S4, available at www.jneurosci.org as supplemental material). These data establish that both SH1 and SH2 are effective in knocking down BACE1 expression and its activity in cultured cells. We then stereotaxically injected LV expressing SH1 to silence BACE1 selectively in the hippocampus of APPswE;PS1ΔE9 mice (Fig. 8A). We confirmed that the LV-SH1 infected neurons efficiently, as judged by GFP-positive cells with neuronal morphology in the dentate gyrus (Fig. 8B). One month after inject-
BACE1 and BACE2/TACE, along with APP, are key determinants of susceptibility of the CNS to Aβ amyloidosis (Wong et al., 2001). We now provide multiple lines of evidence to support this hypothesis. First, BACE1 protein is abundant in the brain compared with other organs (Fig. 1A). Second, neurons are enriched in BACE1 (and APP); moreover, both of these proteins are conspicuous in presynaptic terminals in the hippocampus (Fig. 1D) (Sheng et al., 2003), findings that are consistent with reports documenting that APP is anterogradely transported to synapses.

Discussion

BACE1 is a major determinant of Aβ amyloidosis in the CNS. Previously, we proposed that the distributions and levels of BACE1 and BACE2/TACE, along with APP, are key determinants of susceptibility of the CNS to Aβ amyloidosis (Wong et al., 2001). We now provide multiple lines of evidence to support this hypothesis. First, BACE1 protein is abundant in the brain compared with other organs (Fig. 1A). Second, neurons are enriched in BACE1 (and APP); moreover, both of these proteins are conspicuous in presynaptic terminals in the hippocampus (Fig. 1D) (Sheng et al., 2003), findings that are consistent with reports documenting that APP is anterogradely transported to synapses.

Figure 7. Reduced Aβ burden in young, but not aged, APPswe;PS1ΔE9 BACE1+/−/− mice. A, Analysis of Aβ aggregates using the filter trap assay from brains of 12-month-old APPswe;PS1ΔE9 BACE1+/−/−, APPswe;PS1ΔE9 BACE1+/−/−, and APPswe;PS1ΔE9 BACE1+/−/− mice. As expected, no signals were detected in APPswe;PS1ΔE9 BACE1+/−/− mice. B, Quantitative analysis of the distribution of Aβ in brains of three transgenic mouse lines: APPswe;PS1ΔE9 BACE1+/−/−, APPswe;PS1ΔE9 BACE1+/−/−, and APPswe;PS1ΔE9 BACE1+/−/− mice injected with LV-GFP, which coexpresses a reporter GFP, was stereotaxically injected into dentate gyrus of adult mouse hippocampus. Numerous cells in the dentate gyrus with neuronal morphology were GFP positive. C–F, Coronal hippocampal sections of 18-month-old APPswe;PS1ΔE9 mice immunostained with monoclonal 6E10 specific to Aβ and unilaterally injected with LV-GFP (C, D) or LV-SH1-BACE1 (E, F). C and D are ipsilateral to injection sites, and E and F are contralateral to injection sites. G–J, Coronal hippocampal sections of 13- to 14-month-old APPswe;PS1ΔE9 mice immunostained with antibody specific to ubiquitin and unilaterally injected with LV-GFP (G, H) or LV-SH1-BACE1 (I, J). G and H are ipsilateral to injection sites, and I and J are contralateral to injection sites. K, Unbiased stereological analysis of ubiquitin-stained hippocampal sections of 13- to 18-month-old APPswe;PS1ΔE9 mice. The percentage area covered by neuritic plaques in injected side was normalized to the contralateral side for each mouse. The black bar ± SEM represents LV-SH1-BACE1 (n = 5), and the white bar ± SEM represents LV-SH1-BACE1 (n = 5). Pairwise comparison using Student’s t test, p < 0.0006. L, The percentage area covered by neuritic plaques was determined in the cortex above the hippocampus in the same sections as in K. The black bar ± SEM represents contralateral sides (n = 3), and the white bar ± SEM is ipsilateral sides to LV-SH1-BACE1 injections (n = 3). Pairwise comparison using Student’s t test, p = 0.558.
in the CNS (Buxbaum et al., 1998; Lazarov et al., 2002; Sheng et al., 2002). Although initial reports indicated that BACE1 is anterogradely transported in CNS to a greater extent than in the PNS (Kamal et al., 2001; Sheng et al., 2003), recent studies failed to detect the transport of BACE1 in the PNS (Lazarov et al., 2005). Importantly, BACE1 and APP-βCTF are present in terminal fields of some populations of neurons, and lesions of inputs to these regions reduce levels of APP, βCTFs, and Aβ (Lazarov et al., 2002; Sheng et al., 2002). Third, physiologically high levels of BACE1 in neurons are coupled with low levels of α-secretase and BACE2 activities, which is in contrast to the situation in non-neuronal cells in which levels of active BACE1 are very low and levels of BACE2 and/or α-secretase activities are higher (Fig. 2).

**BACE1 plays critical roles in cognition and emotion**

Our demonstration that BACE1-deficient mice exhibit significant impairments in a number of hippocampus-dependent cognitive tasks provides direct evidence that BACE1 plays a critical role in learning and memory. Because a variety of possible substrates of BACE1 have been identified (Kitazume et al., 2001; Gruninger-Leitch et al., 2002; Lichtenthaler et al., 2003; Li and Sudhof, 2004), it is difficult to ascertain at this juncture which pathways may account for the cognitive deficits observed in BACE1 null mice. However, the observation that transgenic expression of APPswe and PS1ΔE9 is sufficient to restore the memory deficits occurring in BACE1−/− mice suggests the APP processing pathway is a candidate for critical signaling mechanisms influencing cognitive functions. Consistent with this view is the finding that APP-deficient mice appear to exhibit age-associated deficits in tasks that measure spatial reference memory (Dawson et al., 1999).

Although it is not known precisely how APP signaling impacts learning and memory, we postulate that a deficiency in neuronal AICD might contribute to the cognitive deficits observed in BACE1 null animals. The demonstration that a multimeric complex comprising AICD, Fe65, and Tip60 is capable of stimulating heterologous reporter constructs (Cao and Sudhof, 2001) coupled with the identification of a subset of nuclear factor-κB genes regulated by the AICD-mediated transcription complex (Baek et al., 2002) are consistent with the view that AICD plays a signaling role analogous to that of NICD signaling. Recent studies indicate that NICD may play a role in neuronal differentiation through the c-Jun N-terminal kinase pathway (Kimberly et al., 2005). In this model, we suggest that the majority of AICD produced in neurons is BACE1 dependent and, because of the relative abundance of BACE1 enzymatic activities found in these cells (Fig. 2), we hypothesize that only a minor proportion is derived from non-amyloidogenic pathways, i.e., via BACE2 or α-secretase activities. Thus, we envision that BACE1−/− mice have low levels of AICD in neurons leading to misregulation of AICD/Fe65/Tip60-dependent transcription, which results by as yet to be characterized processes leading to memory deficits observed in BACE1−/− mice. Consistent with this concept are observations that cognitive impairments are prevented in APPswe;PS1ΔE9;BACE1−/− mice because the levels of AICD can be compensated by increases in levels of APP (albeit, in current experiments, mutant APP), by greater efficiency of ε-cleavage mediated by PS1ΔE9-dependent γ-secretase activity, or by both mechanisms. Although future investigations will be required to distinguish these possibilities, recent studies show that increased expression of APPswe is capable of preventing mild memory deficits in young BACE1−/− mice (Ohno et al., 2004). Thus, increased levels of APP appear to be sufficient to compensate for the postulated impaired signaling underlying cognitive deficits. Intriguingly, recent studies demonstrated that isoform-specific deletion of Fe65 is associated with memory deficits (Wang et al., 2004). One interpretation of this finding that supports our model is that interactions of AICD with Fe65-dependent pathways are important for normal cognitive function.

Although it is clear that the cognitive deficits observed in BACE1 null mice can be prevented in APPswe;PS1ΔE9; BACE1−/− mice, the emotional deficits occurring in our BACE1 null mice persisted in APPswe;PS1ΔE9;BACE1−/− mice. Consistent with this view are recent demonstrations that increases in motor activity found in BACE1 null mice as assessed by a Y-maze paradigm also occurred in APPswe;BACE1−/− mice (Ohno et al., 2004). Moreover, isoform-specific Fe65 knock-out mice showed cognitive but not emotional deficits (Wang et al., 2004). Our findings strongly support the idea that other potential substrates of BACE1 (other than APP family members) may play critical roles in neuronal circuits that impact aspects of emotion in BACE1−/− mice. These discoveries raise issues regarding mechanism-based alterations in physiology and behavior that may be associated with use of BACE1 inhibitors.

**BACE1 modulates synaptic function in hippocampus**

Although basal synaptic transmission is normal in BACE1−/− mice, we observed that there is an increase in PPF ratios. Changes in PPF have been correlated with probability of presynaptic release (Manabe et al., 1993), which would suggest a presynaptic role of BACE1 (which is present in terminals). However, recent data indicate that PPF can also arise from postsynaptic modifications, namely subunit composition of AMPA receptors (Rozov et al., 1998; Shin et al., 2005). Therefore, we cannot exclude a postsynaptic role for BACE1. Regardless of the exact role BACE1 plays in synaptic functions, the increase in PPF ratios indicates that a lack of BACE1 can increase synaptic transmission at higher frequencies. This observation may explain the larger de-depression in BACE1−/− mice compared with control mice. However, contrary to our expectations, we did not observe any increase in LTP in BACE1−/− mice, which is induced by the same TBS protocol. One possibility is that the expression of LTP and de-depression use different molecular mechanisms (Abeliovich et al., 1993; Lee et al., 2000). In this case, BACE1-dependent pathways may act as a negative regulator of de-depression mechanisms, while leaving LTP mechanisms intact. Our data showing an enhanced summation of responses in BACE1−/− only during de-depression (Fig. 5E) indicates that the BACE1 probably act selectively on the induction mechanisms of de-depression. Regardless of the mechanism, the enhanced transmission of information at high frequencies (attributable to increase in ratio of PPF) suggests that altered BACE1 functions lead to interference with information transmission across synapses. Additionally, the enhanced de-depression (LTD reversal) observed in BACE1−/− mice may prevent stable expression of LTD and hence would interfere with information storage/memory. We speculate that the enhanced PPF and/or de-depression may be responsible for the behavioral deficits seen in our BACE1 null mice by either preventing effective transmission and/or storage of relevant information. In future studies, it will be interesting to clarify whether abnormalities in synaptic plasticity reported here in BACE1−/− mice are dependent on APP processing. Crosses of APP transgenic mice to BACE1−/− mice should be of value in clarifying this issue. Because we have shown that BACE1 also accumulates in CA3 in the hippocampus, it will be important to
define whether deficits in synaptic transmission and plasticity also occur in this area in BACE1−/− mice.

Deletion of BACE1 prevents neuropathological abnormalities and age-associated cognitive deficits occurring in a mouse model of Aβ amyloidosis

Because previous studies demonstrated that BACE1 is the β-secretase required for the secretion of Aβ peptides in the CNS (Fig. 2) (Cai et al., 2001; Luo et al., 2001; Roberds et al., 2001), it was anticipated that the ablation of BACE1 would impact the deposition of Aβ in mouse models of amyloidosis. Indeed, this prediction is confirmed in this investigation documenting that the deletion of BACE1 completely prevented Aβ deposits in brains of aged APPswe;PS1ΔE9 mice (supplemental Fig. S4, available at www.jneurosci.org as supplemental material). This outcome parallels observations showing the absence of Aβ deposits in younger (12 month) APPswe mice lacking BACE1 (Luo et al., 2003). Moreover, we demonstrate that the levels of p3 fragments observed in vitro in BACE1 null cells (Cai et al., 2001) do not seem to contribute to development of Aβ deposition in vivo. These results are consistent with the view that no compensatory mechanisms exist for BACE1-driven Aβ production and indicate that BACE1 might be an excellent therapeutic target for reducing Aβ amyloidosis in AD (Citron, 2002; Luo et al., 2003).

Significantly, our finding that ablation of BACE1 prevented memory deficits in aged APPswe;PS1ΔE9 mice provides strong support for the view that Aβ peptides play important roles in age-associated cognitive impairments occurring in APPswe;PS1ΔE9 mice. Recent studies showed that deficits in a Y-maze alternation task in young APPswe mice (at a time before Aβ deposits were observed) can be prevented in the absence of BACE1 (Ohno et al., 2004), consistent with the hypothesis that oligomeric Aβ (McLean et al., 1999; Walsh et al., 2002), also referred to as Aβ-derived diffusible ligands (Lambert et al., 1998; Holtzman et al., 2000; Klein et al., 2001; Gong et al., 2003), may be important contributors to the cognitive deficits occurring in these mouse models. Together, our results demonstrating that the ablation of BACE1 prevents the neuropathological abnormalities and age-associated cognitive deficits occurring in a mouse model of amyloidosis favor the view that BACE1 inhibitors may prove beneficial in efforts to attenuate Aβ amyloidosis in AD. However, caution is necessary because complete inhibition of BACE1 could lead to memory deficits as occurs in BACE1−/− mice. Analysis of conditional BACE1 knock-out mouse models will be of value in clarifying this issue.

Because these behavioral analyses were performed with aged mutant APP;PS1 mice lacking BACE1 in a hybrid strain background (C57BL/6J and 129sv), it was conceivably that the genetic background was a confounding factor in our Morris water maze study. To address this issue, it was important to verify that baseline performance was intact or to exclude the possibility that strain background impacts significantly the effect of APP;PS1 transgenes. Comparative Morris water maze analysis of mutant APP;PS1 mice on congenic C57BL/6J background showed a similar effect compared with mutant APP;PS1 mice on an F2 C57BL/6J;129sv background (supplemental Figs. S1, S2, available at www.jneurosci.org as supplemental material). Moreover, one of the well known behavioral characteristics of the 129sv strain that may affect the performance in the Morris Water maze is a relatively high floating time (Wolfer et al., 1997). Therefore, we compared time mice spent floating in our cohorts of F2 C57BL/6/

Aβ burden is sensitive to the dosage of BACE1

Because complete inhibition of BACE1 might be associated with mechanism-based toxicities, it may be important to modulate the extent of BACE1 inhibition to minimize potential toxicities. We show that 50% decrease in BACE1 levels significantly ameliorates Aβ deposition in young APPswe;PS1ΔE9;BACE1−/− mice (Fig. 7), establishing that Aβ burden is sensitive to BACE1 dosage. However, in aged APPswe;PS1ΔE9;BACE1−/− mice, the same decrease in BACE1 levels failed to alter Aβ burden, suggesting that BACE1 is no longer a limiting factor in this aged mouse model, possibly attributable to compromised Aβ clearance mechanisms, including those mediated by apolipoprotein E (ApoE), ApoJ (clustrin), insulin degrading enzyme, and neprilysin (Iwata et al., 2001; Leissring et al., 2003; Marr et al., 2003; DeMattos, 2004; Iwata et al., 2004). Future studies will be necessary to clarify the ways by which Aβ clearance mechanisms may be compromised with age. Importantly, our demonstration that silencing BACE1 through RNAi ameliorates Aβ amyloidosis in aged mutant APP;PS1 mice strongly support the view that Aβ deposits can be efficiently cleared by inhibiting BACE1 activity and further validates BACE1 as an excellent therapeutic target for anti-amyloid therapy in AD. During the course of this work, Singer et al. (2005) reported similar findings. Furthermore, they showed that silencing BACE1 through RNAi in the hippocampus attenuated cognitive deficits occurring in mutant APP mice (Singer et al., 2005). Together, our findings emphasize the potential values for development of compounds to inhibit BACE1 as well as drugs that regulate ApoE, clustrin, and Aβ degrading proteases in efforts to reduce Aβ burden.

In summary, although BACE1 is a high-priority therapeutic target, inhibition of BACE1 activity may not be completely free of mechanism-based consequences related to possible roles of BACE1-dependent APP/AICD signaling in cognitive functions. We anticipate that novel mechanism-based treatments such as BACE1 inhibitors will become available in the future. Therefore, it is critical to understand some of the BACE1/APP mechanisms that influence cognition and emotional circuits in the CNS. BACE1 conditional knock-out mouse models will be invaluable for clarifying whether cognitive deficits seen in BACE1−/− mice are related to development or to aging, and whether BACE1 is involved directly in memory formation, as well as for evaluating to what extent Aβ peptide-associated synaptic abnormalities are reversible after reductions of BACE1 activity. Finally, our studies here emphasize the pivotal roles that BACE1 plays in both health and disease, findings that are pertinent to the development of effective and safe anti-amyloid therapies for the treatment of this devastating disease of the elderly.

References


