**ALZHEIMER’S DISEASE**

Axonal organization defects in the hippocampus of adult conditional BACE1 knockout mice

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β-Site APP (amyloid precursor protein) cleaving enzyme 1 (BACE1) is the β-secretase enzyme that initiates production of the toxic amyloid-β peptide that accumulates in the brains of patients with Alzheimer’s disease (AD). Hence, BACE1 is a prime therapeutic target, and several BACE1 inhibitor drugs are currently being tested in clinical trials for AD. However, the safety of BACE1 inhibition is unclear. Germline BACE1 knockout mice have multiple neurological phenotypes, although these could arise from BACE1 deficiency during development. To address this question, we report that tamoxifen-inducible conditional BACE1 knockout mice in which the Bace1 gene was ablated in the adult largely lacked the phenotypes observed in germline BACE1 knockout mice. However, one BACE1-null phenotype was induced after Bace1 gene deletion in the adult mouse brain. This phenotype showed reduced length and disorganization of the hippocampal mossy fiber infrapyramidal bundle, the axonal pathway of dentate gyrus granule cells that is maintained by neurogenesis in the mouse brain. This defect in axonal organization correlated with reduced BACE1-mediated cleavage of the neural cell adhesion protein close homolog of L1 (CHL1), which has previously been associated with axon guidance. Although our results indicate that BACE1 inhibition in the adult mouse brain may avoid phenotypes associated with BACE1 deficiency during embryonic and postnatal development, they also suggest that BACE1 inhibitor drugs developed for treating AD may disrupt the organization of an axonal pathway in the hippocampus, an important structure for learning and memory.

**INTRODUCTION**

Cerebral accumulation of amyloid-β (Aβ) peptide is a defining pathological hallmark of Alzheimer’s disease (AD), and a large body of evidence indicates that Aβ is involved in the pathogenesis of this devastating neurodegenerative disorder (1, 2). Aβ is derived from the proteolytic processing of a large type I membrane protein, the amyloid precursor protein (APP) (3). Two proteases, the β-secretase and the γ-secretase, sequentially cut APP to generate Aβ (4). β-Site APP cleaving enzyme 1 (BACE1) has been identified as the β-secretase protease that cleaves APP to initiate the production of neurotoxic Aβ (5–9). Inhibition of BACE1 and thus Aβ production has therefore emerged as a leading therapeutic intervention for AD. However, the safety of BACE1 inhibition has been questioned because BACE1 has a wide array of substrates, and proper cleavage of these substrates may be necessary for normal physiology (10–12). Germline BACE1 knockout (BACE1−−) mice lack Aβ production (13–15), thereby providing in vivo validation for BACE1 inhibition as a therapeutic approach for AD. However, BACE1−− mice have been reported to exhibit smaller postnatal size and compromised survival (16), hypomyelination (17, 18), spontaneous seizures and abnormal electroencephalograms (EEGs) (19, 20), memory deficits (21, 22), and axon guidance defects (23–26) among other phenotypes (27). Previously, we reported that BACE1−− mice phenocopy the axon guidance defects of close homolog of L1 (CHL1) knockout mice in the hippocampus and olfactory bulb (24), indicating that BACE1 cleavage of CHL1 has a role in axonal targeting in these brain regions. However, because BACE1−− mice are devoid of BACE1 from the moment of conception, the extent to which axon guidance defects and other BACE1-null phenotypes are related to BACE1 deficiency during development versus absence of BACE1 in the adult is unknown. This question has critical implications for the treatment of elderly AD patients with BACE1 inhibitor drugs, several of which are being tested in clinical trials (11, 28).

Here, we show that neurological phenotypes associated with germline BACE1 deficiency are reduced to a great extent, or eliminated altogether, in mice in which the BACE1 gene was inductively deleted in the whole body of the adult. However, adult conditional BACE1 knockout mice exhibited no reduction in the severity of axonal disorganization in the mossy fiber pathway of the hippocampus. The mossy fiber phenotype correlated with deficient BACE1 cleavage of CHL1, which regulates growth cone collapse via semaphorin 3A (26), an axon guidance molecule.

**RESULTS**

Generation and characterization of conditional BACE1 knockout mice

To investigate the consequences of BACE1 inactivation in adults, we generated conditional BACE1 knockout mice in which exon 2 of the
murine BACE1 gene was flanked withloxP sites (BACE1<sup>fl/fl</sup>) by gene
targeting (fig. S1). BACE1<sup>fl/fl</sup> mice were then crossed to either calcium/
calmodulin-dependent protein kinase IIα (CamKIIα)-codon-improved Cre recombinase (iCre) mice (29) that express Cre recombinase in early postnatal forebrain excitatory neurons [BACE1<sup>fl/fl</sup>;CamKIIα-iCre (fig. S2A)] or R26CreER<sup>T2</sup> mice (30) that express from the ROSA26 locus a Cre recombinase fused to the estrogen receptor, thus enabling ubiquitous temporally controlled BACE1 gene deletion with administration of tamoxifen (TAM) [BACE1<sup>fl/fl</sup>;R26CreER<sup>T2</sup>-TAM (fig. S2B)]. We anticipated that BACE1<sup>fl/fl</sup>;CamKIIα-iCre mice should begin excising exon 2 of the BACE1 gene in excitable forebrain neurons around postnatal day 3 (29), with BACE1 exon 2 excision occurring in all cells of the body at any time upon treatment of BACE1<sup>fl/fl</sup>;R26CreER<sup>T2</sup> mice with TAM (30).

We first verified BACE1 ablation in our conditional knockout mice by immunoblotting and immunohistochemistry (Fig. 1 and fig. S3). In BACE1<sup>fl/fl</sup>;CamKIIα-iCre mice, cortical BACE1 in mouse brain dropped below detection beyond postnatal day 4 and was accompanied by accumulation of full-length BACE1 substrates and reduced cleaved fragments (Fig. 1A). In BACE1<sup>fl/fl</sup>;R26CreER<sup>T2</sup> mice treated with TAM at 3 months and analyzed at 12 months of age, we observed ~90 to 95% reduction of BACE1 in the cortex and hippocampus of mouse brain (Fig. 1, D to G). Immunostaining for BACE1 in the mouse brain showed distinct patterns between the postnatal forebrain excitatory neuron knockout in BACE1<sup>fl/fl</sup>;CamKIIα-iCre and the adult whole-body knockout in BACE1<sup>fl/fl</sup>;R26CreER<sup>T2</sup> mice treated with TAM (fig. S3). BACE1 was virtually undetectable in forebrain regions, whereas the cerebellum and brainstem exhibited BACE1 immunostaining in BACE1<sup>fl/fl</sup>;CamKIIα-iCre mouse brain (fig. S3A). In contrast, BACE1<sup>fl/fl</sup>;R26CreER<sup>T2</sup> mice treated with TAM exhibited little BACE1 immunostaining in the cerebellum and brainstem, whereas residual BACE1 immunostaining was observed in subcortical regions and the olfactory bulb. We attribute the small amount of BACE1 immunostaining in BACE1<sup>fl/fl</sup>;R26CreER<sup>T2</sup> TAM-treated mice to slight variability in penetration of TAM into different brain regions. Despite the low residual BACE1 immunostaining detected (for example, in the hippocampus; fig. S3, B and C), our immunoblot analysis confirmed that BACE1<sup>fl/fl</sup>;R26CreER<sup>T2</sup>-TAM-treated mice exhibited robust ~90 to 95% BACE1 reduction in the cortex and hippocampus (Fig. 1, D to G). To ensure that R26CreER<sup>T2</sup> was tightly controlled by TAM treatment, we examined BACE1 by immunoblotting in untreated BACE1<sup>fl/fl</sup>;R26CreER<sup>T2</sup> mice compared to wild-type mice (fig. S4A). Quantification of BACE1 immunosignal showed no change in BACE1 (fig. S4B), demonstrating that R26CreER<sup>T2</sup> was tightly regulated and was completely inactive in the absence of TAM.

After establishing that we were able to induce efficient postnatal forebrain or adult whole-body BACE1 reduction in our conditional knockout mice, we determined the effects of conditional BACE1 gene deletion on the processing of several major BACE1 substrates: APP (5–9), CHL1 (31, 32), neuregulin 1 (NRG1) (17, 18), and Sez6 (32). As expected for BACE1 substrates, in BACE1<sup>fl/fl</sup>;CamKIIα-iCre whole brain and cortex, we observed increased full-length APP, CHL1, Sez6, and α-secretase cleaved APP CTF (α-CTF) and decreased BACE1-cleaved fragments of CHL1, NRG1, Sez6, and APP β-CTF (Fig. 1, A to C). The effect of conditional BACE1 knockout on BACE1 substrates in the cortex and hippocampus of BACE1<sup>fl/fl</sup>;R26CreER<sup>T2</sup>-TAM-treated mice followed similar but less pronounced and more variable changes, displaying increases in full-length APP, α-CTF, full-length CHL1, and full-length Sez6 and decreases in BACE1-cleaved fragments of CHL1, APP, NRG1, and Sez6 (Fig. 1, D to H). The greater inhibition of substrate processing observed in BACE1<sup>fl/fl</sup>;CamKIIα-iCre compared to BACE1<sup>fl/fl</sup>;R26CreER<sup>T2</sup>-TAM-treated mice was consistent with the greater reduction of BACE1 in postnatal forebrain relative to adult whole-body conditional BACE1 knockout. Overall, we did not observe gender differences in the effects of conditional BACE1 knockout on cleavage of BACE1 substrates.

Postnatal lethality and underweight phenotypes are reduced or absent when BACE1 is conditionally deleted

Next, we determined the phenotypic effects of BACE1 reduction in both of our conditional BACE1 knockout mouse lines. BACE1<sup>−/−</sup>-mice have a high mortality rate in early postnatal development and are also smaller than their age-matched heterozygous or wild-type littersmates (16). When BACE1 was ablated in forebrain excitatory neurons at the postnatal stage, lethality and underweight phenotypes were greatly reduced in BACE1<sup>−/−</sup>;CamKIIα-iCre mice (fig. S5, A to C). In a BACE1<sup>fl/fl</sup> × BACE1<sup>fl/fl</sup>;CamKIIα-iCre cross, BACE1<sup>fl/fl</sup>;CamKIIα-iCre offspring survived almost as well as BACE1<sup>fl/fl</sup> control offspring at the expected 50% rate (fig. S5, A and B) and was born underweight but reached almost normal weight by 3 months of age (fig. S5C). In contrast, in a BACE1<sup>−/−</sup> × BACE1<sup>−/−</sup> cross, BACE1<sup>−/−</sup> offspring exhibited marked mortality, and those that survived remained severely underweight. When we conditionally ablated BACE1 in the whole body of the adult, we did not observe any lethality related to BACE1 reduction in BACE1<sup>−/−</sup>;R26CreER<sup>T2</sup>-TAM-treated mice (<em>n</em> = 78) between the ages of 3 and 9 months. Unexpectedly, BACE1<sup>−/−</sup> mice ablated by TAM to adulthood showed a weight gain after adult whole-body conditional BACE1 knockout (fig. SSD). Whereas BACE1<sup>−/−</sup>-mice were reported leaner with enhanced insulin sensitivity (33), glucose and insulin metabolism of BACE1<sup>−/−</sup>;R26CreER<sup>T2</sup>-TAM-treated mice were similar to control BACE1<sup>−/−</sup>-TAM-treated mice 7 months after BACE1 gene deletion (fig. S5, E to H). We also monitored energy metabolism activity in automated phenotyping chambers but found no differences in feeding, drinking, locomotor activity, energy expenditure, and fuel utilization between BACE1<sup>−/−</sup>;R26CreER<sup>T2</sup>-TAM-treated and control BACE1<sup>−/−</sup>-TAM-treated mice (fig. S6). Finally, necropsy of BACE1<sup>−/−</sup>;R26CreER<sup>T2</sup>-TAM-treated mice demonstrated that major tissues were histologically normal (fig. S9). Although the basis of the weight gain observed in BACE1<sup>−/−</sup>;R26CreER<sup>T2</sup>-TAM-treated mice is currently unknown, together, our results suggest that the reduced survival and weight of BACE1<sup>−/−</sup>-mice may be caused mainly by BACE1 deficiency during early development.

Conditional BACE1 knockout mice are cognitively normal but exhibit signs of hyperactivity

We next evaluated learning and memory function in our conditional BACE1 knockout mouse lines because BACE1<sup>−/−</sup>-mice were previously reported to have memory deficits as early as 3 months of age (21, 22). BACE1<sup>−/−</sup>;CamKIIα-iCre mice had normal memory at 6 months of age, despite showing delayed learning in the Morris water maze on day 2 of training (fig. S7). At 9 months of age, delayed learning of BACE1<sup>−/−</sup>;CamKIIα-iCre mice in the Morris water maze became more pronounced (Fig. 2A), yet memory impairment was not observed in the Morris water maze probe trial (Fig. 2B), spontaneous alternation in the Y maze (Fig. 2E), and contextual (Fig. 2G) and cued (Fig. 2I) fear conditioning. As with germline BACE1 knockout
Fig. 1. Conditional BACE1 knockout mice exhibit robust reduction of BACE1 and cleavage of BACE1 substrates. (A) Immunoblots of total cortical homogenates from postnatal BACE1<sup>fl/fl</sup>, CamKII<sub>α</sub>-iCre and BACE1<sup>fl/fl</sup> mice were probed with antibodies against BACE1 and the indicated full-length (FL) BACE1 substrates, BACE1-cleaved substrate fragments, and myelin-associated proteins. PLP, myelin proteolipid protein. (B) Immunoblots of cortical homogenates of 3-month-old BACE1<sup>fl/fl</sup>, CamKII<sub>α</sub>-iCre mice and BACE1<sup>fl/fl</sup> mice were probed with antibodies against BACE1 and the BACE1 substrate seizure 6 (Sez6) in membrane (Sez6 FL) and soluble (Sez6 N-terminal fragment (NTF)) fractions. (C) Immunosignals in (B) were normalized to Ponceau staining and represented as percentage of immunosignal in BACE1<sup>fl/fl</sup> mice. (D and E) Immunoblots of cortical (D) and hippocampal (E) soluble or membrane homogenates from BACE1<sup>fl/fl</sup> and BACE1<sup>fl/fl</sup>;R26CreERT2 mice that were treated with TAM at 3 months and analyzed at 1 year of age. Panels represent immunoblots of membrane [BACE1, APP FL, α/β-CT-F-terminal fragment (CTF), CHL1 FL, Sez6 FL, and myelin basic protein (MBP)] and soluble (CHL1 NTF, Sez6 NTF, and NRG1 NTF) fractions of cortical or hippocampal homogenates. (F and G) Quantification of cortical (F) and hippocampal (G) immunoblot signals for BACE1, BACE1 substrates, and MBP in (D) and (E), respectively. Protein concentrations for BACE1<sup>fl/fl</sup>-TAM and BACE1<sup>fl/fl</sup>;R26CreERT2-TAM mice were compared using unpaired two-way Student’s t tests. P values for the cortex are as follows: α-CTF, P = 0.036; CHL1 NTF, P = 0.024. P values for hippocampus are as follows: APP FL, P = 0.0065; α-CTF: P = 0.00032; CHL1 FL, P = 0.015; CHL1 NTF, P = 0.039; Sez6 FL, P = 0.0003; Sez6 NTF, P = 0.0003. (H) Enlarged APP β-CTF immunoblot bands from lanes 12 and 13 of (D). n.s., not significant.
Fig. 2. Conditional BACE1 knockout mice lack memory deficits and LTP impairment.

BACE<sup>fl/fl</sup>;CamKII<i>α</i>-iCre, BACE<sup>fl/fl</sup>;R26CreER<sup>T2</sup>-TAM, and respective control mice were assessed in the Morris water maze, Y maze, fear conditioning (FC), and LTP tests. (A to D) Testing of conditional BACE1 knockout mice in the Morris water maze. (A and B) Nine-month-old BACE<sup>fl/fl</sup>;CamKII<i>α</i>-iCre mice (n = 23) and BACE<sup>fl/fl</sup> mice (n = 18) were trained using (A) a hidden platform (repeated two-way analysis of variance (ANOVA), P = 0.074, F = 3.382), followed by (B) a probe trial test (one-way ANOVA, F<sub>CKO</sub> = 26.75, F<sub>CONT</sub> = 9.86). (C and D) BACE<sup>fl/fl</sup>;R26CreER<sup>T2</sup> mice (n = 17) and control BACE<sup>fl/fl</sup> mice (n = 14) treated with TAM at 3 months were assessed in the Morris water maze at 9 months of age in (C) the hidden platform (repeated two-way ANOVA, P = 0.089, F = 3.096), followed by the (D) probe trial test (one-way ANOVA, F<sub>CKO</sub> = 18.68, F<sub>CONT</sub> = 17.03).

(E, G, and I) BACE<sup>fl/fl</sup>;CamKII<i>α</i>-iCre and BACE<sup>fl/fl</sup> mice were tested in a Y maze (E) and in tests measuring contextual fear conditioning and cued fear conditioning (G and I, respectively). n = 23 and 18 for Y-maze and n = 24 and 16 for fear conditioning for BACE<sup>fl/fl</sup>;CamKII<i>α</i>-iCre and BACE<sup>fl/fl</sup> mice, respectively.

(F, H, and J) BACE<sup>fl/fl</sup>;R26CreER<sup>T2</sup>-TAM and BACE<sup>fl/fl</sup>-TAM mice were tested in a Y maze (F) and in tests measuring contextual (H) and cued fear conditioning (J). n = 17 and 13 for Y maze and n = 17 and 14 for fear conditioning for BACE<sup>fl/fl</sup>;R26CreER<sup>T2</sup>-TAM and BACE<sup>fl/fl</sup>-TAM mice, respectively.

(K to N) LTP in the CA1 region of the hippocampus. (K) Grouped data showing time course of LTP for 1-year-old BACE<sup>fl/fl</sup>;CamKII<i>α</i>-iCre mice (red, n = 9 slices from five animals) and control BACE<sup>fl/fl</sup> mice (black, n = 15 slices from five animals). (L) Cumulative probability distribution of all LTP recordings in (K). (M and N) Grouped data showing time course of LTP for 9-month-old BACE<sup>fl/fl</sup>;R26CreER<sup>T2</sup>-TAM mice (blue, n = 10 slices from three animals) and control BACE<sup>fl/fl</sup>-TAM mice (black, n = 12 slices from three animals) that were injected with TAM at 3 months of age. (N) Cumulative probability distribution of all LTP recordings in (M). LTP was induced at time = 0 with three trains of 100 Hz, 1-s tetanic stimulation [arrows in (K) and (M)]. LTP statistics were analyzed by unpaired Student’s t test. fEPSP, field excitatory postsynaptic potential.

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control BACE1fl/fl TAM-treated mice (Fig. 3, B and C). These results deletion) showed no seizures or abnormal EEG activity compared to gene 3 months and analyzed at 1 year of age (9 months after Bace1 gene deletion). We observed that they performed comparably to BACE1+/+ TAM-treated mice in all tests but without the delayed learning found in BACE1−/−,CamKIIα-iCre mice in the Morris water maze (Fig. 2, C, D, F, H, and J). Hippocampal CA1 LTP of the BACE1−/−,R26CreER T2 TAM-treated mice was also normal (Fig. 2, M and N). We assessed locomotor activity of the mice in the open-field test, and although there were no differences in basal motor activity for either conditional knockout line (fig. S8E), we observed longer travel distances in the open field (fig. S8C) and greater arm entries in the Y maze for BACE1−/−,CamKIIα-iCre mice (fig. S8E), suggesting novelty-induced hyperactivity as reported previously in BACE1−/− mice (21, 34). BACE1−/−,R26CreER T2 TAM-treated mice also exhibited greater arm entries in the Y maze (fig. S8F) but did not show longer travel distance in the open-field test (fig. S8D). Together, these results suggest that, although conditional Bace1 gene inactivation may be associated with hyperactivity, it does not cause memory deficits if it occurs during postnatal development or in the adult.

**Adult whole-body conditional BACE1 knockout mice lack epileptiform abnormalities and hypomyelination**

Spontaneous seizure and abnormal EEGs are other adverse phenotypes that have been reported in BACE1−/− mice (19, 20). To assess seizure activity in our conditional BACE1 knockout mice, we counted the number of spontaneous seizures and subjected mice to EEG recording and video monitoring. We observed a low penetrance of spontaneous seizures in our BACE1−/−,CamKIIα-iCre colony, in which we counted 18 seizures in 15 mice (3 mice had 2 seizures each) of a total of 83 mice over 2 months of random monitoring. In contrast, over the same period, no spontaneous seizures were observed in 86 BACE1−/−,R26CreER T2 TAM-treated and 62 BACE1+/+ TAM-treated mice. We selected one cohort in our BACE1−/−,CamKIIα-iCre colony for EEG assessment, with eight mice exhibiting spontaneous seizures from a group of 25 at 1 year of age. Of the eight mice that exhibited seizures, seven survived surgery and three had spontaneous seizures captured within the 5-day recording period. An EEG trace of a representative generalized tonic-clonic seizure is presented in Fig. 3A (lower trace) compared to a littermate BACE1−/− control mouse with a normal EEG (upper trace). Both single and polyspikewave discharges were observed in EEGs of BACE1−/−,CamKIIα-iCre mice (Fig. 3B). Overall, BACE1−/−,CamKIIα-iCre mice presented significantly (P = 0.0003) more epileptiform events (3.5 spike-wave discharges per hour) than their BACE1−/− littermate controls (Fig. 3C), although they did not exhibit as many spike-wave discharges per hour as BACE1−/− mice (40 spike-wave discharges per hour) (19, 20), indicating less severe seizure activity in postnatal forebrain neuron conditional BACE1 knockout animals. Notably, we did not observe any spontaneous seizure activity in BACE1−/−,R26CreER T2 TAM-treated mice. EEG recordings from BACE1−/−,R26CreER T2 TAM-treated mice treated with TAM at 3 months and analyzed at 1 year of age (9 months after Bace1 gene deletion) showed no seizures or abnormal EEG activity compared to control BACE1+/+ TAM-treated mice (Fig. 3, B and C). These results suggest that spontaneous seizures and abnormal EEGs derive from BACE1 deficiency during early postnatal development of excitatory forebrain neurons but that conditional BACE1 knockout in adult neurons may not lead to epileptiform activity.

BACE1−/− mice were reported to have hypomyelination from insufficient BACE1 cleavage of NRG1 and impaired downstream signaling (17, 18). To determine whether our conditional BACE1 knockout mice also exhibited hypomyelination, we performed immunoblot analysis of cortex and sciatic nerve homogenates for MBP and PLP and stained brain sections with Luxol fast blue for white matter. Immunoblot analysis revealed reduced MBP and PLP in the brains of postnatal days 21 and 30 BACE1−/−,CamKIIα-iCre mice (Fig. 1A). In contrast, we found no difference in central nervous system myelination in BACE1−/−,R26CreER T2 TAM-treated mice with TAM at 3 months and analyzed at 12 months (9 months after Bace1 gene deletion) either by cortical or hippocampal MBP immunoblotting (Fig. 1, D and E) or by Luxol fast blue staining of brain sections (Fig. 4C). Similarly, although immunoblot analysis of sciatic nerve homogenates from BACE1−/−,R26CreER T2 TAM-treated mice showed undetectable BACE1, we found no change in MBP compared to BACE1+/+ TAM-treated control or BACE1+/+ mice, although BACE1−/− sciatic nerve homogenates displayed a significant reduction of MBP (P = 0.006; Fig. 4, A and B). We further measured myelin thickness and calculated g ratios in semithin sciatic nerve sections of BACE1−/−,R26CreER T2 TAM-treated mice along with BACE1+/+ TAM-treated, BACE1−/−, and BACE1+/+ control animals. Sciatic nerve of BACE1−/− mice, as previously reported (17, 18), exhibited reduced myelin sheath thickness around axons (Fig. 4D) and significantly (P < 0.0001) increased g ratio (Fig. 4E), indicating hypomyelination. In contrast, BACE1+/+,R26CreER T2 TAM-treated mice displayed normal myelin sheath thickness and a g ratio similar to BACE1−/− TAM-treated mice and BACE1+/+ mice (Fig. 4, D and E). Thus, although postnatal forebrain neuron Bace1 gene deletion is associated with hypomyelination, conditional BACE1 knockout in the adult whole body did not seem to affect central or peripheral myelination once development was completed.

**Adult conditional BACE1 knockout mice exhibit axonal organization defects in the hippocampus**

Previously, we determined that BACE1−/− mice exhibited axonal organization defects that correlated with deficient processing of the neural cell adhesion protein CHL1 (24). CHL1 is a substrate that appears to be preferred by BACE1 in neurons (31, 32). Consistent with these observations, BACE1-cleaved CHL1 β-NTF and full-length CHL1 were decreased and increased, respectively, in the brains of BACE1−/−,CamKIIα-iCre mice (Fig. 1A) and BACE1−/−,R26CreER T2 TAM-treated mice (Fig. 1, D to G). Differences in CHL1 β-NTF and full-length CHL1 between BACE1−/−,R26CreER T2 TAM-treated mice and BACE1+/+ TAM-treated control mice were substantial, suggesting that induced deletion of the Bace1 gene in the adult may affect the function of CHL1 even after development is completed.

One of the CHL1-correlated axonal abnormalities that we had observed in the brains of germline BACE1 knockout mice was a disorganized and shortened length of the infrapyramidal bundle (IPB) of the hippocampal mossy fiber pathway and premature crossing of the CA3 pyramidal cell layer (24). To determine whether deficient BACE1 cleavage of CHL1 correlated with axonal defects in adult mice, we measured the length and assessed the organization of the IPB of BACE1−/−,R26CreER T2 TAM-treated mice in coronal brain
sections immunostained for BACE1 and the mossy fiber marker synaptoporin (SPO; Fig. 5A and fig. S15). Similar to BACE1−/− mice, IPB length was shorter in BACE1fl/fl;CamKIIα-iCre mice compared to BACE1fl/fl mice at 9 months of age (Fig. 5, A and C). BACE1fl/fl;R26CreERT2-TAM mice treated with TAM at 3 months and analyzed at 1 year of age (9 months after Bace1 gene deletion) had significantly shorter IPB length (P = 1.4 × 10−5) compared to BACE1fl/fl TAM-treated negative control mice (Fig. 5, A and C). The amount that the IPB was shortened in the adult conditional knockout mice (~30%) was similar to that observed in BACE1−/− mice (Fig. 5C) reported in our previous study (24). In addition, similar to BACE1−/− mice, we observed that the organization of the IPB of BACE1fl/fl;R26CreERT2-TAM-treated mice was severely disrupted compared to that of BACE1fl/fl-TAM-treated mice, exhibiting premature crossing of mossy fibers through the CA3 pyramidal cell layer (Fig. 5B); this is a phenocopy of CHL1 knockout mice (35, 36). Immunostaining with an anti-CHL1 antibody revealed colocalization of CHL1 with presynaptic terminal and mossy fiber bouton markers synaptophysin and calbindin, respectively (Fig. 5B, low-power image in fig. S14), indicating neuronal, and specifically presynaptic, CHL1 localization. As we have previously reported, CHL1 also colocalized with BACE1 in mossy fiber terminals in the hippocampus (24).

Next, we performed immunoblot analysis for CHL1 to determine the extent of BACE1 cleavage of CHL1 in the hippocampus of the adult conditional BACE1 knockout and control mice. As expected, we found that CHL1 β-NTF and full-length CHL1 were decreased and increased in soluble and membrane fractions of hippocampal homogenates, respectively, in BACE1fl/fl;R26CreERT2-TAM-treated mice compared to BACE1fl/fl-TAM-treated mice (Fig. 5, D and E). Linear regression analysis showed that IPB length positively and negatively correlated with soluble CHL1 and the ratio of full-length CHL1 to β-NTF, respectively (Fig. 5, F and G). Together, these results demonstrate that processing of CHL1 by BACE1 is active in the adult brain and is correlated with the maintenance of the normal

Fig. 3. Seizure activity was reduced or absent in conditional BACE1 knockout mice. Seizure activity was measured in postnatal forebrain excitatory neuron conditional BACE1 knockout mice and in adult whole-body conditional BACE1 knockout mice. (A) Electrographic seizure with buildup of generalized fast activity in BACE1fl/fl;CamKIIα-iCre mice at 1 year of age (bottom) compared to age-matched BACE1fl/fl control mice (top). (B) EEG in awake animals showed epileptiform abnormalities, including spike-wave discharges, in BACE1fl/fl;CamKIIα-iCre mice (n = 6) compared to normal EEG in BACE1fl/fl mice (n = 4), BACE1fl/fl-TAM mice (n = 5) and BACE1fl/fl;R26CreERT2-TAM mice (n = 6). (C) Comparison of the frequency of spike-wave discharges in mice with the four indicated genotypes: BACE1fl/fl;CamKIIα-iCre, BACE1fl/fl;R26CreERT2-TAM, and BACE1fl/fl-TAM. **P values are 0.0003 by one-way ANOVA and 0.0011 by Tukey post hoc test between BACE1fl/fl;CamKIIα-iCre mice and BACE1fl/fl mice.

Fig. 4. Central and peripheral hypomyelination is absent in adult whole-body conditional BACE1 knockout mice. (A) Immunoblot analysis of sciatic nerve homogenates showing BACE1 and MBP in BACE1<sup>fl/fl</sup>;R26CreER<sup>T2</sup>-TAM, BACE1<sup>fl/fl</sup>-TAM, BACE1<sup>−/−</sup>, and BACE1<sup>+/+</sup> mice. (B) Quantification of MBP immunoblot signals from (A), normalized to βIII-tubulin immunosignal (one-way ANOVA on MBP, P = 0.0004, F = 15.9; unpaired two-way Student's t test, P = 0.006). (C) Luxol fast blue staining of myelinated fibers in BACE1<sup>fl/fl</sup>;R26CreER<sup>T2</sup>-TAM and control BACE1<sup>fl/fl</sup>-TAM mouse brains. (D) Images of toluidine blue O–stained sciatic nerve thin sections showing myelination in BACE1<sup>fl/fl</sup>;R26CreER<sup>T2</sup>-TAM compared to BACE1<sup>fl/fl</sup>-TAM mice and BACE1<sup>−/−</sup> compared to BACE1<sup>+/+</sup> mice. Scale bar, 20 μm. (E) Quantification of myelination via g ratio between BACE1<sup>fl/fl</sup>;R26CreER<sup>T2</sup>-TAM mice (n = 3) and BACE1<sup>fl/fl</sup>-TAM mice (n = 2) [top; linear regression analysis on slope (F = 0.27, P = 0.6) and on intercepts (F = 1.03, P = 0.31)] and BACE1<sup>−/−</sup> mice (n = 3) and BACE1<sup>+/+</sup> mice (n = 3) [bottom]. One hundred axons per animal [linear regression analysis on slope (F = 0.98, P = 0.32) and on intercepts (F = 233.6, P < 0.0001)].
Fig. 5. Adult conditional BACE1 knockout mice show disorganization of the hippocampal mossy fiber pathway. (A) Shown are coronal brain sections from mice with the indicated genotypes (BACE1−/−, BACE1fl/fl, BACE1fl/fl;CamKIIα-iCre, BACE1fl/fl-TAM, and BACE1fl/fl;R26CreERT2-TAM) revealing the hippocampus colabeled for the mossy fiber marker SPO (green, top) and BACE1 (red, bottom). Arrows delineate the boundaries of the IPB. BACE1fl/fl and BACE1fl/fl;R26CreERT2 mice were treated with TAM at 3 months of age and analyzed at 1 year of age. BACE1−/− and BACE1fl/fl mice at 9 months of age were used as positive and negative controls, respectively. SPB, suprapyramidal bundle; slu, stratum lucidum. Scale bars, 200 μm. DAPI, 4′,6-diamidino-2-phenylindole. (B) Coronal brain sections from BACE1fl/fl-TAM and BACE1fl/fl;R26CreERT2-TAM mice in (A) were coimmunostained with antibodies against CHL1 (red), the mossy fiber bouton and presynaptic terminal markers calbindin (green), and synaptophysin (blue). (C) IPB lengths were normalized to the lengths of the SPB plus slu and were displayed as ratios, that is, IPB/(SPB + slu). The number of mice for each genotype is indicated in each bar of the graph. Error bars indicate SEM; P values for indicated comparisons (lines) using unpaired Student’s t test are shown above the bars. (D) Both soluble and membrane fractions were prepared from the hippocampi of representative BACE1fl/fl-TAM and BACE1fl/fl;R26CreERT2-TAM mice and then were subjected to immunoblot analysis for CHL1 FL and CHL1 β-NTF. The middle panel represents a longer exposure of the upper panel. βIII-tubulin was the loading control. (E) Ratio of CHL1 FL to CHL1 β-NTF intensities in membrane fractions (CHL1 FL/β-NTF) and the intensity of the CHL1 β-NTF band in soluble fractions normalized to βIII-tubulin (sCHL1) displayed as arbitrary units (a.u.) for BACE1fl/fl;R26CreERT2-TAM and BACE1fl/fl-TAM mice (means ± SEM). (F) Correlation of CHL1 FL/β-NTF from (E) plotted against IPB/(SPB + slu) for BACE1fl/fl;R26CreERT2-TAM and BACE1fl/fl-TAM mice ($R^2 = 0.7014, P = 0.0002$). (G) Correlation of sCHL1 from (E) plotted against IPB/(SPB + slu) for BACE1fl/fl;R26CreERT2-TAM and BACE1fl/fl-TAM mice ($R^2 = 0.4117, P = 0.01$).
structure of a major axonal pathway in the hippocampus that is involved in learning and memory.

We and others have previously reported that BACE1 deficiency results in disorganization and mistargeting of olfactory sensory neuron axons in the glomeruli of the olfactory bulb (23–25), which is also a phenotype of CHL1−/− mice (35, 36). Therefore, we determined whether adult conditional BACE1 knockout mice had disrupted olfactory sensory neuron axonal organization in the olfactory bulb. First, we performed immunoblot analysis of olfactory bulb homogenates from the 1-year-old BACE1+/−;R26CreER T2 TAM-treated mice and BACE1+/− mice treated with TAM at 3 months and observed only ~25 to 30% reduction of BACE1 in the olfactory bulbs of adult conditional BACE1 knockout mice (fig. S10, A and B). We hypothesized that this modest BACE1 reduction was insufficient to result in disorganization of olfactory sensory neuron axons in the olfactory bulb. To test this, we immunostained olfactory bulb sections of the BACE1+/−;R26CreER T2 TAM-treated mice and BACE1+/− TAM-treated mice with an antibody against olfactory marker protein, which labels axons and termini of olfactory sensory neurons. We found no evidence of abnormal axonal organization in the olfactory nerve layer or glomeruli of BACE1+/−; R26CreER T2 mice treated with TAM compared to BACE1+/− mice treated with TAM (fig. S10, C and D). An analysis of the circularity of glomeruli of adult conditional BACE1 knockout mice also revealed no differences compared to controls (fig. S10E).

**Axonal organization defects in adult conditional BACE1 knockout mice are not associated with abnormal adult neurogenesis or apoptosis**

Because of the similar IPB phenotypes of CHL1−/−, BACE1−/−, and adult conditional BACE1 knockout mice and because processing of CHL1 by BACE1 regulates growth cone collapse via a pathway involving the axon guidance molecule semaphorin 3A (26), we hypothesized that deficient BACE1 cleavage of CHL1 resulted in an axon guidance defect that caused the shortened and disorganized IPB of the adult conditional BACE1 knockout mice. However, other mechanisms besides axon guidance could contribute to the IPB phenotype associated with BACE1 deficiency. For example, CHL1 has been reported to affect multiple physiological processes, including cell proliferation (37), apoptosis (38), neurite outgrowth (39, 40), neuronal differentiation (37, 41), serotonin receptor signaling (42), and area-specific neuronal positioning and dendrite orientation (43, 44), any of which could potentially influence the organization and length of the IPB. In addition, germine BACE1 knockout mice exhibit increased neurodegeneration with age (19) and an altered balance between hippocampal astrogenesis and neurogenesis (45) that might also contribute to the BACE1-null IPB phenotype.

To begin approaching these questions, we investigated neural progenitor cell proliferation, adult neurogenesis, and apoptosis, which are major processes in the dentate gyrus (DG) that could affect the IPB of adult conditional BACE1 knockout mice. BACE1+/−; R26CreERT2 and BACE1+/− mice treated with TAM at 6 to 9 months were aged to 2 years (15 to 18 months after Bace1 gene deletion) and injected with 5-bromo-2′-deoxyuridine (BrdU), and brains were harvested 24 hours and 1 month after the last BrdU injection. BACE1 immunohistochemistry of hemibrain homogenates from each mouse revealed that BACE1 was reduced by ~90% in BACE1+/−;R26CreER T2 TAM-treated mice (fig. S11A), indicating efficient induction of Bace1 gene deletion in the adult brain. We also verified that the BrdU-labeled BACE1+/−;R26CreER T2 TAM-treated mice exhibited a shortened and disorganized IPB phenotype similar to that present in other adult conditional BACE1 knockout mice (fig. S11B). Coronal sections of the other hemibrain from each mouse were prepared and colabeled with either anti-BrdU antibody and DAPI (24 hours after BrdU) or anti-BrdU and anti-NeuN antibodies (1 month after BrdU) (fig. S12A).

First, we found no differences in either the volume of the DG or the number of BrdU+ cells per cubic millimeter in 24-hour post-BrdU brain sections (fig. S12, B and C), indicating that overall neural progenitor cell proliferation in the subgranular zone and the size of the DG were not affected in adult conditional BACE1 knockout mice. We noted that the rates of neurogenesis in these aged mice were low compared to rates for young mice but were within the range expected for 2-year-old mice (46). Next, we observed that the percentage of BrdU + NeuN double-positive neurons in the granule cell layer of the DG in 1-month post-BrdU brain sections was unchanged in BACE1+/−;R26CreER T2 TAM-treated mice (fig. S12D), suggesting that the differentiation of newly born neurons occurred normally after Bace1 gene ablation in the adult. Because topographic differences in adult neurogenesis are present in the hippocampus (47), we analyzed the regional distributions of dividing cells and newly born neurons in the suprapyramidal blade, infrapyramidal blade, and crest of the DG (fig. S12, F and G). We determined that adult conditional inactivation of BACE1 was not associated with regional differences in either the total estimated number of BrdU+ cells per DG in 24-hour post-BrdU sections (fig. S12F) or the percentage of BrdU + NeuN double-positive neurons in 1-month post-BrdU sections (fig. S12G).

Finally, to assess a potential role for apoptosis in the BACE1-deficient IPB phenotype, we performed terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick end labeling (TUNEL) assays on brain sections from the 24-hour BrdU-labeled mice. Notably, no TUNEL-positive granule cells were observed in sections of the DG of BrdU-labeled BACE1+/−;R26CreER T2 TAM-treated and BACE1+/− TAM-treated mice, despite the presence of TUNEL-positive cells in other areas of the hippocampus (fig. S13).

**DISCUSSION**

Here, we generated conditional Bace1 knockout mice to determine whether BACE1 is required in the adult for normal structure and function. Several of the neurological phenotypes of germline BACE1 knockout mice were absent after adult conditional BACE1 gene deletion. However, when we allowed the brain to develop with BACE1 and then ablated BACE1 after adulthood was achieved, we observed that the IPB of the hippocampal mossy fiber pathway was disorganized and exhibited a shortened length, possibly as the result of a defect in axon guidance. BACE1 is highly concentrated in hippocampal mossy fibers (48, 49), perhaps for the continuing requirement of adult axon guidance (50, 51). CHL1 is a BACE1 substrate (31, 32), and we have shown that BACE1−/− mice phenocopy the axon organization defects of CHL1 knockout mice (24). Moreover, the cleavage of CHL1 by BACE1 regulates the balance between growth cone extension and collapse via the axon guidance molecule semaphorin 3A (26), a process critical for correct axonal targeting. Future studies are necessary to determine whether axon disorganization exists beyond the hippocampus in other regions of the brain of adult conditional BACE1 knockout mice. In addition, treatment of wild-type mice with a BACE1 inhibitor should be performed to determine
whether IPB organizational defects occur after pharmacological BACE1 inhibition.

Neurogenesis of DG granule cells is known to continue in the subgranular zone of the hippocampus during adulthood (52). Consequently, mossy fiber axons of granule cells born in the adult require axon guidance to find their correct target neurons in the hippocampal CA3 region. Mossy fiber axons of newly born granule cells in the infrapyramidal blade of the DG grow for a distance along the ventral side of the CA3 pyramidal cell layer, thus forming the IPB. The axons then invade the cell layer and cross to the dorsal side where they merge into the stratum lucidum and join other mossy fiber axons that come from the suprapyramidal blade of the DG. The factors that determine the distance that mossy fibers grow before crossing the CA3 pyramidal cell layer, and therefore the length of the IPB, are not yet fully elucidated but correlate with CHL1 processing by BACE1 (24). The length of the IPB varies between different inbred strains of mice, and longer IPB lengths correlate with better spatial orientation learning in the radial maze (53). Given this finding, it was unexpected that we did not observe memory deficits in adult conditional BACE1 knockout mice in our study. In future studies, it will be important to test adult conditional BACE1 knockout mice in more sensitive and varied behavioral paradigms, including the radial maze and Barnes maze that avoid the stress of swimming, to detect potential deficits in spatial orientation and other types of learning or memory with age. Moreover, germline BACE1 knockout mice have been reported to exhibit impaired mossy fiber–CA3 LTP (54, 55). Therefore, it will be important to determine whether adult conditional BACE1 knockout mice have a mossy fiber–CA3 LTP abnormality and, if so, understand its relationship to the IPB and behavioral phenotypes.

Although we favor the hypothesis that deficient BACE1 cleavage of CHL1 causes defective axon guidance resulting in the short and disorganized IPB of adult conditional BACE1 knockout mice, other mechanisms, either involving CHL1 or not, could be responsible. Our BrdU and TUNEL experiments indicated that alterations in adult neurogenesis including overall or regional neural progenitor cell proliferation, neuronal differentiation, and apoptosis in the DG were unlikely to produce the phenotype. However, we could not exclude other potential causes, such as abnormal neurite outgrowth, serotonin receptor signaling, area-specific neuronal positioning and dendrite orientation, neurodegeneration, or defective activity-dependent axonal refining, among others.

It is possible that a combination of mechanisms may influence the length and organization of the IPB. For example, impaired axon guidance together with axonal degeneration as observed in germ-line BACE1 knockout mice (19) may affect the IPB. A study reported that only ~10% of DG neurons are newly generated by adult neurogenesis in mice, although the authors noted that this number is an underestimate (56). The same authors also observed that relatively few DG neurons die and that the total number of granule cells continues to increase in the adult DG. These results suggest that defective axon guidance of newly generated mossy fibers, together with degeneration of axons formed during development, may contribute to the shortened and disorganized IPB of adult conditional BACE1 knockout mice. Because BACE1 cleavage of CHL1 regulates growth cone collapse (26), it is possible that both axon guidance and mossy fiber degeneration are affected by CHL1 processing in the adult DG. It will be important to conduct additional studies to investigate the potential roles of these and other mechanisms in determining the characteristics of the IPB.

Our conditional BACE1 knockout mice have been useful for deciphering the roles of BACE1 in neurons during development versus the adult. Postnatal excitatory forebrain neuron conditional BACE1 knockout mice were partially protected from reduced survival, growth retardation, seizures, EEG abnormalities, and hypomyelination and were fully protected from memory deficits, whereas adult whole-body conditional BACE1 knockout mice completely lacked these adverse phenotypes. Therefore, we conclude that many of the neurological phenotypes of the germline BACE1 knockout mice are the result of BACE1 deficiency during nervous system development and do not result from absence of BACE1 function in the adult. This conclusion is also supported by the observation that BACE1 and some of its substrates such as NRG1, and BACE1 itself, are highly expressed in the developing organism, especially in the nervous system (17, 18) implying critical functions for BACE1-cleaved substrates during neuronal proliferation, determination, differentiation, or matura-

In conclusion, our results with adult conditional BACE1 knockout mice demonstrate that BACE1 continues to function after development to maintain normal axonal organization in the adult brain, at least in the mossy fiber pathway of the hippocampus. Regarding BACE1 inhibitors for AD, although these drugs have advanced into clinical trials, safety data from human and animal studies are just becoming available (59, 60). By comparing previously known phenotypes in BACE1+− mice to our conditional BACE1 knockout mice, our study provides valuable preclinical safety and tolerability information of relevance to the current BACE1 inhibitor trials and has also shown that several of the neurological phenotypes of the germline BACE1 knockout mice are the result of BACE1 deficiency during development rather than in the adult. On the other hand, the current human clinical trials of BACE1 inhibitors include doses that lower cerebrospinal fluid (CSF) Aβ up to ~90% (59, 61, 62). This represents strong inhibition of BACE1 comparable to the BACE1 reduction in the cortex and hippocampus of our adult conditional BACE1 knockout mice. The human trials will treat subjects for 18 to 24 months, although some prevention trials will treat for 5 years. Because of the short lifespan of the mouse, the adult conditional BACE1 knockout...
mice analyzed after 6 to 9 months of BACE1 deficiency represent a longer relative duration compared to the treatment times of the current clinical trials when normalized to human lifespan. Therefore, our experiments suggest that BACE1 inhibition may cause mechanism-based side effects that might not be revealed within the relatively short time frame of the clinical trials. In contrast to the mouse, studies of hippocampal neurogenesis in humans indicate that most DG neurons are exchanged in the adult (63). This suggests that the disorganization of DG mossy fiber axons may be even more severe in humans exposed to chronic BACE1 inhibitor treatment than in adult mice induced by BACE1 inactivation. Because a patient will likely require chronic treatment with a BACE1 inhibitor drug for the remainder of life, our results strongly caution against aggressive BACE1 inhibition or otherwise risk mechanism-based side effects in the adult hippocampus.

MATERIALS AND METHODS

Study design
This study used a loxP-Cre recombinase genetic approach to conditionally delete the Bace1 gene in mice to determine whether BACE1 is required in the adult animal to provide insights into mechanism-based side effects of BACE1 inhibitors in human clinical trials. Two conditional BACE1 knockout mouse strains were generated in this study: excitatory forebrain neuron BACE1fl/fl;CamKIIα-iCre mice and adult TAM-inducible whole-body BACE1fl/fl;R26CreERT2-TAM mice. To model patients under long-term BACE1 inhibition, all mice analyzed had at least 6 months of conditional BACE1 knockout. Previously published phenotypes associated with germ-line BACE1 knockout mice were analyzed in conditional BACE1 knockout mice including metabolism, seizures, axonal organization defects, behavioral abnormalities, and learning/memory deficits.

For all experiments, sample sizes were determined on the basis of our previous experience with germ-line BACE1 knockout mice and the literature, which proved to be sufficient to allow detection of statistically significant differences. Experimental methods chosen in the study were either previously published for analysis of germ-line BACE1 knockout mice or widely accepted and well-established approaches. Outliers were excluded only in behavioral analyses and only when the values were beyond two SDs of the mean. The number of biological replicates for each experiment is specified in the figure legends and elaborated in Materials and Methods. Mice were randomized in genotypes and sexes during behavioral testing. For behavioral, EEG, and LTP studies, experimenters were blinded during data acquisition and unblinded for data analysis.

Antibodies
The antibodies used are as follows: rabbit anti-actin (#926–42210, LI-COR), rabbit anti-APP (#ab32136, Abcam), mouse anti-BACE1 (3D5), rabbit anti-BACE1 (#ab108394, Abcam), rat anti-Brdu (#MA1-82088, Thermo Fisher Scientific), rabbit anti-calbindin (#2173, Cell Signaling Technology), goat anti-CHL1 (#AF2147, R&D Systems), rat anti-MBP (#ab7349, Abcam), rabbit anti-neuregulin type III (#AB5551, Millipore), chicken anti-NeuN (#ABN91, Millipore), goat anti-olfactory marker protein (OMP) (#544–1001, WAKO), mouse anti-βIII-tubulin (TuJ1) (a gift from L. Binder), rabbit anti-PLP (#ab28486, Abcam), goat anti-SPO (#sc21212, Santa Cruz Biotechnology), mouse anti-synaptophysin (#MAB5258, Millipore), and rat anti-Sez6 (a gift from S. Lichtenenthaler).

Animals
BACE1 embryonic stem (ES) cell clones in which BACE1 gene exon 2 (BACE1-floxed) was flanked by loxP sites were obtained from the European Mouse Mutant Cell Repository (ES cell line JM8A3.N1) and were grown under standard conditions by the Northwestern University Transgenic and Targeted Mutagenesis Laboratory. Southern blot–verified clones were then microinjected to C57Bl/6 blastocysts. Chimeric mice were verified by tail DNA polymerase chain reaction (PCR) using primers (5′-TGATTTCTTTATTAAAGG-3′ and 5′-TTAAGCTTATGTTGCCCATC-3′). BACE1-floxed mice (BACE1fl/fl) were generated by crossing chimeric mice to CAG-FLPe mice to remove the flip recombinase target (FRT)–flanked lacZ and neomycin cassette (fig. S1). For the forebrain conditional BACE1 knockout study, BACE1fl/fl mice were crossed to heterozygous CamKIα-iCre mice (29) to generate BACE1fl/fl;CamKIα-iCre heterozygous or BACE1fl/fl;CamKIα-iCre–negative littermates, which served as controls for the study. For the adult whole-body inducible BACE1 knockout study, BACE1fl/fl mice were crossed to homozygous R26CreER T2 mice (30) to generate BACE1fl/fl;R26CreER T2 homozygous mice. A separate cohort of BACE1fl/fl mice treated with TAM was used as controls. TAM (#156738, MP Biomedicals) was dissolved in corn oil at 25 mg/ml. Three-month-old BACE1fl/fl;R26CreER T2 mice were intraperitoneally injected with TAM at a dose of 100 mg/kg body weight daily for 5 days, followed by 3 days of break (one cycle). Complete treatment consisted of three cycles of TAM injection. Unless indicated, no significance was noted between the genders, and the data presented were the means of both male and female animals. All animal work was performed in accordance with Northwestern University Institutional Animal Care and Use Committee approval.

Behavioral testing
Mice were allowed to acclimate to the testing environment at least 0.5 hours before tests. Mice were randomized by gender and genotypes to which the experimenter was blinded during the tests. Behavioral tests were performed in the order described below.

Open field
The open-field arena was a box with the following dimensions: 55 cm (width) × 55 cm (length) × 30 cm (height). The mouse was placed in the middle of the box and was allowed to explore the box for 15 min. Ambulation activity was tracked and quantified by LimeLight software (Actimetrics).

Y maze
The maze contained three 42-cm-long identical arms spaced 120° apart radiating from a central triangular area. The mouse was placed at the base of the arm forming the stem of Y and was allowed to freely traverse the maze for 8 min. The alternation score was calculated as the number of alternations divided by the number of possible alternations multiplied by 100, with a 50% score being random selection. Travel pathway was tracked by LimeLight software (Actimetrics).

Morris water maze
An 8.5-cm-diameter platform was submerged in opaque water in a quadrant of a 1.8-m-diameter pool. Water remained at room temperature (22° ± 1°C) through all trials. Distinct visual cues were present in both hidden and probe trials. Hidden platform training was performed by giving three trials a day with 20- to 30-min intervals between trials for 5 days. The probe trial for memory of the position of the hidden platform took place 24 hours after the last hidden platform training trial. Three visible platform trials were done
right after the probe trial on the same day to test for visual acuity. Tests were monitored and analyzed using ANY-maze software (San Diego Instruments).

**Fear conditioning**
A mouse was allowed to freely explore a testing chamber equipped with shock and tone (Harvard Instruments) for 2 min, and then, the mouse was subjected to a tone of 2.5 kHz for 30 s, during the last 2 s of which a 0.8-mV shock was applied. The mouse was then removed from the chamber 30 s after the shock and returned to the home cage. Contextual fear conditioning was tested 24 hours after training in which the mouse was reexposed to the chamber for 2 min, except that no shock was applied. Forty-eight hours after initial conditioning, cue fear conditioning was tested in which the mouse was exposed to a new environment with a novel odor for 2 min (pre-tone), and then, the tone but no shock was applied for another 2 min (tone). Freezing behavior was recorded and scored using ANY-maze software.

**Tissue extraction and immunoblot analysis**
Mice were deeply anesthetized by intraperitoneal injection of xylazine (15 mg/kg) and ketamine (100 mg/kg), perfused with ice-cold phosphate-buffered saline (PBS) with phenylmethylsulfonyl fluoride (20 μg/ml), leupeptin (0.5 μg/ml), sodium orthovanadate (20 μM), and dithiothreitol (0.1 mM), followed by decapitation and brain removal. The hemibrain was dissected on ice into the cortex, hippocampus, olfactory bulb, cerebellum, and the rest of the brain and then snap-frozen in liquid nitrogen and stored at −80°C. Tissues were homogenized in tris-buffered saline, followed by centrifugation at 20,800g for 20 min at 4°C. The supernatant was collected as the soluble fraction, the pellet was extracted again in radioimmunoprecipitation assay (RIPA) buffer, and the resulting supernatant was referred to as the total extract. All buffers contained protease inhibitor cocktail III (#353140, Millipore) and Halt phosphatase inhibitor (#78420, Thermo Fisher Scientific). Alternatively, tissues were directly homogenized in RIPA buffer, followed by centrifugation. The resulting supernatant was referred to as total extraction. Protein concentration was determined using bicinchoninic acid assay (BCA) assay (#23225, Thermo Fisher Scientific). Equal amount of protein was separated under reduced and denatured conditions, transferred onto a polyvinylidene difluoride membrane, and stained using the avidin-biotin complex (ABC) method (Vector Laboratories).

**Fast blue staining**
Deparaffinized sections on glass slides were incubated in Luxol fast blue solution in a 60°C oven overnight, destained with 95% ethanol and then distilled water, and differentiated in lithium carbonate solution and 70% ethanol. After rinsing with water, slides were dehydrated in graded ethanol series and mounted in Permount.

**Measurement of the IPB**
Detailed procedures for IPB length analysis have been described previously (24). Briefly, 30-μm coronal sections were labeled with antibodies against the mossy fiber marker SPO and BACE1. Confocal microscopy images of the mossy fiber pathway were taken from sections near the rostral-caudal midline, and the interblade line was made, connecting the tips of the superior and inferior blades of the DG. The length of the SPB (beginning from the interblade junction to the point where SPB begins to taper outward), slu (from the point where SPB begins to taper outward to the distal end of the slu), and IPB (from the end of pyramidal cell layer to the distal point of uninterrupted SPO staining beneath the pyramidal cell layer) was measured by free-hand tracing along the middle line of SPO staining in ImageJ. Three sections from each brain were measured and averaged.

**Electrophysiology**
Horizontal hippocampal slices were prepared, as previously described (64). Briefly, animals were deeply anesthetized by intraperitoneal injection of xylazine (10 mg/kg) and ketamine (100 mg/kg), and cardiac perfusion was performed with an ice-cold sucrose artificial cerebrospinal fluid (ACSF) solution containing 85 mM NaCl, 2.5 mM KCl, 1.25 mM NaH₂PO₄, 25 mM NaHCO₃, 25 mM glucose, 75 mM sucrose, 0.5 mM CaCl₂, and 4 mM MgCl₂ with 0.01 μM DL-2-amino-5-phosphonoveric acid (DL-APV) and 0.1 μM kynurenic acid equilibrated with 95% O₂ and 5% CO₂. The brain was quickly removed, and horizontal sections (350 μm thick) were prepared in the same ice-cold sucrose ACSF using a Vibratome VT1200 (Leica Microsystems Inc). Slices were transferred to a recovery chamber containing the same sucrose ACSF for ~15 min at 30°C, and the solution was gradually exchanged for a recovery ACSF containing 125 mM NaCl, 2.4 mM KCl, 1.2 mM Na₂PO₄, 25 mM NaHCO₃, 25 mM glucose, 1 mM CaCl₂, and 2 mM MgCl₂ with 0.01 μM DL-APV and 0.1 μM kynurenic acid at room temperature. Individual slices were transferred to a recording chamber and visualized using Dottt contrast optics. During recordings, slices were continuously perfused with a normal ACSF containing 125 mM NaCl, 2.4 mM KCl, 1.2 mM Na₂PO₄, 25 mM NaHCO₃, 25 mM glucose, 2 mM CaCl₂, and 1 mM MgCl₂. Recording electrodes were manufactured from borosilicate glass pipettes and had tip resistances of 3 to 5 megohms when filled with ACSF. Extracellular fEPSPs were evoked using a monopolar electrode filled with ACSF placed in the stratum radiatum. fEPSPs were recorded within the CA1 subregion of the hippocampus. After a stable fEPSP had been recorded for at least 10 min at a frequency of 0.05 Hz (using a response about 50% of the maximum), LTP was induced with three trains of 100 Hz for 1 s with an intertrain interval of 20 s. Data were collected and analyzed using pCLAMP 10 software (Molecular Devices).

**Animal surgery and EEG recording**
An EEG was recorded using Pinnacle 2EEG/1EMG mouse EEG hardware with synchronized video and was acquired using the Pinnacle Sirenia Software Suite (Pinnacle Technology Inc.). Briefly, under isoflurane anesthesia, EEG/electromyogram (EMG) headmounts were
attached to the skull with four screws that serve as surface EEG electrodes and then were secured with dental acrylic. During the same surgery, two EMG electrodes were placed into the trapezius muscle. Animals were allowed to recover for 1 week before EEG recording. All animals were maintained on a 12-hour light/12-hour dark cycle and received 4 or 5 days of continuous EEG recording with synchronized video (with infrared lighting for capture of video during dark phase). EEG data were visualized using the Sirenia Software (Pinnacle Technology Inc.). To aid with seizure detection, additional signal processing was performed using MATLAB r2016a and the EEGLAB toolbox (Swaartz Center for Computational Neuroscience). The entire 5-day EEG recording was reviewed for electrographic seizures, with further review of the video for any periods suspicious for seizure. The total number of spike-wave discharges was determined over a 48-hour epoch. Single spikes were identified as sharp activity with an amplitude of at least twice the baseline and associated with an aftercoming slow wave, whereas polyspikes were identified as multispike complexes with a spike-wave morphology and amplitude at least twice the baseline. Comparison of the EEG signal to the EMG signal was used to exclude movement artifacts.

Sciatic nerve g-ratio analysis
Samples were fixed in 2% paraformaldehyde (PFA) and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3), post-fixed with unbuffered 2% osmium tetroxide, rinsed with distilled water, en bloc stained with 3% uranyl acetate, rinsed with distilled water, dehydrated in ascending grades of ethanol, transitioned with propylene oxide, embedded in resin mixture from the Embed 812 Kit, and cured in a 60°C oven. Samples were sectioned on a Leica Ultracut UC6 ultramicrotome. One-micrometer-thick sections were collected and stained with toluidine blue O. Images were taken under 100×, and Feret’s diameters were determined from hand-traced axons using the ImageJ built-in function. One hundred axons were randomly sampled from each animal. g ratio was calculated by dividing the inner axon diameter by the outer diameter including the myelin sheath.

Glucose tolerance and insulin tolerance tests
Mice were fasted for 16 hours before the glucose tolerance test. Glucose at 2 g/kg was intraperitoneally injected and blood glucose was determined using a standard glucose meter (Precision Xtra glucose meter, Abbott). For the insulin tolerance test, mice were intraperitoneally injected with 0.75 U/kg regular human insulin (Lilly). Blood glucose was determined at 0, 15, 30, 60, and 120 min by a Precision Xtra glucose meter (Abbott) and compatible glucose strips.

TSE LabMaster automated phenotyping
Mice were singly housed in their home cages in an enclosed environmental chamber of the TSE Automated Phenotyping System (TSE Systems Inc.) with controlled temperature under 12-hour light/12-hour dark cycle at the Northwestern University Comprehensive Metabolic Core. Data collection began after a 3-day acclimation period. Food and fluid intake were continuously recorded via feeding/drinking sensors. Locomotor activities in three dimensions were monitored via infrared beam breaks through frames mounted on the perimeter of the metabolic cages, and CO2 production and O2 consumption were used to assess energy expenditure and respiratory exchange ratio. Data were averaged and plotted in a 24-hour duration because no differences between light and dark cycles were observed.

Necropsy
Necropsy was performed by the Northwestern University Mouse Histology and Phenotyping Laboratory. Briefly, mice were anesthetized with a lethal dose of ketamine/xylazine cocktail and perfused with equal volume of PBS and 4% PFA. Tissues were harvested, fixed in 10% formalin, and processed for paraffin embedding. Four-micrometer paraffin sections on glass slides were used for hematoxylin and eosin staining, and images were obtained on a standard light microscope.

Cell proliferation, neurogenesis, and cell death analysis
BACE1f/f and BACE1f/f;R26CreER2 mice received TAM injections at 6 to 9 months of age, were aged to 2 years, and then were treated with daily BrdU (50 μg/g) intraperitoneal injection for seven consecutive days. Half of mice were collected 24 hours and the rest 1 month after the last BrdU injection. Mice were perfused and processed for free-floating brain section staining as described under the “Tissue extraction and immunoblot analysis” and “Immunohistochemistry” sections. Every sixth section was used for measuring cell proliferation. DG granule cell layer (GCL) area was obtained by hand-tracing on 10× images in ImageJ. Volume was estimated by multiplying the area with the distance spaced between sections. The number of BrdU+ cells was estimated by (number of BrdU+ cells counted)/(GCL volume).

For neurogenesis experiments, every third section was colorabeled for BrdU and NeuN. A minimum of 10 BrdU+ cells was counted. Colocalization of BrdU and NeuN was confirmed by confocal microscopy. For regional analyses, the GCL was subdivided into crest defined as the first one-fourth, in length of suprapyramidal and infrapyramidal blades at the junction, and the rest of the suprapyramidal and infrapyramidal blades minus crest. The same BrdU+ cells counted in previous cell proliferation and neurogenesis experiments were divided into these three regions based on their location. For an estimate of total number of BrdU+ cells in subregions of the DG, the previously counted number of BrdU+ cells in each subregion was multiplied by 6 (that is, the number of intervening sections; fig. S12F). For cell death detection, sections were permeabilized with Triton X-100 for an hour, followed by a 1-hour incubation of reaction mix from the In Situ Cell Death Detection Kit, TMR Red (#12156792910 Roche, Sigma-Aldrich) at 37°C.

Statistics
Individual-level data is supplied in table S1. Statistics were calculated using Prism 5 (GraphPad Software). Repeated two-way ANOVA was used in hidden platform training in the Morris water maze test (Fig. 2, A and C). One-way ANOVA was applied in the Morris water maze probe trials (Fig. 2, B and D), spike-wave discharge per hour (Fig. 3C), and sciatic nerve MBP (Fig. 4B). Linear regression analyses were used in g ratio analysis (Fig. 4E) and correlation between CHL1 and IPB lengths (Fig. 5, F and G). Chi-squared analysis was applied for BACE1f/f;CamKII-tCre postnatal lethality (fig. S5A). Unpaired two-way Student’s t test was used in the rest of the data analysis. Numbers of replicates and P values are stated in each figure legend. All data are plotted as means ± SEM. Significance was concluded when the P value was less than 0.05, indicated by *P < 0.05, **P < 0.01, ***P < 0.001. N.S. (not significant) denotes P > 0.05.

SUPPLEMENTARY MATERIALS
www.sciencetranslationalmedicine.org/cgi/content/full/10/459/eaao5620/DC1
Fig. S1. BACE1f/f gene targeting strategy and Southern blot and PCR screening.

Fig. 52. Diagram of experimental timeline.

Fig. 53. Conditional BACE1 knockout confirmed by immunohistochemistry of brain sections from BACE1iCre,R26CreER T2 and BACE1fl/fl,R26CreER T2 TAM-treated mice.

Fig. 54. CreER T2 recombinase does not induce the excision of the BACE1 gene in the absence of TAM in BACE1fl/fl,R26CreER T2 mice.

Fig. 55. BACE1+/− postnatal lethality and underweight phenotypes were greatly reduced and completely absent in postnatal forebrain excitatory neuron and adult whole-body conditional BACE1 knockout mice, respectively.

Fig. 56. BACE1fl/fl,R26CreER T2 TAM-treated mice showed normal metabolism and basal activity after long-term BACE1 knockout.

Fig. 57. BACE1fl/fl,R26CreER T2 mice at 6 months of age had normal memory performance but showed slightly slower learning in the Morris water maze.

Fig. 58. Both BACE1fl/fl,R26CreER T2 and BACE1iCre,R26CreER T2 TAM-treated mice displayed novelty-induced hyperactivity.

Fig. 59. Necropsy indicated no gross differences in major organs or tissues in BACE1fl/fl,R26CreER T2 TAM-treated mice as a result of conditional BACE1 knockout in the adult whole body.

Fig. 60. No morphology changes observed in olfactory glomeruli of BACE1fl/fl,R26CreER T2 TAM-treated mice.

Fig. 61. Verification of BACE1 knockdown and the IPB phenotype of BACE1fl/fl and BACE1fl/fl,R26CreER T2 TAM-treated mice.

Fig. 62. No difference observed in cell proliferation and regional neurogenesis between BACE1fl/fl and BACE1fl/fl,R26CreER T2 TAM-treated mice.

Fig. 63. Minimum cell death in hippocampus in aged BACE1fl/fl and BACE1fl/fl,R26CreER T2 TAM-treated mice.

Fig. 64. Low-power hippocampal images of calbindin (green), CHL1 (red), and synaptophysin (blue) immunostaining of 1-year-old BACE1fl/fl and BACE1fl/fl,R26CreER T2 TAM-treated mice.

Table S1. Individual-level data for all figures.

REFERENCES AND NOTES


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38. Integrins, and the plasminogen activator inhibitor-2 promotes CHL1-induced neurite

39. Close homolog of L1 modulates area-specific neuronal positioning and dendrite

40. Orientation in the neocortex via PTP

41. Interaction between CHL1 and serotonin receptor 2c regulates signal transduction and

42. mouse hippocampus.

43. Neurons born in the adult dentate gyrus form functional synapses with target cells.


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Axonal organization defects in the hippocampus of adult conditional BACE1 knockout mice

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Getting back to BACEics
Drugs that block the β-secretase BACE1 are in clinical trials for Alzheimer’s disease. However, their safety is unclear because mice with germline loss of BACE1 exhibit neurological phenotypes, although these could arise from BACE1 deficiency during development. Ou-Yang et al. now have generated conditional BACE1 knockout mice, in which mice underwent development with BACE1 present, and then, the Bace1 gene was ablated in the mature adult animal. Although these adult conditional BACE1 knockout mice largely lacked the phenotypes observed in germline knockout animals, they did exhibit a Bace1-null phenotype involving disorganization of an axonal pathway in the hippocampus, a brain region critical for memory. This suggests that BACE1 inhibitor drugs for treating Alzheimer’s disease could potentially disturb hippocampal axonal pathways.