Abstract: The Alzheimer BACE1 enzyme cleaves numerous substrates, with largely unknown physiological consequences. We have previously identified the contribution of elevated BACE1 activity to voltage-gated sodium channel Na(v)1.1 density and neuronal function. Here, we analyzed physiological changes in sodium channel metabolism in BACE1-null mice. Mechanistically, we first confirmed that endogenous BACE1 requires its substrate, the -subunit Na(v) (2), to regulate levels of the pore-forming -subunit Na(v)1.1 in cultured primary neurons. Next, we analyzed sodium channel -subunit levels in brains of BACE1-null mice at 1 and 3 months of age. At both ages, we found that Na(v)1.1 protein levels were significantly decreased in BACE1-null versus wild-type mouse brains, remaining unchanged in BACE1-heterozygous mouse brains. Interestingly, levels of Na(v)1.2 and Na(v)1.6 -subunits also decreased in 1-month-old BACE1-null mice. In the hippocampus of BACE1-null mice, we found a robust 57% decrease of Na(v)1.1 levels. Next, we performed surface biotinylation studies in acutely dissociated hippocampal slices from BACE1-null mice. Hippocampal surface Na(v)1.1 levels were significantly decreased, but Na(v)1.2 surface levels were increased in BACE1-null mice perhaps as a compensatory mechanism for reduced surface Na(v)1.1. We also found that Na(v) (2) processing and Na(v)1.1 mRNA levels were significantly decreased in brains of BACE1-null mice. This suggests a mechanism consistent with BACE1 activity regulating mRNA levels of the -subunit Na(v)1.1 via cleavage of cell-surface Na(v) (2). Together, our data show that endogenous BACE1 activity regulates total and surface levels of voltage-gated sodium channels in mouse brains. Both decreased Na(v)1.1 and elevated surface Na(v)1.2 may result in a seizure phenotype. Our data caution that therapeutic BACE1 activity inhibition in Alzheimer disease patients may affect Na(v)1 metabolism and alter neuronal membrane excitability in Alzheimer disease patients.

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Reduced Sodium Channel Na$_{\text{v}}$1.1 Levels in BACE1-null Mice*§

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The Alzheimer BACE1 enzyme cleaves numerous substrates, with largely unknown physiological consequences. We have previously identified the contribution of elevated BACE1 activity to voltage-gated sodium channel Na$_{\text{v}}$1.1 density and neuronal function. Here, we analyzed physiological changes in sodium channel metabolism in BACE1-null mice. Mechanistically, we first confirmed that endogenous BACE1 requires its substrate, the β-subunit Na$_{\text{v}}$β$_2$, to regulate levels of the pore-forming α-subunit Na$_{\text{v}}$1.1 in cultured primary neurons. Next, we analyzed sodium channel α-subunit levels in brains of BACE1-null mice at 1 and 3 months of age. At both ages, we found that Na$_{\text{v}}$1.1 protein levels were significantly decreased in BACE1-null versus wild-type mouse brains, remaining unchanged in BACE1-heterozygous mouse brains. Interestingly, levels of Na$_{\text{v}}$1.2 and Na$_{\text{v}}$1.6 α-subunits also decreased in 1-month-old BACE1-null mice. In the hippocampus of BACE1-null mice, we found a robust 57% decrease of Na$_{\text{v}}$1.1 levels. Next, we performed surface biotinylation studies in acutely dissociated hippocampal slices from BACE1-null mice. Hippocampal surface Na$_{\text{v}}$1.1 levels were significantly decreased, but Na$_{\text{v}}$1.2 surface levels were increased in BACE1-null mice perhaps as a compensatory mechanism for reduced surface Na$_{\text{v}}$1.1. We also found that Na$_{\text{v}}$β$_2$ processing and Na$_{\text{v}}$1.1 mRNA levels were significantly decreased in brains of BACE1-null mice. This suggests a mechanism consistent with BACE1 activity regulating mRNA levels of the α-subunit Na$_{\text{v}}$1.1 via cleavage of cell-surface Na$_{\text{v}}$β$_2$. Together, our data show that endogenous BACE1 activity regulates total and surface levels of voltage-gated sodium channels in mouse brains. Both decreased Na$_{\text{v}}$1.1 and elevated surface Na$_{\text{v}}$1.2 may result in a seizure phenotype. Our data caution that therapeutic BACE1 activity inhibition in Alzheimer disease patients may affect Na$_{\text{v}}$1 metabolism and alter neuronal membrane excitability in Alzheimer disease patients.

Alzheimer disease (AD)$^3$ is a devastating neurodegenerative disorder characterized by progressive memory loss. In the late stages of the disease, neuropsychiatric symptoms include depression, aggressiveness, agitation, and generalized anxiety (1). Pathological hallmarks of AD patient brains consist of extracellular amyloid deposits (senile plaques), neurofibrillary tangles, and loss of neurons and synapses in the hippocampus and the cerebral cortex (2, 3). The main component of amyloid deposits is a short amyloid β (Aβ) peptide most commonly of 40–42 amino acids, which derives from the amyloid precursor protein (APP). To generate Aβ, APP is first cleaved by β-site APP-cleaving enzyme 1 (BACE1) and subsequently by γ-secretase. Because of their central role in Aβ generation, it is not surprising that the two secretases are among the prime drug targets for AD treatment (4, 5).

BACE1 is a transmembrane aspartic protease that is ubiquitously expressed with the highest levels in the brain (6, 7). BACE1 levels and activity are increased in AD patient brains (8–10). The reason why BACE1 is elevated in AD brains is unknown, although it has been shown that depletion of the adaptor protein GGA3 and phosphorylation of the translation initiation factor eIF2α increase BACE1 levels (11, 12). Cellular and oxidative stresses as well as energy starvation have an effect on the enzyme function (11, 13, 14). Currently, only a few proteins are known as physiological substrates of BACE1 (15–19). To understand the physiological function of BACE1, it is essential to characterize BACE1-mediated cleavage of its neuronal substrates.

Voltage-gated sodium channels (Na$_{\text{v}}$1s) consist of one pore-forming α-subunit, associated with one or two β-accessory subunits (20). Currently, 10 α- and 4 β-subunits have been reported (21). The β-subunits are type I single-transmembrane proteins that regulate the localization, trafficking, and inactivation of the α-subunits by direct interaction (20, 22–24). Of the four β-subunits, the β2-subunit (Na$_{\text{v}}$β$_2$) appears to have a major role in the regulation of the total and cell surface density of sodium channels in neurons (25–27). Na$_{\text{v}}$β$_2$-null mice show altered response to pain and a reduced threshold for seizures because of defects in electrical excitability in neurons (26, 27).

BACE1-null mice have revealed abnormalities in behavior, cognitive, and emotional functions (28–31). An imbalance in sodium channel function may contribute to some of these phenotypes. Indeed, the auxiliary β2-subunit of voltage-gated sodium channels undergoes sequential processing by BACE1 and γ-secretase similar to APP (18, 19). We found that BACE1-mediated cleavage of Na$_{\text{v}}$β$_2$ alters neuronal activity (32). Moreover, elevated levels of the pore-forming α-subunit Na$_{\text{v}}$1.1 are observed in AD brains with elevated BACE1 expression and also in mice overexpressing human BACE1 (32). The β2-intra-
cellular domain (β2-ICD), derived from BACE1 and γ-secretase-mediated cleavage of β2, regulates Na\textsubscript{1.1} expression. However, Na\textsubscript{1.1} trafficking to the cell membrane is impaired resulting in reduced Na\textsubscript{1.1} surface levels, which leads to reduced sodium current densities (32).

Here, we asked whether endogenous BACE1 activity regulates Na\textsubscript{1} metabolism in neuronal cells under physiological conditions. First, we found that Na\textsubscript{1,β2} is essential for BACE1 activity-dependent regulation of Na\textsubscript{1.1} mRNA and protein levels in cultured embryonic neurons. Next, we also found that endogenous BACE1 activity regulates total and surface levels of Na\textsubscript{1.1} especially in adult hippocampus. Interestingly, Na\textsubscript{1,2} appears to compensate for decreased surface levels of Na\textsubscript{1.1}.

**EXPERIMENTAL PROCEDURES**

**Reagents and Antibodies**—N-[N-3,5-Difluorophenacyl]-l-alanyl-S-phenylglycine t-butyl ester and BACE inhibitor IV were purchased from Calbiochem. BACE1 monoclonal antibody (3D5) was a kind gift from Dr. Robert Vassar (Northwestern University), and anti-Na\textsubscript{1,β2} directed against the C terminus of endogenous Na\textsubscript{1,β2} was from Dr. Nobuyuki Nukina (RIKEN Brain Science Institute). The following antibodies were purchased for this study: anti-GAPDH (Chemicon); anti-Na\textsubscript{1,1} antibody (University of California, Davis/National Institutes of Health NeuroMab Facility); anti-Na\textsubscript{1,2}, anti-Na\textsubscript{1,3}, and anti-Na\textsubscript{1,6} (Alomone Labs); anti-Na\textsubscript{1,5} (Sigma); anti-N-cadherin (BD Transduction Laboratories); and anti-synaptophysin (StressGen).

**Primary Neuronal Cultures**—Mouse primary hippocampal/cortical neuronal cultures were prepared and maintained in Neurobasal media (Invitrogen) supplemented with B27 and 0.5 mM l-glutamine as described previously (18). The neuronal cultures were maintained at 37 °C in a humidified 5% CO\textsubscript{2} atmosphere for 14 days. For rat primary neuronal culture, cryopreserved rat hippocampal/cortical regions were purchased from BrainBits (Springfield, IL), dissociated, and plated on poly-L-lysine/laminin-coated plates. The neuronal cultures were maintained for 14–17 days in Neurobasal media (Invitrogen) supplemented with B27 and 0.5 mM l-glutamine.

**Breeding**—All animal procedures were approved by the Subcommittee on Research Animal Care on the Use and Care of Animals, Massachusetts General Hospital. BACE1-null and wild-type control mice (C57BL/6J background) were purchased from The Jackson Laboratory (Bar Harbor, ME). BACE1-heterozygous knock-out mice were generated by crossing BACE1-null and wild-type control mice. Genotypes were determined by genomic PCR using the Allele-in-One mouse tail direct PCR system (Allele Biotechnology, San Diego) using the following primers: 5′-AGGCAGCTTTTGTGGAGATGGT-3′, 5′-CGGGAATGGAAAGGCTACTCC-3′, and 5′-TGATGTGAATGTGTCGG-3′. Na\textsubscript{1,β2}-null and control mice were kindly provided by Dr. Lori L. Isom (University of Michigan). Generation and genotyping of Na\textsubscript{1,β2}-null mice were described previously (26).

**Preparation of Mouse Brain Lysates**—One- to 3-month-old mice were sacrificed, and brains were immediately removed and stored at −80 °C until use. To prepare total brain lysates, the frozen brains were powdered under liquid nitrogen with the use of mortar and pestle. The aliquots were stored at −80 °C for individual experiments. For total brain extraction, 50–100 μg of brain powder was extracted in 5 volumes of SDS extraction buffer (10 mM Tris–HCl (pH 6.8), 2% SDS, 1 mM EDTA, 150 mM NaCl, a protease inhibitor mixture (Roche Applied Science), 2 mM 1,10-phenanthroline, 2 μM MG132, and 1 mM PMSF). After incubation on ice for 10 min, samples were sonicated at 4 °C for 5 min, passed through 0.22-μm pore-sized filter in spin-x centrifuge tubes (Costar, Corning, NY), and centrifuged for 45 min at 14,000 rpm. To prepare crude brain membrane fractions, the brain powder was resuspended in TBS extraction buffer (10 mM Tris–HCl (pH 6.8), 1 mM EDTA, 150 mM NaCl, a protease inhibitor mixture (Roche Applied Science), 2 mM PNT, 2 μM MG132, and 1 mM PMSF) and passed through 22-gauge needle three times. After brief centrifugation (500 × g, 5 min), the supernatant was then centrifuged again at 10,000 × g for 1 h. The membrane pellet was then resuspended in TBS extraction buffer, and the protein level was determined by BCA assay. For SDS-PAGE analysis, the crude membrane fractions were directly mixed with 4× lithium dodecyl sulfate sample buffer (Invitrogen) supplemented with 8% (v/v) β-mercaptoethanol and heated at 95 °C for 5 min. To prepare hippocampal brain lysates, brains were microdissected under microscope immediately after the brains were removed. Hippocampi were then directly lysed in SDS extraction buffer (10 mM Tris–HCl (pH 6.8), 1.5% SDS, 1 mM EDTA, 150 mM NaCl, a protease inhibitor mixture (Roche Applied Science), 2 mM PNT, 2 μM MG132, and 1 mM PMSF) and the protein level was determined by BCA assay. For SDS-PAGE analysis, the crude membrane fractions were directly mixed with 4× lithium dodecyl sulfate sample buffer (Invitrogen) supplemented with 8% (v/v) β-mercaptoethanol and heated at 95 °C for 5 min. To prepare hippocampal brain lysates, brains were microdissected under microscope immediately after the brains were removed. Hippocampi were then directly lysed in SDS extraction buffer (10 mM Tris–HCl (pH 6.8), 1.5% SDS, 1 mM EDTA, 150 mM NaCl, a protease inhibitor mixture (Roche Applied Science), 2 mM PNT, 2 μM MG132, and 1 mM PMSF). RNeasy mini kit (Qiagen) was used to extract total RNA from the brains. Approximately 30 mg of brain powder was dissolved in 600 μl of buffer RLT containing 1% β-mercaptoethanol by vortexing. Lysates were applied to QiAshredder (Qiagen). The instructions for RNeasy mini kit (Qiagen) were followed.

**Confocal Immunofluorescence Staining of Hippocampal Sections**—BACE1-null, BACE1 heterozygote knock-out, and wild-type control mice were perfused with 12 ml of PBS and 1% paraformaldehyde, 1% sucrose solution, respectively. Brains were rapidly removed, further fixed in 1% paraformaldehyde, 1% sucrose solution for 3 h at 4 °C, transferred to 30% sucrose solution, and incubated for 48 h at 4 °C. 40-μm sections were cut, washed three times with PBS, and incubated with blocking solution containing 0.1% Triton X-100 and 5% purified goat IgG (Jackson ImmunoResearch) for 12 h. The sections were then incubated with primary antibody solution containing anti-Na\textsubscript{1,1} antibody (monoclonal, 1:20 dilution, University of California, Davis/National Institutes of Health NeuroMab Facility), 0.1% Triton X-100, and 5% goat IgG for 24 h at 4 °C. After washing three times, sections were incubated with secondary antibody solution with anti-mouse Alexa Fluor488 (Invitrogen) for 3 h at room temperature. Immunostained sections were mounted and analyzed by Olympus IX70 fluorescence microscope equipped with a confocal Disk Spinning Unit (Olympus). Images were captured and analyzed by IPLab software.

**Western Blot Analysis**—20–100 μg of protein were resolved on 4–12% gradient BisTris gels, 12% BisTris gels, or 16% Tricine gels (Invitrogen), depending on the individual experiment as described. The blots were visualized by enhanced chemiluminescence (ECL). The images were captured by using BioMax

Redacted Na\textsubscript{1,1} Levels in BACE1-null Mice
Reduced Na\textsubscript{v}1.1 Levels in BACE1-null Mice

film (Eastman Kodak Co.) or VersaDoc imaging system (Bio-Rad) and quantitated by using QuantityOne software (Bio-Rad).

Quantitative RT-PCR—Levels of sodium channel genes were analyzed by quantitative RT-PCR performed in a Light Cycler PCR system (Bio-Rad). 1 \mu g of total RNA was used for the synthesis of cDNA using oligo(dT) primers following the protocol of the SuperScript first-strand synthesis system for RT-PCR (Invitrogen). Relative quantification of mRNA expression of Scn1a (Na\textsubscript{v}1.1) compared with endogenous expression of GAPDH was analyzed by TaqMan real time PCR. Primers were purchased from Applied Biosystems (catalogue numbers Mn01329052-m1, Mn00488110-m1, and Mn01270368-m1). Expression analysis was performed with 40 two-step cycles for 30 s at 95 °C and 60 s 60 °C as described in the supplier’s protocol (Applied Biosystems) using a Bio-Rad iCycler. Sodium channel mRNA was normalized to GAPDH mRNA by the comparative \textit{CT} method.

Acute Hippocampal Slice Preparation—Hippocampal neurons from BACE1-transgenic and control mice (1–2 month) were acutely isolated using standard procedures (26, 33). Mice were decapitated under deep isoflurane anesthesia, and brains were rapidly removed and iced. Slices (400 \mu m) were cut and transferred to a low calcium buffer containing 15 mM of Nav1.1 (32). To test whether endogenous BACE1 activity localized to the nucleus and regulates mRNA and protein levels and incubated with 100 \mu M of BACE1 activity was conducted according to a method described by Thomas-Crusells et al. (34) with slight modifications. The slices were prepared as described earlier and incubated with 100 \mu M Sulfo-NHS-Biotin (Pierce) in Earle’s balanced salt solution bubbled with 95% O\textsubscript{2} and 5% CO\textsubscript{2} at room temperature. Surface Biotinylation—Surface biotinylation of acutely prepared hippocampal slices was performed according to a method described by Thomas-Crusells et al. (34) with slight modifications. The slices were prepared as described earlier and incubated with 100 \mu M Sulfo-NHS-Biotin (Pierce) in Earle’s balanced salt solution bubbled with 95% O\textsubscript{2} and 5% CO\textsubscript{2} for 80 min under dark conditions. The reaction was stopped by washing three times with cold Earle’s balanced salt solution containing 100 \mu M L-lysine. Hippocampal regions were rapidly separated from the individual slices under a dissecting microscope. The isolated hippocampi were extracted with a lysis buffer containing 10 mM Tris-HCl (pH 6.8), 1 mM EDTA, 150 mM NaCl, 0.2% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, and protease inhibitors, followed by a spin at 16,000 \times g.

Biotinylated proteins were captured by Neutravidin beads (Pierce) at 4 °C overnight.

Statistical Analyses—All statistical analyses were performed using a two-tailed Student’s \textit{t} test or one-way ANOVA followed by post hoc Tukey’s test. Error bars represented in graphs denote the means ± S.E.

RESULTS

Na\textsubscript{v}1.1 BACE1 Mediates the Effect of BACE1 on Na\textsubscript{v}1.1 Levels in Primary Neurons—Previously, we found that BACE1 and γ-secretase sequentially cleave Na\textsubscript{v}1.2 to release the intracellular domain of Na\textsubscript{v}1. β2 (β2-ICD). In neuroblastoma cells, β2-ICD is localized to the nucleus and regulates mRNA and protein levels of Na\textsubscript{v}1.1 (32). To test whether endogenous BACE1 activity regulates Na\textsubscript{v}1.1 through the processing of Na\textsubscript{v}1.2 in \textit{vivo}, we treated cultured rat cortical/hippocampal neurons with BACE1 inhibitors and analyzed Na\textsubscript{v}1.1 levels. We found that \textit{dR9} treatment decreased Na\textsubscript{v}1.1 levels up to 50% in a dose-dependent manner (Fig. 1A). Interestingly, we also found that Na\textsubscript{v}1.2 levels were dose-dependently increased upon \textit{dR9} treatment, suggesting potential compensatory changes (supplemental Fig. 1, \textit{A} and \textit{B}). Previously, we have shown that recombinant β2-ICD expression significantly elevates Scn1a (Na\textsubscript{v}1.1) mRNA levels in rat neuroblastoma cells (32). Similarly to Na\textsubscript{v}1.1, Na\textsubscript{v}1.2 may also be regulated at the level of β2-ICD. Therefore, we tested the effect of recombinant β2-ICD on Scn2a (Na\textsubscript{v}1.2) mRNA levels. Unlike Scn1a (Na\textsubscript{v}1.1), Scn2a (Na\textsubscript{v}1.2) mRNA levels did not change in a statistically meaningful way under the same conditions (supplemental Fig. 1C). These results suggest that a potential compensatory change of Na\textsubscript{v}1.2 may not be directly regulated by β2-ICD.

To explore whether Na\textsubscript{v}1.2 is required for BACE1 activity-dependent Na\textsubscript{v}1.1 decrease, we took advantage of primary neuronal cultures from Na\textsubscript{v}1.2-null mice (26). Cultured hippocampal/cortical neurons (14 days \textit{in vitro}) from wild-type and Na\textsubscript{v}1.2-null mice were treated with BACE1 inhibitors, followed by analysis of Na\textsubscript{v}1.1 mRNA and protein levels. As expected, in wild-type neurons we found that two different BACE inhibitors, \textit{dR9} or inhibitor IV, decrease Na\textsubscript{v}1.1 protein levels by 42–70% (Fig. 1, \textit{B} and \textit{C}). Scn1a (Na\textsubscript{v}1.1) mRNA levels were also decreased by \textit{dR9} or inhibitor IV (supplemental Fig. 1D). These data show that BACE1 activity is required for maintaining Na\textsubscript{v}1.1 levels in cultured rat and mouse neurons. In contrast, in Na\textsubscript{v}1.2-null neurons Na\textsubscript{v}1.1 levels were unaffected by the same BACE1 inhibitor treatments (Fig. 1, \textit{D} and \textit{E}). These data show that Na\textsubscript{v}1.2 is required for BACE1-dependent regulation of Na\textsubscript{v}1.1 levels in cultured embryonic neurons.

Reduced Na\textsubscript{v}1.1 Levels in BACE-null Mice—Next, we investigated whether endogenous BACE1 activity is required for maintaining normal Na\textsubscript{v}1.1-α-subunit levels in adult brains. Brain samples were prepared from 1- and 3-month-old BACE1-null (BACE1-KO), BACE1-heterozygous knock-out (BACE1-HE), and aged-matched wild-type control mice (WT). One month of age was chosen because it is the minimum age for analyzing adult Na\textsubscript{v}1.1s and is also age-compatible with acute preparation of hippocampal slices without inducing significant neuronal death. All animals were healthy; however, BACE1-null mice were generally smaller in size than wild-type and BACE1-HE animals as reported previously (28). Furthermore, increased mortality was observed among BACE1-null mice.

We compared total brain Na\textsubscript{v}1.1-α-subunits levels in 1-month-old BACE1-null and BACE1-heterozygous knock-out with age-matched wild-type mice. To avoid degradation of protease-prone Na\textsubscript{v}1.1-α-subunits, brains were rapidly removed, powdered under liquid nitrogen, aliquoted, and stored at −80 °C until extraction. Because Na\textsubscript{v}1.1-α-subunits are hydrophobic integral membrane proteins, extraction conditions with strong ionic detergents were required to solubilize α-subunits from insoluble membrane fractions. We compared several extraction conditions, and we found that extraction with 2% SDS showed the most consistent results, tightly comparable with a method based on direct boiling with SDS-PAGE sample.
loading buffer (Fig. 2; for detailed buffer formulation and conditions, please see “Experimental Procedures”).

Western blot analysis showed more than 50% decrease of Na\(_{\alpha1.1}\) levels in brain extracts from BACE1-null as compared with age-matched wild-type control mice, although no significant difference was detected between BACE1-heterozygous knock-out and wild-type controls (Fig. 2). These data suggest that more than 50% decrease in BACE1 expression is required for decreasing Na\(_{\alpha1.1}\) levels in adult mouse brains. Interestingly, we also observed significant decreases in Na\(_{\alpha1.2}\) and Na\(_{\alpha1.6}\) levels, two additional major CNS Na\(_{\alpha}\) subunits (Fig. 2). Levels of Na\(_{\alpha1.3}\), an embryonic Na\(_{\alpha}\) subunit, did not change (data not shown). The supplemental Fig. 2 shows a littermate analysis of BACE1-null and BACE1-heterozygous knock-out mice. As expected, we found more than 50% decrease of Na\(_{\alpha1.1}\) levels in BACE1-null mice as compared with BACE1-heterozygous knock-out littermate controls at the same age.

Finally, we investigated whether the changes in Na\(_{\alpha1.1}\) levels were also seen in older mice. For this purpose, Na\(_{\alpha1}\) levels were analyzed in whole brain extracts from 3-month-old BACE1-null and wild-type control mice (Fig. 3A). Similar to 1-month-old mice, we observed a significant ~20% decrease in Na\(_{\alpha1.1}\) and Na\(_{\alpha1.2}\) levels in BACE1-null mice but no significant changes in Na\(_{\alpha1.6}\) levels (Fig. 3B). Interestingly, decreases in Na\(_{\alpha1}\) subunit levels were less pronounced at 3 months versus 1 month of age, suggesting the possibility that the lack of BACE1 activity affects Na\(_{\alpha1}\) subunit levels more prominently at early ages (Fig. 3, A and B). High expression of BACE1 in early postnatal stages may also explain the strong effects of BACE1 knock-out on Na\(_{\alpha1}\) subunit levels in young mouse brains (15). Na\(_{\alpha1.3}\) levels were hardly detectable, because this subunit is predominantly expressed in early developmental stages (data not shown). Together, these data indicate that endogenous BACE1 activity regulates Na\(_{\alpha}\) subunit levels in adult neurons.

**Na\(_{\alpha1.1}\) Protein Levels in the Hippocampus of BACE-null Mice—**

We then investigated the effect of BACE1 ablation in the hippocampus, which is a highly affected brain region in patients with Alzheimer disease. Hippocampal regions were dissected from the brains of 3-month-old BACE1-null and wild-type control mice, extracted, and analyzed for Na\(_{\alpha1}\) subunits by Western blot analysis. We observed a robust 57% decrease of Na\(_{\alpha1.1}\) levels in BACE1-null as compared with wild-type control mice (Fig. 4, A and B). Interestingly, we could not detect any significant changes in Na\(_{\alpha1.2}\) or Na\(_{\alpha1.6}\) levels in these samples, suggesting that Na\(_{\alpha1.1}\) is the major Na\(_{\alpha}\) subunit affected by BACE1 activity in the hippocampal region (Fig. 4, A and B).

We also investigated Na\(_{\alpha1.1}\) distribution in the hippocampal region of 3-month-old BACE1-null mice by immunofluorescence (Fig. 4C). Using a monoclonal Na\(_{\alpha1.1}\) antibody (Neuro-mab), we mainly detected Na\(_{\alpha1.1}\) in neuronal cell bodies of hippocampal neurons (35, 36). As shown in Fig. 4C, we found that Na\(_{\alpha1.1}\) levels were significantly decreased in the dentate gyrus, hilus, and CA3 regions (Fig. 4C, upper and middle panels). At higher magnification, we confirmed that somatic Na\(_{\alpha1.1}\) staining is specifically decreased in BACE1-null mice in the same regions (Fig. 4C, bottom panels). We also found similar but
more dramatic decreases of Na,v1.1 in 1-month-old BACE1-null mice as compared with age-matched controls (supplemental Fig. 3). These data show that ablation of BACE1 significantly decreases Na,v1.1 levels in adult hippocampal neurons.

Altered Surface Na,v1 Levels in BACE1-null Brain Slices—In neurons, newly synthesized Na,v1 α-subunits accumulate inside the cell body, and only a small fraction of active channels reaches the cell surface (22). To explore whether endogenous BACE1 activity modulates surface expression of Na,v1 α-subunits, we performed surface biotinylation analysis using acute hippocampal slices prepared from BACE1-HE and BACE1-null mice as described previously (32). The biotinylated surface proteins were captured, and total and surface Na,v1 α-subunit levels were analyzed by Western blot analysis (Fig. 5A). As expected, total levels of Na,v1.1 were largely decreased in BACE1-null as compared with BACE1-HE (Fig. 5A, left panel). Similar to the total Na,v1.1 levels, surface levels of Na,v1.1 also largely and significantly decreased in BACE1-null as compared with BACE1-HE (Fig. 5A, right panel). When quantitated, we found 32% decrease in BACE1-null as compared with BACE1-HE control mice (Fig. 5B). Surface expression of Na,v1.2 showed a significant increase, in contrast with Na,v1.1 (Fig. 5, A and C). This suggests a compensatory increase in Na,v1.2 sodium channel density in adult mice. We also compared Na,v1.1 surface levels between BACE1-null with wild-type control mice and found a similar 35% decrease (Fig. 5, D and E). Our data show that endogenous BACE1 activity regulates surface expression of Na,v1 α-subunit levels in adult neurons and suggest a compensatory regulation between Na,v1.1 and Na,v1.2 surface levels.

In addition to Na,v1 α-subunit surface levels, we also investigated surface expression of full-length Na,β2 in acute brain slices from BACE1-HE and BACE1-null mice. Interestingly, we found that surface levels of full-length Na,β2 increased by 1.92-fold in BACE1-null as compared with BACE1-HE (±0.06, Fig. 6A). Similarly, the APP, another well known physiological substrate of BACE1, also showed increased surface expression of its full-length mature form in BACE1-null mice (Fig. 6A). These surface level changes suggest that ablation of BACE1 decreased the cleavage of these substrates at the cell surface because
Reduced Na\textsubscript{v}1.1 Levels in BACE1-null Mice

BACE1 is known to cleave its substrates at the cell surface or in the endosomal recycling compartment (37).

Previously, we found that BACE1 and γ-secretase sequentially cleave Na\textsubscript{v}β\textsubscript{2} to release β2-ICD (32). As shown in our previous study and supplemental Fig. 1C, β2-ICD is localized to the nucleus and regulates mRNA and protein levels of Na\textsubscript{v}1.1 in neuroblastoma cells. To test whether decreased Na\textsubscript{v}1.1 proteins levels correspond to reduced Na\textsubscript{v}β\textsubscript{2} processing, we assessed levels of the BACE1 cleavage product Na\textsubscript{v}β\textsubscript{2} C-terminal fragment (Na\textsubscript{v}β\textsubscript{2}-CTF). Consistent with previous reports (18, 19), we found that ablation of BACE1 reduced generation of Na\textsubscript{v}β\textsubscript{2}-CTF by 40% both in total brain extract from 1-month-old BACE1-KO and in hippocampal regions from 3-month-old BACE1-KO mice (Fig. 6B). We were not able to directly detect β2-ICD because the Na\textsubscript{v}β\textsubscript{2} antibody was not sensitive enough to detect β2-ICD, which was present in very low levels. However, together these data strongly suggest that endogenous BACE1 activity modulates Na\textsubscript{v}β\textsubscript{2} processing, and ablation of BACE1 decreases Na\textsubscript{v}β\textsubscript{2}-CTF and possibly β2-ICD.

Next, we investigated whether decreased Na\textsubscript{v}β\textsubscript{2} processing also results in reduced Scn1a (Na\textsubscript{v}1.1) mRNA levels in brains of BACE1-null mice (32). In brain samples from 1-month-old WT, BACE1-HE, and BACE1-null, we measured Scn1a mRNA levels by the TaqMan real time RT-PCR method. Similar to its protein levels, we found a significant decrease of Scn1a (Na\textsubscript{v}1.1) mRNA in BACE1-null as compared with wild-type mice (Fig. 6C). Interestingly, we were not able to detect significant changes of Scn2a (Na\textsubscript{v}1.2) or Scn8a (Na\textsubscript{v}1.6) mRNA levels, suggesting that changes at the protein level may be responsible for decreases in Na\textsubscript{v}1.2 or Na\textsubscript{v}1.6 in BACE1-null mice (supplemental Fig. 4). Together, our mechanistic data strongly suggest that decreased Na\textsubscript{v}β\textsubscript{2} processing is responsible for the decrease of Na\textsubscript{v}1.1 protein levels by decreasing scn1a mRNA in BACE1-null mice.

DISCUSSION

Here, we show that expression levels of three major brain Na\textsubscript{v}1 channels are strongly affected by BACE1 deletion during early postnatal brain development. Levels of the Na\textsubscript{v}1.1 α-subunit showed the most dramatic decrease. These changes are predicted to have significant effects on the development of excitability. Ablation of BACE1 results in ∼40–50% decrease of Na\textsubscript{v}1.1 protein and mRNA levels in whole brains of 1-month-old mice and up to 56% in the hippocampus of 3-month-old mice (Figs. 2–4 and supplemental Figs. 2–4). We also found that the decrease in Na\textsubscript{v}1.1 in BACE1-null mice was less pronounced at 3 months versus 1 month of age, suggesting the possibility that the lack of BACE1 activity affects Na\textsubscript{v}1.1 α-subunit levels more prominently at early ages. Na\textsubscript{v}1.2 α-subunit levels were also decreased in whole brain samples from 1- and 3-month-old mice but not in the hippocampus. In addition, we found that Na\textsubscript{v}1.6 α-subunit levels were also decreased in brains of 1-month-old but not in 3-month-old mice. These data demonstrate that BACE1 regulates adult brain Na\textsubscript{v}1.1 α-subunit levels in physiological conditions, possibly in an age-dependent and/or region-specific manner.

Previously, we reported that BACE1/γ-secretase activities promote the release of the β2-ICD, which localizes to the nucleus and increases mRNA and protein levels of the pore-forming Na\textsubscript{v}1.1 α-subunit in neuroblastoma cells (18, 32). Elevated BACE1 activity also increases Na\textsubscript{v}β\textsubscript{2} cleavages and Na\textsubscript{v}1.1 levels in brains of BACE1-transgenic mice and AD patients (32). Although BACE1 cleaves all four β-subunits in vitro, only Na\textsubscript{v}β\textsubscript{2} and Na\textsubscript{v}β\textsubscript{4} were reported as physiological substrates in mouse brains (19). Consistent with our previous findings, we found a significant decrease of Na\textsubscript{v}β\textsubscript{2} C-terminal fragment levels in the hippocampus of BACE1-null mice (Fig. 6B). Therefore, endogenous BACE1 activity is essential for Na\textsubscript{v}β\textsubscript{2} cleavage in the hippocampus in vivo. Fig. 1 also suggests a crucial role of Na\textsubscript{v}β\textsubscript{2} in BACE1-dependent regulation of Na\textsubscript{v}1.1 levels at least in the embryonic neuronal cells. Although BACE1 inhibitors largely decreased Scn1a (Na\textsubscript{v}1.1) mRNA and protein levels in cultured cortical/hippocampal neurons, the same inhibitors did not change Na\textsubscript{v}1.1 levels in Na\textsubscript{v}β\textsubscript{2}-null neurons (Fig. 1 and supplemental Fig. 1, A, B, and D). Together, these data support the notion that Na\textsubscript{v}β\textsubscript{2} and its processing are essential for regulating Na\textsubscript{v}1.1 levels under physiological conditions. Interestingly, we were not able to detect significant changes of Scn2a (Na\textsubscript{v}1.2) or Scn8a (Na\textsubscript{v}1.6) mRNA levels, although Na\textsubscript{v}1.2 and Na\textsubscript{v}1.6 protein levels are significantly decreased in the brains from 1-month-old BACE1-null mice. This suggests an alterna-
Reduced Na	extsubscript{v}1.1 Levels in BACE-null Mice

A Western blot analysis of Na	extsubscript{v}1.1, Na	extsubscript{v}1.2, and Na	extsubscript{v}1.6 from WT and BACE1-KO mice. B Quantitative analysis of Na	extsubscript{v}1.1, Na	extsubscript{v}1.2, and Na	extsubscript{v}1.6 levels in hippocampal brain extracts from BACE1-KO and WT mice (Student’s t-test; * p < 0.01; n = 3 for WT and n = 4 for BACE1-KO). C Confocal immunofluorescence analysis of Na	extsubscript{v}1.1 (green) in hippocampal regions of 3-month-old WT or BACE1-KO mice. Top and middle panels, low magnification (×50) images of hippocampal region stained with Na	extsubscript{v}1.1 staining (green). The red dotted box indicates the area of high magnification shown in the bottom panels. Bottom panels, high magnification (×200) images with Na	extsubscript{v}1.1 staining (bottom left panel WT; bottom right panel, BACE1-KO).

The reduced Na	extsubscript{v}1.1 levels in BACE-null mice may reflect a compensatory mechanism between Na	extsubscript{v}1.2 and Na	extsubscript{v}1.6. Altered processing of other neuronal BACE substrates may contribute to these Na	extsubscript{v}1.1 α-subunit changes. Further studies will be required to fully characterize the underlying molecular mechanism of Na	extsubscript{v}1.1 α-subunit changes.

As shown in Fig. 5, ablation of BACE1 results in ~40% decrease of surface Na	extsubscript{v}1.1 levels in hippocampal neurons, which is comparable with the decreased levels of total Na	extsubscript{v}1.1 in the same region. Therefore, surface expression of Na	extsubscript{v}1.1 is regulated by the decrease of total Na	extsubscript{v}1.1 levels in BACE1-null mice in physiological conditions. On the contrary, we found that Na	extsubscript{v}1.2 surface levels are significantly increased (Fig. 5, A and D). This suggests a compensatory mechanism between Na	extsubscript{v}1.1 and Na	extsubscript{v}1.2 surface levels. However, disconnected total versus cell surface distributions of Na	extsubscript{v}1.2 in BACE1-null mice are reminiscent of Na	extsubscript{v}1.1 distribution in BACE1-overexpressing systems. In neuroblastoma cells and adult hippocampal neurons from BACE1-transgenic mice, overexpressed BACE1 paradoxically reduced cell surface expression of Na	extsubscript{v}1.1 α-subunits, although total Na	extsubscript{v}1.1 α-subunits levels increased (32). The increased Na	extsubscript{v}1.1 was retained inside the cells and accumulated in HSP70-positive punctate structures, suggesting that Na	extsubscript{v}1.1 trafficking to cell surface was inhibited by BACE1 overexpression (32). Together, these data indicate that expression of Na	extsubscript{v}1.1 α-subunits on the cell surface is under tight regulation, and changes in intracellular α-subunit levels may not always reflect surface expression of each channel. Decreased BACE-mediated cleavage of surface Na	extsubscript{v}1.2 may differentially affect Na	extsubscript{v}1.1 and Na	extsubscript{v}1.2 surface trafficking. Further studies will be required to clarify how BACE1-mediated cleavage of Na	extsubscript{v}1.2 can differentially regulate the trafficking of each α-subunit.

Neuronal Na	extsubscript{v}1 channels are known to be up-regulated or redistributed upon nerve injury or demyelination (38–41). In experimental allergic encephalomyelitis, an animal model of multiple sclerosis, levels and distribution of Na	extsubscript{v}1.2 and Na	extsubscript{v}1.6 are altered in demyelinated axons within acute multiple sclerosis plaques (39–41). BACE1-null mice also show mild but significant hypomyelination in the CNS and peripheral nervous system, and these might contribute to the elevated Na	extsubscript{v}1.2 surface levels shown in BACE1-null mice (15, 31, 42). Interestingly, a recent study showed that Na	extsubscript{v}1.2 also mediates demyelination-induced Na	extsubscript{v}1.1 α-subunit regulation in the same multiple sclerosis model (43). Together with our data, this study suggests the interesting possibility that in BACE1-null mice Na	extsubscript{v}1.2 not only is an essential Na	extsubscript{v}1.1 α-subunit in regulating changes in Na	extsubscript{v}1.1 α-subunit levels via generation of its ICD but also indirectly via reduced myelination, which is a well characterized phenotype of BACE-null mice. Further studies are required to clarify whether Na	extsubscript{v}1.2 also plays a role in regulating Na	extsubscript{v}1.1 α-subunit levels under mild hypomyelination in addition to demyelinating conditions.
Deletion of the Nav1.1 gene does not significantly affect sodium current density in hippocampal pyramidal neurons because of high expression of Nav1.2 and other /H9251-subunits (35, 36, 44). However, Nav1.1 plays a major role in regulating sodium currents in hippocampal GABAergic interneurons. Indeed, 50% decrease of Nav1.1 dramatically reduced sodium current in GABAergic interneurons (36). Similarly, 40–50% decrease of total and surface Nav1.1 levels in BACE1-null mice may decrease sodium current in hippocampal GABAergic interneurons. This would result in electrical imbalances and the vulnerability to epileptic seizures as previously shown in Nav1.1-null mice (36). Increase of Nav1.2 surface expression may also contribute to the electrical imbalances by selectively increasing sodium currents in hippocampal pyramidal neurons.

While we were revising this manuscript, two independent groups reported that BACE1-null mice showed increased seizure susceptibility and spontaneous behavioral seizure phenotypes (45, 46). These results clearly support our prediction, based on altered Na1.1 and Na1.2 surface expression in BACE1-null mice (Fig. 5). Hu et al. (45) showed that surface levels of Na1.2 are increased in cultured hippocampal neurons from BACE1-null mice, which is also consistent with our results on increased Na1.2 surface levels in adult hippocampus from BACE1-null mice (Fig. 5). In addition to Na1.2 changes, we also found reduced surface levels of Na1.1, which allow us to propose a novel Na1.1-based mechanism for the increased seizure susceptibility of BACE1-null mice (Fig. 6D). Indeed, mutations of the Na1.1 /H9251-subunit are frequently associated with inherited familial epileptic seizures in humans (44).

Early electrical deficits may also explain the enhanced lethality of BACE1/2-null mice at very early ages and their body weight loss (28, 46). Na1.1-null and -heterozygous mice show severe and fatal seizure phenotypes (36, 44). A selective loss of Na1.1-mediated sodium currents in inhibitory neurons is proposed as a molecular mechanism underlying these severe phenotypes. Interestingly, all the major Na1.1 /H9251-subunits are decreased in BACE1-null mice (Fig. 2) and may not be selective to one neuronal population. Therefore, neuronal excitability in BACE1-null mice may be generally reduced all over the brain rather than in specific circuits, which may not lead to a severe and fatal electrical imbalance in neural circuits. This may explain why BACE1-

FIGURE 5. Surface Na1.1 /H9251-subunit levels are altered in BACE1-null hippocampal slices. Surface biotinylation experiments were performed on acutely prepared hippocampal slices from WT, BACE1-HE, and BACE1-KO mice. 2–3 matching slice pairs from similar locations of the brains were selected for the experiments. The slices with the same numbers are matching pairs. A, representative Western blot showing total and biotin-captured surface Na1.1 and Na1.2 levels in hippocampal slices (Slice #1–3) from BACE1-HE and BACE1-KO. B, representative Western blot showing total and biotin-captured surface Na1.1 levels in hippocampal slices (Slice #1–3) from wild-type control (WT) and BACE1-KO. C, graph showing quantitated surface Na1.1 levels in BACE1-HE and BACE1-KO (Student’s t test; *, p < 0.01; n = 7 slices from three BACE1-HE and n = 8 from three BACE1-KO mice). Surface N-cadherin levels were used to normalize the surface Na1.1 and Na1.2 levels. D, graph showing quantitated surface Na1.2 levels in BACE1-HE and BACE1-KO (Student’s t test; *, p < 0.01; n = 7 slices from three BACE1-HE and n = 8 from three BACE1-KO mice). E, graph showing quantitated surface Na1.1 levels in WT and BACE1-KO (Student’s t test; *, p < 0.01; n = 8 slices from three WT and BACE1-KO mice, respectively).
null mice do not show a fatal seizure phenotype as predicted by the drastic Na\textsubscript{\textalpha},1.2-subunit decreases at the early age (Fig. 2).

Na\textsubscript{\textalpha},1.2-subunits are large hydrophobic membrane proteins with multiple transmembrane domains (24). It is technically challenging to analyze total Na\textsubscript{\textalpha},1.2-subunits because they are highly insoluble in mild detergent extraction conditions. To overcome this technical problem, we used several extraction conditions and compared the results (Fig. 2). We found that 1.5–2% of SDS extraction conditions showed the most consistent results, tightly comparable with a method based on direct boiling of membrane fractions in the presence of SDS-PAGE sample loading buffer. The direct boiling method has been commonly used for sodium channel analysis by other groups (27, 36). In these conditions, we found that Na\textsubscript{\textalpha},1.2 levels are consistently decreased especially in young adult BACE1-null mice as compared with BACE-HE and wild-type controls (Fig. 2). These data are consistent with the results from Hu et al. and Hitt et al. (45, 46). However, Hitt et al. (46) reported that Na\textsubscript{\textalpha},1.2 and Na\textsubscript{\textalpha},1.6 levels were not significantly changed in BACE1-null mice (45). The discrepancy regarding Na\textsubscript{\textalpha},1.2 may derive from differential extraction conditions (2% SDS versus 1% Triton X-100) or different ages of the mice. Indeed, we found that the decrease in Na\textsubscript{\textalpha},1.2 is much less pronounced (\textless 20%) in 3-month-old mice as compared with 1-month-old mice (\textgreater 40–50%) (Figs. 2 and 3). Consistent with the data from Hitt et al. (46), we found that Na\textsubscript{\textalpha},1.2 levels were not significantly changed in the hippocampal region of 3-month-old mice, although Na\textsubscript{\textalpha},1.1 shows a dramatic decrease in the same samples (Fig. 4).

Because the vast majority of sodium channels is retained inside the cells, total Na\textsubscript{\textalpha},1.2 levels may not reflect changes at the cell surface. We found that Na\textsubscript{\textalpha},1.1 levels decrease at the cell surface, although Na\textsubscript{\textalpha},1.2 levels increase in agreement with Hu et al. (45). Taken together, these data argue that the lack of BACE1 activity dynamically modulates changes in Na\textsubscript{\textalpha},1 \&\& subunit levels depending on

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**FIGURE 6.** Na\textsubscript{\textalpha},2 processing and scn1a (Na\textsubscript{\textalpha},1.1) mRNA levels are decreased in brains of BACE1-null mice. A, surface Na\textsubscript{\textalpha},2 and APP levels are largely decreased in brain slices from BACE1-null (BACE1-KO) as compared with BACE1-heterozygous knock-out (BACE1-HE). Slice biotinylation experiments were performed as described in Fig. 5. B, upper panel, representative Western blot showing full-length Na\textsubscript{\textalpha},2 and C-terminal fragment (Na\textsubscript{\textalpha},2-CTF) levels in hippocampal brain extracts prepared from WT and BACE1-KO mice. Lower panel, quantitative analysis of Na\textsubscript{\textalpha},2 CTF levels in total brain extract from 1-month-old WT and BACE1-KO mice and hippocampal extracts from 3-month-old WT and BACE1-KO mice (left) and hippocampal extracts from 1-month-old WT and BACE1-KO mice (right). Student’s t test; *, p \textless 0.01; n = 3 per each condition. C, TaqMan real time RT-PCR analysis of scn1a (Na\textsubscript{\textalpha},1.1) mRNA levels in 1-month-old WT, BACE1-HE, and BACE1-KO (ANOVA followed by a post hoc Tukey’s test; *, p \textless 0.05; n = 5 for WT, n = 8 for BACE1-HE, and n = 6 for BACE1 KO). GAPDH levels were used to normalize Scn1a (Na\textsubscript{\textalpha},1.1) mRNA levels. D, diagram summarizing altered sodium channel metabolism in BACE1-null mice.
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age/brain region and that changes in cell surface Na⁺,1 subunit levels are likely to cause epileptic seizures in BACE1-null mice. Further studies will be required to fully address BACE1 effects on Na⁺,1 subunit levels in vivo.

BACE1 is one of the prime drug targets for therapeutic treatment of AD patents. Genetic ablation of BACE1 showed decreased Aβ levels and ameliorated cognitive function in animal models (47). Our findings that the ablation of BACE1 leads to decreased sodium channel levels suggest that complete block of BACE1 is likely to cause side effects through altered sodium current densities. However, our studies on Na⁺,1 levels in BACE1-heterozygous mice show that 50% decrease of BACE1 activity does not significantly decrease Na⁺,1 levels (Fig. 1), although partial reduction of BACE1 results in dramatic reduction in Aβ plaques, neuritic burden, and synaptic deficits in older mice (48). Therefore, it will be important to find a therapeutic window for inhibiting BACE1 activity to block Aβ burden, without significantly affecting Na⁺,1 metabolism.

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