Sequential processing of amyloid precursor protein (APP) by membrane-bound proteases, BACE1 and γ-secretase, plays a crucial role in the pathogenesis of Alzheimer disease. Much has been discovered on the properties of these proteases; however, regulatory mechanisms of enzyme-substrate interaction in neurons and their involvement in pathological changes are still not fully understood. It is mainly because of the membrane-associated cleavage of these proteases and the lack of information on new substrates processed in a similar way to APP. Here, using RNA interference-mediated BACE1 knockdown, mouse embryonic fibroblasts that are deficient in either BACE1 or presenilins, and BACE1-deficient mouse brain, we show clear evidence that β subunits of voltage-gated sodium channels are sequentially processed by BACE1 and γ-secretase. These results may provide new insights into the underlying pathology of Alzheimer disease.

Alzheimer disease is a progressive neurodegenerative disorder and the most common form of age-dependent dementia. The major pathological features of Alzheimer disease are senile plaques and neurofibrillary tangles, which are the deposits of amyloid β peptide (Aβ) and hyperphosphorylated tau, respectively. It is widely accepted that the sequential processing of APP, a type I membrane protein, by β- and γ-secretases in the brain is crucial for the accumulation of Aβ and disease pathogenesis (1, 2). Although β-site APP-cleaving enzyme (BACE1) has been identified to be the β-secretase (3–6), a growing body of evidence favors presenilins-1 and -2 as the catalytic core of γ-secretase (7). Although the properties of both proteases as APP processing enzymes are relatively well established, the regulatory mechanisms of sequential cleavage by both proteases in neurons are not completely clear. This is partly because of the fact that APP and its family proteins are still the only substrates identified for both β- and γ-secretases, although a number of integral membrane proteins have been reported to be processed either by BACE1 (8, 9) or γ-secretase (10). Identifying new substrates for both β- and γ-secretases in neurons would therefore be useful to further explore the precise mechanism by which BACE1 and γ-secretase function in cohort.

Recently, our laboratory has been focusing on examining the role of voltage-gated sodium channel (VGSC) β in the pathogenesis of Huntington disease and the regulation of APP processing in lipid rafts. VGSC is a large, multimeric complex that consists of an α subunit and one or more β subunits. To date, nine functional α subunits and four β subunits have been identified (11, 12). Although VGSCβ subunits are not essential to the basic operation of sodium channels, they are considered to be important auxiliary subunits, because co-expression of β subunits are required to reconstitute full properties of the native sodium channel and to modify channel properties and intracellular localization (11, 13). In the course of analyzing the VGSCβ, we found that these subunits are preferentially associated with Lubrol-WX-resistant membranes in the mouse brain and possess putative BACE1 cleavage sites juxtaposed to the transmembrane region (14). In this study, we explored the possibility that VGSCβ subunits could be processed sequentially by BACE1 as well as γ-secretase. Here we show direct evidence that these subunits are indeed novel substrates of both proteases.

**EXPERIMENTAL PROCEDURES**

Reagents and Antibodies—DAPT and Lubrol-WX were purchased from Calbiochem and Serva Electrophoresis, respectively. Antibodies used in this study were as follows. For mouse monoclonal antibodies, anti-human BACE1 ectodomain (MAB9311, R & D Systems), anti-c-Myc (9E10, Santa Cruz Biotechnology), anti-FLAG (M2, Sigma), anti-GFP (Roche Applied Science), anti-GM130 (Transduction Laboratories), anti-GM130 (Transduction Laboratories), anti-β-amyloid (Aβ, Amylin, Biochemical Technology), anti-α-synuclein (

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anti-β-tubulin (Sterner-Brown Monoclon), and anti-V5 (Invitrogen) were used. For rabbit polyclonal antibodies, anti-BACE1 (JBL), anti-human BACE1 (BioSource), anti-FLAG (Sigma), anti-prion protein (FL-253, Santa Cruz Biotechnology), anti-PS1 (BD Biosciences, clone 6E10, and anti-PS2 (clone 6G8, Zymed Laboratories Inc.) were used. Mouse/rabbit Alexa Fluor® 488/546-conjugated secondary antibodies were from Molecular Probes, and horseshad peroxidase-conjugated secondary antibodies were from Amersham Biosciences.

Cell Cultures—The cerebra from C57BL newborn mice were enzymatically digested in papain solution (20 units/ml, Worthington) for 20 min at 37 °C. The tissues were then triturated in a detergent-free DMEM/F12 medium (Invitrogen) and mechanically dissociated with fire-polished glass pipettes. The cells were subsequently seeded on polylysine (Sigma; M, 70,000–150,000)-coated culture dishes and maintained in Neurobasal medium containing 1× B27 supplement, 0.5 μM t-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (PIS) (Invitrogen) in a 5% CO2 incubator at 37 °C. HEK cells, N2a cells, MEFs, and HTB-148/H4 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum (Sigma), 2 μg/ml t-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen) in a 5% CO2 incubator at 37 °C.

Generation of BACE1 Knock-out Mice—A BACE1 knock-out mouse line was generated by inserting a neomycin expression cassette from pMC1neoA (Thomas and Capel, 1987) into exon 1 (glutamate 19 of the BACE1 cDNA). The insertion of the neo-cassette into the coding sequence leads to the production of a truncated gene (Fig. 1A) that is predicted to generate a truncated BACE1 protein.

Mammalian Expression Vectors—Plasmids encoding full-length (FL) VGSCβ1–β4 subunits (GenBank accession numbers: β1, U85788.2; β2, XM134787; β3, AY949036; and β4, BK010031) were generated by PCR from mouse brain cDNAs. The resulting PCR products were subcloned into pV5-His-TOPO (Invitrogen). Truncated versions of mouse VGSCβ1–β4 (VGSCβ1–β4/2, Fig. 3A) were also constructed via PCR, and the resulting PCR products were subcloned into pScetag2/HyGrb (Invitrogen). The sequences of all the constructs were verified by sequencing.

Generation of C-Terminal-specific Anti-VGSCβ Antibodies—Rabbit polyclonal antibodies to mouse VGSCβ1, -2, -3, or -4 were raised against the C terminus of each subunit (β1, ITSESKENCTGQVQAV, β2, GEGNARDGTK, β3, IPSENKSENSVVPPVE, β4, GLPGSKEAEPKPTVK, and underlined as shown in Fig. 1B). Keyhole limpet hemocyanin-conjugated C-terminal peptide for each VGSCβ subunit was used for immunization, and antibodies were affinity purified by the peptide immobilized on SulfoLink coupling gel (Pierce). The specificity of each antibody was verified by immunoblot analysis.

Production of End-specific VGSCβ4 Antibodies—A cleavage site-directed antibody was generated as described above with minor modifications (15). Briefly, we synthesized a peptide corresponding to the predicted C-terminal region of C-terminal fragment (CTF) β4 from in vitro cleavage assay (NH₂-HVYDKLEK) with C-terminal Cys. The peptide was conjugated with keyhole limpet hemocyanin and injected into rabbits. For antibody purification, a shorter peptide, NH₂-HVYDNLK, and acetyl-QVVDKLC were synthesized and coupled with SulfoLink coupling gel (Pierce). The antisera was purified by affinity chromatography on the immobilized NH₂-HVYDKLEK. To obtain N-terminal end-specific antibody, the affinity-purified antibody was further adsorbed against the immobilized acetyl-QVVDKLC. We designated these new antibodies as QV6 antibody.

Preparation of Detergent-soluble Brain Membrane Extracts—Various regions of the mouse brain were dissected from BDF1 (wild type) or C57/B6 × 120SV (BACE1-deficient) mice, and the tissues were homogenized in 10 volumes of dissection buffer supplemented with 1 μM NaCl and 1% Triton X-100 and incubated at 4 °C for 30 min with constant rocking. Detergent-soluble protein extract was collected as the supernatant after spinning at 40,000 × g for 20 min. Preparation of Detergent-resistant Membranes (DRMs) from Primary Cultures—Primary cortical neuron cultures were lysed with 1% Lubrol WX at 4 °C in MES-buffered saline (pH 6.5) supplemented with Complete™ protease inhibitor mixture (Roche Applied Science). After lysis, the detergent-resistant membranes were prepared essentially as previously described (16).

RNA Interference—BACE1 small interfering RNA (siGENOME™ SMARTPool, Dharmacan) was transfected into MEFs or H4 cells with Lipofectamine reagent (Invitrogen) according to the manufacturer’s instructions. The cells were collected for further analysis after 2–3 days.

Phosphatase Assay—Full-length VGSCβ subunit cDNAs were cloned into pPATag-4 (GenHunter Corporation) to express VGSCβ subunits fused with soluble human placentalk alkaline phosphatase (Alp) at their N termini (AP-β). Each AP-β and BACE1 were transfected into HEK cells with β-galactosidase to normalize the expression levels. Alkaline phosphatase activity in the medium was measured after 24 h in essentially the same way as described previously (9).

In Vitro Cleavage Assay and Matrix-assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOP) Mass Spectrometry Analysis—Synthetic peptides (50 μM) in 0.1 M sodium acetate, pH 4.5, were incubated with 25 μg/ml recombinant human BACE1 (R & D Systems) at 30 °C for 12 h. Monoisotopic mass values of the peptides were measured by a Bios I MALDITOF mass spectrometer (Beckman Coultronics) in the reflector mode. The peptide solution was desalted with ZipTip C18 (Millipore) and co-crystallized with equal volumes of 10 mg/ml α-cyano-4-hydroxycinnamic acid (Fluka) matrix in 50% acetonitrile/0.1% trifluoroacetic acid. All MALDI spectra were calibrated externally using a peptide standard. Cleavage sites were searched by calculating monoisotop masses of possible peptides with PAWS software (Accelrys Solutions, 6.2.1.9). Spectra of peptides were processed, because BACE1 and γ-secretase cleavage sites are essentially the same way as described previously (9).

Immunofluorescence Microscopy—Cells grown on coverslips or growth chambers were fixed with 2% paraformaldehyde in PBS at room temperature for ~3 min. The cells were then blocked with 5% nonfat milk in 1 h in PBS, washed extensively, and incubated with the appropriate primary antibody at 4 °C overnight. After primary antibody incubation, the cells were washed three times with PBS and subsequently incubated with fluorescence-conjugated secondary antibodies at room temperature for 1 h in the dark. The cells were finally washed three times with PBS and mounted in VECTASHIELD® (Vector) and then analyzed by confocal microscopy (the spectral confocal scanning system TCS SP2 from Leica).

Western Blotting Analysis—Cells that were ready to be analyzed by Western blotting were rinsed with cold PBS and lysed in an appropriate volume of lysis buffer (8.6% sucrose, 1 ml EDTA, 1 ml sodium orthovandate (NaVO₃), 10 ml sodium fluoride (NaF), 50 ml Tris, 0.0386% EGTA, 1% Triton X-100 and Complete™). Protein concentration was measured with the bicinchoninic acid Protein Assay Kit (Sigma) according to the manufacturer’s instructions. Appropriate volumes of protein lysates were then mixed with 4× SDS gel-loading buffer (200 μl Tris-HCl, pH 6.8, 400 μg dithiothreitol, 8% SDS, 40% glycerol, and an appropriate amount of bromphenol blue), boiled at 100 °C for 5 min, and resolved in 7.5–15% polyacrylamide gels (Atto). Proteins were subsequently transferred onto polyvinylidene difluoride membrane (0.22 μm, Schleicher & Schuell), and the membrane was blocked with 5% nonfat milk in TBS-T (10 ml Tris, pH 7.5, 50 ml NaCl, and 0.1% Tween 20, Sigma) for 1 h. The blots were then incubated with appropriately diluted primary and horseshad peroxidase-conjugated secondary antibodies, each for 1 h at room temperature, with extensive washing with TBS-T after each incubation. Finally, the blots were developed with ECL solutions and exposed onto Hyperfilm™ MP (Amersham Biosciences).

RESULTS

VGSCβ Subunits Are Preferentially Associated with Lipid Rafts—DRMs prepared with Lubrol-WX (Lubrol rafts) have been shown to be especially useful for the analysis of APP processing, because BACE1 and γ-secretase can be efficiently recovered (16, 17). To confirm the association of VGSCβ subunits with Lubrol rafts in neuronal cells, we fractionated mature postnatal primary cortical neuron cultures (4 weeks) with Lubrol-WX and examined their expression patterns by Western blotting (WB). As shown in Fig. 1A, each of the VGSC subunits was substantially and prominently recovered in fraction
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3 of the preparation that was also enriched in prion protein, a marker of lipid raft domains, as well as BACE1, CTFs of presenilin-1/2, and the mature form of APP. These results indicate that VGSC\(\beta\) subunits are preferentially associated with lipid rafts in neurons. Indeed, based on the preferential amino acid residues flanking the BACE1 cleavage site (14), we found several putative BACE1 cleavage sites juxtaposed to the transmembrane region on each VGSC\(\beta\) subunit (Fig. 1B, arrows (\(\uparrow\))). The recovery of these proteins in the prion protein-enriched fraction and the presence of putative BACE1 cleavage sites led us to examine whether VGSC\(\beta\) subunits are physiological substrates for both proteases.

**Fig. 1. Identification of VGSC\(\beta\) subunits as possible physiological substrates of BACE1.**

A, association of VGSC\(\beta\) subunits with DRMs. Primary neurons were lysed with Lubrol-WX and fractionated on a sucrose gradient (see “Experimental Procedures”). Equal volumes from each fraction were resolved by 15% SDS-PAGE, and blots were probed with the antibodies indicated in the diagram. GM130, a cis-medial Golgi SNARE protein, serves as a marker for non-raft fractions. The blots were also stained with 0.25% Coomassie Brilliant Blue to show protein loading. To represent the relative distribution of each protein graphically, total protein expression was set at 100%, and the expression of each protein in any individual fraction was compared with the total accordingly.

B, potential cleavage sites of BACE1 on each VGSC\(\beta\) subunit. Mouse VGSC\(\beta\) subunits were aligned using ClustalW (EMBL-EBI). Arrows indicate the potential proteolytic sites by BACE1, and boxes represent the transmembrane domain. C-terminal peptides used to generate antibodies are underlined.
VGSCβs Are Substrates of BACE1 and γ-Secretase

FIG. 2. VGSCβ subunits are substrates of BACE1. A, processing of full-length VGSCβ subunits by BACE1. Cell lysates from HEK cells transiently transfected with GFP or each VGSCβ subunit with or without BACE1 were collected, resolved on a 15% SDS-PAGE gel, and blots were probed with antibodies as indicated. The generation of CTFβs was confirmed by anti-V5. β-tubulin serves as a loading control in this experiment. B, inhibition of endogenous BACE1 expression reduces the production of CTFβs of VGSCβ subunits. HTB-148/H4 cells were first transiently transfected with each VGSCβ subunit and allowed to express the VGSCβ subunit for 24 h. One hundred nM BACE1 small interfering RNA was subsequently applied to the cells, and the expression of CTFβs was analyzed after 48 h by immunobots. Expression of CTFβs and BACE1 was quantified, normalized to that of β-tubulin, and presented graphically in the lower panel. Inhibition of endogenous BACE1 enhanced the accumulation of full-length protein. Mean ± S.E., n = 3 from three independent experiments is shown. C, reduction of expression of CTFβs in BACE1-deficient MEFs. GFP serves as a transfection control. Expression of CTFβs was quantified, normalized to that of GFP, and presented graphically in the right panel. Mean ± S.E., n = 3 from three independent experiments. *, p < 0.05, (Student’s t test).
but not FL-β4, and 3) anti-BACE1 antibodies that recognize the N-terminal extracellular region of BACE1. As shown in Fig. 3C [a’], there were virtually no FLAG immunoreactivities detected on the cell surface membrane when β4–1 and BACE1 co-expressed at the same time, indicating that amino acid residues 143–161 contained a putative BACE1 cleavage site. To show the processing in an alternative way, we made use of the newly generated QV6 antibody that detected only the CTF produced by BACE1 (see Fig. 3C, right lower panel, for specificity). Using this cleavage site-directed antibody, we found a co-localization between cleaved β4 and BACE1 (Fig. 3C, [b–b’]). In contrast, FLAG-labeled β4–2 proteins showed in-
tense co-localization with BACE1 on the cell surface, (Fig. 3C, [c-c’]), and there was virtually no immunoreactivities detected by the QV6 antibody (Fig. 3C, [d]). Furthermore, to show the N termini of VGSCβs were actually released from the cell surface, we fused an Alp tag to the N terminus of each FL-β subunit and measured the Alp activity in the medium 24 h after transfection. As shown in Fig. 3D, there was a significant increase of Alp activity in the conditioned medium upon co-expression of BACE1 and Alp-tagged FL VGSCβ, compared with the VGSCβ alone-transfected condition. In summary, these data clearly indicate that the sequence adjacent to the transmembrane domain on each VGSCβ subunit contains a putative BACE1 cleavage site(s), and the N-terminal part of VGSCβ is shed and released similar to that of APP.

**Confirmation of the Predicted Putative BACE1 Cleavage Sites on VGSCβ Subunits**—To demonstrate that BACE1 is able to cleave the VGSCβ subunit directly and to confirm the putative cleavage sites that we predicted in Fig. 1B on each VGSCβ subunit, we employed an in vitro cleavage assay using the recombinant BACE1 catalytic domain and peptides corresponding to the extracellular domain of each VGSCβ subunit containing putative BACE1 cleavage sites (β1, amino acids 122–160; β2, amino acids 128–157; β3, amino acids 121–159; β4, amino acids 132–161) (Figs. 3A and 4). Peptides were synthesized, purified by high pressure liquid chromatography, and incubated with or without recombinant BACE1. The APP peptide (662–679, KTEEISEVKMDEAFRHDSS) was also included in this assay to confirm the activity of recombinant BACE1 (data not shown). The resulting peptides were analyzed by MALDI-TOF mass spectrometry. It is worth noting that only the masses that can be assigned to the sequence of each peptide were labeled in the MALDI spectra (Fig. 4, A–D), and we cannot exclude the possibility that other relatively minor processing sites exist. Furthermore, some of the peptides may not be properly recovered in this assay (for example, the TSVVSE peptide for VGSCβ3). As shown in Fig. 4, peptides incubated without BACE1 (Fig. 4, A–D, Con) exhibited peaks of 4616.0, 3504.0, 4522.1, and 3475.2 arbitrary intensity, corresponding to the calculated masses of ex-VGSCβ1–4, respectively (Fig. 4E). Co-incubation of BACE1 with ex-VGSCβ peptides (Fig. 4, A–D, +BACE1) led to the production of new peaks, indicating that ex-VGSCβ peptides were processed by BACE1. Fig. 4E summarizes all of the peptide fragments and cleavage sites on each ex-VGSCβ peptide that are preferentially processed by BACE1. Importantly, all of the processing sites revealed by this in vitro cleavage assay are included in the putative BACE1 cleavage sites that we predicted and indicated in Fig. 1B.

**CTFβs of VGSCβ Subunits Are Further Processed by γ-Secretase**—Next we tested whether CTFβs could be further processed by γ-secretase similar to that of the APP family (1, 18). As a first step to examining whether CTFβs are substrates of γ-secretase, we incubated HEK cells transfected with VGSCβ subunits and BACE1 with a γ-secretase inhibitor, DAPT. As shown in Fig. 5, A and B, co-incubation with DAPT led to a significant accumulation of CTFβ1, -2, and -3. In contrast, a small but consistent increase was observed for CTFβ4. Similar results were also obtained by the addition of another γ-secretase inhibitor, L-685, 458 (data not shown). Overexpression of BACE1 also led to the production of
small immunoreactive bands for VGSCβ1, -2, and -4, which were DAPT-inhibitable (Fig. 5C). Overexpression indicates that these smaller immunoreactive bands were possibly the intracellular domains and that their production depended on the γ-secretase activity. To further confirm the role of γ-secretase in processing these CTFβs, we transfected BACE1 and VGSCβ subunits into the MEFs that are either deficient in presenilin-1 (PS1 knock-out), presenilin-2 (PS2 knock-out) or both presenilins (double knock-out) (19). Compared with that of wild type MEFs, the lack of PS1 increased the expression of all CTFβs being examined by 2-fold, whereas the absence of PS2 resulted in a 1.5–2-fold increase in the expression of CTFβ1 and CTFβ3 and a 3–4-fold increase in CTFβ2 and CTFβ4. Interestingly, the lack of both PS1 and PS2 (double knock-out) produced a synergistic accumulation of all CTFβs, because a >6-fold increase was observed (Fig. 5D, and quantified in E). This observation is supported by pub-

**FIG. 5.** CTFβs of VGSCβ subunits are substrates of γ-secretase. A, inhibition of γ-secretase activity increases expression of CTFβs. HEK cells transiently transfected with each VGSCβ subunit and BACE1 were incubated with 1 μM DAPT for 10–12 h before cell lysis. Expression levels of CTFβs were subsequently analyzed by WB with anti-VGSCβ, and results were confirmed by blotting with anti-V5 antibody. B, quantification of the expression of CTFβs in A. Mean ± S.E., n = 4 from four independent experiments. *, p < 0.05; #, p > 0.05 (Student’s t test). C, inhibition of putative intracellular domain expression by γ-secretase inhibitor. Mouse Neuro2a cells were transfected with various VGSCβ-V5 subunits and BACE1 as indicated in the diagram. Lactacystin (Lac) at 10 μM and DAPT at 1 μM were added to the cells 4 h and 36 h after transfection, respectively. Cell lysates were collected after 48 h, and expression of VGSCβ subunits was analyzed using anti-V5 with Western blotting. D, presenilins are required to further process CTFβs. Mouse embryonic fibroblasts (MEFs) deficient in PS1, PS2, or both were transiently transfected with BACE1 and each VGSCβ subunit. The expression levels of CTFβs were analyzed by immunoblots with the corresponding anti-VGSCβ. Blots were reprobed with anti-PS1 and -PS2 to confirm the lack of presenilins. wt, wild type; KO, knock-out; DKO, double knock-out. Asterisks indicate nonspecific bands. E, quantification of the expression of CTFβs in D. Mean ± S.E., n = 3 from three independent experiments. F, accumulation of CTFβ2 and -4 in mature primary neurons in the presence of γ-secretase inhibitor. Pure primary neuron cultures (6 weeks old) were incubated with 5 μM DAPT for 10 h before collection of protein lysates. Expression levels of CTFβ were subsequently analyzed with the corresponding anti-VGSCβ by immunoblots and quantified, normalized, and presented graphically on the right panel. Mean ± S.E., n = 3 from three independent experiments. *, p < 0.05 (Student’s t test).
lished data demonstrating that PS1 and PS2 are functionally redundant and the complete deficiency in both presenilins results in a more severe phenotype (20). Furthermore, as shown in Fig. 5F, the addition of DAPT also led to a significant accumulation of CTFβ2 and CTFβ4 (2–2.5-fold increase, right panel) in the mature primary cortical neuron cultures, indicating that these C-terminal fragments were also processed in a similar manner in neurons (of note, there were no CTFβ1 and CTFβ3 detected in primary neurons). These results confirm that CTFβ2 and CTFβ4 are present in neuronal cells and suggest that the inability of γ-secretase inhibitors to produce a significant accumulation of CTFβ4 in Fig. 5A is likely due to the inhibitor-specific effect on VGSCβ4 in the overexpression system.

In Vivo Cleavage of VGSCβ by BACE1—The lack of CTFβ1 and CTFβ3 expression in the primary neurons led us to investigate the processing of VGSCβ subunits in the mouse brain. First, we examined the expression pattern of VGSCβ subunits in protein lysates prepared from wild type mouse brain, because it has high BACE1 activity. Essentially the same as in the primary neuron system, CTFβ2 and CTFβ4 (but not CTFβ1 and CTFβ3) were readily detected in protein extracted from the cerebral cortex (CX), cerebellum (CB), spinal cord (SP), and subcortical area (SC) of adult mice (Fig. 6A, left panels). Next, to confirm that BACE1 is the protease that cleaves VGSCβ2 and VGSCβ4 in vivo, we examined the expression levels of CTFβ2 and CTFβ4 in the cerebrum (Fig. 6A, CERE) and striatum (STR) of BACE1-deficient mice. Consistent with results obtained in our in vitro overexpression system, the absence of BACE1 in the mouse brain led to reduced expression of both CTFβ2 and CTFβ4 (Fig. 6A, right panels, and quantified in B). We also detected a reduction of CTFβ4 expression in the cerebrum and striatum in the absence of BACE1 using the QV6 antibody. These results indicate that BACE1 is indeed the protease that cleaves VGSCβ2 and VGSCβ4 in the nervous system. Other processing enzyme(s) may exist, as the expression of CTFβ2 and CTFβ4 is not completely absent in the BACE1-deficient mouse brain. Furthermore, the reduction of CTFβ4 expression in the striatum also led to a consistent and significant increase (20–25%) of FL striatal VGSCβ4 in the BACE1 knock-out mice (Fig. 6C). The lack of significant increase for that of cerebral β2/4 and striatal β2 might be explained by the fact that CTFβ2 expresses at relatively low levels in the mammalian nervous system (compare with the overexpression system); therefore a 40% reduction in expression levels may not be able to be reflected via a significant increase of the full-length protein. It may also be that the processing of VGSCβ subunits is limited to specific types of neuronal populations in the cerebrum, thus an overall significant increase was not easily observed. In summary, all of the results indicate that VGSCβ subunits are indeed the physiological substrate of BACE1 in the mammalian brain.

**DISCUSSION**

In this article, we report the finding of VGSCβ subunits as a second group of substrates that can be sequentially processed by both BACE1 and γ-secretase. First, we found that VGSCβ subunits, similar to BACE1 and PS1/2, associated with DRMs prepared with Lubrol-WX and that each VGSCβ subunit contained putative BACE1 cleavage sites (Fig. 1). We subsequently employed an overexpression system to show that the production of CTFβ2, resulting from overexpression of BACE1, could be specifically recognized by antibodies raised against the C terminus of each VGSCβ subunit. The reduction or lack of BACE1 expression led to the reduction of CTFβ expression (Fig. 2). We further showed that N termini of VGSCβ subunits were released from the cell surface upon BACE1 expression (Fig. 3) and determined the putative cleavage sites that are preferentially recognized by BACE1 through an in vitro cleavage assay (Fig. 4), confirming the putative cleavage sites that we predicted in Fig. 1. Interestingly, in addition to being the substrates of BACE1, VGSC-CTFβ2β4 were further processed by γ-secretase activity, as demonstrated in the overexpression system, presenilin knock-out MEFs, and primary neurons (Fig. 5). More importantly, we confirmed the processing of VGSCβ2 and VGSCβ4 by BACE1 in the mammalian nervous system by
employing newly generated BACE1 knock-out mice and the in vivo cleavage site detected by the QV6 antibody (Fig. 6).

Up to this moment, no protein is found to be processed sequentially by both β and γ-secretases in a manner similar to that of APP family proteins (1, 2, 18, 21–23). In keeping with the current belief that BACE1 recognizes substrate sequences based on residue preference instead of stringent consensus sequence, we initially predicted and subsequently confirmed by an in vitro cleavage assay that each VGSCβ subunit contains putative BACE1 cleavage sites juxtaposed to the transmembrane region. Our findings correlate well with previous studies, demonstrating the residue preference for subsites of BACE1 using combinatorial inhibitor libraries (14, 24); that is, BACE1 preferentially recognizes bulky hydrophobic residues, such as leucine (for VGSCβ-1, -2, and -4) or phenylalanine (for VGSCβ3) at the P1 site. Indeed, as an additional group of BACE1 substrates, the processing site of each VGSCβ subunit is also comparable with that of rat β-galactoside α2,6-sialyltransferase (ST6GalI) and P-selectin glycoprotein ligand-1, the two known BACE1 substrates that are also cleaved by BACE1 after leucine residue (8, 9, 25, 26). Furthermore, it is also worth noting that other protease(s), such as BACE2 (27, 28), may also process VGSCβ at the same (or very close) site, because the lack of BACE1 in MEFs and mouse brain only led to the reduction of CTFβ production (Figs. 2C and 6, A and B).

To investigate the processing of voltage-gated sodium channels by BACE1 is important, because VGSCs are one of the most fundamental and abundant types of ion channels that are responsible for the initiation and propagation of action potentials in neurons. Recently, it has been proposed that neural activity is able to regulate the production of AD through β- and γ-secretase and that the β secretase depresses synaptic transmission and, hence, suppresses neuronal activity (29). Given that CTFβ2 and CTFβ4 are expressed in various areas of the nervous system (Fig. 6a), the turnover of membrane-localized functional sodium channels by sequential processing by BACE1 and γ-secretase in wild type neurons may be involved in such a feedback mechanism as to depress synaptic transmission and, hence, synaptic transmission and, hence, activity, and to induce other more subtle cognitive and behavioral changes. As it is likely that other physiological substrates for both BACE1 and γ-secretase exist, it is therefore important to develop therapeutic strategies that specifically target APP processing and AD production to prevent the possible adverse side effects that might have arisen in Alzheimer disease patients.

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