Glu11 Site Cleavage and N-Terminally Truncated Aβ Production upon BACE Overexpression[†]

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ABSTRACT: Amyloid β peptides (Aβ) are generated by the proteolytic processing of the amyloid β precursor protein (APP). The newly identified β-site APP-cleaving enzyme (BACE) cleaves APP at Asp1 as well as between Tyr10 and Glu11 of Aβ, producing C-terminal fragments (CTFs) C99 and C89, respectively. Subsequent cleavage by γ-secretase gives rise to Aβ1−40/42 and Aβ11−40/42. Although both full-length and Aβ peptides truncated at residue 11 have been identified in amyloid plaques in the AD brain, the relative proportion of these two cleavage products produced by BACE and secreted into the medium by cultured cells is unknown. Using cell lines stably overexpressing BACE, we found that Aβ11−40 and Aβ11−42 are major Aβ cleavage products generated by BACE. We further showed that BACE utilizes both full-length APP as well as C99 as substrates for the production of C89, and that Aβ11−40/42 can be generated by sequential cleavage of single APP molecules by BACE and γ-secretase. Taken together, the abundance of Aβ11−40/42 produced by BACE suggests that their roles in AD pathogenesis may be underestimated.

The deposition of amyloid β peptide (Aβ)[1] is an early event in the pathogenesis of Alzheimer's disease (AD), one of the leading causes of dementia. Aβ is generated by the proteolytic processing of amyloid β precursor protein (APP) through sequential cleavages by β- and γ-secretases. BACE (for β-site APP-cleaving enzyme; also known as BACE1, Asp 2, or memapsin 2) is a type I integral membrane glycoprotein which exhibits all of the properties expected of a β-secretase (*1*−*4*). For example, its optimum pH is mildly acidic, it is localized in cellular compartments where Aβ is generated, and it cleaves synthetic APP substrates at the β-cleavage site with a higher affinity for Swedish APP (swAPP), a familial AD (FAD)-associated APP mutation known to increase Aβ production. In addition, its transfection into 293 cells stably expressing APP increases β-cleavage products sAPPβ and C99, while antisense oligonucleotides have the opposite effect. Finally, it is present in Aβ-secreting cells and is highly expressed in neurons. BACE also competes with α-secretase for APP, consistent with previous studies (*5*).

In addition to cleaving APP between its Met596 and Asp597 sites (i.e., Asp1 of Aβ), BACE also cleaves APP between Tyr10 and Glu11 of Aβ in vitro (*2*). The shorter Aβ11−40 and Aβ11−42 fragments produced have been detected in conditioned medium of primary rat neuronal cell cultures and mouse N2a cells, suggesting that they are normal APP cleavage products generated in neurons (*6*, *7*). Significantly, these shorter Aβ fragments have also been identified as major species in AD brains and normal aging brains by biochemical analysis (*8*) as well as in Down syndrome brains with AD pathology by immunohistochemistry studies (*9*). However, little is known about the generation of Aβ11−40 and Aβ11−42 from APP or any of its proteolytic fragments, such as C99, nor are the factors that govern the relative efficiencies with which BACE cleaves APP at Asp1 or Glu11 well understood. The identification of BACE provides an opportunity for direct examination of Aβ11−40 and Aβ11−42 production, as well as their role in AD pathogenesis relative to full-length Aβ1−40 and Aβ1−42.

Here we investigated BACE cleavage of APP at Glu11 by using HEK293 cells that stably expressed different levels of BACE. We found that Aβ11−40 and Aβ11−42 were major species secreted in the conditioned medium of these cells, and that their production was directly related to BACE expression levels. Second, concomitant with increased secretion of Aβ11−40 and Aβ11−42, a decrease in the levels of Aβ1−40 and Aβ1−42 was observed with increased BACE

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[1] Abbreviations: Aβ, amyloid β peptide; Aβ1−40 and Aβ1−42, 40 and 42 amino acid long forms of Aβ, respectively; Aβ11−40 and Aβ11−42, Aβ peptides cleaved at Glu11; AD, Alzheimer's disease; APP, amyloid β precursor protein; wtAPP, wild-type human APP695 protein; swAPP, APP 695 containing the KM/NL FAD Swedish mutation; BACE, β-site APP-cleaving enzyme; FAD, familial Alzheimer's disease; HA, influenza hemagglutinin; SFV, Semliki Forest virus; ELISA, enzyme-linked immunosorbent assay; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; RIPA buffer, radioimmunoprecipitation assay buffer [0.5% sodium deoxycholate, 0.1% SDS, 1% NP-40, 5 mM EDTA in TBS (pH 8.0)]; TGN, trans Golgi network; ER, endoplasmic reticulum; mAbs, monoclonal antibodies.

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expression, suggesting that increased BACE expression or prolonged association between BACE and APP might result in enhanced production of Aβ11−40 and Aβ11−42 at the expense of Aβ1−40 and Aβ1−42. Finally, we found that both full-length APP as well as C99 are substrates for BACE cleavage at Glu11, indicating that a single APP molecule can be cleaved by BACE either once (at Asp1 or Glu11) or twice (at Asp1 and Glu11). These findings indicate that the deposition of Aβ11−40 and Aβ11−42 in the CNS and their potential role in AD pathogenesis need to be reevaluated.

EXPERIMENTAL PROCEDURES

Generation of APP Mutant Constructs. Primers for creating APP mutations were designed as follows: 5′-GAGATCTCTGAAGTGAAGgTGGATGCAGAATTCCGAC-3′ for M596V forward, 5′- CGACATGACTCAGGATATaAAGTTCATCATCAAAAATTGGTG-3′ for E607K forward, and 5′-CGACATGACTCAGGATATGtAGTTCATCATCAAAAATTGGTG-3′ for E607V forward. wtAPP695 in pcDNA3 was used as the template to create the mutations with the Quikchange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). Mutations were confirmed by sequencing, and DNA was purified by maxiprep (Qiagen, Valencia, CA). BACE constructs containing a C-terminal influenza hemagglutinin antigenic tag (HA), i.e., BACE-HA, as well as C99 were prepared as described (*10*, *11*).

Transfection of HEK293 Cells. Cells were grown and passaged 3 times per week in DMEM (Life Technologies, Inc., Rockville, MD) containing 10% fetal bovine serum and 1% penicillin/streptomycin. HEK293 cell lines stably expressing different levels of BACE-HA were generated by transfection with the BACE-HA construct followed by selection in 0.6 mg/mL G418 and subcloning. Stable expression of BACE-HA in HEK293 cell lines was maintained by the inclusion of 0.2 mg/mL G418 in the culture medium according to standard protocols. For transient transfection, cells were plated in 6-well tissue culture plates at 70−80% confluency the day of transfection, and 4 μg of DNA and 20 μL of Geneporter (Gene Therapy Systems, San Diego, CA) reagent were added in 1 mL of serum-free medium for each well in 6-well plates. After 3 h of incubation, the transfection medium was replaced with growth medium, and the cells were cultured for an additional 22 h.

Preparation of Semliki Forest Virus and Infection of HEK293 Cells. Semliki Forest virus (SFV) vectors expressing wild-type APP695 (wtAPP-SFV) or Swedish APP which contains the KM/NL mutations (swAPP-SFV) were prepared and titered as described previously (*12*−*15*). Control 293 cells and 293 cells stably expressing BACE were infected in serum-free medium at a multiplicity of infection of 10. After 1 h, complete growth medium was replaced, and infection was allowed to proceed overnight.

Antibodies for Immunoprecipitation and Immunoblotting. The antibodies used in this study and their epitope specificities are summarized in Figure 2. Briefly, Karen is a goat polyclonal antiserum raised to the large secreted N-terminal fragment of APP, and antibody 2493 is a rabbit polyclonal antibody raised to a synthetic peptide corresponding to the last 40 amino acid residues at the C-terminus of APP. C5A4 is a rabbit polyclonal antibody raised to a synthetic peptide corresponding to the amino acid sequence CSEVKM, and this antibody binds specifically to the free C-terminus of sAPPβ. Also used in this study were several mouse monoclonal antibodies (mAbs) to Aβ that are specific for residues 1−10 (Ban50), 11−28 [BNT77 (*16*)], and 17−24 (4G8), as well as mAbs that are specific for the free C-terminus of Aβx-40 (BA27) or Aβx-42 (BC05). Finally, to detect BACE-HA expression in the 293 cells, we used rabbit polyclonal antibodies against the C-terminus of BACE (ProSci, Poway, CA) and the influenza hemagglutinin tag located at the C-terminus of BACE-HA (Covance, Richmond, CA).

Metabolic Labeling, Immunoprecipitation, and Gel Electrophoresis. 293 cells were preincubated in methionine- and cysteine free-medium for 30 min, after which they were pulse-labeled with [^{35}S]methionine (300−500 μCi/mL) in methionine/cysteine-free Dulbecco's modified Eagle's medium, 5% dialyzed fetal bovine serum, and glutamine (NEN Life Science Products) for 1 h or 90 min and chased with complete growth medium for different lengths of time as indicated in the figure legends. The peptide aldehyde protease inhibitor MG-132 (200 μM in DMSO; Peptides International) was added in a similar manner for 30 min prior to chase as well as during the chase when used in specific experiments. At the conclusion of the experiments, medium was collected, and cells were washed once in phosphate-buffered saline and lysed in 800 μL of radioimmunoprecipitation assay (RIPA) buffer (0.5% sodium deoxycholate, 0.1% SDS, 1% Nonidet P-40, 5 mM EDTA in Tris-buffered saline, pH 8.0) with a mixture of protease inhibitors (1 μg/mL each of pepstatin A, leupeptin, L-1-tosylamido-2-phenylethyl chloromethyl ketone, 1-chloro-3-tosylamido-7-amino-2-heptanone, soybean trypsin inhibitor, and 0.5 mM phenylmethylsulfonyl fluoride). After brief sonication, medium and cell lysates were centrifuged at 40 000 rpm (Beckman TLA45) for 20 min at 4 °C, and the supernatant was collected and subjected to immunoprecipitation with various antibodies. APP C-terminal fragments were separated on 10/16% step gradient Tris−Tricine gels and Aβ peptides on 20% Tris−Tricine gels, and APP, sAPPα, sAPPβ′, and sAPPβ were resolved on 7.5% Tris−glycine gels. Gels were fixed in 50% methanol, dried, and placed on PhosphorImager plates overnight (*15*). Alternatively, proteins were transferred to nitrocellulose membranes and detected by western blot analysis using horseradish peroxidase (HRP)-conjugated secondary antibodies followed by visualization with Enhanced Chemiluminescence reagent (NEN Life Science Products, Boston, MA).

Sandwich ELISA. Sandwich ELISA was performed as previously described using mAbs specific for different species of Aβ (*17*, *18*). BAN50 or BNT77 were used as capturing antibodies, and HRP-conjugated BA27 or BC05 were used as reporter antibodies. The Ban50 sandwich ELISA was used to determine the levels of Aβ1−40 and Aβ1−42 whereas the BNT77 ELISA was used to measure the levels of Aβ1−40 and Aβ1−42 as well as Aβ11−40 and Aβ11−42. Synthetic Aβ1−40 and Aβ1−42 peptides (Bachem Bioscience Inc., King of Prussia, PA) were used to generate standard curves. The BAN50, BNT77, BA27, and BC05 mAbs were developed and characterized as described previously (*16*, *18*, *19*).

Immunoprecipitation and Mass Spectral (IP/MS) Analysis of Aβ. Overnight conditioned medium from 6-well plates of 293 and S22 cells expressing swAPP or APP M596V (1.5 mL/well) was harvested, and protease inhibitor was added

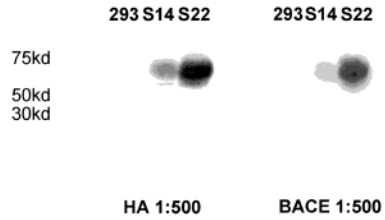

FIGURE 1: QBI 293A cell lines stably overexpressing HA-tagged BACE. QBI 293A human embryonic kidney cell lines stably expressing BACE with a HA antigenic tag at the C-terminus of the protein were subcloned by limiting dilution, and two subclones (S14 with medium BACE expression and S22 with high BACE expression) were chosen for further study. Untransfected 293 cells, which contain low levels of endogenous BACE (293), served as controls. To demonstrate different BACE expression levels, 20 μg of cell lysate was loaded per lane, resolved on 7.5% SDS−PAGE, and transferred to a nitrocellulose membrane. A polyclonal antibody against the HA tag was first used for the blot (left panel). After development, the membrane was stripped and blotted with a polyclonal antibody against the C-terminus of BACE (right panel).

before centrifugation at 16000*g* for 5 min at 4 °C using a Sorvall Biofuge Pico centrifuge (Kendro Laboratory Products, Newtown, CT) to remove cellular debris. Supernatant was collected and incubated with 1.0 μL of mAb 4G8 [3.7 mg/mL, anti-Aβ-(17−24)] and 3 μL of Protein A/G Plus-agarose (Santa Cruz Biotechnology) in a rotator at 4 °C for 18 h. The immunoprecipitated complex was collected by centrifugation at 8000*g* for 2 min, and the supernatant was aspirated. The agarose beads were washed once with ice-cold RIPA buffer and twice with 0.5 M Tris-HCl, pH 6.8. Each sample was prepared in duplicate and was combined into a 0.5 mL Eppendorf tube (*7*). Immunoprecipitated Aβs were extracted with a 3 μL matrix (a saturated solution of α-cyano-4-hydroxycinnamic acid in 0.1% trifluoroacetic acid/50% acetonitrile). Samples were sonicated briefly, and 1.5 μL of the extraction solution was loaded onto a matrix polycrystalline film on the mass spectrometer sample probe and dried at ambient temperature. The spotted sample probe was rinsed briefly with water and allowed to dry again. Mass spectra were measured using an UV-laser desorption/ionization time-of-flight mass spectrometer Micromass Tof-spec 2E at the University of Pennsylvania Protein Chemistry Laboratory.

RESULTS

Generation of BACE-HA Stable Cell Lines. To study the relative efficiencies with which BACE cleaves APP at Asp1 or Glu11 and the factors that may modulate this process, we generated QBI 293A human embryonic kidney cell lines stably expressing BACE engineered to contain a HA antigenic tag at the C-terminus of the protein. We have previously shown that the presence of the HA tag at this location has no effect on the normal metabolism and function of BACE (*10*). The stable BACE-expressing cell lines were generated by subcloning using limiting dilution, and two subclones (S14 with medium BACE expression and S22 with high BACE expression) were chosen for further study (Figure 1). Untransfected 293 cells, which contain low levels of endogenous BACE (293), served as controls.

Processing of APP and Secretion of Aβ11−40/42 upon BACE Expression. To study the consequences of variable BACE expression on APP processing, we monitored secreted

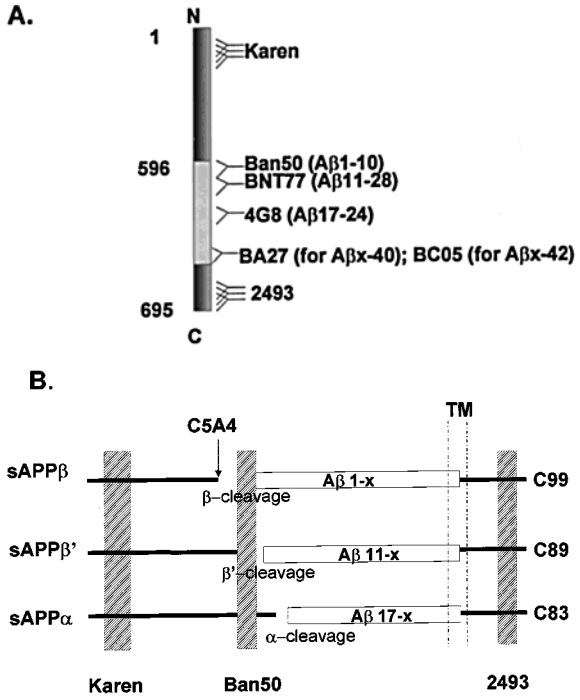

FIGURE 2: Diagram of antibodies used in this study. (A) Polyclonal antibodies Karen and 2493 are directed against the N-terminus and C-terminus of APP, respectively. mAbs Ban50 and BNT77 recognize the N-terminus of Aβ and were used as capturing antibodies for Aβ 1-x (Ban50) and Aβ 1/11-x (BNT77) in this study. mAb 4G8 is specific for the middle region of Aβ and was used to immunoprecipitate all Aβ species. mAbs BA27 and BC05 are C-terminal end-specific for Aβ40 and Aβ42, and HRP-conjugated BA27 and BC05 were used as reporting antibodies for Aβ40 and Aβ42, respectively. (B) Upon β- or α-secretase cleavage, different sAPP species are generated with corresponding CTFs. Polyclonal antibody Karen, being raised against the ectodomain of APP, is able to recognize all sAPP species. Polyclonal antibody 2493 is against the extreme C-terminus of APP and was used to capture all CTFs. mAb Ban50 binds to Aβ1−10 and therefore recognizes sAPPα, and possibly sAPPβ'. Polyclonal antibody C5A4 is a C-terminal end-specific antibody for sAPPβ. TM stands for transmembrane region.

forms of APP (sAPP) which are the products of α, β (Asp1), or β' (Glu11) cleavages that are designated as sAPPα, sAPPβ, and sAPPβ', respectively. Cells were transiently transfected with wtAPP695, and after incubation overnight, the conditioned medium was collected and immunoprecipitated with antiserum generated against the ectodomain of APP (Karen, see Figure 2). sAPPs were detected by western blots using an antibody that is specific for sAPPβ (C5A4, Figure 2) or an antibody that reacts with both sAPPβ' and sAPPα (Ban50, see Figure 2). As shown in Figure 3A, cells expressing endogenous levels of BACE appeared to produce far more sAPPα+β' than sAPPβ. However, since different antibodies were used to detect these species, it is not possible to state this unequivocally. Nonetheless, other studies have shown that APPα is the predominant form of sAPP secreted by nonneuronal cells. In contrast to cells expressing endogenous levels of BACE, cells expressing intermediate levels of BACE produced more sAPPβ under steady-state conditions with correspondingly less sAPPα+β'. This shift was somewhat more pronounced in cells expressing the highest levels of BACE.

Differences in the relative amounts of sAPPα+β' and sAPPβ recovered from the medium could be due to differ-

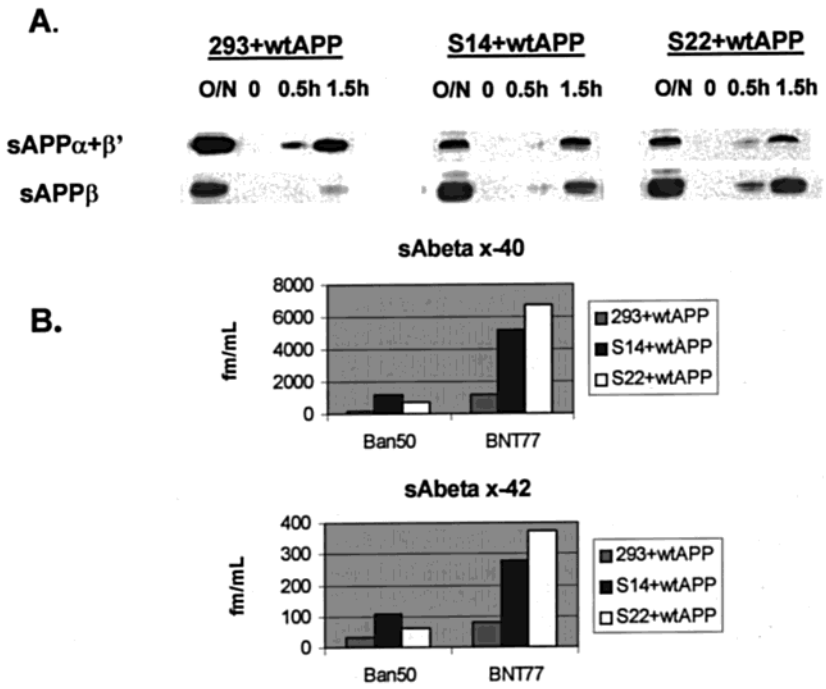

FIGURE 3: APP processing and Aβ secretion are affected by BACE expression levels. (A) 293, S14, or S22 cells were transfected with a wtAPP695 expression plasmid. The next day, conditioned medium was collected (labeled O/N), and the cells were placed in fresh medium (time 0). Additional aliquots of medium were collected at the indicated time points, after which the samples were immunoprecipitated with a polyclonal antiserum directed against the APP ectodomain (Karen) and analyzed by SDS−PAGE. Proteins were transferred to nitrocellulose membranes, and sAPPβ was detected by C5A4. The same blot was then stripped and reprobed with Ban50, which binds to both sAPPα and sAPPβ'. (B) Overnight conditioned medium from independent experiments was analyzed by sandwich ELISA to measure Aβ levels. Cells were transfected with a wtAPP-expressing plasmid. Conditioned medium was collected the next day. Ban50 or BNT77 were used as capturing antibodies for Aβ 1-x or Aβ 1/11-x, and HRP-conjugated BA27 or BC05 were used as reporting antibodies for Aβx-40 or Aβx-42, respectively. Data from a representative experiment are shown here. Similar trends were observed repeatedly with slight variance in values.

ences in their production, turnover after secretion, or a combination of the two. To evaluate these possibilities, cells were washed and placed in fresh medium, and the amount of sAPP species secreted after 0.5 and 1.5 h was assessed (Figure 3A). The results paralleled what was observed under steady-state conditions: the amount of sAPPβ correlated with the amount of BACE expression, and there was an inverse correlation between the amounts of sAPPα+β' and BACE expression levels. Thus, BACE expression levels are limiting in APP-transfected 293 cells. With increased BACE expression, increased amounts of sAPPβ are produced.

To examine the Aβ profile in the conditioned medium as a result of Asp1 and Glu11 cleavages, we performed a highly sensitive Aβ sandwich ELISA analysis using two different capturing antibodies. Ban50 was used since it recognizes only full-length Aβ species (Aβ 1-x, Figure 2), while BNT77 captures N-terminally truncated Aβ species in addition to full-length Aβ (Aβ *n*-x) (Figure 2). The use of the HRP-conjugated reporting antibodies BA27 and BC05 made it possible to distinguish Aβ40 from Aβ42. Thus, subtracting the amount of Aβ captured by Ban50 from that captured by BNT77 allows us to quantify the amount of Aβ11−40 and Aβ11−42.

Conditioned medium from all three cell lines was analyzed. Ban50 ELISA data revealed 5-fold more Aβ1−40 from the S14 subclone, which expresses moderate levels of BACE, than in parental 293 cells expressing endogenous levels of this enzyme. By contrast, only 3-fold more Aβ1−40 was recovered from the S22 subclone, which expresses high levels of BACE. However, Aβ1/11−40 data from the BNT77 ELISA demonstrated a dose-dependent increase corresponding to BACE levels. Although in both S14 and S22 cells this is likely a mixture of both Aβ1−40 and Aβ11−40, there was clearly more Aβ1−40 in S14 cells and more Aβ11−40 in S22 cells. Aβ1−42 and Aβ1/11−42 demonstrated similar patterns (Figure 3B). Therefore, the BACE to APP expression ratio appears to correlate with both Aβ production and the type of Aβ produced. While a moderate level of BACE expression in S14 cells leads to a significant increase of Aβ1−40/42, a high level of BACE expression in S22 cells leads to only a small increase of Aβ1−40/42 but a dramatic increase of Aβ11−40/42. Furthermore, although increased BACE expression in HEK293 cells resulted in increased sAPPβ secretion, this was not correlated with secreted Aβ1−40/42 levels since these levels are actually decreased in cells expressing very high levels of BACE.

Analysis of C-Terminal Fragments of APP upon BACE Expression. The experiments described above suggested that increased BACE expression results in enhanced APP cleavage, with cleavage at position 11 predominating at very high levels of BACE expression. However, the antibodies available provided an imperfect measure of the various APP and Aβ species. In addition, the use of several antibodies makes direct quantitative comparisons uncertain. Therefore, we examined APP C-terminal fragments (CTFs) that result from BACE and α-secretase cleavages using quantitative immunoprecipitations with a polyclonal antibody (2493, Figure 2) that recognizes the C-terminus of APP. Cleavage of APP by BACE at position 1 results in C99, cleavage at position 11 results in C89, while cleavage by α-secretase

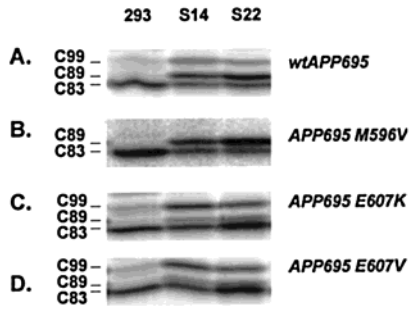

FIGURE 4: Specificity of Glu11 site cleavage versus Asp1 site cleavage of APP. 293, S14, or S22 cells were transfected with expression plasmids for either wtAPP695, APP695 M596V, APP695 E607V, or APP695 E607K as indicated. After overnight incubation, cells were metabolically labeled with [^{35}S]methionine for 90 min and chased for 1 h in the presence of the γ-secretase inhibitor MG132 in order to increase the intensity of the signal. CTFs in cell lysates were immunoprecipitated with polyclonal antibody 2493. Proteins were then separated on 10−16% Tris−Tricine gels, fixed in 50% methanol, dried, and placed on PhosphorImager plates overnight for signal detection.

results in C83, all of which can be resolved by SDS−PAGE (Figures 2 and 4). Subsequent cleavage of these CTFs by γ-secretase will give rise to Aβ1−40/42, Aβ11−40/42, and Aβ17−40/42.

Cells expressing wtAPP were metabolically labeled for 90 min and chased for 1 h prior to lysis and immunoprecipitation with the polyclonal antibody 2493. To enhance recovery of CTFs, the γ-secretase inhibitor MG132 was included in the chase medium. Immunoprecipitates were analyzed on 10/16% Tris−Tricine gels. In 293 cells, C83, the product of α-secretase cleavage and the precursor of p3, was the major CTF (Figure 4A), consistent with the fact that in 293 cells and other nonneuronal cell types α-secretase cleavage is the main APP processing pathway. By contrast, in both S14 and S22 cells, we observed a band corresponding to C99, the precursor of Aβ1−40/42. Consistent with the ELISA results, the highest intensity of C99 was observed in S14 cells. However, a band corresponding to C89, the precursor of Aβ11−40/42, was the major band detected both in S14 and in S22 cells (Figure 4A). The identities of these bands were confirmed by the mutagenesis studies discussed below (Figure 4B−D). Together with the ELISA results, our data suggest that Glu11 is a major cleavage site for BACE upon overexpression in QBI 293A cell lines, and Aβ11−40/42 is one of the major components detected in the conditioned medium.

Specificity of Glu11 Site versus Asp1 Site Cleavages of APP by BACE. To confirm the sequence specificity of the Glu11 site cleavage as well as the identities of the CTF bands, artificial mutations around the Asp1 and Glu11 sites were generated. M596V (APP695 numbering) has been shown to abolish the Asp1 cleavage site (*20*), while E607K and E607V were designed to diminish the Glu11 site cleavage by substituting the negatively charged glutamic acid with the positively charged lysine or hydrophobic valine. Upon transient transfection, CTFs were examined as before. There was a complete loss of C99 when cells expressed M596V, with a corresponding increase of C89 in both S14 and S22 cells (Figure 4B). This indicates that BACE can utilize full-length APP as a substrate to generate C89. The intensity of C83 also increased significantly when C99 production was blocked, providing further evidence that BACE competes with α-secretase cleavage for APP.

Both E607K and E607V led to a significant decrease in the production of C89. The intensity of C99, on the other hand, increased with the highest intensity detected in S14 cells (Figure 4C,D). The E607K and E607V mutations also led to increased C83, especially in S22 cells, again consistent with both α-secretase and BACE competing for the APP substrate. The increased recovery of C99 when cleavage at position 11 was largely blocked as a consequence of these mutations suggests that C99, like full-length APP, can be a substrate for BACE, resulting in the production of C89.To correlate the effects of these mutations on CTF production with Aβ secretion, we used the sandwich Aβ ELISA. Cells

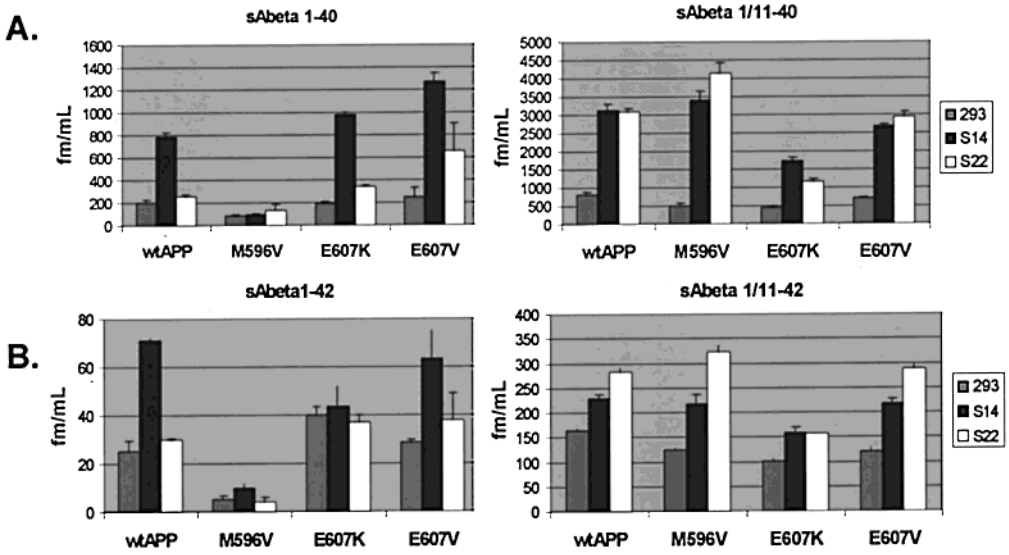

FIGURE 5: Quantification of secreted Aβ from APP mutants upon BACE overexpression. 293, S14, and S22 cells were transfected with expression plasmids for either wtAPP695, M596V, E607K, or E607V. The next day, conditioned medium was collected for sandwich ELISA analysis. Ban50 and BNT77 were used as capturing antibodies for Aβ 1-x and Aβ 1/11-x, and HRP-conjugated BA27 and BC05 were used as reporting antibodies for Aβx-40 and for Aβx-42, respectively. Data from three independent experiments, each performed in duplicate, were averaged, and the SEM is shown.

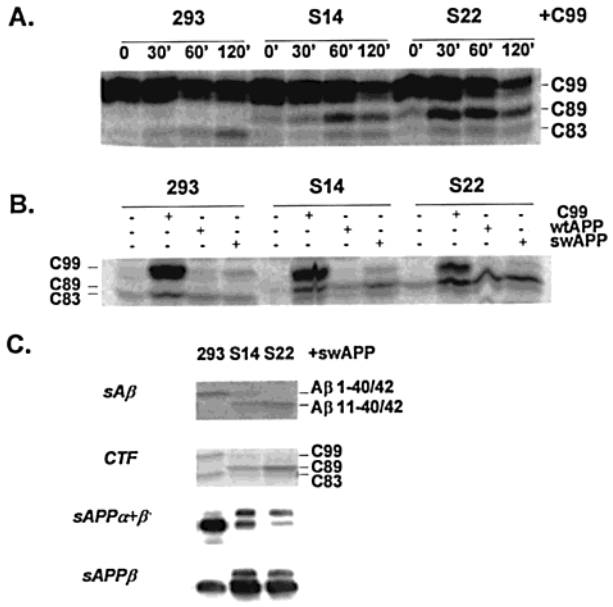

FIGURE 6: C99 is a substrate for Glu11 site cleavage by BACE. (A) Pulse−chase examination of C99 processing upon BACE overexpression. 293, S14, or S22 cells were transfected with an expression plasmid for C99. After overnight incubation, cells were metabolically labeled with [^{35}S]methionine for 1 h and chased in cold medium for the indicated time. CTFs were then immunoprecipitated with polyclonal antibody 2493, separated on 16% Tris−tricine gels, and revealed by PhosphorImager analysis. (B) Processing of C99, wtAPP, and swAPP upon BACE overexpression. 293, S14, or S22 cells were transfected with C99 or infected with either wtAPP695 SFV or swAPP695 SFV. After overnight incubation, cells were labeled with [^{35}S]methionine for 90 min and chased for 1 h. CTFs were then immunoprecipitated with 2493 and resolved on 16% Tris−tricine gels. (C) Processing of swAPP. 293, S14, or S22 cells were infected with swAPP SFV and labeled with [^{35}S]-methionine overnight. Conditioned medium was immunoprecipitated with monoclonal antibody 4G8 for all secreted Aβ species, and proteins were separated on 20% Tris−tricine gels. CTFs were immunoprecipitated with polyclonal antibody 2493 and were resolved on 16% Tris−tricine gels. To detect sAPPs, 293, S14, and S22 cells were transfected with an expression plasmid for swAPP. After overnight incubation, conditioned medium was immunoprecipitated with mAb Ban50 for sAPPα and β'. After two rounds of Ban50 immunoprecipitation for complete removal of sAPPα and β', medium was then immunoprecipitated with polyclonal antibody Karen for the remaining sAPPβ. All samples were resolved on 7.5% SDS−PAGE, transferred to nitrocellulose membranes, blotted with Karen, and visualized by the Enhanced Chemiluminescence reagent.

expressing the APP M596V mutant, which eliminated BACE cleavage at position 1 but resulted in enhanced cleavage at position 11, secreted basal levels of Aβ1−40/42 and high levels of Aβ1/11−40/42 (Figure 5). By contrast, cells expressing APP E607K or E607V secreted higher levels of Aβ1−40/42 and lower levels of Aβ1/11−40/42, consistent with the CTF results (Figure 5).

C99 as a Substrate for BACE Glu11 Site Cleavage. Our results show that full-length APP, and perhaps C99 as well, can be cleaved by BACE at Glu11. If correct, then we would expect production of C89 upon expression of C99 in vitro. Indeed, when an artificial construct encoding C99 with an N-terminal signal peptide was expressed in either S14 or S22 cells, C89 was produced (Figure 6A). A pulse−chase experiment demonstrated that C89 was produced from C99 with relatively rapid kinetics, with maximal production occurring from 30 to 60 min after synthesis (Figure 6A). A small amount of C83 was also observed, and could be due to the presence of endogenous full-length APP or from α-secretase cleavage of C99. The ability of BACE to cleave C99 at Glu11 raises the possibility that APP may be subjected to sequential BACE cleavage events, being first cleaved at Asp1 to generate C99, followed by a second BACE cleavage event to produce C89. To examine this possibility, we studied the processing of Swedish APP (swAPP), a FAD-associated mutation known to promote both Aβ1−40 and Aβ1−42 production (*21*). We reasoned that while swAPP is a better substrate for BACE Asp1 cleavage than wtAPP (*22*), there is little reason to suspect it would be a better substrate for BACE Glu11 cleavage since the swAPP mutations are at positions 595K and 596M (Figure 2). Thus, a larger amount of C99 should be produced upon swAPP expression. If C99 can be cleaved by BACE at Glu11, then a larger amount of C89 should be produced as well.

We found that expression of swAPP in 293 cells led to increased C99 generation relative to the amounts observed upon expression of wtAPP, consistent with previous results (Figure 6B) (*2*). By contrast, in S14 and S22 cells the increase of C89 from swAPP processing was more striking than that of C99. This was especially evident in S22 cells, where C99 was barely detectable. Since BACE efficiently cleaved swAPP at Asp1, we conclude that under the conditions of overexpression examined here BACE can sequentially cleave APP, resulting in the production of C89. To determine if sequential APP cleavage ultimately results in the production of Aβ11−40/42, we expressed swAPP in 293 cells using a recombinant SFV vector in order to obtain the high levels of APP expression needed to obtain readily detectable Aβ1−40/42 and Aβ11−40/42 by immunoprecipitation. Conditioned medium from the SFV-infected cells was immunoprecipitated with mAb 4G8 (see Figure 2) or 2493. 4G8 detected mainly Aβ1−40/42 in 293 cells, both Aβ1−40/42 and Aβ11−40/42 in S14 cells, and mostly Aβ11−40/42 in S22 cells (Figure 6C), consistent with both ELISA (data not shown) and CTF data (Figure 6C). These observations support the hypothesis that swAPP is first cleaved by BACE at the Asp1 site to produce C99. A sequential Glu11 cleavage then generates C89, which serves as the precursor for Aβ11−40/42.

To this point our analyses of Aβ species relied on the use of well-characterized antibodies. To confirm the identity of these Aβ peptides by an independent method, we performed mass spectrum analysis of immunorecipitates from conditioned medium. 293 and S22 cells were transfected with expression plasmids for swAPP and APP M596V. Overnight conditioned medium was immunoprecipitated with mAb 4G8, which should detect all secreted Aβ species, and the immunoprecipitates were subjected to mass spectral analysis. Synthetic Aβ1−40 and Aβ11−40 were spiked into the conditioned medium of nontransfected 293 cells as a positive control (Figure 7A). Consistent with our ELISA and CTF data, Aβ1−40 was the predominant Aβ species in conditioned medium from 293 cells expressing swAPP (Figure 7B). In contrast, Aβ species beginning at position 11 were the predominant forms in medium from S22 cells (Figure 7C). When APP M596V was expressed in S22 cells, only Aβ species beginning at position 11 were detected (Figure 7D). In all cases, the levels of Aβ42 were below

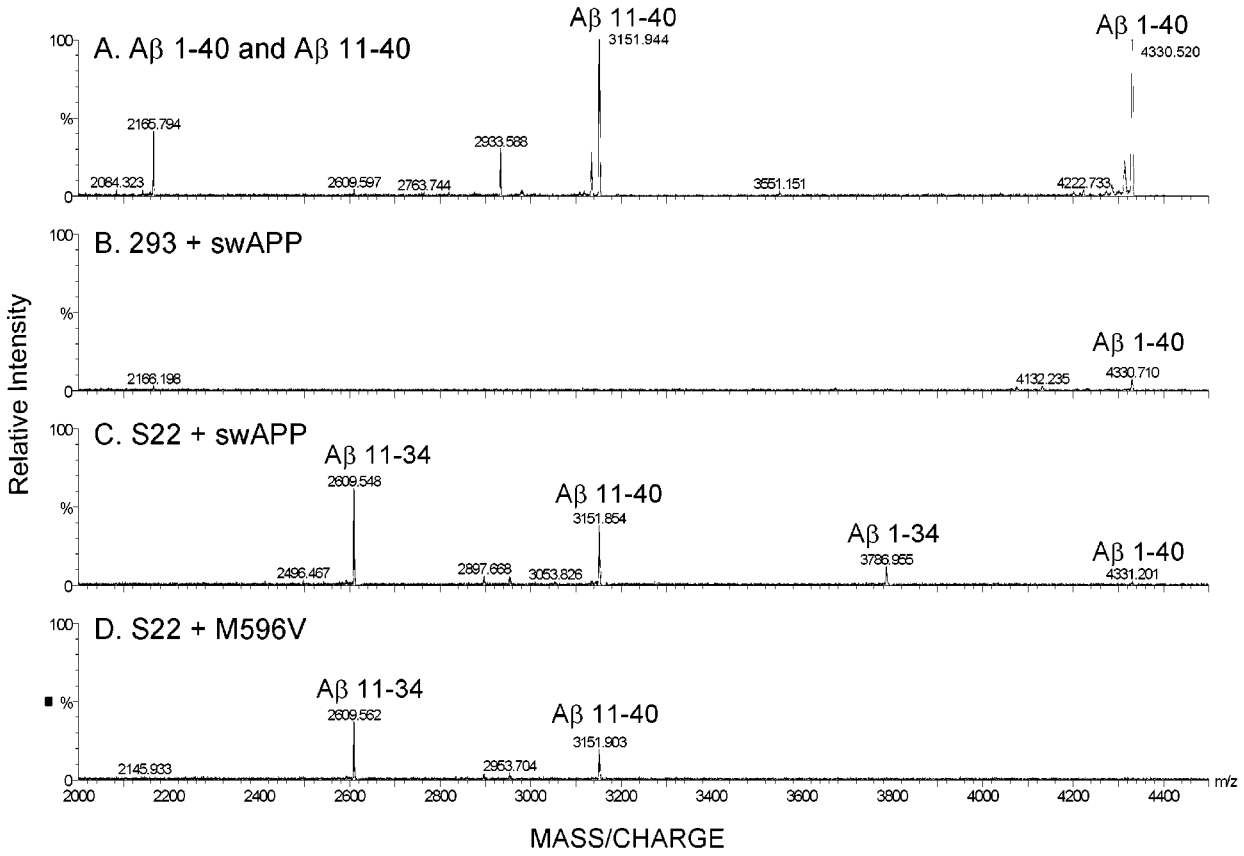

FIGURE 7: Mass spectral analysis of secreted Aβ species. 293 and S22 cells were transfected with expression plasmids for swAPP or APP M596V. Overnight conditioned medium was collected and immunoprecipitated with mAb 4G8. Each sample was prepared in duplicate and was combined before mass spectral analysis. For a positive control, 1 μg samples of synthetic Aβ1−40 (Bachem Bioscience Inc., King of Prussia, PA) and Aβ11−40 (Dr. D. B. Teplow) peptides were spiked into 1.5 mL of conditioned medium of nontransfected 293 cells and were subjected to IP/MS analysis (A). Alternatively, medium from 293 cells expressing swAPP (B) or S22 cells expressing swAPP (C) or APP APP M596V (D) was collected, immunoprecipitated, and analyzed by mass spectroscopy. Results were repeated with two independent transfections and IP/MS analyses with similar results.

detection. Interestingly, Aβ1/11−34 was also detected as a major component of secreted Aβ from transfected S22 cells. This is consistent with a recent report from Vandermeeren et al., which has attributed the C-terminal cleavage to direct or indirect involvement of γ-secretase (*23*). Thus, consistent with our ELISA and CTF results, overexpression of BACE results in enhanced cleavage of Aβ at position 11.

DISCUSSION

Senile plaques composed of Aβ peptides are a central and invariant feature of AD. A variety of Aβ peptides have been detected both in vivo and in vitro, though quantitative analysis has proven difficult due to the lack of specific immunologic reagents and differential solubility resulting from slight differences in peptide length. Aβ1−42, for example, aggregates more readily than Aβ1−40 in vitro (*24*) and is the more prevalent species in senile plaques (*25*). The importance of this 42 residue long form of Aβ is underscored by the fact that a number of mutations in APP and in PS1 that are associated with familial AD increase the production of Aβ1−42 relative to that of Aβ1−40 (*25*).

In addition to variation at the C-terminus of Aβ, variation at the N-terminus has been noted as well. While most in vitro studies have, perhaps because of the specificity of antibodies such as Ban50, focused on Aβ species beginning at residue 1 (i.e., cleaved by BACE between Met596 and Asp597), N-terminally truncated forms of Aβ have also been described. For example, Aβ11−40/42 has been detected as one of the major Aβ species in AD brains (*8*), DS brains with AD pathology (*9*), normal aging brains (*8*), as well as conditioned medium of primary rat and mouse neuronal cultures and mouse N2a cells (*6, 7, 26*). While 10 amino acids shorter than Aβ1−40/42, Aβ11−40/42 still contains the core sequence needed for fibril formation (*27, 28*). In fact, in vitro studies indicate that N-terminal deletions enhance Aβ aggregation in vitro (*29*). Thus, just as the addition of several amino acids at the C-terminus of Aβ may enhance its aggregation potential, so too might truncations at the N-terminus of this peptide.

While Aβ species beginning at position 11 have been detected in brain extracts and in the media of some cell types, it has not been clear if Aβ11−40/42 is produced intracellularly as a primary cleavage product of APP or if it represents degraded forms of Aβ1−40/42 produced extracellularly. The identification of BACE makes it possible to address this question by demonstrating that this enzyme is responsible for APP cleavage at position Glu11. Our results show that APP can be readily cleaved by BACE at position 11 by two pathways: BACE can cleave APP directly at position 11, or it can first cleave APP at position 1 to generate C99, which can then be cleaved to produce C89 (Figure 8). Work by Creemers et al. also suggested that sequential cleavage of APP may occur (*30*). We were able to show

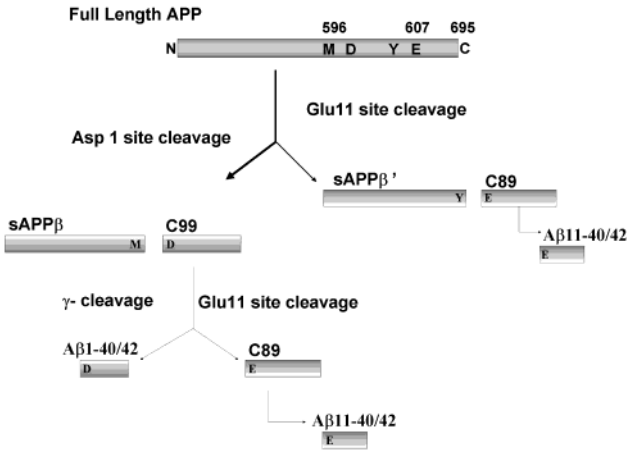

FIGURE 8: Model for Asp1 and Glu11 cleavages of APP by BACE. BACE cleaves full-length APP at Asp1 to generate sAPPβ and C99. Subsequent γ-cleavage of C99 produces Aβ 1−40/42. Alternatively, accumulated C99 can serve as a substrate for Glu11 cleavage by BACE to produce C89, which will give rise to Aβ11−40/42 upon γ-cleavage. On the other hand, BACE is also able to cleave full-length APP at Glu11 to generate C89, although it does not seem to be the preferred pathway when compared with Asp1 cleavage.

this directly by expressing C99 in cells: abundant levels of both C89 and Aβ11−40/42 were produced. In addition, expression of swAPP gave rise to increased levels not only of C99, as previously reported, but also of C89 as well. However, cleavage at Asp1 is not required for cleavage at Glu11: expression of an APP mutant that abrogated cleavage at Asp1 still gave rise to abundant C89 and Aβ11−40/42.

If BACE can cleave APP at two distinct sites, what governs the relative ratios of these cleavage products and what might the consequences be for Aβ production and AD pathogenesis? It is evident that BACE expression levels impact cleavage activity. When BACE levels are high, cleavage at Glu11 predominates; when BACE levels are low, cleavage at Asp1 occurs more frequently (*30*). In our study, there was a direct relationship between levels of C99 and Aβ1−40/42 and between C89 and Aβ11−40/42. Thus, we conclude that cleavage at Glu11 is fully compatible with subsequent γ-secretase cleavage and Aβ production. In vivo, other factors may impact the ability of BACE to cleave APP at Glu11. Prolonged association of BACE with APP could lead to enhanced cleavage at Glu11 since C99 is also a substrate for BACE. To fully document the efficiency with which this occurs, however, more detailed measures of the half-lives of C99, C89, and C83 will be needed. Finally, our recent studies indicate that intracellular pH does not obviously impact the efficiency with which BACE cleaves APP at position 11 (Huse, Liu, Püak, Carlin, Lee, and Doms, unpublished experiments).

Accurate measurements of Glu11 cleavage products in vivo have not been made. Since most cell types express relatively low levels of BACE, Aβ1−40/42 are the most common species recovered. However, neurons express higher levels of BACE (*2*, *31*), and produce much higher levels of Aβ11−40/42 in culture (*6*, *26*). It will be important to determine if neurons produce higher levels of Aβ11−40/42 than other cell types in vivo and whether Glu11 cleavage of C99 is part of this mechanism. Studies with primary neurons may be made more difficult by the fact that the APP Glu11 site cleavage seems to be species-specific: mouse BACE is unable to cleave human APP at this position (*7*, *26*). This may help to explain the absence of hAβ11−40/42 in mouse N2a cells transfected with hAPP (*7*) as well as the absence of hAβ11−40/42 in transgenic mice overexpressing human APP FAD mutants (*32*). Given this, it is apparent that the presently available transgenic models cannot be used to study the interaction between Aβ11−40/42 and Aβ1−40/42 in plaque deposition and other aspects of neuropathology.

The ability of BACE to cleave APP at Glu11 calls for a reevaluation of the role of Aβ11−40/42 in AD pathogenesis. If Aβ species beginning at Glu11 prove to be more insoluble than those beginning at Asp1, then quantitative measurements will be made more difficult, and intracellular, insoluble pools of Aβ will have to be examined. Recently, several groups have reported the identification of several FAD-associated APP or PS1 mutations which specifically increase N-terminally truncated Aβ production (*33*−*35*). All of these mutations are involved in interference of γ-secretase cleavage, which may explain the specific increase of Aβx-42 versus Aβx-40 in some cases (*33*, *35*). Although more quantitative studies are needed, it is intriguing how these γ-secretase cleavage-related mutations might increase N-terminally truncated Aβ production. Development of N-terminal end specific antibodies for Aβ11−40/42 together with more thorough cell culture studies on γ-secretase cleavage-related mutations will help to address this question. Finally, hBACE-overexpressing mice crossed with hAPP-overexpressing mice will help to make it possible to investigate whether Aβ11−40/42 is a major species deposited in vivo upon BACE expression and how it influences the formation of plaques in the presence of full-length Aβ. This will help to elucidate the role of the Glu11 β'-secretase cleavage in AD development.

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