Secretion and Intracellular Generation of Truncated Aβ in β-Site Amyloid-β Precursor Protein-cleaving Enzyme Expressing Human Neurons*

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Edward B. Lee, Daniel M. Skovronsky, Farhad Abatian, Robert W. Doms§, and Virginia M.-Y. Lee¶

From the Center for Neurodegenerative Disease Research, Department of Pathology and Laboratory Medicine, and ¶Department of Microbiology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

Insoluble pools of the amyloid-β peptide (Aβ) in brains of Alzheimer’s disease patients exhibit considerable N- and C-terminal heterogeneity. Mounting evidence suggests that both C-terminal extensions and N-terminal truncations help precipitate amyloid plaque formation. Although mechanisms underlying the increased generation of C-terminally extended peptides have been extensively studied, relatively little is known about the cellular mechanisms underlying production of N-terminally truncated Aβ. Thus, we used human NT2N neurons to investigate the production of Aβ11–40/42 from amyloid-β precursor protein (APP) by β-site APP-cleaving enzyme (BACE). When comparing undifferentiated human embryonal carcinoma NT2– cells and differentiated NT2N neurons, the secretion of sAPP and Aβ correlated with BACE expression. To study the effects of BACE expression on endogenous APP metabolism in human cells, we overexpressed BACE in undifferentiated NT2– cells and NT2N neurons. Whereas NT2N neurons produced both full-length and truncated Aβ as a result of normal processing of endogenous APP, BACE overexpression increased the secretion of Aβ11–40/42 and Aβ11–40/42 in both NT2– cells and NT2N neurons. Furthermore, BACE overexpression resulted in increased intracellular Aβ11–40/42 and Aβ11–40/42. Therefore, we conclude that Aβ11–40/42 is generated prior to deposition in senile plaques and that N-terminally truncated Aβ peptides may contribute to the downstream effects of amyloid accumulation in Alzheimer’s disease.

Alzheimer’s disease (AD) is characterized by the accumulation of aggregated Aβ peptides in senile plaques and vascular deposits. Aβ has classically been described as a 4-kDa peptide, derived from proteolytic processing of APP, varying in length from 40 to 42 amino acids due to C-terminal heterogeneity. The generation of the longer Aβ1–42 peptide is specifically increased by several mutations linked to familial Alzheimer’s disease (1). Furthermore, Aβ1–42 is more fibrillogenic than Aβ1–40 in vitro (2), consistent with the finding that both diffuse and senile plaques are composed of primarily Aβ peptides that terminate at position 42 (3). However, several N-terminal truncations are also found in Aβ peptides derived from AD brains, demonstrated as early as the first biochemical isolation of Aβ peptides from senile plaques (4). The relative importance of N-terminally truncated Aβ peptides in the pathogenesis of AD is unknown. Interestingly, some individuals with sporadic AD (5), familial AD (5, 6), and Down’s syndrome (7) preferentially accumulate N-terminally truncated Aβ species. Also, overexpression of APP harboring disease-associated mutations within the Aβ domain (i.e., A692G and E693G) results in increased secretion of Aβ11–40/42 (8, 9).

Whereas some truncations may be due to the partial degradation of full-length Aβ after secretion of peptides into the extracellular milieu, Aβ peptides beginning with glutamine at position 11 are derived from the membrane-bound β-site APP-cleaving enzyme (BACE) (10–12). To generate full-length Aβ, BACE cleaves APP between the methionine and aspartate at position 1 (Asp-1) of the N terminus of Aβ (β-cleavage), resulting in the secretion of a large N-terminal ectodomain, sAPPβ, and the retention of a 99-amino acid C-terminal fragment, C99. To generate N-terminally truncated Aβ, BACE cleaves APP between tyrosine and glutamate at position 11 (Glu-11) within the Aβ domain (β’-cleavage), resulting in the secretion of a slightly larger N-terminal ectodomain, sAPPβ’, and the retention of an 89-amino acid C-terminal fragment, C89. C89 can also be produced by proteolysis of C99 by BACE at Glu-11 (11). Alternatively, APP may also be cleaved at position 16 by α-secretase (13, 14), resulting in secretion of the N-terminal sAPPs along with the retention of an 83-amino acid C-terminal fragment, C83. Membrane-bound C-terminal fragments are subjected to further proteolysis within the transmembrane domain.

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¶ John H. Ware III professor of Alzheimer’s research. To whom correspondence should be addressed: Center for Neurodegenerative Disease Research, Dept. of Pathology and Laboratory Medicine, Maloney 3, HUP, Philadelphia, PA 19104-4283. Tel.: 215-662-6427; Fax: 215-349-0939. E-mail: vmyttc@neuro.med.upenn.edu

† The abbreviations used are: AD, Alzheimer’s disease; Aβ, amyloid-β peptide; Aβ11–40, Aβ11–42, 40- and 42-amino acid forms of Aβ respectively; Aβ11–40, Aβ11–42, N-terminally truncated Aβ peptides starting at position Glu-11; ADAM, a disintegrin and metalloprotease; APP, amyloid-β precursor protein; APP95, 95-amino acid isoform of APP; APP751/770, 751- and 770-amino acid isoforms of APP; BACE, β-site APP-cleaving enzyme; C83, α-secretase derived C-terminal fragment of APP; C89, β-secretase derived C-terminal fragment beginning at Glu-11; C99, β-’secretase derived C-terminal fragment beginning at Asp-1; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; GFP, green fluorescent protein; NT2–, undifferentiated embryonal car

cinoma NTera2/c1.D1; NT2N, differentiated neuron derived from NTera2/c1.D1; PMA, phorbol 12-myristate 13-acetate; RIPA buffer, radioimmune precipitation assay buffer; sAPP, total secretase-derived N-terminal ectodomain of APP; sAPPα, α-cleavage-derived N-terminal ectodomain of APP; sAPPβ, β-cleavage-derived N-terminal ectodomain of APP; TAPI, (N-R-(2-hydroxyaminocarbonyl)methyl)-4-methylpentanoyl-l-naphthylalanylt-l-alanine 2-aminoethyl amide; Tricine, N-[2-hydroxyl-1,1-bis(hydroxymethyl)ethy]glycine; VSV-G, vesicular stomatitis virus surface glycoprotein; DMEM, Dulbecco’s modified Eagle’s medium; MADLI-TOF, matrix-assisted laser desorption ionization/time of flight; CSF, cerebral spinal fluid.

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main by γ-secretase, with cleavage typically occurring at either position 40 or 42 within the Aβ region. Whereas all of the components of γ-secretase have not been identified, the presenilin proteins are necessary for secretion of Aβ peptides and have been postulated to contain the active site of γ-secretase (1). However, multiple γ-secretases may exist as presenilin is not needed for Aβ1–42 production early in the secretory pathway (15).

Untransfected non-neuronal cells and murine cells are not well suited toward the study of BACE-derived N-terminally truncated Aβ. Many non-neuronal cells preferentially use the α-secretase pathway at the expense of β-cleavage of APP (13, 14), although the secretion of full-length and N-terminally truncated Aβ can be increased upon overexpression of either APP or BACE (11, 12, 16, 17). Furthermore, due to low BACE activity and low APP expression, intracellular Aβ is difficult to detect in non-neuronal cells (16, 18). In contrast, neuronal cells cleave a larger proportion of APP by BACE (19–21), although overexpression of human APP in rodent cells does not lead to the generation of Aβ1–40/42 from human APP (22), consistent with evidence that β′-cleavage is species-specific (23). Furthermore, rodent neuronal cells preferentially cleave endogenous APP at position 11, whereas the presence of β′-cleavage in human neuronal cells has not been adequately addressed. Therefore, rodent cell culture models may not accurately reflect the proteolytic processing of APP in human neurons.

NT2N neurons are a post-mitotic, terminally differentiated human neuronal cell culture model derived from retinoic acid treatment of the human embryonal carcinoma cell line NTERa2/c1.D1 (NT2–) (24–27). Their high APP expression and β-secretase activity make them amenable to biochemical analysis of both neuronal specific and human-specific characteristics of APP processing (18, 21). We have shown that NT2N neurons generate Aβ1–40/42 intracellularly prior to secretion and that endogenous secretion of Aβ1–40/42 from NT2N neurons increases with age in culture (19, 20). Furthermore, a detergent-insoluble pool of intracellular Aβ accumulates with time in NT2N neurons (28). In this study, we found that sAPP and Aβ secretion increase upon neuronal differentiation of NT2N neurons, correlating with BACE expression. Given a recent report (29) that BACE protein expression and activity are increased in AD, we sought to refine further our understanding of APP metabolism in NT2N neurons by examining the effect of exogenous expression of BACE in undifferentiated NT2– cells and differentiated NT2N neurons. We found that this increase in Aβ production due to BACE overexpression was more pronounced in NT2N neurons than in non-neuronal NT2– cells. Furthermore, we found that Aβ11–40/42 is produced endogenously by NT2N neurons and that BACE overexpression increases the secretion of both full-length and truncated Aβ peptides. We further demonstrate that Aβ11–40/42 is generated intracellularly, indicating that Aβ11–40/42 is produced prior to deposition in senile plaques. The effect of BACE expression on the generation of both full-length and N-terminally truncated Aβ underscores the role of BACE activity in the generation of Aβ peptides and indicates that N-terminally truncated Aβ peptides may contribute to the pathogenesis of AD.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Undifferentiated NTERa2/c2.D1 cells were maintained as described previously (26, 27) in Opti-MEM (Invitrogen) containing 5% fetal bovine serum (FBS), 100 units/ml penicillin, and 10 μg/ml streptomycin sulfate. Cells were differentiated by twice weekly 10 μM retinoic acid treatments for 5 weeks and then replated (replated 2 neurons) in DMEM with high glucose, 5% FBS, and mitotic inhibitors (10 μM uridine, 10 μM 5-fluor-2′-deoxyuridine, 1 μM cytosine arabinoside, Sigma) to obtain nearly pure NT2N neurons (28). Greater than 99% pure neurons (replated 3 neurons) (27) were isolated by mechanical separation of neurons after brief trypan blue staining of mixed cultures and replated onto 6-well plates coated with Matrigel and poly-L-lysine.

**Western Blot Analysis**—Cell lysates were collected in RIPA buffer (0.5% sodium deoxycholate, 0.1% SDS, 1% Nonidet P-40, 5 mM EDTA in TBS, pH 8.0) in the presence of protease inhibitors (1 μg/ml each of pepstatin A, leupeptin, 1-1-tosylamido-2-phenylthyl chloromethyl ketone, 1-chloro-3-oxo-2-laminido-7-amin-2-heptanone, soybean trypsin inhibitor, and 0.5 mM phenylmethylsulfonyl fluoride) and briefly sonicated. Protease inhibitors were also added to conditioned media samples. Samples were centrifuged at 100,000 × g for 20 min at 4 °C, electrophoresed on 7.5% Tris-glycine acrylamide gels, and transferred to nitrocellulose. When indicated, samples were immunoprecipitated with Karen, a goat polyclonal antibody raised against sAPP, prior to Western analysis. APP and total sAPP were probed with Karen (1:2000), and sAPPβ was probed with Ban50, a mouse monoclonal antibody recognizing Aβ residues 1–11. The specificity of C5a4 and C10a4 was determined by their lack of immunoreactivity with full-length APP or C-terminal APP fragments, and by blocking experiments in which only peptides with the corresponding free C terminus are able to block immunoreactivity (data not shown). Immunoblots were visualized with enhanced chemiluminescence (PerkinElmer Life Sciences) after application of species-specific horseradish peroxidase-conjugated anti-IgG antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). However, for quantification of APP and sAPP, Karen immunoblots were labeled with [35S]methionine (250 μCi/ml in methionine-free DMEM supplemented with 5% dialyzed FBS; PerkinElmer Life Sciences) for 90 min, and chased for 1 h. Cells were treated with 10 μM TAPi in Me2SO (Sigma) during the chase period and/or with 10 μM TAPI in Me2SO (Peptides International, Louisville, KY) for 1 h prior to and during the chase period. Protease inhibitors were added to conditioned media, and sAPP was immunoprecipitated with Ban50 prior to electrophoresis on 7.5% Tris-glycine acrylamide gels. For C-terminal fragment analysis, cells were labeled for 2 h with [35S]methionine in the presence of 200 μg/ml cycloheximide, rinsed, and lysed in 1000 μl of RIPA buffer containing protease inhibitors for immunoprecipitation. Lysates were briefly sonicated, and both lysates and media were cleared by centrifugation at 100,000 × g for 20 min at 4 °C. C-terminal fragments of secreted APP were immunoprecipitated with 2493, a rabbit polyclonal antibody recognizing the C-terminal region of APP, and resolved on 10/16.5% step gradients of Tris-Tricine gels. Gels were fixed in 50% methanol, 5% glycerol, dried, and exposed to PhosphorImager plates for visualization.

**Metabolic Labeling and Immunoprecipitation**—Cells were incubated in methionine-free DMEM (Invitrogen) for 30 min, labeled with [35S]methionine (250 μCi/ml in methionine-free DMEM supplemented with 5% dialyzed FBS; PerkinElmer Life Sciences) for 90 min, and chased for 1 h. Cells were treated with 10 μM TAPi in Me2SO (Sigma) during the chase period and/or with 10 μM TAPI in Me2SO (Peptides International, Louisville, KY) for 1 h prior to and during the chase period. Protease inhibitors were added to conditioned media, and sAPP was immunoprecipitated with Ban50 prior to electrophoresis on 7.5% Tris-glycine acrylamide gels. For C-terminal fragment analysis, cells were labeled for 2 h with [35S]methionine in the presence of 200 μg/ml cycloheximide, rinsed, and lysed in 1000 μl of RIPA buffer containing protease inhibitors for immunoprecipitation. Lysates were briefly sonicated, and both lysates and media were cleared by centrifugation at 100,000 × g for 20 min at 4 °C. C-terminal fragments of secreted APP were immunoprecipitated with 2493, a rabbit polyclonal antibody recognizing the C-terminal region of APP, and resolved on 10/16.5% step gradients of Tris-Tricine gels. Gels were fixed in 50% methanol, 5% glycerol, dried, and exposed to PhosphorImager plates for visualization.

Finally, to detect both secreted and intracellular Aβ, two 10-cm dishes of replate 2 NT2N neurons were infected with recombinant Semliki Forest virus encoding wild type APP695, prepared as described previously (18, 30). Cells were infected in serum-free medium for 1 h, cultured in complete growth medium for 14 h, and then labeled with [35S]methionine (500 μCi/ml) for 8 h. Conditioned media and RIPA cell lysates were collected, cleared by centrifugation, and immunoprecipitated with 4G8 (Senetek, Maryland Heights, MO) prior to electrophoresis in 10/16.5% step gradient Tris-Tricine gels.

**Northern Analysis**—Total RNA was extracted from NT2– cells and NT2N neurons with the Trizol Reagent (Invitrogen) as per manufacturer’s protocol. RNA concentrations were determined by optical density readings, and equal amounts of RNA were separated on 1% agarose gels.
rose-formaldehyde gels. RNA was transferred to a nitrocellulose membrane (Amersham Biosciences) and hybridized with a radiolabeled probe generated by either PstI digestion or AccI-HincII double digestion of a BACE cDNA. The blot was washed and exposed to a PhosphorImager plate for visualization. Glyceraldehyde-3-phosphate dehydrogenase levels were obtained by using a glyceraldehyde-3-phosphate dehydrogenase probe purchased from Ambion (Austin, TX). 28 S and 18 S ribosomes were visualized by staining a duplicate gel with ethidium bromide.

Stable Transduction of NT2—Cells—NT2— cells were stably transduced with a vescicular stomatitis virus surface glycoprotein (VSV-G) pseudotyped self-inactivating lentiviral vector. To generate the virus, QBI 293A cells were plated on poly-L-lysine-coated 10-cm dishes. Cells were transfected with pMD.G (containing the VSV-G envelope glycoprotein), pCMVΔR8.2 (containing viral structural, enzymatic, and accessory genes), and SIN-EFp-GFP/SIN-EFp-BACE (containing minimal human immunodeficiency virus-based viral sequences, the elongation factor 1-α promoter, and either a GFP or BACE cDNA) using standard CaPO4 techniques. Media conditioned with viral particles were harvested over 3 days, centrifuged at 1000 rpm for 5 min, and passaged through a 0.45-μm filter to remove any cellular debris. Viral supernatants were added to NT2—cells, and transduced NT2— cultures were subcloned by limited dilution into 96-well plates. Uniform expression was verified either by direct fluorescence for GFP-expressing cells or indirectly (36), supplied by Dr. M. Mercken (Janssen Research Foundation, Perspective (Framingham, MA) Voyager DE-PRO MALDI-TOF instrument in the positive-ion mode at the Protein Microchemistry/Mass Spectrometry Facility of the Wistar Institute (Philadelphia). Samples were spotted to a 100-well plate using a cyano-4-cinnamic acid matrix (Sigma) at 10 μg/ml. Reflector mode with the accelerating potential at 20 kV was used. External calibration was performed on all samples.

Sandwich ELISA Analysis—Secreted Aβ was detected using ELISA protocols described previously (20, 33). Briefly, Ban50 (anti-Aβ1–10) or BNT77 (anti-Aβ11–28) were used as capturing antibodies. After application of culture media samples, horseradish peroxidase-conjugated BA-27 and BC-05 were used to report Aβ species ending at position 40 and 42, respectively. For quantification of Aβ levels, specific values relative to NT2—cells. D, Aβ1–40 levels were quantified by Ban50/BA-27 ELISA, normalized to intracellular APP expression levels, and shown as mean values relative to NT2— cells. E, sAPP/APP ratio, corrected for intracellular APP expression, and shown as mean values relative to NT2— cells ± S.E. One-way analysis of variance revealed p < 0.0001; post hoc analysis showed p < 0.01 (*) compared with NT2— cells. F, sAPP/APP ratio, corrected for intracellular APP expression, and shown as mean values relative to NT2— cells ± S.E. One-way analysis of variance revealed p < 0.0001. Post hoc analysis showed p < 0.01 (*) compared with NT2—cells.

RESULTS

sAPP and Aβ Production Correlates with Neuronal Differentiation—APP processing differs between non-neuronal and neuronal cells, underscoring the importance of using neuronal systems to study APP metabolism and Aβ generation (18–20, 28). Prior to studying BACE activity and β-cleavage in NT2N neurons, we quantified the differences in the expression and proteolytic processing of APP between NT2— cells and NT2N neurons. Whereas both NT2— cells and NT2N neurons express high levels of APP, the two cell types express different isoforms of APP (Fig. 1A). We have shown previously (19) that NT2—cells predominantly express the 751- and 770-amino acid isoforms of APP (APP751/770) that appear as a doublet corresponding to immature (~110 kDa) and mature N- and O-glycosylated APP (~125 kDa). NT2N neurons predominantly express the shorter 695-amino acid isoform of APP (APP695), the majority of which is immature APP695 (~95 kDa) with relatively less mature APP695 (~110 kDa). Despite the difference in isoform expression, densitometric quantification indicated that when normalized for total protein content, NT2— cells expressed APP at 98% ± 5 (S.E.) compared with NT2N neurons. However, despite equivalent total APP expression, we found that proteolytic processing of APP was more efficient in NT2N neurons compared with NT2— cells. To measure total sAPP, derived from both α- and β-secretase cleavage of APP, media conditioned by NT2— cells or NT2N neurons for 24 h were immunoprecipitated with a polyclonal antibody raised

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against the N-terminal domain of APP. We found that NT2N neurons secreted more total sAPP relative to NT2− cells (Fig. 1B). Densitometric quantification of total sAPP indicated that NT2N neurons secreted over 5-fold more sAPP than NT2− cells (Fig. 1C). Differentiation had an even larger effect on Aβ secretion, as NT2N neurons secreted over 20-fold more Aβ than NT2− cells (Fig. 1D), determined by sandwich ELISA for Aβ1–40. Because differences in APP expression cannot explain the increase in Aβ secretion, differences in either β- or γ-secretase activity are likely to be responsible for the more efficient proteolysis of APP in NT2N neurons.

TAPI-insensitive sAPP Production in NT2N Neurons—The increased production of sAPP secretion by NT2N neurons indicated that BACE and/or α-secretase activity might be elevated in NT2N neurons upon neuronal differentiation of the NT2− cells. To distinguish between these possibilities, we first addressed the presence of α-secretase by testing the pharmacologic responses of NT2− cells and NT2N neurons upon α-secretase inhibition. α-Secretase has been attributed to members of a family of proteases that contain a disintegrin and a metalloprotease domain (ADAM), including ADAM10 and tumor necrosis factor-α converting enzyme. α-Cleavage is enhanced by phorbol ester-induced stimulation of tumor necrosis factor-α converting enzyme via protein kinase C and can be inhibited by metalloprotease inhibitors. Therefore, we treated metabolically labeled NT2− cells and NT2N neurons with either phorbol 12-myristate 13-acetate (PMA) or the specific metalloprotease inhibitor, TAPI. sAPP from media samples was immunoprecipitated using Ban50, a monoclonal antibody that recognizes the first 11 amino acids of AβC5A4/2, a polyclonal antibody that specifically recognizes the C terminus of sAPPBACE-derived Aβ peptides in human neurons, we characterized BACE expression in NT2− cells and NT2N neurons. Expression of BACE mRNA was determined by Northern analysis of NT2− cells and NT2N neurons. BACE mRNA expression was clearly present in NT2N neurons, as seen by the presence of 7.0-, 4.4-, and 2.6-kb bands (Fig. 3A). NT2− cells, however, showed markedly less BACE expression, most notably demon-

**Fig. 2. TAPI-insensitive secretion of sAPP in NT2N neurons.** A, NT2− cells and replicate 3 NT2N neurons were metabolically labeled for 90 min and chased for 60 min. Cells were treated with 10 μM PMA during the chase period and/or 10 μM TAPI 30 min prior to and throughout the chase period. Conditioned media were immunoprecipitated with Ban50 and separated on 7.5% Tris-glycine gels. A representative gel out of three separate experiments is shown. B, sAPPα/sAPPβ’ levels were quantified by PhosphorImager and shown as mean values ± S.E. normalized to control, untreated cells. C, total sAPP was immunoprecipitated with Karen from media conditioned for 24 h by NT2− cells and replicate 3 NT2N neurons, corrected for cell lysate concentration or intracellular APP expression. Samples were then separated on a 7.5% Tris-glycine gel and immunoblotted with Ban50 (top panel), NAB228 (2nd panel), C5A4/2 (3rd panel), or C10A4 (bottom panel). The specific sAPP fragments recognized by these antibodies are labeled.

Stratifying that TAPI is able to inhibit PMA-induced α-secretase in both non-neuronal and neuronal cells. However, the inability of TAPI to decrease sAPP generation by NT2N neurons below base-line levels indicated that a relatively small proportion of APP is normally proteolysed by α-secretase in NT2N neurons.

Because Ban50 recognizes both sAPPα and sAPPβ’, the ability of Ban50 to immunoprecipitate TAPI-insensitive sAPP from NT2N neurons suggested that NT2N neurons may produce sAPPβ’ endogenously. Therefore, to demonstrate more directly the presence of BACE-derived sAPP fragments, we used a panel of antibodies that recognize different sAPP species to analyze media conditioned for 24 h by NT2− cells and NT2N neurons. To better compare the relative abundance of sAPP species, media samples were corrected for either lysate protein concentration or intracellular full-length APP levels prior to analysis. Despite preferential utilization of α-cleavage over β-cleavage in non-neuronal cells (13, 14), we detected less sAPP with Ban50 from NT2− cells compared with NT2N neurons (Fig. 2C, top panel), consistent with the possibility that NT2N neurons produce sAPPβ’ endogenously. This result was confirmed with a second monoclonal antibody, NAB228, that recognizes the first 11 amino acids of Aβ (Fig. 2C, 2nd panel). C5A4/2, a polyclonal antibody that specifically recognizes the C terminus of sAPPβ, showed the presence of sAPPβ in media conditioned by NT2N neurons (Fig. 2C, 3rd panel). In contrast, the amount of sAPPβ in media conditioned by NT2− cells was undetectable. Finally, a polyclonal antibody that specifically recognizes the C terminus of sAPPβ’ demonstrated the presence of endogenous sAPPβ’ produced by NT2N neurons (Fig. 2C, bottom panel). Therefore, not only is α-secretase activity relatively low in NT2N neurons, as determined pharmacologically, but BACE cleavage at both Asp-1 and Glu-11 is readily detected as a product of normal APP metabolism from NT2N neurons.

**BACE Expression in NT2− Cells and NT2N Neurons**—To better understand the secretion and intracellular generation of BACE-derived Aβ peptides in human neurons, we characterized BACE expression in NT2− cells and NT2N neurons. Expression of BACE mRNA was determined by Northern analysis of NT2− cells and NT2N neurons. BACE mRNA expression was clearly present in NT2N neurons, as seen by the presence of 7.0-, 4.4-, and 2.6-kb bands (Fig. 3A). NT2− cells, however, showed markedly less BACE expression, most notably demon-
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Fig. 3. BACE expression in NT2- cells and NT2N neurons. A, mRNA from NT2- cells and 3 replicate NT2N neurons were electrophoresed on formaldehyde-agarose gels and hybridized with a radiolabeled Pro1 BACE cDNA fragment and visualized by PhosphorImager. Equal mRNA loading was determined by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression and by 28 S and 18 S ribosome levels. B, crude membrane fractions from stably transduced NT2- cells and replicate 2 NT2N neurons expressing BACE (NT2-NT2B30-/-NT2B17-), undifferentiated cells; NT2N/NT2B30N/NT2B17N, neurons) were separated on a 7.5% Tris-glycine gel and immunoblotted for BACE. C, total sAPP from media conditioned for 24 h from NT2- cells or replicate 3 NT2N neurons was immunoprecipitated with Karen, electrophoresed on a 7.5% Tris-glycine gel, and immunoblotted for sAPPβ with C5A4/2, showing sAPPβ derived from either APP751/770 or APP695. D, C-terminal APP fragments were immunoprecipitated with 2493 from NT2- cells and replicate 2 NT2N neurons that were metabolically labeled for 2 h, separated on a 10/16.5% discontinuous gradient Tris-Tricine gel, and exposed to a PhosphorImager screen. The bands corresponding to C99, C89, and C83 are labeled.

Secrecion of Full-length and N-terminally Truncated β—Given the differences in endogenous BACE expression between NT2- cells and NT2N neurons, we sought to determine the effect of overexpressing BACE on β production in these cells and to demonstrate the presence of N-terminally truncated β peptides derived from BACE cleavage of APP at Glu-11. Media conditioned for 24 h were subjected to sandwich ELISA analysis and normalized for APP expression. Two sandwich ELISA systems were used in which either Ban50 (anti-β11–10) or BNT77 (anti-β11–28) monoclonal antibodies were used to capture β. The Ben50 ELISA was used to detect full-length β, whereas the BNT77 ELISA was used to detect both full-length and N-terminally truncated β species. Both ELISA systems demonstrated the production of β in NT2- cells upon the overexpression of BACE (Fig. 4A) at levels similar to that secreted by untransduced NT2N neurons. However, the effect of BACE overexpression was more pronounced in NT2N neurons, increasing β secretion 5–8-fold over untransduced NT2N neurons. Furthermore, the differences in β concentration as detected by Ban50 or BNT77 indicated that a large proportion of secreted β peptides are N-terminally truncated. To identify N-terminally truncated β peptides secreted by NT2N neurons, conditioned media were immobilized by 4G8 and subjected to MALDI-TOF mass spectrometry. A control mixture of synthetic β11–40 and β11–10 yielded peaks of expected mass (4329.27 and 3152.29 Da, respectively; Fig. 4B). Furthermore, both β11–40 and β11–40 were readily detected from untransduced NT2N neurons (4328.56 and 3151.57 Da, respectively; Fig. 4C) and BACE-overexpressing NT2N neurons (4329.91 and 3150.94 Da, respectively; Fig. 4D), consistent with the ELISA data. Other peaks corresponding to C-terminally truncated β (β11–34, β11–37, β11–38, and β11–39 with masses of 3784.71, 4076.02, 4132.14, and 4230.10 Da, respectively) were also identified in the mass spectra of neuronal medium (Fig. 4, C and D). However, since these C-terminally truncated β peptides contain intact N termini, the increased concentration detected by BNT77 is primarily due to the presence of β11–40/42 in culture media.

Intracellular Generation of Truncated β—Previous analysis of several cell lines indicated that both high expression of APP and high β-secretase activity were co-requisites for intracellular β detection (18, 28). We therefore tested whether BACE overexpression increases intracellular β in NT2N neurons. Because the BNT77 ELISA did not have the sensitivity required for accurate intracellular β quantification, a more...
sensitive ELISA was utilized in which Aβ peptides from cell lysates were captured with either JRF/cAβ40 or JRF/cAβ42, specific for Aβ40 and Aβ42, respectively. Captured peptides were detected with either JRF/AβN, recognizing Aβ1–7 (to measure full-length Aβ) or m266, recognizing Aβ13–28 (to measure full-length and N-terminally truncated Aβ). BACE overexpression resulted in the presence of intracellular Aβ from undifferentiated NT2B30 cells and NT2B17 cells, in contrast with NT2− and NT2G7− cells (Fig. 5). The amount of intracellular Aβ from BACE-expressing non-neuronal cells was comparable with the amount of intracellular Aβ from untransduced NT2N neurons. Similar to secreted Aβ, BACE overexpression resulted in a marked increase in intracellular Aβ in NT2N neurons. Furthermore, N-terminally truncated Aβ peptides comprised a large proportion of intracellular Aβ. The difference between Aβ concentration as determined by JRF/AβN and m266 indicated that N-terminally truncated Aβ is present intracellularly not only in BACE-overexpressing cells but in untransduced NT2N neurons as a result of normal metabolism of endogenous APP.

To demonstrate further the presence of intracellular N-terminally truncated Aβ species, NT2N, NT2B30N, and NT2B17N neurons were metabolically labeled, and both media and cell lysates were immunoprecipitated with 4G8. Due to the lower sensitivity of 4G8 immunoprecipitation, we overexpressed APP in NT2N neurons to increase the production of Aβ. As shown in Fig. 6A, overexpression of APP in NT2N neurons resulted in a large increase in secreted Aβ. However, by using both Ban50 and BNT77 ELISAs, we found that the relative amount of truncated Aβ secreted by NT2N neurons upon APP overexpression was reduced. That is, although 24.5% of Aβ produced from endogenous APP is truncated, only 8.7% of Aβ is truncated upon APP overexpression. Despite this relative decrease in truncated Aβ, immunoprecipitates from NT2N media revealed three bands (Fig. 6B) corresponding to Aβ1–40/42 (upper 4-kDa band), Aβ11–40/42 (middle 3.2-kDa band), and p3 (Aβ17–40/42, lower 3-kDa band). As expected, Aβ1–40/42 and Aβ11–40/42 were increased in a dose-dependent manner upon BACE expression, consistent with both the ELISA and mass spectral analysis. Immunoprecipitates from neuronal lysates demonstrated that full-length Aβ increased as a result of BACE expression. Furthermore, Aβ11–40/42 was also recovered from BACE-expressing NT2N cells lysates in a dose-dependent manner, as shown by the presence of a 3.2-kDa band that co-migrated with the Aβ11–40/42 recovered from media. Importantly, p3 was not detected from NT2N neuron lysates, indicating that p3 does not accumulate intraneuronally and that lysates were not contaminated with media. Thus, although the relative amount of truncated Aβ recovered by 4G8 immunoprecipitation was altered by APP overexpression, these results nonetheless confirm that N-terminal heterogeneity of Aβ is present in the intracellular processing pathway of APP and not solely due to partial degradation of secreted Aβ peptides.

**DISCUSSION**

Insoluble amyloid deposits from AD brains are heterogeneous in morphology and composition. Although the seeding and maturation of senile plaques in vivo is not well understood, increased β-cleavage has been implicated in the pathogenesis of AD, either due to a pathogenic mutation (K595N/M596L, APPsw) at the β-cleavage site of APP in familial AD (41–43) or by increased BACE expression in sporadic AD (29). Alternatively, other familial AD-associated mutations in either APP or presenilin increase the production of Aβ1–42 relative to Aβ1–40 (1). Despite the predominance of Aβ1–40 in cerebral spinal fluid (CSF) (44), Aβ1–42 is more abundant in senile formations.
plagues (3), consistent with its ability to aggregate more readily than Aβ1–40 in vitro (2). A third class of familial AD mutations, located in the middle of the Aβ domain, has been postulated to increase the amyloidogenicity of Aβ peptides (9). Interestingly, these mutations also appear to increase the production of Aβ11–40/42 (8, 9). Similar to C-terminal extensions to Aβ, N-terminal truncations have been shown to reduce solubility although increasing sedimentation and β-pleated sheet structure of Aβ peptides relative to full-length Aβ (45–47). Furthermore, cyclization of the N-terminal glutamate in Aβ11–40/42 protects the peptide from degradation by most aminopeptidases (48). Therefore, N-terminally truncated Aβ peptides may accelerate the seeding and maturation of senile plaques and thus exacerbate the progression of Alzheimer’s disease, particularly in some genetic settings.

Until recently, the mechanism whereby Aβ peptides are N-terminally truncated has been unclear. Aβ peptides beginning at Glu-11 were first identified from purification of Aβ peptides from human CSF (37) and are found in insoluble fractions from AD brain (5, 12). The discovery of BACE led to the realization that these cleavages are site specific (10, 23). Therefore, although rodent neuronal cells preferentially cleave endogenous APP at position 11 (22, 49), overexpression of human APP in rodent cells does not result in the formation of human Aβ11–40/42 (22). Many modified Aβ peptides accumulate in brains of tg2576 mice, a transgenic mouse model overexpressing APPsw, including isomerized Asp-1 (1, iso-Asp), stereoisomerized Asp-1 (rectus Asp), and pyroglutamated Glu-3. However, pyroglutamated Glu-11 is conspicuously absent from tg2576 brains (50). Therefore, although many of the mechanisms for N-terminal modification of human Aβ are present, the generation of Aβ11–40/42 is currently missing from both rodent cell culture and transgenic models. Given the limitations of rodent models, we investigated the generation of Aβ11–40/42 in human NT2N neurons. We found that the secretion of sAPP and Aβ correlates with the expression of BACE that occurs upon neuronal differentiation. The increased secretion of sAPP was predominantly due to the secretion of sAPPβ and sAPPβ′. Additionally, NT2N neurons produce the N-terminally truncated Aβ11–40/42 from normal metabolism of endogenous APP. Furthermore, exogenous BACE expression increased the secretion and intracellular generation of both Aβ1–40/42 and Aβ11–40/42. Interestingly, increasing APP expression decreased the relative amount of truncated Aβ produced by NT2N neurons. In contrast, non-neuronal cells with higher levels of BACE overexpression than reported here resulted in the preferential generation of N-terminally truncated Aβ over full-length Aβ (11, 12). Taken together, the ratio of APP to BACE expression may dictate the extent of β′-cleavage. Regardless, the intracellular generation of Aβ11–40/42 from normal APP processing in NT2N neurons indicates that this N-terminally truncated peptide is generated prior to deposition into insoluble aggregates in AD.

Additional Aβ peptides were recovered from NT2N neuron medium with truncated C termini. These C-terminally truncated Aβ species are also found in human CSF (51) and AD brain homogenates (5, 12), indicating that they may also contribute to amyloid formation. The close correlation between Aβ peptides found in NT2N neuronal medium and human CSF further validates the NT2N neuronal culture system as a useful model to study the generation of N- and C-terminally truncated Aβ peptides. Immunoprecipitation of intracellular Aβ from BACE-expressing NT2N neurons yielded a faint band slightly smaller than full-length Aβ (see Fig. 6). Although obscured somewhat by the intense signal derived from full-length Aβ, this truncated Aβ peptide appeared to be present in NT2N media samples, indicating that it corresponds to one of the C-terminally truncated Aβ peptides identified by mass spectrometry. APP and presenilin mutations that are known to affect C-terminal γ-secretase cleavage may influence each other. Importantly, the magnitude of the increase in Aβ production upon BACE expression indicates that the level of endogenous BACE expression in NT2– cells and NT2N neurons is rate-limiting in terms of Aβ generation. Although γ-secretase activity was not addressed directly in these experiments, the modest effect of BACE expression in non-neuronal NT2– cells compared with the effect of BACE expression in NT2N neurons indicates that γ-secretase cleavage is enhanced in neurons.

Although secretion of Aβ from neuronal cells is high relative to non-neuronal cells, the concentration of Aβ in human CSF is below the threshold for Aβ aggregation in vitro (2). The stability and insolubility of intraneuronal Aβ lead to the hypothesis...
that intracellular Aβ may be the source of Aβ aggregates that seed senile plaques. Indeed, insoluble Aβ accumulates in NT2N neurons with age in culture (28), and SDS-stable oligomeric Aβ is found intracellularly prior to secretion (52). Furthermore, prior to the presence of amyloid pathology, Aβ can be detected biochemically from tg2576 mice (50) and patients with early cognitive dysfunction (53). Intracellular Aβ has been found in affected brain regions in AD brains (54–56) and in animal models of AD amyloid pathology (57–60). Finally, mRNA iso-

lated from senile plaques is predominantly neuronal (61). These reports suggest that the nidus for senile plaque formation may be intraneuronal Aβ. Interestingly, although various N-terminally truncated Aβ species, including Aβ11–40/42, are readily detected from detergent-insoluble preparations from AD brain, p3 is not detected (5). p3 is a major component of diffuse plaques in AD (62, 63) and in diffuse plaques in the cerebellum of Down’s syndrome patients (64). However, cerebellar diffuse plaques of Down’s syndrome patients do not progress to form neuritic senile plaques even though in vitro studies of the p3 peptide indicate that it is highly hydrophobic, capable of forming fibrils, and has the tinctorial properties of amyloid as determined by thioflavin T and Congo Red staining (64). We could not detect intracellular p3 from NT2N neurons, consistent with the generation of p3 at or near the plasma membrane (65, 66). These observations are also consistent with the hypothesis that the intracellular environment is necessary to convert fibrillogenic Aβ peptides into a nidus for senile plaque formation.

Multiple subcellular sites are responsible for the production of different Aβ peptides. The trans-Golgi network produces predominantly Aβ1–40 (67, 68), although the endoplasmic reticulum/intermediate compartment produces Aβ1–42 (28, 31, 68, 69). Endoplasmic reticulum/intermediate compartment-derived Aβ1–42 is not secreted but rather is retained intracellularly and contributes to the accumulation of a pool of insoluble Aβ that can be recovered with formic acid. Unfortunately, the sandwich ELISAs used in this study either do not have the sensitivity (BNT77) or are incompatible (JRF/A6N and m266) with formic acid lysates. However, the increased production of intracellular Aβ upon BACE expression is expected to increase the accumulation of insoluble full-length and truncated Aβ peptides. Production of endoplasmic reticulum/intermediate compartment-derived Aβ is independent of presenilin, indicating that multiple γ-secretases may be responsible for γ-secretase cleavage in different subcellular organelles (15). In contrast, BACE-deficient untransduced NT2− cells do not have appreciable levels of intracellular Aβ, although BACE overexpression increases intracellular Aβ. Therefore, BACE appears to be responsible for both secreted and intracellular Aβ. The extent of β-cleavage is also dependent on the subcellular localization of BACE and APP in 293 cells (12). The engineering of BACE-overexpressing NT2N neurons allows for future investigations into the subcellular site of Aβ11–40/42 generation in neuronal cells. However, the downstream effect of Aβ11–40/42 generation on plaque formation awaits the engineering of transgenic mice co-expressing human BACE and human APP.

Increased BACE expression has been implicated in the pathogenesis of AD (29). Interestingly, BACE expression had a more profound effect on Aβ generation in NT2N neurons than in non-neuronal NT2− cells. Therefore, even modest increases in BACE expression may precipitate amyloid formation due to overproduction of Aβ. Conversely, mild inhibition of BACE activity may have a large effect on Aβ generation, underscoring the possibility of using BACE inhibitors as a therapy for AD. However, given the endogenous production of Aβ11–40/42 by human NT2N neurons, the effect of BACE inhibitors on both full-length and N-terminally truncated Aβ peptides needs to be determined.

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