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Human neurons derived from a teratocarcinoma cell line express solely the 695-amino acid amyloid precursor protein and produce intracellular β-amyloid or A4 peptides

(amyloid/NTera2 cells/Alzheimer disease/cell culture)

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ABSTRACT **The β-amyloid or β/A4 peptides that accumulate as filamentous aggregates in the extracellular space of Alzheimer disease (AD) brains are derived from one or more alternatively spliced amyloid precursor proteins (APPs). The more abundant APPs in the central nervous system are the 695- (APP695), 751- (APP751), and 770- (APP770) amino acid isoforms, and each could be the source of β/A4 peptide that accumulates in the AD brain. It is plausible that altered metabolism of these APPs by central nervous system neurons could lead to the release and deposition of β/A4 peptide in brain parenchyma. Thus, we examined the expression and processing of the three major brain APPs in nearly pure human neurons (NT2N cells) derived from a teratocarcinoma cell line (NTera2/c1.D1 or NT2 cells) after retinoic acid treatment. NT2N neurons expressed almost exclusively APP695, whereas NT2 cells expressed predominantly APP751/770. Furthermore, the processing of the APPs in NT2N cells was distinct from NT2 and nonneuronal cells. Most significantly, the NT2N neurons but not the NT2 cells constitutively generated intracellular β/A4 peptide and released it into the culture medium. This work demonstrates the intracellular production of β/A4 peptide and suggests that cultured NT2N cells may provide a unique model system for understanding the contribution of neurons and APP695 to amyloidogenesis in the AD brain.**

Alzheimer disease (AD), the most common human neurodegenerative disorder, is characterized by the formation of amyloid plaques containing deposits of the β-amyloid or A4 peptide (β/A4 peptide) in selected regions of the brain (1). The β/A4 peptide is derived from one or more amyloid precursor proteins (APPs), all of which are integral membrane, tyrosine-sulfated, glycoproteins that span the membrane once and have an extracytoplasmic N terminus (2–5). The β/A4 peptide is a 39- to 43-amino acid internal sequence that extends from within the transmembrane domain into the extracytoplasmic domain of each APP. The three major central nervous system (CNS) APP isoforms are encoded by the same gene on chromosome 21 (6), and alternative mRNA splicing generates 695- (APP695), 751- (APP751), and 770- (APP770) amino acid APPs (7–12). The two larger isoforms contain a 56-amino acid insert that is homologous to a protease inhibitor of the Kunitz type (KPI) (13). APP770 contains an additional 19-amino acid insert adjacent to the KPI motif. Although APP mRNA is found outside the nervous system, the brain is the richest source of APPs and APP695 is restricted almost exclusively to the CNS and the peripheral nervous system (7, 8, 14).

To understand how amyloid plaques develop in the AD brain, it is important to identify the pathway(s) that leads to generation of β/A4 peptide from APPs. Recent studies indicate that the "endosomal/lysosomal" pathway generates a number of C-terminal APP fragments *in vivo* and *in vitro* and some of these fragments contain the entire β/A4 peptide (15–18). Furthermore, a 4-kDa peptide identical to the β/A4 peptide found in AD amyloid has been recovered from medium conditioned by transfected neural and nonneuronal cells as well as from the cerebrospinal fluid (19, 20). This implies that the secretion of β/A4 peptide is part of a normal constitutive processing pathway. In addition, transfection of a familial AD APP mutant gene into neural and nonneuronal cells resulted in the increased release of β/A4 peptide (21, 22). Despite the recovery of β/A4 peptide from medium cultured by transfected cells, intracellular β/A4 peptide has not been detected in any cell systems analyzed thus far.

Neurons produce large amounts of APP mRNA, particularly the KPI-free APP695 form, and APPs undergo fast axonal transport (23). Since it is reasonable to propose that neurons may be the source of the β/A4 peptide deposited as amyloid in AD, it is important to study APP expression and processing in neurons. Furthermore, AD only occurs in humans and not in any other species such as rodents. Thus, it is important to study APP processing in human neurons. However, postmitotic mature human neurons are difficult to isolate and maintain in culture for such studies.

To circumvent this difficulty, we have investigated APP expression and processing in NTera2/c1.D1 (NT2) cells, a human teratocarcinoma cell line that is induced by treatment with retinoic acid to commit irreversibly to a neuronal phenotype (24–26). These cells (NT2N cells) were shown previously to resemble primary cultures of rodent CNS neurons (27). For example, they are permanently postmitotic and develop functional dendrites and axons (27). Although previous studies with the P19 mouse teratocarcinoma cell line demonstrate changes in C-terminal fragment expression and a shift from APP751/770 to APP695 upon treatment with retinoic acid, these cultures usually contain, in addition to neurons, astrocytes and cells of other phenotypes (28–30). In contrast, NT2N cells can be purified to yield >99% pure postmitotic and differentiated immature human neurons in culture (27), and they can be produced in large quantities required for biochemical studies of APP processing. Thus, comparison of differentiated NT2N and undifferentiated NT2 cells affords a unique opportunity to gain insight into the

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Abbreviations: AD, Alzheimer disease; β/A4 peptide, β-amyloid or A4 peptide; APP, amyloid precursor protein; CNS, central nervous system; KPI, Kunitz type protease inhibitor; RT, reverse transcriptase.

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changes in APP expression and processing that are associated with the human neuronal phenotype.

MATERIALS AND METHODS

Cell Culture. The NT2 cells were grown and maintained as described (27). For studies involving NT2N cells, only Replate no. 3 cells (>99% pure neuronal cultures) were used. This step is essential in order to eliminate any contamination with NT2 cells that may confound the interpretation of APP processing in NT2N cells.

Western Blot Analysis. Whole cell homogenates of NT2 cells were prepared by washing culture dishes twice in phosphate-buffered saline before treatment with hot homogenizing buffer (Laemmli sample buffer) and before they were scraped into microcentrifuge tubes. The samples were subsequently boiled for 10 min, and protein concentrations were determined by a Coomassie blue dye binding assay (Pierce). These samples were run on SDS/7.5% polyacrylamide gels, electroblotted to nitrocellulose membranes (Schleicher & Schuell), and probed with antibodies as described (27, 31).

PCR Analysis of Reverse-Transcribed RNA. Isolation of RNA, reverse transcription, and PCR amplifications were performed as described (7). Total RNA from NT2 cells, NT2N neurons, human meninges, or human hippocampus was reverse transcribed by using Moloney murine leukemia virus reverse transcriptase (MLV RT; BRL). In each reaction mixture, 5 μg of RNA, 500 pmol of random hexamers (Boehringer Mannheim), and 10 μl of 5× RT buffer (BRL) were mixed in a total vol of 40 μl. The mixture was then heated to 95°C for 2 min and cooled on ice. Subsequently a 10-μl mixture containing dNTPs, MLV RT, and RNasin (Promega) was added such that the final 50-μl reaction mixture contained 1000 units of MLV RT, 500 μM dNTPs, and 20 units of RNasin. After mixing, the mixture was incubated for 10 min at 23°C and 50 min at 42°C. Finally, the RT was inactivated by heating at 65°C for 10 min. Amplifications were carried out in the presence of 50 μM dNTPs, 0.5 μCi of [α-^{32}P]dATP per ml (3000 Ci/mmol; 1 Ci = 37 GBq), 0.5 μM each specific primer (APP −1213 and APP +958; ref. 7), and 0.25 unit of *Taq* I polymerase per ml. The reaction buffer was 50 mM KCl/10 mM Tris·HCl, pH 8.3/1.5 mM $MgCl_2$. The reaction volume was 50 μl and the reaction mixture was overlaid with 100 μl of mineral oil to prevent evaporation. Amplifications (25 cycles) were performed in an Ericomp thermal cycler with 1 min of denaturation at 94°C, followed by 30 sec of annealing at 60°C and extension at 72°C for 1 min 30 sec.

Metabolic Labeling and Immunoprecipitation. Cultured NT2 cells were incubated in Dulbecco's modified Eagle's medium (DMEM) (GIBCO) without methionine or serum for 20 min before the addition of DMEM with 100 μCi of [^{35}S]methionine per ml (ICN). In the pulse–chase studies of full-length APPs, NT2 and NT2N cells were labeled for 15 min, washed twice, chased in complete DMEM for 0–8 hr, and then lysed as described (16). For the detection of β/A4 in NT2N cells and in medium, NT2 and NT2N cells were labeled continuously for 24 hr and both the cells and the medium were processed separately. The conditioned medium from the cells was centrifuged for 30 min at 100,000 × *g* and proteins in the supernatant were precipitated with equal volumes of saturated ammonium sulfate at 4°C overnight. After a high-speed spin, the pellet was resuspended in 1× RIPA buffer (16), followed by immunoprecipitation as described (16). Full-length APP and C-terminal APP fragments were separated by standard SDS/7.5% PAGE as well as with 16.5% high resolution Tris/Tricine gels (16). The gels were stained with Coomassie blue R (Pierce), treated with EN^{3}HANCE (NEN), dried, and then placed on PhosphorImager (Molecular Dynamics) plates for 72 hr to quantitate the amount of radioactivity. Alternatively, gels were transferred to Immobilon membranes (Schleicher & Schuell) for Western blot analysis. The gels were also analyzed by routine autoradiographic methods (27). Quantification of the amount of radioactivity in a given protein band was performed with the IMAGEQUANT software provided with the PhosphorImager.

RESULTS

NT2N Cells Decrease Expression of APP751/770 mRNAs and Predominantly Express APP695 After Treatment with Retinoic Acid. Using a modification of the method described by Golde *et al.* (7), we examined the relative abundance of the various APP mRNAs in NT2 and NT2N cells by reverse transcribing total RNA and then amplifying APP695, APP751, and APP770 cDNAs with primers flanking the adjacent alternatively spliced 168- and 57-nucleotide inserts found in APP751 and APP770 mRNAs. For comparison, human hippocampal RNA, which contains predominantly the APP695 form, and human meningeal RNA, which contains predominantly APP751/770 forms, were examined in parallel. This analysis (Fig. 1) showed that induction of the neuronal phenotype switches expression from predominantly APP751/770 to predominantly APP695 mRNA.

APP Processing in NT2 and NT2N Cells. Full-length APP in cell lysates and APP derivatives secreted into the medium were analyzed on immunoblots labeled with the monoclonal antibody LN21 (14, 32), which specifically recognizes the N terminus of all three forms of APP. As expected from our analysis of APP mRNAs, NT2 cells secreted a large ≈115-kDa APP derivative similar to that released by cells transfected with APP751 expression constructs and NT2N cells secreted an ≈95-kDa derivative similar to that released by cells expressing APP695 (Fig. 2*B*).

Examination of NT2N cell lysates showed a prominent ≈95-kDa band similar to the immature full-length APP695 that has been identified in many cell lines and a far less abundant ≈110-kDa form. The ≈110-kDa (Fig. 2*A*) form probably represents immature APP751/770 present in contaminating nonneural NT2 cells because its level correlated with the percentage of contaminating cells. However, some of it is also likely to be mature fully glycosylated APP695 in NT2N cells. The paucity of mature APP695 in NT2N cells is noteworthy because previous studies have shown this form to be abundant in the other cell lines that express APP695 (5, 16, 17, 33). In undifferentiated NT2 cells, the ≈95- and ≈110-kDa

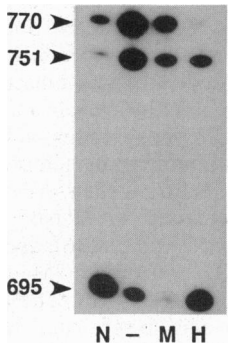

FIG. 1. NT2N neurons downregulate APP mRNA containing the KPI domain when they are induced to differentiate from NT2 cells with retinoic acid. cDNAs representing the APP695 (87 bp), APP751 (255 bp), and APP770 (312 bp) mRNAs were obtained by reverse transcription and amplification of APP mRNAs with primers flanking the adjacent, alternatively spliced 168- and 57-nucleotide inserts. Note that NT2N cells (lane N) are similar to human hippocampus (lane H) in that they express primarily APP695 mRNA, whereas NT2 cells (lane −) are similar to human meninges (lane M) and express predominantly APP751 and APP770 species.

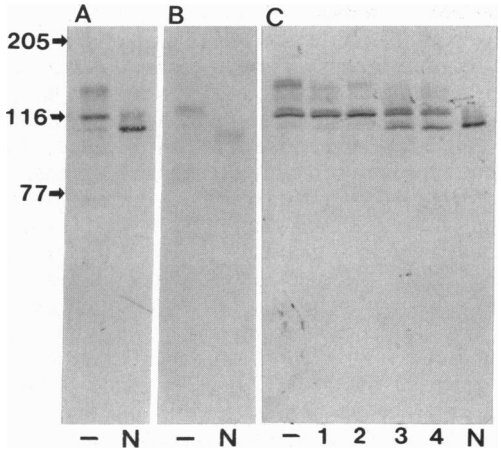

FIG. 2. NT2 cells and NT2N neurons display a different set of holo-APP glycoforms. (*A*) Twenty micrograms of NT2 (lane –) and NT2N (lane N) cell homogenates immunoblotted with LN21. (*B*) Ammonium sulfate precipitates of conditioned medium from ≈2 × 10^6 NT2 and NT2N cells immunoblotted with LN21. NT2N cells display predominantly N-glycosylated APP695, while NT2 cells display all three isoforms of APP in both N-glycosylated and fully mature states. (*C*) Immunoblot analysis with LN21 of APP glycoforms in NT2 (lane –) cells, during 4 consecutive weeks of retinoic acid treatment (lanes 1–4) and in NT2N (lane N) neurons at 20 μg of total cell homogenate per lane. During neuronal induction of NT2 cells with retinoic acid, nascent NT2N neurons begin to produce relatively abundant quantities of APP695 by ≈3 weeks, and this occurs concomitant with reduced glycosylation of the larger isoforms. All lanes are from SDS/7.5% polyacrylamide gels. Molecular mass markers are in kDa as indicated.

forms were also present but immature APP695 (≈95 kDa) was detected in trace amounts only, consistent with the results of our mRNA analysis, and the ≈110-kDa species, presumably immature APP751/770, was the major form detected. In addition, NT2 cells contained a 125-kDa APP similar to the mature, fully glycosylated APP751/770 form identified in other cells expressing KPI-containing APPs. Analysis of lysates with two other N-terminal-specific monoclonal antibodies, LN27 and LN39, and with antibodies specific to the C terminus of APP yielded similar results (data not shown). Thus, all of the 95- to 125-kDa proteins in NT2 and NT2N cells (Fig. 2*A*) are full-length APPs.

Analysis of the time course of the effect of retinoic acid on APP expression and processing (Fig. 2*C*) showed a dramatic increase in APP695 by 3 weeks, which correlated with the increased number of neuron-like NT2N cells. In contrast, mature APP751/770 (≈125 kDa) diminished dramatically after 1 week of treatment.

The Half-Life of APPs in the NT2N Cells Is Prolonged. To further analyze APP processing, NT2 and NT2N cells were pulse labeled with [^{35}S]methionine for 15 min and chased for up to 8 hr (Fig. 3). Radiolabeled APPs were then immunoprecipitated from whole-cell lysates using LN21, separated by SDS/7.5% polyacrylamide gels, and analyzed quantitatively with a PhosphorImager system (Molecular Dynamics). In NT2 cells, maturation of APP751/770 to its fully glycosylated 125-kDa form was evident in the first hour of chase, then there was substantial degradation between 1 and 4 hr of chase, and APP was barely detectable by 8 hr of chase (Fig. 3*A*). In contrast, APP processing in the NT2N cells was strikingly different. Virtually all of the APP labeled in 15 min was the 95-kDa immature APP695, and only trace amounts matured to a 110-kDa form during the first hour of chase (Fig. 3*B*). Turnover of the 95-kDa form was slow, with 75% remaining after 1 hr of chase and detectable levels remained even after 8 hr. Quantitative analysis indicated that APP

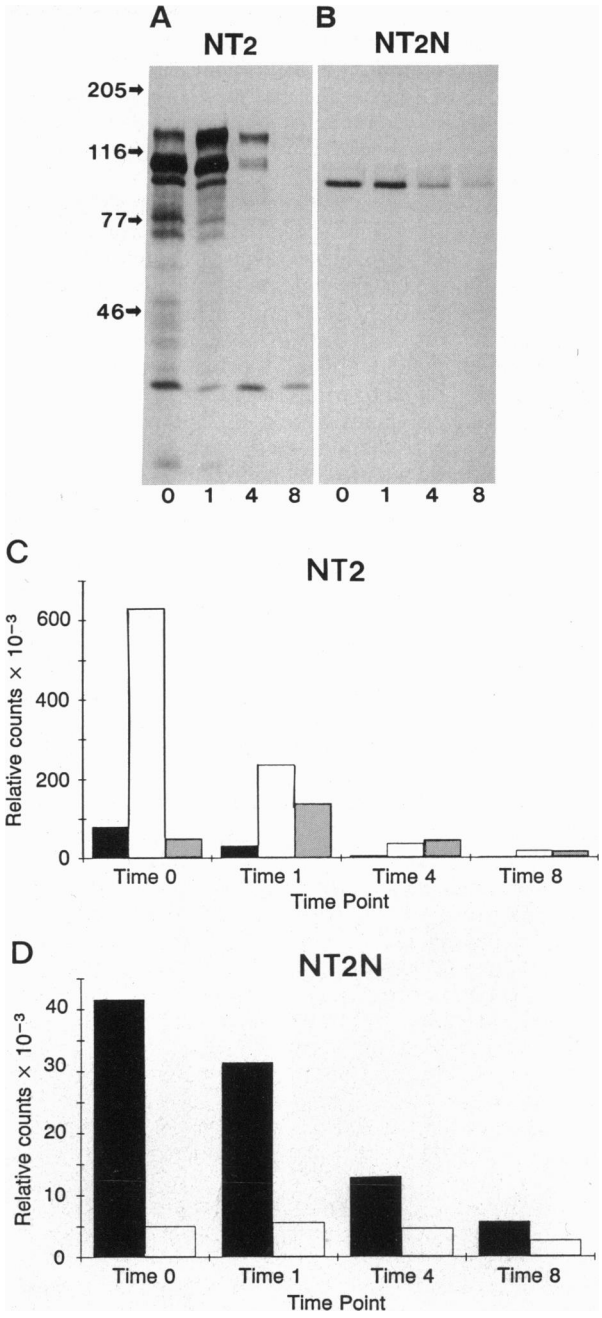

FIG. 3. Determination of half-life and glycosylation state of holo-APPs in NT2 cells and NT2N neurons. Tissue culture dishes (6 cm) of NT2 and NT2N cells containing ≈0.7 × 10^6 cells were metabolically radiolabeled with 100 μCi of [^{35}S]methionine per ml during a 15-min pulse followed by a chase in complete medium for times that extended up to 8 hr as indicated below each lane. APP glycoforms were immunoprecipitated with LN21 and goat anti-mouse IgG coupled to agarose beads. Samples were separated on SDS/7.5% polyacrylamide gels. (*A*) O-Glycosylation of APP751/770 in the NT2 cells is evident at time 0 before chasing as represented by the 125-kDa band. O-Glycosylation of APP695 is not discernible due to the comigration of N-glycosylated APP751/770 at 110 kDa. N-Glycosylated APP695 is visualized at 95 kDa. The half-life of all species is ≈1 hr. (*B*) NT2N cells display almost exclusively N-glycosylated APP695 throughout the time course with only minor O-glycosylation. The half-life of APP695 is ≈3 hr. (*C* and *D*) Summary of pulse–chase in NT2 and NT2N cells, respectively. Ordinate values indicate average PhosphorImager volume integration counts of specific bands averaged over three separate experiments; standard deviations were consistently <7.5%. Solid bars, 95 kDa; open bars, 110 kDa; shaded bars, 125 kDa.

holoprotein turns over with a half-life of ≈1 hr in NT2 cells and ≈3 hr in NT2N cells (Fig. 3 *C* and *D*). We also estimated the amount of secreted APP N-terminal fragments accumulated after a 15-min pulse and an 8-hr chase and found that only low levels of radiolabeled N-terminal derivatives are secreted (data not shown). These results are similar to those reported for microglia and astrocytes (34).

A β/A4-like Fragment Is Recoverable from NT2N Neuron Cell Lysates and Conditioned Medium. To determine whether NT2N or NT2 cells produce and release a soluble 4-kDa β/A4 peptide (19, 20), these cells were metabolically labeled for 24 hr and the presence of β/A4 peptide in conditioned medium and cell lysate was analyzed. In both medium and lysates from NT2N cells, we identified an ≈4-kDa protein immunoprecipitated by an antiserum raised to $\beta/A4_{1-40}$ peptide (SGY2134; Fig. 4*B*, lanes 3′ and 6′) but not by LN23, a monoclonal antibody that detected amino acid residues just beyond the N terminus of β/A4 peptide (Fig. 4*B*, lane 4′) or anti-C15, an antiserum to the last 15 amino acids in the APP (Fig. 4*B*, lane 5′). The antiserum SGY2134 was shown previously to bind most avidly to amino acids 1–17 of β/A4 peptide and was used to immunoprecipitate authentic β/A4 peptide from human cerebrospinal fluid as well as from conditioned medium from cultured cells (20, 21). Furthermore, β/A4 peptide immunoprecipitated from NT2N cells comigrated with synthetic $\beta/A4_{1-40}$ and $\beta/A4_{1-42}$ peptides since we cannot resolve the differences in the electrophoretic mobility of these two peptides in our gel system (data not shown). Five other antisera raised to synthetic $\beta/A4_{1-42}$ peptide were also capable of immunoprecipitating β/A4 peptide from medium and lysate from NT2N cells, and we have observed the production and release of this 4-kDa β/A4 peptide in four identical experiments. Furthermore, the 4-kDa β/A4 peptide can be detected by Western blot analysis (data not shown). Although similar numbers of NT2 cells were examined, β/A4 peptide could not be detected in either conditioned medium or cell lysates (Fig. 4, compare lanes 2 and 2′ with lanes 3, 3′, 6, and 6′). Thus, our data indicate that induction of the neuronal phenotype in the NT2 line alters APP processing and dramatically increases the synthesis and the release of the 4-kDa soluble β/A4 peptide. PhosphorImager analysis showed that the 4-kDa β/A4 peptide accumulated over a 24-hr period represents 7.8% ± 4.2% of the total [^{35}S]methionine (n = 12; Fig. 4*B*, lanes 3′ and 6′; data not shown) released by the NT2N cells. Since β/A4 peptide contains only one methionine residue and the N terminus derivatives contain 17 methionine residues, this suggests that extraordinary amounts of β/A4 peptide are released into the medium.

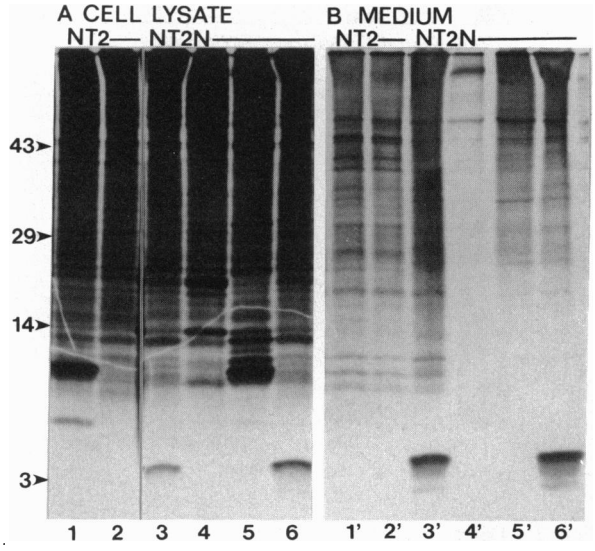

FIG. 4. NT2N neurons secrete a β/A4-like fragment. Tissue culture dishes (10 cm) containing ≈2 × 10^6 cells of NT2 and NT2N cells were metabolically radiolabeled with 100 μCi of [^{35}S]methionine per ml for 24 hr. Proteins from conditioned medium were recovered by adding an equal volume of saturated ammonium sulfate and incubating at 4°C for 2 hr followed by centrifugation at 100,000 × *g* for 30 min. APP isoforms and fragments were immunoprecipitated with SGY2134, LN23, or anti-C15 antiserum. Proteins were separated on 16.5% Tris/Tricine gels, which were then analyzed by PhosphorImager screens and conventional autoradiography. Molecular mass markers are in kDa. (*A*) Cell lysates. (*B*) Ammonium sulfate-precipitated conditioned medium. Lanes: 1 and 1′, NT2 cells immunoprecipitated with anti-C15 antiserum; 2 and 2′, NT2 cells immunoprecipitated with SGY2134; 3 and 3′, NT2N neurons immunoprecipitated with SGY2134; 4 and 4′, NT2N neurons immunoprecipitated with LN23; 5 and 5′, NT2N neurons immunoprecipitated with anti-C15 antiserum; 6 and 6′, NT2N neurons immunoprecipitated with SGY2134. The culture of NT2N neurons used in lane 3 was 6 weeks old, whereas the cultures used in lanes 4–6 were 5 weeks old.

DISCUSSION

This study demonstrates the intracellular generation of β/A4 peptide in cultured human neurons—i.e., NT2N cells. This is significant since no other cell line has previously been shown to produce β/A4 peptide intracellarly, and neurons are one of the major CNS cell types suspected to be the source of amyloidogenic β/A4 peptide. Furthermore, we also provide evidence that β/A4 peptide is released into the culture medium by NT2N cells. Our observation that lower levels of β/A4 peptide are recovered inside NT2N cells and higher amounts of this peptide are secreted into the medium suggests that β/A4 peptide is a cleavage by-product in NT2N cells that is released rapidly once it is produced. These observations together with the exclusive expression of APP695 in the postmitotic NT2N cells and other characteristics of these cells (27) suggest that the NT2N cells faithfully recapitulate the phenotype of human neurons *in vivo* and hence may be a unique model system in which to dissect the role of neurons in amyloidogenesis.

Our data on β/A4 metabolism suggest that the intracellular production of β/A4 peptide and its subsequent release is part of a constitutive APP processing pathway in neurons. It is unclear at the present time where β/A4 peptide is generated in the NT2N neurons prior to secretion. Recent evidence suggests that β/A4 peptide could be produced by the endosomal/lysosomal pathway in neural and nonneuronal cells (20). However, a second secretory pathway may be involved in β/A4 peptide genesis (35). The generation of β/A4 peptide by both of these pathways would involve rapid O-glycosylation and efficient translocation of mature APPs to the cell surface followed by either membrane cleavage or reinternalization of the holo-APP into endosomes. Our observation that the O-glycosylation of APP695 proceeds rather slowly in NT2N cells and that most of the APP695 is degraded before becoming fully glycosylated suggests that most neuronal APP695 may not be translocated to the cell surface but is routed almost entirely within the neuron until it reaches its site(s) of proteolysis. Future studies need to directly address whether or not β/A4 peptide is generated by cell-surface secretase cleavage, by the endosomal/lysosomal pathway that requires reinternalization of the mature protein, or by other yet to be identified pathways. Our ability to detect intracellular β/A4 peptide in NT2N cells would provide a unique system to address where and through what mechanism β/A4 peptide is generated before it is released into the medium.

Our analysis of APP expression and processing in differentiated NT2N and undifferentiated NT2 cells suggests that the NT2/NT2N system should be useful for our understand-

ing of APP metabolism. NT2 cells express mostly mature APP751/770, whereas NT2N neurons express predominantly immature APP695. APP751/770 in NT2 cells turns over 3 times faster than APP695 in NT2N cells. Although both NT2 and NT2N cells secrete low levels of N terminus derivatives, NT2N but not NT2 cells produce and secrete β/A4 peptide. However, the low level of secretion of N terminus derivatives from both NT2 and NT2N cells suggests that much of the APP holoproteins in these cells may be processed intracellularly.

Finally, our observation that normal human NT2N neurons are capable of secreting β/A4 peptide suggests that age-related or genetic perturbation of this normal pathway like the familial AD mutant reported recently could lead to increased secretion of β/A4 peptide into the extracellular space and the subsequent aggregation of β/A4 peptide into senile plaques. This implies that APP695 in neurons may be the source of the β/A4 peptide that accumulates as SPs. It is unclear at the present time exactly where β/A4 peptide is generated in the NT2N neurons prior to secretion. *In vivo* studies indicate that the APPs synthesized in neurons are transported within axons to synaptic terminals as full-length molecules where they are cleaved (33). Whether or not β/A4 peptide in NT2N cells is generated and secreted at the most distal region of axons remains to be determined. However, it is tempting to speculate that in the AD brain, β/A4 peptide is released at nerve terminals at sites where SPs are found. If this is the case, then the NT2N cells will provide an ideal model system in which to test this hypothesis.

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