

Detection of a Novel Intraneuronal Pool of Insoluble Amyloid β Protein that Accumulates with Time in Culture

Daniel M. Skovronsky, Robert W. Doms, and Virginia M.-Y. Lee

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

Abstract. The amyloid- β peptide ($A\beta$) is produced at several sites within cultured human NT2N neurons with $A\beta$ 1-42 specifically generated in the endoplasmic reticulum/intermediate compartment. Since $A\beta$ is found as insoluble deposits in senile plaques of the AD brain, and the $A\beta$ peptide can polymerize into insoluble fibrils in vitro, we examined the possibility that $A\beta$ 1-40, and particularly the more highly amyloidogenic $A\beta$ 1-42, accumulate in an insoluble pool within NT2N neurons. Remarkably, we found that formic acid extraction of the NT2N cells solubilized a pool of previously undetectable $A\beta$ that accounted for over half of the total intracellular $A\beta$. $A\beta$ 1-42 was more abundant than $A\beta$ 1-40 in this pool, and most of the insoluble $A\beta$ 1-42 was gen-

erated in the endoplasmic reticulum/intermediate compartment pathway. High levels of insoluble $A\beta$ were also detected in several nonneuronal cell lines engineered to overexpress the amyloid- β precursor protein. This insoluble intracellular pool of $A\beta$ was exceptionally stable, and accumulated in NT2N neurons in a time-dependent manner, increasing 12-fold over a 7-wk period in culture. These novel findings suggest that $A\beta$ amyloidogenesis may be initiated within living neurons rather than in the extracellular space. Thus, the data presented here require a reexamination of the prevailing view about the pathogenesis of $A\beta$ deposition in the AD brain.

ALZHEIMER'S disease (AD)¹ is characterized by accumulation of fibrillar amyloid- β peptides ($A\beta$) in senile plaques. That the accumulation of $A\beta$ is essential for the pathogenesis of AD is supported by genetic studies showing that mutations in the amyloid- β precursor protein (APP) (which gives rise to $A\beta$ through proteolytic processing) are linked to a subset of familial AD (FAD) cases with autosomal penetrance, and alter $A\beta$ production (reviewed in Selkoe, 1997). For example, the double mutation found in a Swedish FAD kindred leads to overproduction of $A\beta$, while other mutations alter the relative levels of the two major forms of $A\beta$, resulting in an increased $A\beta$ 1-42/1-40 ratio (Citron et al., 1992; Scheuner et al., 1996). Previous studies have shown that $A\beta$ 1-42 is more insoluble than the more abundant $A\beta$ 1-40, and that it is

the most prevalent $A\beta$ species found in senile plaques (Iwatsubo et al., 1994). Other FAD mutations that account for the majority of early-onset FAD cases have been linked to the *Presenilin 1* (*PS1*) and *Presenilin 2* (*PS2*) genes (Levy-Lahad et al., 1995; Sherrington et al., 1995). Mutations in these genes, like some of those in the APP gene, also increase the $A\beta$ 1-42/1-40 ratio (Borchelt et al., 1996; Duff et al., 1996; Scheuner et al., 1996).

Since genetic studies have established a role for $A\beta$ in the pathogenesis of AD, it is essential to understand how $A\beta$ is produced from APP. For example, it has been shown that APP is cleaved by β -secretase(s) to generate the NH₂ terminus of $A\beta$, and by γ -secretase(s) to generate the COOH terminus of $A\beta$ (Haass et al., 1992; Shoji et al., 1992). These cleavages may occur in a variety of subcellular locations, including the endoplasmic reticulum/intermediate compartment (ER/IC; Chyung et al., 1997; Cook et al., 1997; Hartmann et al., 1997; Xu et al., 1997), the trans-Golgi network (TGN; Xu et al., 1997), and the endosomal/lysosomal system (Koo and Squazzo, 1994). Whereas $A\beta$ produced by these pathways may be secreted (as has been shown for TGN-generated $A\beta$) or may remain intracellular (as has been shown for $A\beta$ generated by the ER/IC pathway), the relative roles of intracellular and secreted $A\beta$ in the pathogenesis of AD remain to be determined.

Address all correspondence to Dr. Virginia M.-Y. Lee, Center for Neurodegenerative Disease Research, Department of Pathology and Laboratory Medicine, Maloney 3, HUP, Philadelphia, PA 19104-4283. Tel.: 215-662-6427; Fax: 215-349-5909; E-mail: vmylee@mail.med.upenn.edu

1. *Abbreviations used in this paper:* $A\beta$, amyloid- β peptide; AD, Alzheimer's disease; APP, amyloid- β precursor protein; BHK, baby hamster kidney; ER/IC, endoplasmic reticulum/intermediate compartment; FAD, Familial Alzheimer's disease; SFV, Semliki Forest Virus; SFV-APPwt, SFV expressing wild-type APP695; TGN, trans-Golgi network.

While numerous studies have documented that nonneuronal cells engineered to express APP secrete both A β 1-40 and A β 1-42, intracellular A β is not commonly seen in these cells (Forman et al., 1997; Xu et al., 1997). However, intracellular A β can be detected readily in human NT2N neurons after metabolic labeling, and its production precedes that of secreted A β (Wertkin et al., 1993; Turner et al., 1996). Analysis of intracellular A β by ELISA indicates that intracellular and secreted A β are composed of different ratios of A β 1-42/1-40, with A β 1-40 being more prevalent in secreted material (Turner et al., 1996). In addition to being produced by mechanisms with different time courses, and being composed of different proportions of A β 1-40 and A β 1-42, intracellular and secreted A β can be produced by different pathways in NT2N neurons. Recent studies have shown that A β 1-42, but not A β 1-40, is produced by an ER/IC pathway, and that this pathway does not contribute to the secreted pool of A β (Cook et al., 1997). Finally, secretion of A β by NT2N neurons increases with time in culture (Turner et al., 1996). An age-dependent increase in A β secretion by neurons *in vivo* may play a role in the deposition of A β into senile plaques in the extracellular space of the brain during normal aging and in AD, as well as in the cortex and hippocampus of transgenic mice that overexpress mutant forms of APP (Games et al., 1995; Hsiao et al., 1996).

In addition to forming insoluble extracellular plaques, A β may also accumulate intracellularly in an aggregated insoluble pool. For example, exogenous A β 1-42 added to culture medium can be taken up by cells, after which it can be solubilized only by formic acid extraction (Knauer et al., 1992; Yang et al., 1995). Thus, these findings raise the possibility that endogenously produced intracellular A β may aggregate within neurons as well. Because formic acid is required to solubilize A β from senile plaques, we sought to detect the presence of insoluble A β within NT2N neurons and other cell lines by formic acid extraction, and found that a significant fraction of the total intracellular A β , particularly A β 1-42, was retained as an insoluble pool within these cells. Further, this insoluble pool of A β increased 12-fold in postmitotic NT2N neurons over a period of 7 wk in culture. Since the prevailing view of amyloidogenesis in AD is that plaque formation is initiated in the extracellular space by secreted A β , our findings challenge this assumption by implicating the intracellular compartment as a site where A β may accumulate in an insoluble form.

Materials and Methods

Cell Culture

NT2 cells derived from a human embryonal carcinoma cell line (Ntera2/cl.D1) were grown and passaged as described previously (Pleasure et al., 1992; Pleasure and Lee, 1993). Cells were differentiated by two weekly retinoic acid treatments (10 μ M) for 5 wk, and were replated (replate 2 cells) in the presence of mitotic inhibitors to yield nearly pure NT2N neurons (Pleasure et al., 1992). To obtain 99% pure neurons (replate 3 cells), replat 2 cells were removed enzymatically and mechanically, and were replated in 10-cm dishes (Pleasure et al., 1992). Cultures of Replate 2 or Replate 3 NT2N cells were used for experiments when they were 3–4 wk old unless otherwise indicated. CHO Pro5 cells were grown and passaged three times per week in Alpha-MEM (Life Technologies, Inc., Gaithersburg, MD) containing 10% FBS and penicillin/streptomycin. Baby ham-

ster kidney (BHK-21) cells were grown and passaged three times per week in Glasgow MEM (Life Technologies, Inc.) supplemented with 10% tryptose phosphate, 5% FBS, and 0.02 M Hepes. CHO-695 cells were obtained from Dr. S.S. Sisodia, and were grown and passaged as described above for CHO Pro5 cells with the addition of 0.2 mg/ml of G418 to the culture medium.

Preparation of Semliki Forest Virus and Infection of Cultured Cells

Semliki Forest Virus (SFV) expressing wild-type APP695 (SFV-APPwt) or an APP mutant in which the third and fourth amino acids from the carboxyl terminus of APP have been changed to lysines (SFV-APP Δ KK) were prepared and titered as previously described (Chyung et al., 1997; Cook et al., 1997). CHO-Pro5, BHK-21, NT2, and NT2N cells were infected in serum-free medium at a multiplicity of infection of \sim 10. After 1 h, complete growth medium was replaced and infection was allowed to proceed for 12 h.

Metabolic Labeling, Immunoprecipitation, and Gel Electrophoresis

Cultured NT2N cells were methionine-deprived by incubation in methionine-free DMEM (Life Technologies, Inc.) for 30 min before adding [35 S]methionine (500 μ Ci/ml in methionine-free DMEM + 5% dialyzed FBS; DuPont-NEN, Boston, MA) for a 12-h labeling period. Cells were washed twice in PBS and lysed in 600 μ l RIPA buffer (0.5% sodium deoxycholate, 0.1% SDS, 1% NP40, 5 mM EDTA in TBS, pH 8.0) with a cocktail of protease inhibitors (1 μ g/ml each of Pepstatin A, Leupeptin, TPCK, TLCK, STI, and 0.5 mM PMSF). After brief sonication, cell lysates were centrifuged at 40,000 g for 20 min at 4°C, and the supernatant was subjected to immunoprecipitation with 6E10 (a monoclonal antibody specific for A β 1-17; Kim et al., 1988) as previously described (Turner et al., 1996). The remaining pellets were resuspended in 100 μ l 70% formic acid and sonicated until clear. For direct extraction into formic acid, cells were scraped in 1 ml PBS, pelleted by centrifugation, and lysed in 100 μ l of 70% formic acid with sonication. Formic acid from both directly extracted and sequentially extracted samples was removed by vacuum centrifugation for 40 min, and the resulting dry pellet was resuspended in 100 μ l of 60% acetonitrile. RIPA buffer (1.9 ml) was added to each of the samples before they were subjected to immunoprecipitation with 6E10. Immunoprecipitated A β was resolved on a 10/16.5% step gradient Tris-Tricine gel, fixed in 60% methanol, dried, and placed on PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA) plates for 72 h.

Trypsin Treatment of CHO Cells

CHO Pro5 cells were infected with SFV-APPwt for 12 h, rinsed twice in PBS, and incubated on ice for 20 min in either PBS alone, 10 μ g/ml of trypsin (Life Technologies, Inc.) in PBS, or 10 μ g/ml trypsin plus 0.1% Triton X-100 in an adaptation of a previously described technique (Turner et al., 1996; Chyung et al., 1997). Trypsin was then inactivated by adding 100 μ g/ml soybean trypsin inhibitor. The treated cells were then washed with ice-cold PBS, scraped into PBS buffer, centrifuged at 2,000 g for 2 min, resuspended in 100 μ l formic acid, sonicated, and centrifuged at 40,000 g for 20 min at 4°C. The supernatant was neutralized with 1.9 ml of 1 M Tris base and diluted 1:3 in H₂O for quantification of A β 1-40 and A β 1-42 by sandwich-ELISA.

Lysis of Cells and Sandwich ELISA

For serial extraction in RIPA and formic acid, cells were washed twice in PBS and then lysed in 600 μ l RIPA buffer and centrifuged for 20 min at 40,000 g at 4°C. Supernatant was subjected directly to sandwich ELISA, and the pellet was resuspended in 100 μ l 70% formic acid with sonication until clear. Formic acid samples were then neutralized by adding 1.9 ml 1 M Tris base and diluted 1:3 in H₂O before quantifying A β by sandwich-ELISA.

For direct extraction into formic acid, cells were scraped in PBS after washing twice with PBS. Cells were pelleted by centrifugation at 2,000 g for 2 min, and were then lysed in 100 μ l formic acid. Insoluble material was pelleted by centrifugation at 40,000 g at 4°C for 20 min, and the supernatant was neutralized by adding 1.9 ml 1 M Tris base and diluted 1:3 in H₂O before quantification of A β by sandwich-ELISA.

For extraction into PBS, cells were scraped in PBS after washing twice

with PBS. Cells were lysed by sonication, and insoluble material was pelleted by centrifugation at 40,000 *g* at 4°C for 20 min, and A β in the soluble fraction was quantitated by sandwich-ELISA.

Sandwich-ELISA was performed as described previously using mAbs specific for different species of A β (Suzuki et al., 1994; Turner et al., 1996). BAN-50 (a mAb specific for the first 10 amino acids of A β) was used as a capturing antibody, and horseradish peroxidase-conjugated BA-27 (a mAb specific for A β 1-40) and horseradish peroxidase-conjugated BC-05 (a mAb specific for A β 1-42) were used as secondary antibodies. To calibrate the sensitivity of the ELISA for detecting A β after formic acid extraction and neutralization, synthetic A β 1-40 and A β 1-42 peptides (Bachem Bioscience Inc., King of Prussia, PA) used to generate the standard curves were treated with formic acid and neutralized in the same manner as the cell lysates. Under these conditions, the sandwich ELISA had a detection limit of <1 femtomole of synthetic A β per sample. The BAN50, BA-27, and BC-05 mAbs were prepared and characterized as described previously (Suzuki et al., 1994).

Cycloheximide Treatment

For experiments involving cycloheximide treatments, NT2N cells were incubated in media containing 150 μ g/ml cycloheximide for various time points up to 24 h. Cells were harvested and extracted sequentially in RIPA and formic acid as described above. Samples were then subjected to sandwich ELISA analysis.

Western Blot Analysis of APP Levels

RIPA-extracted cell lysates (15 μ g as determined by BCA assay) were resolved on a 7.5% Tris-glycine acrylamide gel and transferred to nitrocellulose for immunoblotting with Karen (a goat anti-APP antibody) at a 1:1,000 dilution (Turner et al., 1996; Chyung et al., 1997). After application of a rabbit anti-goat IgG linker, [¹²⁵I]Protein A was applied, and radiolabeled APP was quantitated by PhosphorImager analysis.

Results

Neurons Contain Insoluble Amyloid β Peptide

To evaluate the possibility that A β exists in multiple intracellular pools with different solubility characteristics, NT2N neurons were sequentially extracted in aqueous buffer (PBS), detergent buffer (RIPA), and then 70% formic acid. The levels of A β 1-40 and A β 1-42 present in each fraction were quantified by sandwich-ELISA. Previous studies have shown that nonionic detergents liberate intracellular A β , but not A β deposited in senile plaques or fibrillar A β formed in vitro (Selkoe et al., 1986; Burdick et al., 1992; Harigaya et al., 1995; Turner et al., 1996). However, more rigorous solubilization methods using 70% formic acid liberate A β from these insoluble aggregates. Sonication of cells in PBS in the absence of detergent failed to release any soluble A β (data not shown). By contrast, significant levels of A β 1-40 and A β 1-42 were solubilized by RIPA buffer. Nonetheless, RIPA buffer released only a fraction of the total intracellular A β since subsequent extraction of the detergent-insoluble material with 70% formic acid revealed a much larger pool of both A β species (Fig. 1 A). Since increased production of A β 1-42 relative to A β 1-40 has been associated with AD (Borchelt et al., 1996; Duff et al., 1996; Scheuner et al., 1996), we examined the ratios of these A β species in the detergent-soluble and -insoluble pools in NT2N neurons. The ratio of A β 1-42/1-40 in the RIPA soluble pool was 1.0 ± 0.1 (Fig. 1 B), consistent with previous studies in a variety of experimental systems (Cook et al., 1997; Forman et al., 1997). However, A β 1-42 was more abundant in the detergent-insoluble pool, with an A β 1-42/1-40 ratio of 2.7 ± 0.3 (Fig. 1 B).

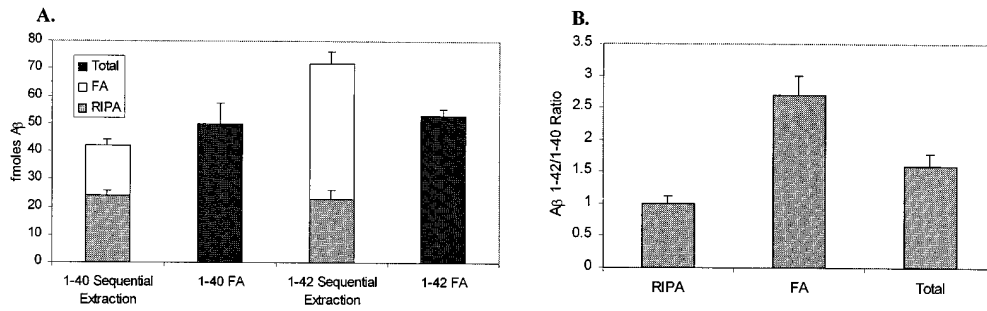
This finding is consistent with the reduced solubility of A β 1-42 relative to A β 1-40 in vitro, and the predominance of A β 1-42 in insoluble deposits in the AD brain (Jarrett et al., 1993a; Iwatsubo et al., 1994).

The identification of a large and previously undetected pool of insoluble A β in NT2N neurons prompted us to establish precise conditions for reproducible recovery of the maximum amount of formic acid-extractable A β . Sonication was found to be necessary for efficient A β extraction, and a volume of 100 μ l formic acid was found to extract A β optimally from cell lysates containing \sim 1 mg of total protein. However, longer incubation times in formic acid (up to 24 h) or high incubation temperatures (up to 37°C) did not increase A β recovery (data not shown). To confirm that formic acid-extracted A β was present in intracellular compartments and not attached to the cells or culture dish, cells were treated with trypsin in the presence or absence of 0.1% Triton X-100. We found that formic acid-solubilized intracellular A β was resistant to trypsin digestion in the absence of detergent, but sensitive to trypsin digestion after solubilization by Triton X-100 (data not shown). This finding indicates that the formic acid-soluble pool of A β is located intracellularly, and is accessible to trypsin only when cell membranes are first permeabilized by detergent. Finally, we found that cells extracted directly into formic acid yielded amounts of A β similar to the sum of RIPA-soluble and RIPA-insoluble A β (Fig. 1 A). From these studies, we concluded that neurons contain at least two major pools of intracellular A β : a detergent soluble pool, and a larger formic acid soluble pool that is enriched in A β 1-42.

Insoluble A β is Present in a Range of APP-Expressing Cell Types

To determine if insoluble intracellular A β is present in cell types other than neurons, NT2, CHO Pro5, and BHK-21 cells were sequentially extracted with RIPA followed by formic acid, and A β levels were measured by sandwich-ELISA (Fig. 2). To evaluate the consequences of increased APP production on the generation of soluble and insoluble intracellular A β , each cell type was also infected with a recombinant SFV vector that led to the expression of high levels of APP695. Additionally, A β levels in stably transfected CHO cells expressing APP695 (CHO-695) were examined (Fig. 2 A). Steady-state APP levels present in each cell type were determined by Western blotting in order to correlate the levels of intracellular A β with APP (Fig. 2 B).

In contrast to NT2N neurons, retinoic acid naive NT2 cells did not produce significant amounts of A β , despite expressing nearly equivalent levels of APP (Fig. 2, A and B). This observation is consistent with previous experiments that have demonstrated that NT2 cells do not efficiently process APP by the β -secretase pathway, and thus generate only low levels of A β (Wertkin et al., 1993; Forman et al., 1997). Furthermore, the engineered expression of APP695 in NT2 cells at levels similar to those found in NT2N neurons resulted in only a modest increase in intracellular A β levels (Fig. 2, A and B), indicating that the lack of intracellular A β in NT2 cells relative to NT2N neurons was not due to differential expression of APP iso-



Mean results and standard errors are shown (six separate experiments, each done with duplicate samples). (B) 1-42/1-40 ratios were calculated for the RIPA soluble pool of A β , the RIPA insoluble (FA soluble) pool of A β , and the total intracellular pool of A β for these NT2N neurons, demonstrating that the insoluble intracellular pool of A β consists mainly of A β 1-42, while the soluble pool contains similar amounts of A β 1-40 and A β 1-42.

Figure 1. FA extraction of NT2N neurons reveals a large pool of insoluble intracellular A β . (A) 10-cm dishes of NT2N cells (replate 2, 4 wk old) were either sequentially extracted in RIPA followed by FA, or lysed directly into FA. A β 1-40 and A β 1-42 levels in the RIPA and FA samples were quantified by sandwich ELISA.

forms in the two cell types (APP751/770 in NT2 cells vs. APP 695 in NT2N cells), but to differential processing of APP. In addition, the fact that only low levels of A β were detected by sandwich-ELISA in this cell line further confirms that this assay is highly specific for A β , and does not significantly cross-react with other cellular proteins, including full-length APP, other A β -containing carboxy-terminal fragments, or non-A β APP-derived fragments.

CHO Pro5 and BHK-21 cells expressed barely detectable levels of APP, and they did not produce detectable levels of soluble or insoluble A β , further confirming the specificity of the A β ELISA. CHO-695 cells, however, did produce intracellular A β , 22 \pm 3% of which was insoluble (Fig. 2 A). Likewise, infection of CHO Pro5 cells and BHK-21 cells with SFV-APPwt led to a markedly increased production of APP as well as intracellular A β , of which up to 74 \pm 5% was insoluble. This dramatic increase in A β production over a relatively short period of time could favor A β aggregation, resulting in a decrease in A β solubility. Indeed, CHO cells stably expressing APP contained a much lower proportion of insoluble A β than did SFV-APP-infected CHO cells (Fig. 2 A).

These findings indicate that in addition to cell type-specific factors, the level of APP expression also governs deposition of insoluble A β . In cells that efficiently use the β -secretase pathway to generate A β , increased APP expression generally resulted in increased levels of both soluble and insoluble A β . However, while CHO-695 cells and NT2N neurons both expressed similar levels of APP and produced similar levels of soluble A β , NT2N neurons accumulated significantly higher levels of insoluble A β (Fig. 2; compare tracks labeled NT2N vs. CHO-695). This difference may be due to the higher metabolic rate of CHO-695 cells, which may result in increased turnover of A β , thus hindering aggregation. Alternatively, A β aggregation in CHO-695 cells may be impeded by continual dilution due to cell division. In postmitotic neurons, A β may accumulate intracellularly over time, and thus favor the formation of insoluble aggregates.

Taken together, these results indicate that while NT2N neurons accumulate intracellular insoluble A β as a consequence of endogenous APP production, other cell types also exhibit this property when they overexpress APP. It is interesting to note that increased expression of APP in NT2N neurons as a consequence of SFV-APPwt infection

did not result in increased levels of intracellular A β 1-42, consistent with some of our previous work indicating that γ -secretase cleavage in the ER/IC pathway is rate-limiting (Cook et al., 1997). By contrast, increased expression of APP in NT2N neurons resulted in increased levels of intracellular A β 1-40. That this increase was due solely to increased levels of soluble A β 1-40 is consistent with this form of A β being produced late in the secretory pathway, and being recovered from cells before secretion.

A β Can be Immunoprecipitated from an Insoluble Pool

To further confirm that the material recovered by extraction with formic acid and measured by sandwich ELISA was indeed A β , SFV-APPwt-infected NT2N and CHO cells were metabolically labeled with [³⁵S]methionine for 12 h, and A β was immunoprecipitated using 6E10. As shown in Fig. 3, a band of ~4 kD was immunoprecipitated by an A β -specific antibody in the RIPA-soluble cell lysate. Additional A β was immunoprecipitated from the RIPA-insoluble (formic acid-extracted) cellular fraction, thus confirming that a pool of A β remained insoluble in RIPA buffer, and could be extracted by formic acid (Fig. 3). However, the yield of A β after formic acid extraction was lower than that predicted by A β sandwich ELISA. To determine if formic acid extraction compromised the recovery of A β by immunoprecipitation, [³⁵S]methionine-labeled SFV-APPwt-infected cells were extracted directly into formic acid. Direct extraction of cells into formic acid would be expected to yield amounts of A β equal to the sum of A β extracted in the RIPA-soluble and -insoluble pools. However, lower levels of A β than expected were recovered by this method (Fig. 3; compare lane 3 with lanes 1 and 2, and lane 6 with lanes 4 and 5). Thus, immunoprecipitation of formic acid-extracted cells was not quantitative, and resulted in only partial recovery of A β . This low recovery of A β may have been due to incomplete resolubilization of A β in acetonitrile after lyophilization, or re-aggregation of A β during immunoprecipitation. To evaluate the contribution of each of these factors to the incomplete recovery of A β by immunoprecipitation, we measured A β levels in the formic acid-extracted cell lysate before and after lyophilization and immunoprecipitation by sandwich-ELISA. We found that ~43% of formic acid-extracted A β could be resolubilized in acetonitrile

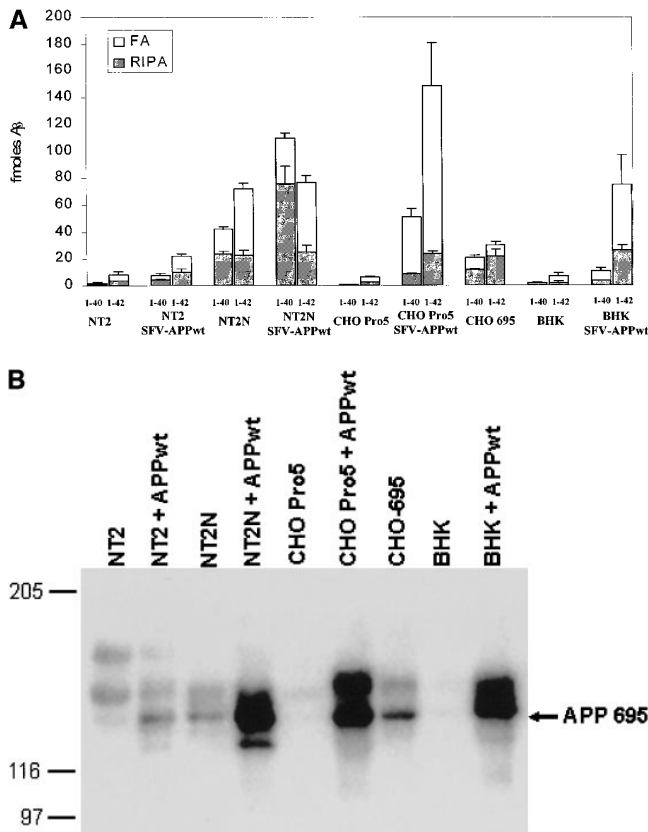


Figure 2. FA extraction of a variety of cell lines reveals the presence of varying levels of insoluble intracellular A β . (A) Uninfected and SFV-APPwt-infected NT2 cells, NT2N cells (replate 2, 4 wk old), CHO Pro5 cells, CHO-695 cells (uninfected only), and BHK-21 cells were sequentially extracted in RIPA followed by FA. A β 1-40 and 1-42 levels in the RIPA and FA samples were quantified by sandwich ELISA. Means and standard errors (four separate experiments done in triplicate) of A β levels are shown. (B) Samples from RIPA cell lysates of each of these cell lines (both uninfected and SFV-APPwt infected) were resolved on a 7.5% Tris-glycine acrylamide gel, and immunoblotted with Karen antibody; bands were detected by PhosphorImager after using an 125 I-labeled secondary antibody.

after lyophilization, and ~45% of this resolubilized A β could be captured by immunoprecipitation with the antibody 6E10 (data not shown). Nevertheless, despite the shortcomings of the immunoprecipitation protocol as compared with the A β sandwich-ELISA, these data confirm that the formic acid-extracted pool does indeed contain A β .

Differential Production of Insoluble A β 1-40 and A β 1-42 in Subcellular Compartments

While it has been shown that secreted A β is mainly produced in the TGN, intracellular A β 1-42, but not A β 1-40, is produced in the ER/IC (Cook et al., 1997). To determine if A β 1-42 produced in the ER/IC enters the insoluble pool, NT2N neurons and CHO Pro5 cells were infected with SFV-APPwt or SFV-APP Δ KK (an APP mutant containing the dilysine ER retrieval sequence). Infection of both cell types with SFV-APP Δ KK gave similar results: almost a complete abrogation of A β 1-40 production, with

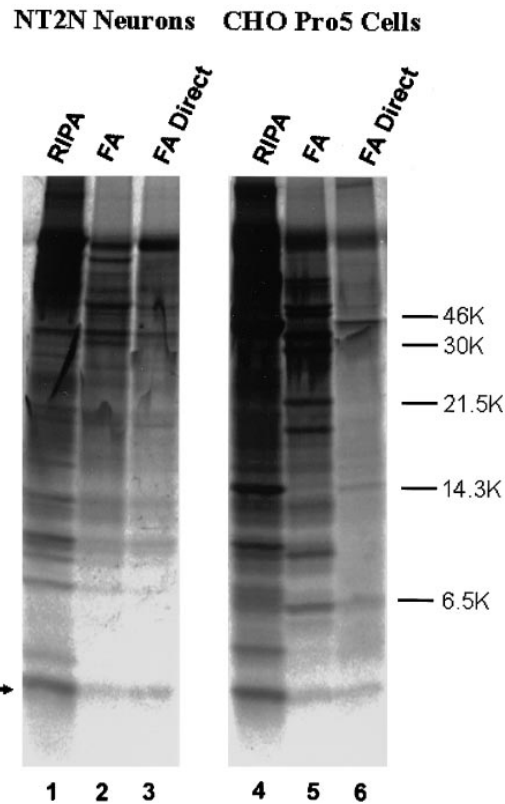


Figure 3. A β can be recovered from RIPA-soluble and RIPA-insoluble (FA-solubilized) fractions of cell lysate. SFV-APPwt-infected NT2N cells and CHO Pro5 cells were metabolically labeled for 12 h before being lysed in either FA or RIPA, followed by extraction of insoluble material by FA. RIPA and FA samples were subjected to immunoprecipitation with 6E10, and were resolved on a 10/16.5% step gradient Tris-tricine gel. Molecular weight standards and A β bands are labeled.

no diminution of A β 1-42 production relative to SFV-APPwt infected cells (Fig. 4, A and B). Importantly, the levels of insoluble A β 1-42 were the same in SFV-APPwt and SFV-APP Δ KK-infected cells. These results demonstrate that A β 1-42 produced in the ER/IC pathway represents the bulk of the insoluble A β 1-42 inside cells. By contrast, insoluble A β 1-40 is produced by a post-ER/IC pathway. Finally, these results also prove that insoluble A β can accumulate in the absence of secretion, and they provide additional evidence that the A β solubilized by formic acid is intracellular.

Time-dependent Accumulation of Insoluble A β

Our previous studies have shown that secretion of A β 1-40 and A β 1-42 by the NT2N neurons increases with time in culture without an increase in APP synthesis (Turner et al., 1996). However, a time-dependent increase in intracellular A β was not detected. Conversely, we found that retention of APP in the ER/IC resulted in continued production of A β 1-42, but without either secretion or intracellular accumulation (Cook et al., 1997). Our observation here that intracellular A β (particularly the A β 1-42 species produced in the ER/IC) forms an insoluble pool provided a

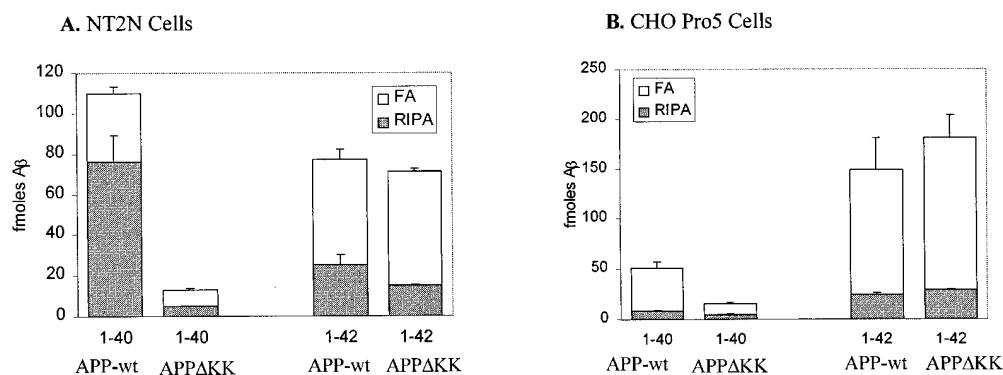


Figure 4. Insoluble A β 1-42 can be produced by APP Δ KK expressing NT2N and CHO cells. (A) NT2N cells and (B) CHO Pro5 cells were infected with SFV-APPwt or SFV-APP Δ KK for 12 h before sequential extraction in RIPA, followed by FA. A β in each sample was quantified by sandwich-ELISA. Means and standard errors (two separate experiments each done in triplicate) are shown.

possible explanation for both of these earlier findings. To test the hypothesis that insoluble A β can accumulate intracellularly over time, NT2N neurons were analyzed at various time points after replating by sequential extraction in RIPA and formic acid, followed by sandwich-ELISA for A β quantitation. We found a dramatic increase (12-fold over 7 wk in culture) in the levels of formic acid-extractable intracellular A β 1-40 and A β 1-42 in NT2N cells concomitant with increased time in culture (Fig. 5, A and B). In addition to an increase in the absolute amount of insoluble A β with longer times in culture, an increase in the fraction of insoluble A β was also observed. For example, at 4 wk, ~58% of A β was insoluble, while at 7 wk ~78% of A β was insoluble (Fig. 5, A and B). This result suggests that the equilibrium of soluble to insoluble A β may be shifted to favor insoluble A β in NT2N cells that were cultured longer (i.e., older neurons).

The Intracellular Accumulation of A β Over Time in Culture is Due to the Slow Turnover of Insoluble A β

The time-dependent accumulation of insoluble intracellular A β in neurons could be due to several factors, including the slow turnover of insoluble A β . To examine this possibility, we treated NT2N cells with cycloheximide to

prevent protein synthesis, and measured endogenous levels of A β in the soluble and insoluble pools over time in culture. This approach was needed (rather than a standard pulse-chase analysis) because immunoprecipitation of A β after formic acid extraction was not quantitative (Fig. 3). Fig. 6 shows that over the 24-h cycloheximide treatment, soluble A β 1-40 and A β 1-42 decreased by ~63% and ~77%, respectively. Assuming a constant rate of degradation, we calculated half-lives of ~18 h and ~12 h for the decay of intracellular soluble A β 1-40 and A β 1-42, respectively. By contrast, insoluble A β 1-40 and A β 1-42 levels did not decrease significantly over 24 h. The slow turnover of the insoluble pool of A β precluded an accurate estimate of the half-life of this pool. In addition, this analysis is complicated by two factors. First, although no new APP will be synthesized in the presence of cycloheximide, existing pools of APP continue to be processed to generate A β . However, the half-life of APP in NT2N neurons is ~3 h. Thus, de novo production of A β from existing pools of APP is unlikely to contribute significantly to intracellular A β pools, especially at later time points. Second, soluble A β 1-40 and A β 1-42 may enter the insoluble pool over time, again making accurate estimates of turnover rates difficult. Nevertheless, our results show that intracellular insoluble A β is very long-lived, and that this long life is

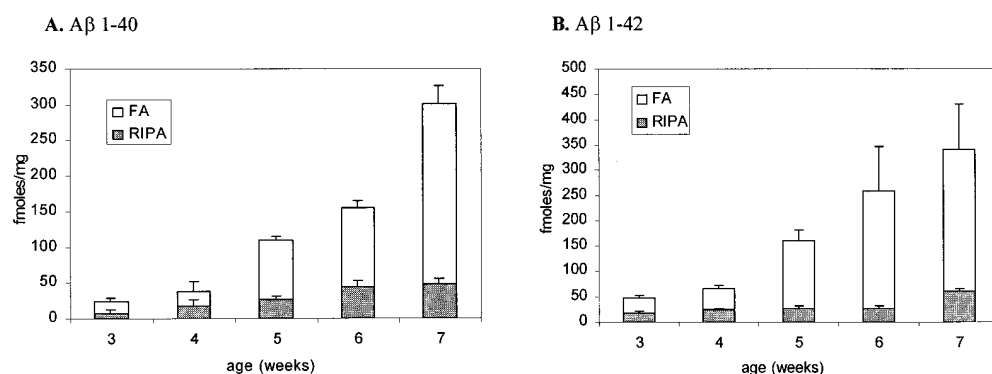


Figure 5. Insoluble intracellular A β accumulates over time within NT2N cells. 10-cm dishes of Replate 2 NT2N cells were harvested each week 3–7 wk after replating. Cells were lysed in RIPA buffer, and insoluble material was resuspended in FA. A β 1-40 (A) and 1-42 (B) in the soluble and insoluble intracellular pools were quantified by sandwich-ELISA. Data were normalized for total protein present (in mg). The experiment was repeated three times, and means and standard errors (three to five samples per time point) are shown for a representative experiment.

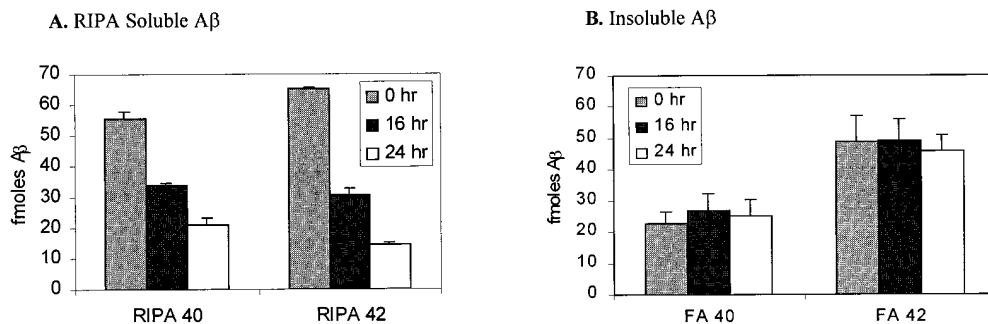


Figure 6. The insoluble pool of intracellular A β is stable over 24 h, while the soluble pool of cellular A β turns over more rapidly. 10-cm dishes of Replate 2 NT2N cells were treated with 150 μ g/ml cyclohexamide for the times indicated before being sequentially extracted in RIPA and FA. A β 1-40 and 1-42 in the soluble (A) and insoluble intracellular pools (B) were quantified by sandwich-ELISA. The experiment was repeated three times, and means and standard errors (three samples per time point) are shown for a representative experiment.

likely to play an important role in the time-dependent accumulation of insoluble A β we observed in NT2N cells over weeks in culture.

Discussion

The presence of insoluble aggregates of A β in senile plaques is a well-characterized feature of AD (Selkoe, 1997). A β is composed of two major species that terminate at residues 40 and 42 of the intact A β sequence. Both species can be recovered from the CSF of normal and AD individuals, with A β 1-40 being approximately 10-fold more abundant than A β 1-42 (Citron et al., 1992). However, A β 1-42 is the major A β species present in senile plaques, with A β 1-40 being only a minor constituent (Iwatsubo et al., 1994). That alterations in APP processing can lead to development of AD has been shown by several FAD-associated APP mutations that, when expressed in vitro or in transgenic animals, lead to either an overall increase in A β production or an increase in the amount of A β 1-42 relative to A β 1-40 (Borchelt et al., 1996; Duff et al., 1996). The differential production of A β 1-40 and A β 1-42 as a consequence of AD-associated APP mutations as well as the preferential deposition of A β 1-42 in senile plaques raises important questions as to the intracellular sites of A β 1-42 generation, the origin of A β that is recovered from senile plaques, and the factors that control its deposition.

Both A β 1-40 and A β 1-42 are constitutively produced and secreted from cells in vitro and in vivo as judged by their recovery from conditioned medium and CSF (Shoji et al., 1992; Tamaoka et al., 1996). Since FAD-associated APP mutations lead to increased secretion of A β , and senile plaques are extracellular lesions, it is possible that secreted A β is ultimately deposited in senile plaques, even though the factors controlling its deposition are obscure. However, we have recently discovered that retention of APP in the ER/IC induced by a variety of methods leads to continued production of intracellular A β 1-42, but not A β 1-40 (Cook et al., 1997). While A β 1-42 is constitutively produced by this novel pathway in NT2N neurons, other cell types can also process APP to generate A β in the ER/

IC after overexpression of APP (Wild-Bode et al., 1997). A β 1-42 has also been shown to be localized to the ER/IC by immunoelectron microscopy and by cell fractionation (Hartmann et al., 1997; Wild-Bode et al., 1997). Interestingly, this compartment also is the site where PS1 and PS2 are localized (Cook et al., 1996; Kovacs et al., 1996). Since mutations in PS1 and PS2 account for the majority of early-onset FAD cases, and FAD-associated PS1 and PS2 mutations have been shown to result in an increased ratio of A β 1-42/1-40 (Borchelt et al., 1996; Duff et al., 1996; Scheuner et al., 1996), colocalization of the presenilins with a major site of constitutive A β 1-42 production raises the possibility that alterations in A β production by the ER/IC pathway may play an important role in AD pathogenesis.

While retention of APP in the ER/IC resulted in continued and selective production of A β 1-42, we were unable to document either secretion of this material or its intracellular accumulation (Cook et al., 1997). Taken at face value, this result indicates that the production and turnover of A β 1-42 by the ER/IC pathway are in equilibrium. However, given the propensity of A β 1-42 to aggregate in vivo and in vitro, we asked whether A β 1-42 also aggregated intracellularly. Since formic acid has been shown to effectively solubilize aggregated A β present in senile plaques, we solubilized cell lysates in formic acid. Using this approach, we found that a considerable fraction of total intracellular A β 1-42, and to a lesser extent A β 1-40, could be solubilized by formic acid, but not by a variety of detergents. Currently, we do not know whether or not the formic acid-extractable A β self-aggregates or coaggregates with other proteins. Our observation that none of the cell-associated A β (including that targeted for secretion) can be extracted with aqueous buffer suggests that it may be bound to other cellular proteins. On the other hand, in vitro studies of A β aggregation suggest that A β is prone to self-aggregation. Future ultrastructural studies on the accumulated intracellular A β will help to resolve this issue. Intracellular insoluble A β was recovered in a number of different APP-expressing cell lines. Overexpression of APP generally resulted in increased production of insoluble A β . However, insoluble A β was produced most efficiently

in NT2N neurons. Thus, while aggregation of intracellular A β is not cell type-specific, the subcellular environment in neurons appears to favor this process.

Identification of a novel form of intracellular A β that has previously escaped detection could explain our failure to detect secretion or intracellular accumulation of A β 1-42 produced by the ER/IC pathway (Cook et al., 1997). To test this possibility, we expressed APP bearing an ER/IC retrieval signal in the cytoplasmic domain in NT2N neurons and in CHO cells. Production of intracellular soluble and insoluble A β 1-40 was almost completely inhibited after expression of this construct, while levels of soluble and insoluble intracellular A β 1-42 were unchanged by ER retention. Thus, almost all of the formic acid-soluble A β 1-42 can be derived from the ER/IC pathway. By extension, insoluble A β 1-40 must be produced by a post-ER/IC compartment. Production of insoluble A β 1-40 and A β 1-42 in different subcellular compartments may help explain the predominance of A β 1-42 in the intracellular pool. A β 1-40 is produced late in the biosynthetic pathway, and may spend relatively little time in the cell before secretion, thereby minimizing the opportunity for aggregation. By contrast, the bulk of the intracellular A β 1-42 is produced by the ER/IC pathway. This fact represents an environment distinct from that in which A β 1-40 is produced, and one that does not result in A β 1-42 secretion. The long-lived nature of A β 1-42, its continued production at an intracellular site from which it cannot be secreted, and the fact that it is intrinsically less soluble than A β 1-40 all may contribute to its propensity to enter a stable, intracellular pool of insoluble material. It will be important to define further the factors that govern A β deposition in this insoluble pool, and to more carefully study its physical state.

During the course of our experiments, we found that recovery of insoluble A β from NT2N neurons was somewhat variable. However, we found that this result was due to a time-dependent accumulation of A β . Specifically, we found that A β levels increased by 12-fold as the NT2N neurons aged over 7 wk in culture. While insoluble A β 1-40 and A β 1-42 accumulated at similar rates, more detailed kinetic studies are needed to determine if production of insoluble A β 1-40 and A β 1-42 is contemporaneous, or if generation of insoluble A β 1-42 seeds subsequent polymerization of A β 1-40, as has been reported in vitro. In any event, time-dependent accumulation of insoluble A β could be due to increased production, decreased turnover, or stable accumulation of A β at a relatively constant rate. We found that intracellular insoluble A β was exceptionally stable. Thus, even slow addition of A β to the insoluble pool over weeks in culture could result in steady accumulation of A β seen in the insoluble pool over time. This observation may have implications for AD pathogenesis, where it is thought that accumulation of A β occurs slowly over decades. Since AD is an age-dependent disease, the data presented here suggest that gradual accumulation of intracellular A β may be a factor in the slow onset and progression of AD. It will be important to determine if accumulation of intracellular insoluble A β is simply the result of the stability of this form of A β , or if other time-dependent factors (such as altered APP processing or neurotoxic insults) contribute to this process.

Although intracellular β -amyloid fibrils have been ob-

served in the AD brain (Kim et al., 1988) as well as in a transgenic mouse model of AD (Masliah et al., 1996), it is unclear whether A β fibrils can form within neurons from endogenously produced A β . The experiments presented here demonstrate that significant levels of A β are insoluble within neurons. The observation that A β can accumulate with time in a relatively stable insoluble pool may explain how A β deposition in senile plaques can begin despite relatively low levels of secreted and CSF-soluble A β 1-42. Concentrated intracellular A β 1-42 could rapidly nucleate fibril formation, and intracellularly produced A β 1-42 and A β 1-40 could add to these fibrils over time, thus serving as a nidus for a developing senile plaque.

We gratefully thank Dr. N. Suzuki and Tekeda Pharmaceutical for providing the monoclonal antibodies for the A β sandwich-ELISA. We also thank Dr. J. Wang for her help with neuronal aging experiments, and Dr. J.Q. Trojanowski for critically reading the manuscript.

This work was supported by grants from the National Institute on Aging. D.M. Skovronsky is the recipient of a Medical Scientist Training Program Predoctoral Fellowship from the National Institutes of Health, and R.W. Doms is the recipient of a Paul Beeson Faculty Scholar Award.

Received for publication 20 January 1998 and in revised form 31 March 1998.

References

- Borhelt, D.R., G. Thinakaran, C.B. Eckman, M.K. Lee, F. Davenport, T. Ratovitsky, C.M. Prada, G. Kim, S. Seekins, D. Yager, et al. 1996. Familial Alzheimer's disease-linked presenilin 1 variants elevate Abeta1-42/1-40 ratio in vitro and in vivo. *Neuron*. 17:1005-1013.
- Burdick, D., B. Soreghan, M. Kwon, J. Kosmoski, M. Knauer, A. Henschen, J. Yates, C. Cotman, and C. Glabe. 1992. Assembly and aggregation properties of synthetic Alzheimer's A4/beta amyloid peptide analogs. *J. Biol. Chem.* 267:546-554.
- Chyung, A.C., B.D. Greenberg, D.G. Cook, R.W. Doms, and V.M.-Y. Lee. 1997. Novel beta-secretase cleavage of beta-amyloid precursor protein in the endoplasmic reticulum/intermediate compartment of NT2N cells. *J. Cell Biol.* 138:671-680.
- Citron, M., T. Oltsersdorf, C. Haass, L. McConlogue, A.Y. Hung, P. Seubert, C. Vigo-Pelfrey, I. Lieberburg, and D.J. Selkoe. 1992. Mutation of the beta-amyloid precursor protein in familial Alzheimer's disease increases beta-protein production. *Nature*. 360:672-674.
- Cook, D.G., M.S. Forman, J.C. Sung, S. Leight, D.L. Kolson, T. Iwatsubo, V.M.-Y. Lee, and R.W. Doms. 1997. Alzheimer's A beta(1-42) is generated in the endoplasmic reticulum/intermediate compartment of NT2N cells. *Nat. Med.* 3:1021-1023.
- Cook, D.G., J.C. Sung, T.E. Golde, K.M. Felsenstein, B.S. Wojczyk, R.E. Tanzi, J.Q. Trojanowski, V.M.-Y. Lee, and R.W. Doms. 1996. Expression and analysis of presenilin 1 in a human neuronal system: localization in cell bodies and dendrites. *Proc. Natl. Acad. Sci. USA*. 93:9223-9228.
- Duff, K., C. Eckman, C. Zehr, X. Yu, C.M. Prada, J. Perez-tur, M. Hutton, L. Buee, Y. Harigaya, D. Yager, et al. 1996. Increased amyloid-beta42(43) in brains of mice expressing mutant presenilin 1. *Nature*. 383:710-713.
- Forman, M.S., D.G. Cook, S. Leight, R.W. Doms, and V.M.-Y. Lee. 1997. Differential effects of the Swedish mutant amyloid precursor protein on beta-amyloid accumulation and secretion in neurons and nonneuronal cells. *J. Biol. Chem.* 272:32247-32253.
- Games, D., D. Adams, R. Alessandrini, R. Barbour, P. Berthelette, C. Blackwell, T. Carr, J. Clemens, T. Donaldson, and F. Gillespie. 1995. Alzheimer-type neuropathology in transgenic mice overexpressing V717F beta-amyloid precursor protein. *Nature*. 373:523-527.
- Haass, C., M.G. Schlossmacher, A.Y. Hung, C. Vigo-Pelfrey, A. Mellon, B.L. Ostaszewski, I. Lieberburg, E.H. Koo, D. Schenk, and D.B. Teplow. 1992. Amyloid beta-peptide is produced by cultured cells during normal metabolism. *Nature*. 359:322-325.
- Harigaya, Y., M. Shoji, T. Kawarabayashi, M. Kanai, T. Nakamura, T. Iizuka, Y. Igeta, T.C. Saido, N. Sahara, and H. Mori. 1995. Modified amyloid beta protein ending at 42 or 40 with different solubility accumulates in the brain of Alzheimer's disease. *Biochem. Biophys. Res. Commun.* 211:1015-1022.
- Hartmann, T., S.C. Bieger, B. Bruhl, P.J. Tienari, N. Ida, D. Allsop, G.W. Roberts, C.L. Masters, C.G. Dotti, K. Unsicker, and K. Beyreuther. 1997. Distinct sites of intracellular production for Alzheimer's disease A beta40/42 amyloid peptides. *Nat. Med.* 3:1016-1020.
- Hsiao, K., P. Chapman, S. Nilsen, C. Eckman, Y. Harigaya, S. Younkin, F. Yang, and G. Cole. 1996. Correlative memory deficits, Abeta elevation, and

- amyloid plaques in transgenic mice. *Science*. 274:99–102.
- Iwatsubo, T., A. Odaka, N. Suzuki, H. Mizusawa, N. Nukina, and Y. Ihara. 1994. Visualization of A beta 42(43) and A beta 40 in senile plaques with end-specific A beta monoclonals: evidence that an initially deposited species is A beta 42(43). *Neuron*. 13:45–53.
- Jarrett, J.T., E.P. Berger, and P.T.J. Lansbury. 1993a. The carboxy terminus of the beta amyloid protein is critical for the seeding of amyloid formation: implications for the pathogenesis of Alzheimer's disease. *Biochemistry*. 32: 4693–4697.
- Jarrett, J.T., and P.T.J. Lansbury. 1993b. Seeding "one-dimensional crystallization" of amyloid: a pathogenic mechanism in Alzheimer's disease and scrapie? *Cell*. 73:1055–1058.
- Kim, K.S., D.L. Miller, V.J. Sapienza, C.-M. Chen, C. Bai, I. Grundke-Iqbal, J.R. Currie, and H.M. Wisniewski. 1988. Production and characterization of monoclonal antibodies reactive to synthetic cerebrovascular amyloid peptide. *Neurosci. Res. Commun.* 2:121–130.
- Knauer, M.F., B. Soreghan, D. Burdick, J. Kosmoski, and C.G. Glabe. 1992. Intracellular accumulation and resistance to degradation of the Alzheimer amyloid A4/beta protein. *Proc. Natl. Acad. Sci. USA*. 89:7437–7441.
- Koo, E.H., and S.L. Squazzo. 1994. Evidence that production and release of amyloid beta-protein involves the endocytic pathway. *J. Biol. Chem.* 269: 17386–17389.
- Kovacs, D.M., H.J. Fausett, K.J. Page, T.W. Kim, R.D. Moir, D.E. Merriam, R.D. Hollister, O.G. Hallmark, R. Mancini, K.M. Felsenstein, et al. 1996. Alzheimer-associated presenilins 1 and 2: neuronal expression in brain and localization to intracellular membranes in mammalian cells. *Nat. Med.* 2:224–229.
- Levy-Lahad, E., W. Wasco, P. Poorkaj, D.M. Romano, J. Oshima, W.H. Pettigell, C.E. Yu, P.D. Jondro, S.D. Schmidt, and K. Wang. 1995. Candidate gene for the chromosome 1 familial Alzheimer's disease locus. *Science*. 269: 973–977.
- Masliah, E., A. Sisk, M. Mallory, L. Mucke, D. Schenk, and D. Games. 1996. Comparison of neurodegenerative pathology in transgenic mice overexpressing V717F beta-amyloid precursor protein and Alzheimer's disease. *J. Neurosci.* 16:5795–5811.
- Pleasure, S.J., and V.M.-Y. Lee. 1993. NTera 2 cells: a human cell line which displays characteristics expected of a human committed neuronal progenitor cell. *J. Neurosci. Res.* 35:585–602.
- Pleasure, S.J., C. Page, and V.M.-Y. Lee. 1992. Pure, postmitotic, polarized human neurons derived from NTera 2 cells provide a system for expressing exogenous proteins in terminally differentiated neurons. *J. Neurosci.* 12:1802–1815.
- Scheuner, D., C. Eckman, M. Jensen, X. Song, M. Citron, N. Suzuki, T.D. Bird, J. Hardy, M. Hutton, and W. Kukull. 1996. Secreted amyloid beta-protein similar to that in the senile plaques of Alzheimer's disease is increased in vivo by the presenilin 1 and 2 and APP mutations linked to familial Alzheimer's disease. *Nat. Med.* 2:864–870.
- Selkoe, D.J. 1997. Cellular and molecular biology of the beta-amyloid precursor protein and Alzheimer's disease. In *The Molecular and Genetic Basis of Neurological Disease*. R.N. Rosenberg, S.B. Prusiner, S. DiMauro, and R.L. Barchi, editors. Butterworth-Heinemann, Boston, MA. 601–612.
- Selkoe, D.J., C.R. Abraham, M.B. Podlisy, and L.K. Duffy. 1986. Isolation of low-molecular-weight proteins from amyloid plaque fibers in Alzheimer's disease. *J. Neurochem.* 46:1820–1834.
- Sherrington, R., E.I. Rogae, Y. Liang, E.A. Rogaeva, G. Levesque, M. Ikeda, H. Chi, C. Lin, G. Li, and K. Holman. 1995. Cloning of a gene bearing missense mutations in early-onset familial Alzheimer's disease. *Nature*. 375: 754–760.
- Shoji, M., T.E. Golde, J. Ghiso, T.T. Cheung, S. Estus, L.M. Shaffer, X.D. Cai, D.M. McKay, R. Tintner, and B. Frangione. 1992. Production of the Alzheimer amyloid beta protein by normal proteolytic processing. *Science*. 258:126–129.
- Suzuki, N., T.T. Cheung, X.D. Cai, A. Odaka, L.J. Otvos, C. Eckman, T.E. Golde, and S.G. Younkin. 1994. An increased percentage of long amyloid beta protein secreted by familial amyloid beta protein precursor (beta APP717) mutants. *Science*. 264:1336–1340.
- Tamaoka, A., T. Fukushima, N. Sawamura, K. Ishikawa, E. Oguni, Y. Komatsuzaki, and S. Shoji. 1996. Amyloid beta protein in plasma from patients with sporadic Alzheimer's disease. *J. Neurol. Sci.* 141:65–68.
- Turner, R.S., N. Suzuki, A.S. Chyung, S.G. Younkin, and V.M.-Y. Lee. 1996. Amyloids beta40 and beta42 are generated intracellularly in cultured human neurons and their secretion increases with maturation. *J. Biol. Chem.* 271: 8966–8970.
- Wertkin, A.M., R.S. Turner, S.J. Pleasure, T.E. Golde, S.G. Younkin, J.Q. Trojanowski, and V.M.-Y. Lee. 1993. Human neurons derived from a teratocarcinoma cell line express solely the 695-amino acid amyloid precursor protein and produce intracellular beta-amyloid or A4 peptides. *Proc. Natl. Acad. Sci. USA*. 90:9513–9517.
- Wild-Bode, C., T. Yamazaki, A. Capell, U. Leimer, H. Steiner, Y. Ihara, and C. Haass. 1997. Intracellular generation and accumulation of amyloid beta-peptide terminating at amino acid 42. *J. Biol. Chem.* 272:16085–16088.
- Xu, H., D. Sweeney, R. Wang, G. Thinakaran, A.C. Lo, S.S. Sisodia, P. Greenberg, and S. Gandy. 1997. Generation of Alzheimer beta-amyloid protein in the trans-Golgi network in the apparent absence of vesicle formation. *Proc. Natl. Acad. Sci. USA*. 94:3748–3752.
- Yang, A.J., M. Knauer, D.A. Burdick, and C. Glabe. 1995. Intracellular A beta 1-42 aggregates stimulate the accumulation of stable, insoluble amyloidogenic fragments of the amyloid precursor protein in transfected cells. *J. Biol. Chem.* 270:14786–14792.