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Short Communication

Intraneuronal Aβ42 Accumulation in Human Brain

Gunnar K. Gouras,*†‡ Julia Tsai,‡ Jan Naslund,*† Bruno Vincent,*† Mark Edgar,§ Frederic Checler,¶ Jeffrey P. Greenfield,*† Vahram Haroutunian,‖ Joseph D. Buxbaum,‖ Huaxi Xu,*† Paul Greengard,*† and Norman R. Relkin‡

From the Laboratory of Molecular and Cellular Neuroscience and Fisher Center for Research on Alzheimer's Disease,† The Rockefeller University; the Departments of Neurology and Neuroscience‡ and Pathology,‖ Weill Medical College of Cornell University; Mount Sinai School of Medicine,‖ New York, New York; and Institute de Pharmacologie Moleculaire et Cellulaire,¶ Valbonne, France*

Alzheimer's disease (AD) is characterized by the deposition of senile plaques (SPs) and neurofibrillary tangles (NFTs) in vulnerable brain regions. SPs are composed of aggregated β-amyloid (Aβ) 40/42(43) peptides. Evidence implicates a central role for Aβ in the pathophysiology of AD. Mutations in βAPP and presenilin 1 (PS1) lead to elevated secretion of Aβ, especially the more amyloidogenic Aβ42. Immunohistochemical studies have also emphasized the importance of Aβ42 in initiating plaque pathology. Cell biological studies have demonstrated that Aβ is generated intracellularly. Recently, endogenous Aβ42 staining was demonstrated within cultured neurons by confocal immunofluorescence microscopy and within neurons of PS1 mutant transgenic mice. A central question about the role of Aβ in disease concerns whether extracellular Aβ deposition or intracellular Aβ accumulation initiates the disease process. Here we report that human neurons in AD-vulnerable brain regions specifically accumulate γ-cleaved Aβ42 and suggest that this intraneuronal Aβ42 immunoreactivity appears to precede both NFT and Aβ plaque deposition. This study suggests that intracellular Aβ42 accumulation is an early event in neuronal dysfunction and that preventing intraneuronal Aβ42 aggregation may be an important therapeutic direction for the treatment of AD. ***(Am J Pathol 2000, 156:15–20)***

Alzheimer's disease (AD) neuropathology is classically characterized by the accumulation of senile plaques (SPs) and neurofibrillary tangles (NFTs) in vulnerable brain regions. SPs are composed of parenchymal and cerebrovascular aggregates of β-amyloid (Aβ) 40/42(43) peptides. Increasing evidence indicates that Aβ plays a central role in the pathophysiology of AD. Individuals with Down's syndrome (DS) have an extra copy of chromosome 21, where the gene encoding the β-amyloid precursor protein (βAPP) is localized, and invariably develop AD pathology at an early age. Mutations in βAPP segregate with some forms of autosomal dominant familial AD (FAD). Transgenic mice bearing FAD βAPP mutations develop striking AD-like senile plaque pathology.[1] FAD mutations in βAPP and presenilin 1 (PS1) lead to elevated secretion of Aβ, especially the more amyloidogenic Aβ42. In addition, immunohistochemical studies have underscored the importance of Aβ42 as the initiator of plaque pathology in AD and DS.[2,3]

Over the past few years cell biological studies support the view that Aβ is generated intracellularly[1,4–10] from the endoplasmic reticulum (ER)[1,7,8] to the trans-Golgi network (TGN),[4] and the endosomal-lysosomal system.[10] Recently, endogenous Aβ42 staining was demonstrated within cultured primary neurons by confocal immunofluorescence microscopy[9] and within neurons of human PS1 mutant transgenic mice by immunocytochemical light microscopy.[11] A central question on the role of Aβ in AD is whether extracellular Aβ deposition or intracellular Aβ accumulation is initiating the disease process. Several groups had postulated the presence of intraneuronal Aβ immunostaining. However, the Aβ immunoreactivity observed in these studies was compromised by that of full-length βAPP, because these Aβ antibodies also recognize full-length βAPP.[12–14] In addition, NFTs had previously been reported to be immunoreactive to Aβ.[15–16] This association of Aβ with NFTs was subsequently believed to be the result of artifactual "shared" epitopes.[17]

We now report that human neurons in AD-vulnerable brain regions specifically accumulate γ-cleaved Aβ42

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Address reprint requests to Dr. Gunnar K. Gouras, Laboratory of Molecular and Cellular Neuroscience, The Rockefeller University, 1230 York Avenue, New York, NY 10021. E-mail: gkgouras@mail.med.cornell.edu.

but not the more abundantly secreted Aβ40. We also demonstrate intraneuronal Aβ42 staining in neurons in both the absence and presence of NFTs. Our observations in adjacent sections of intraneuronal Aβ42 staining and hyperphosphorylated tau staining suggest that neuronal Aβ42 staining is more abundant and therefore may precede NFTs, which would exclude the possibility of cross-reactivity of shared epitopes. Furthermore, we observe the earliest Aβ42 immunoreactive SPs developing along the projections and at terminals of early Aβ42 accumulating neurons, suggesting a mechanism for the previously hypothesized regional specificity of AD disease progression within the brain.[18]

Materials and Methods

Antibodies

Polyclonal rabbit Aβ40 (RU226) and Aβ42 (RU228) C-terminal specific antibodies were generated at Rockefeller University (RU). Polyclonal rabbit Aβ40 and Aβ42 C-terminal antibodies were also obtained commercially (QCB). The results obtained with these two sets of antibodies were similar and were confirmed using well-characterized polyclonal rabbit Aβ40 (FCA3340) and Aβ42 (FCA3542) antibodies[19] (kindly provided by F. Checler). Antibody 4G8 recognizes amino acids 17–24 of Aβ (Senetek). Hyperphosphorylated tau was recognized by antibody AT8 (Polymedco). ApoE was visualized with a mouse monoclonal anti-ApoE antibody (Boehringer-Mannheim).

Immunocytochemistry

Postmortem brain tissue was examined from representative neurologically normal controls (ages 3 months and 3, 30, 44, 58, and 79 years); elderly nursing home residents without dementia (Clinical Dementia Rating (CDR) 0; ages 64, 69, 71, 72, 82, and 91 years) or with mild cognitive dysfunction (CDR 0.5; ages 67, 81, 87, 93, and 94 years) or mild (CDR 1; ages 79, 83, 84, 87, and 90 years), moderate (CDR 2; ages 83, 85, 90, 93, 94, and 94 years), or severe dementia (ages 64, 72, 79 years); and subjects with DS of varying ages (3 months and 3, 12, 13, and 24 years). The normal control and DS tissue were from New York Hospital, and the CDR tissue was from Mt. Sinai Medical Center. Postmortem intervals ranged from 6 to 18 hours. Ten percent formalin-fixed, paraffin-embedded brain sections (8 μm) were deparaffinized, washed in phosphate-buffered saline (PBS), incubated for 30 minutes at room temperature in 90% formic acid, washed again in PBS, incubated in 0.4% Triton X-100 (Tx) for 30 minutes, quenched for endogenous peroxidase with 3% hydrogen peroxide for 5 minutes, and preincubated in 3% serum from the species of the secondary antibody in 0.1% Tx/PBS for 1 hour to prevent nonspecific staining. Thereafter, slides were incubated with the appropriate antibody in 3% serum from the species of the secondary antibody/0.1%Tx/PBS overnight: anti-ApoE antibody (1:500), AT8 antibody (1:500), anti-Aβ40, or 42 C-terminal specific antibodies (typically 1:500 for RU and 1:100 for QCB antibodies). Slides were washed with PBS and incubated with secondary antibody (anti-primary antibody species antibody) (Vectastain ABC kit; Vector) in 1.5% serum from the species of the secondary antibody/0.1%Tx/PBS at room temperature for 1 hour. Slides were incubated with avidin-biotin and developed with diaminobenzidine (DAB) (ABC kit) for 2 minutes. Except for some representative sections counterstained with hematoxylin and eosin (H&E), most sections were not counterstained, so as not to obscure the immunohistochemical staining.

Primary Neuronal Cultures

Primary neuronal cultures were derived from the cerebral cortices of embryonic day 15 (E15) CD1 mice (Charles River) as previously described.[20] Brains were removed, cortices were isolated, and the meninges were removed. Cortices were triturated in glass pipettes until cells were dissociated. Cells were counted in a hemocytometer and plated in serum-free Neurobasal media with N2 supplement (Gibco) and 0.5 mmol/L L-glutamine on poly-D-lysine-treated (0.1 mg/ml; Sigma) 100-mm dishes.

Metabolic Labeling and Immunoprecipitation

Cortical cultures plated 3–4 days previously or murine N2a neuroblastoma cells doubly transfected with human βAPP_{695} and the Δ10e FAD mutant human PS1[21] were washed with PBS and incubated at 37°C for 20–30 minutes in methionine-free/glutamine-free Dulbecco's minimum essential medium (Gibco). Cells were labeled with 750 μCi/ml [^{35}S]methionine (NEN/Dupont) (1 Ci = 37 GBq) in methionine-free medium supplemented with N2 and L-glutamine for 4 hours. Cells were scraped into ice-cold PBS with a rubber policeman. The supernatant was aspirated after brief centrifugation, and lysis buffer (100 μl) (0.5% deoxycholate, 0.5% NP-40, Trasylol (5 μg/ml), leupeptin (5 μg/ml), and phenylmethylsulfonyl fluoride (0.25 mmol/L)) was added. The lysate was subjected to agitation, repeat centrifugation, and collection of supernatant. Samples were treated with 0.5% sodium dodecyl sulfate, and the solutions were heated for 2 minutes at 75°C. Samples were adjusted to 190 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 8.3), 6 mmol/L EDTA, and 2.5% Triton X-100. Samples were incubated overnight with either antibody 4G8 or Aβ40/42 antibodies, followed by secondary rabbit anti-mouse antibody (Cappell) for 1 hour and protein A-Sepharose (Pharmacia) beads for 2 hours (all at 4°C). Proteins were analyzed with 10–20% tricine sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by autoradiography on Kodak X-OMAT AR5 film.

Sucrose gradients used to prepare ER- and Golgi-enriched fractions were prepared as previously described.[9] Metabolically labeled cells were homogenized in 0.25 mol/L sucrose, 10 mmol/L Tris-HCl (pH 7.4), 1 mmol/L $MgAc_2$, and a protease inhibitor cocktail (Boehringer-Mannheim). The homogenate was loaded on a

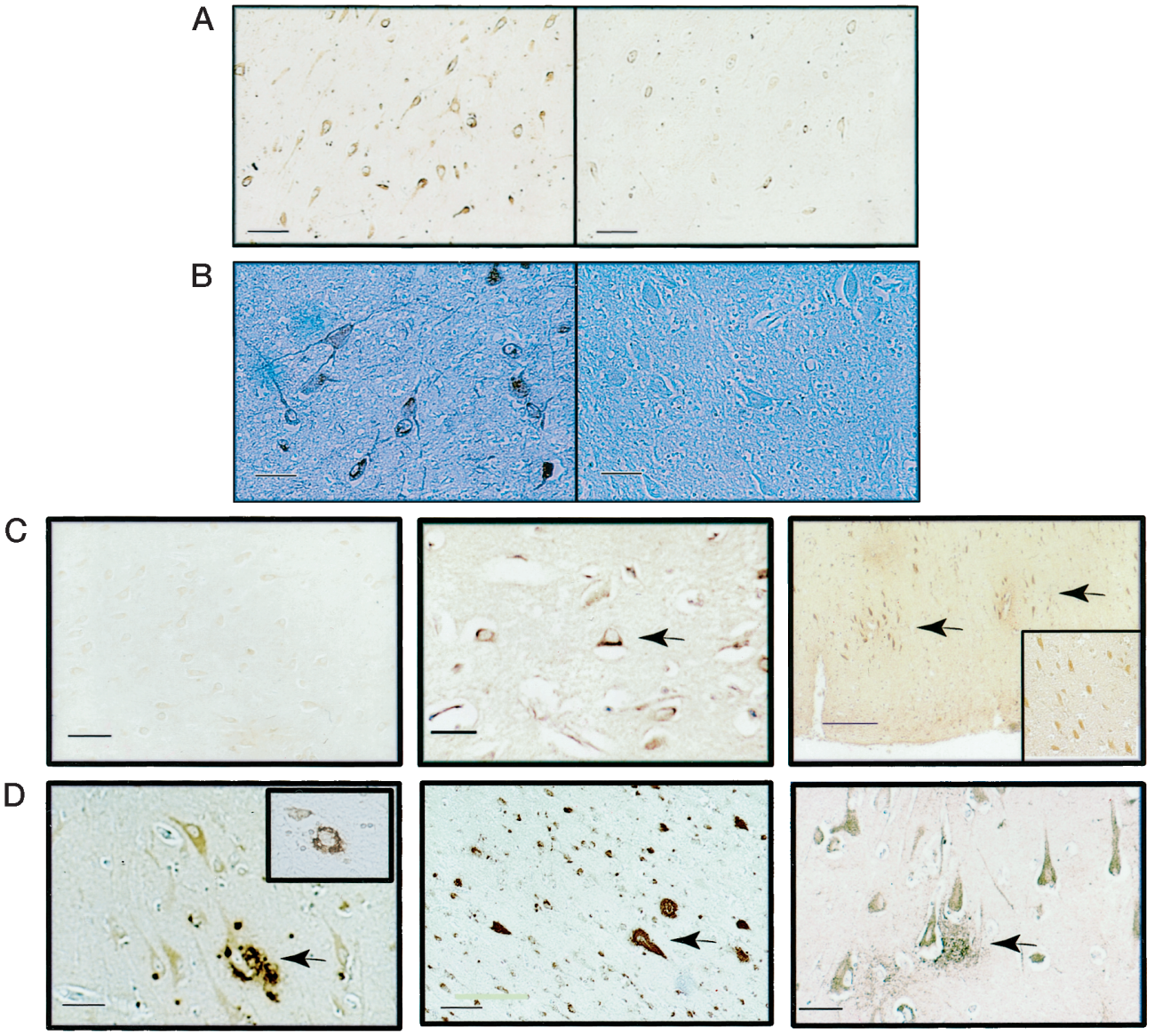

Figure 1. Intraneuronal Aβ42 accumulation occurs in AD-vulnerable neurons before the formation of senile plaques. **A, Left:** Neuronal Aβ42 staining (RU antibody) in the CA1 region of hippocampus derived from a 64-year-old patient with mild (CDR 0.5) cognitive dysfunction. **Right:** Aβ40 staining from the same CA1 region shows only slight immunoreactivity compared with the more pronounced intracytoplasmic staining seen with Aβ42. Antibody concentrations and time of development were equivalent. Bar = 60 μm. **B, Left:** Aβ42 immunoreactivity (RU antibody) in basal forebrain magnocellular neurons. **Right:** This staining is abolished by Aβ1–42 peptide competition; a blue filter was used to highlight negatively staining neurons. Bar = 60 μm. **C, Left:** Aβ42 staining (QCB) in the CA4 region of hippocampus from a neurologically normal 3-year-old patient (control); only faint neuronal staining can be seen (**left**), Bar = 60 μm. **Center:** Pronounced CA4 Aβ42 immunoreactivity (QCB) in a 3-year-old with Down's syndrome. The **arrow** indicates a neuron with intracellular staining. Bar = 40 μm. **Right:** Aβ42 staining (QCB) in a 79-year-old without dementia indicates marked Aβ42 intracellular staining in layer II neurons (**arrows**) of the entorhinal cortex. Bar = 100 μm. **D, Left:** In this 83-year-old cognitively impaired subject (CDR1), the absence of intranuclear Aβ42 staining is evident in neurons stained for Aβ42 (RU). Early Aβ42 aggregates appear to be present within a neuron marked by an **arrow**; the **inset** provides another example of such seemingly intracellular Aβ42 accumulation in (RU) in a 94-year-old CDR 2 case. Bar = 40 μm. **Center:** "Neuronal" shaped SP (**arrow**) adjacent to a more conventional spherical SP in a 72-year-old subject with advanced AD (RU Aβ42). Bar = 60 μm. **Right:** The CA1 region of a 79-year-old cognitively impaired subject (CDR1) demonstrates both intraneuronal Aβ42 immunoreactivity (QCB) and apparent extraneuronal diffuse plaque-like staining (**arrow**) adjacent to a few neurons. Bar = 40 μm.

step gradient of 2 mol/L, 1.3 mol/L, 1.16 mol/L, and 0.8 mol/L sucrose. Gradients were centrifuged for 2.5 hours at 100,000 × *g*. Fractions were collected from the top of each gradient, immunoprecipitated with Aβ40/42 antibodies, and visualized as described above.

Results

Brain tissue from a 64-year-old representative subject with mild cognitive impairment (Clinical Dementia Rating Scale 0.5 (CDR 0.5); $n = 5$), stained with antibodies specific to the C-terminus of Aβ42, revealed significant amounts of region-specific intraneuronal immunoreactivity (Figure 1a, left), compared with relatively little Aβ40 immunoreactivity (Figure 1a, right). This intraneuronal Aβ42 staining was especially evident within pyramidal neurons of areas such as the hippocampus/entorhinal cortex, which are prone to developing early AD neuropathology. Aβ42 staining was less evident in sections from brain regions less affected by AD, such as primary sen-

sory and motor cortices. The non-β-pleated nature of this intracellular Aβ42 is supported by a lack of Bielschowsky silver staining, the absence of Congo red birefringence under polarized light, the lack of thioflavin S staining, and the presence of Aβ42 immunostaining without formic acid pretreatment. The Aβ42 immunoreactivity was seen equivalently by three different Aβ42 antibodies, was abolished by synthetic Aβ1–42 peptide competition (Figure 1b), and was not detected with the use of preimmune serum or in the absence of the primary antibody (data not shown). These Aβ42 antibodies have negligible cross-reactivity to full-length βAPP. Intraneuronal Aβ42 immunoreactivity in a representative normal 3-month-old brain (Figure 1c, left) was of markedly less intensity than that in the brain of a 3-year-old with DS (Figure 1c, center) or the brain of a nondemented 76-year-old (Figure 1c, right). Thus neurons from neurologically normal controls ($n = 6$, ages 3 months to 79 years) showed intraneuronal Aβ42 staining that appeared to increase in relation to the subject's age at death. Analogous to the variability of SP deposition that exists among anatomical regions in an individual and between the same anatomical regions of different subjects, there was variability in the degree of intraneuronal Aβ42 immunoreactivity between anatomical regions (ie, CA1 compared with CA4) within and between individuals.

Because intraneuronal Aβ42 accumulation occurs with early AD pathology, it is possible that extracellular Aβ plaques may develop from this intraneuronally accumulating pool of Aβ42. Consistent with this possibility, we observed instances where Aβ42 appears to aggregate within the cytoplasm of neurons (Figure 1d, left) and where Aβ plaque staining was neuronal in shape (Figure 1d, center). As has been described by others, we also observed diffuse plaque-like Aβ42 immunoreactivity that appears to be located directly outside neurons (Figure 1d, right). Early Aβ42 immunoreactivity was observed along the axonal projections (perforant path) of early Aβ42 accumulating neurons of the entorhinal cortex and at their terminal fields, the outer molecular layer of the dentate gyrus.

It is of particular interest that with increasing cognitive dysfunction and Aβ plaque deposition (CDR 2 subjects, $n = 6$, and severe AD, $n = 3$), we observed that intraneuronal Aβ42 immunoreactivity tended to become less apparent. For example, in layer 2 neurons (islands of Calleja) of the entorhinal cortex from a CDR 1 patient, marked intraneuronal Aβ42 immunoreactivity was observed (Figure 2a), whereas in the patient with more advanced CDR 2 this staining was lost, presumably resulting from death or severe dysfunction of these neurons. In contrast, the emergence of Aβ40 immunoreactive plaques can be seen in the patient with more advanced CDR 2 compared to the CDR1 patient, which is known to occur with disease progression.

In an attempt to elucidate whether Aβ42 immunoreactivity may precede NFT formation, we stained representative sections, taken from several subjects with marked intraneuronal Aβ42 immunoreactivity, with antibody AT8 for hyperphosphorylated tau, the principal component of NFTs. Neurons with Aβ42 immmunoreactivity were more

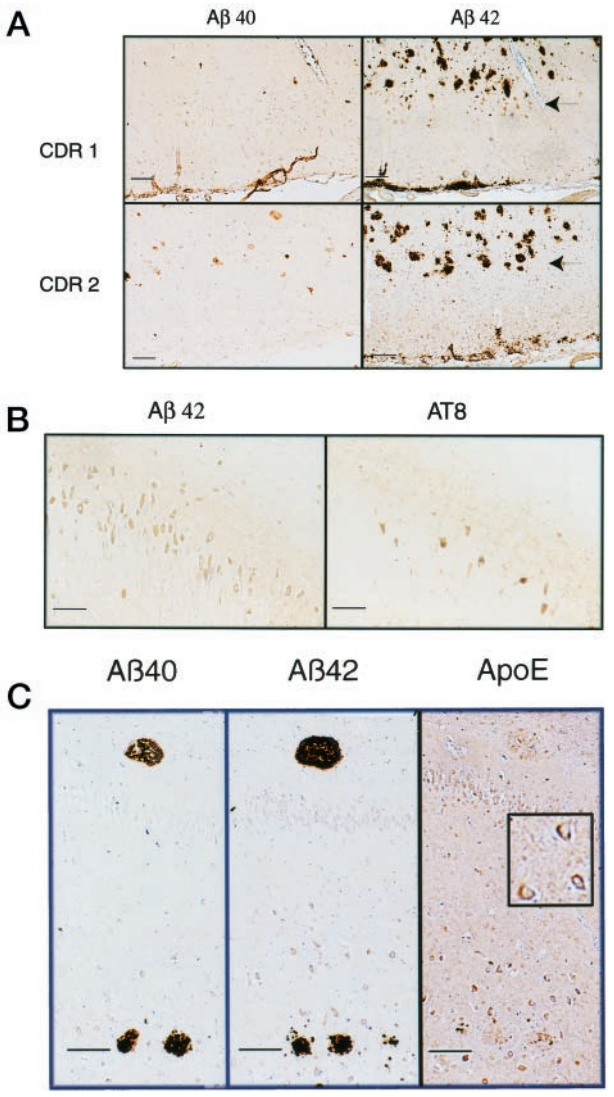

Figure 2. A: Intraneuronal Aβ42 immunoreactivity (QCB) in layer II (islands of Calleja) of the entorhinal cortex (**arrow**) in a 90-year-old CDR1 patient, compared with the absence of staining (**arrow**) in an 83-year-old CDR2 patient; Aβ42 immunoreactive plaques can be seen above. In the CDR 2 patient, note the emergence of Aβ40 SPs. Bar = 100 μm. **B:** Abundant Aβ42 immunoreactivity (RU) compared with only occasional AT8 staining for hyperphosphorylated tau in the CA1 region of a 94-year-old patient (CDR 2). Bar = 60 μm. **C:** Adjacent sections of CA4 (below) and dentate gyrus (above) immunostained with antibodies to Aβ40, Aβ42 (QCB), and apoE in an 83-year-old cognitively impaired patient (CDR 2). Noticeable intraneuronal apoE staining is evident (**inset**, enlarged ×5). Bar = 100 μm.

numerous than those with hyperphosphorylated tau staining (Figure 2b), suggesting that Aβ42 accumulation may occur in the absence of appreciable tau pathology. In agreement with previous reports describing the presence of intraneuronal apoE,[14] we also observed that neurons with marked intracellular Aβ42 immunoreactivity also seemed to stain positively for apoE (Figure 2c), suggesting a possible involvement of apoE in these intracellular events.

To corroborate our light microscopic observations of intraneuronal Aβ42 immunoreactivity, we used metabolic labeling-immunoprecipitation to demonstrate endogenous Aβ42 in primary rodent neuronal cultures. Pulse-labeling of these neuronal cultures, followed by immuno-

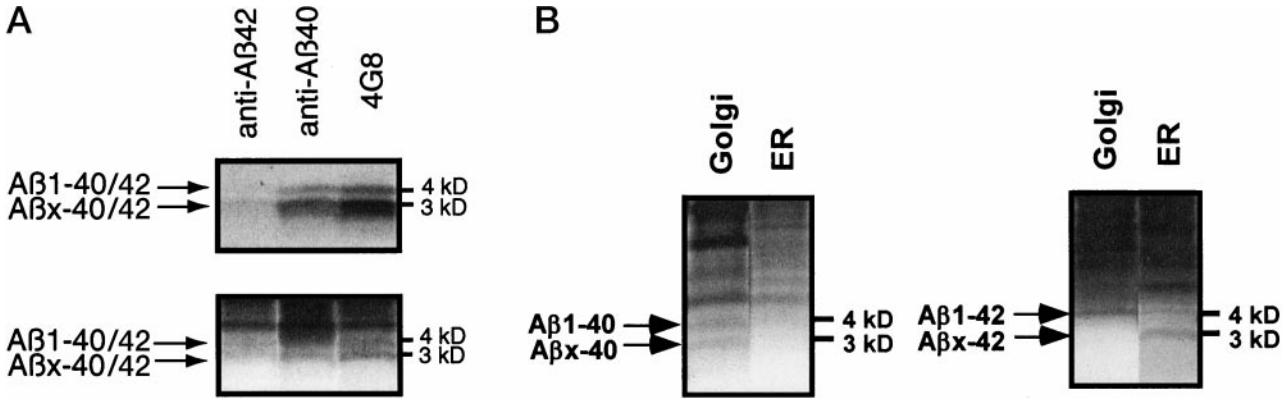

Figure 3. Metabolic labeling and immunoprecipitation of intraneuronal Aβ40 and Aβ42. **A:** Primary mouse neuronal cultures. **Top:** IP of conditioned medium indicates significantly lower secretion of Aβ42 compared with Aβ40. **Bottom:** Comparable amounts of Aβ40 and Aβ42 species in neuronal cell lysate. **B:** Aβ40 and Aβ42 species in sucrose density gradients from neuroblastoma cells harboring the Δ10eFAD PS1 mutation. Aβ1–40, Aβx-40, and Aβ1–42 species predominate in the Golgi-enriched fraction, whereas Aβx-42 predominates in the ER-enriched fraction.

precipitation of conditioned media by the use of Aβ40 and Aβ42 C-terminus-specific antibodies, revealed the expected predominance of secreted Aβ40 over secreted Aβ42 species (Figure 3a, top). In agreement with observations made using Aβ40/Aβ42 enzyme-linked immunosorbent assay in NT2 cells,[6] we observed relatively greater ratios of intracellular Aβ1–42/Aβ1–40 and of Aβx-42/Aβx-40 in neuronal lysates than in conditioned media. In fact, almost equal amounts of Aβx-40 and Aβx-42 species were detected with the use of a standard detergent lysis buffer (Figure 3a, bottom). To more readily detect intracellular Aβ42, we used a murine neuroblastoma N2a cell line harboring the human Δe10 FAD PS1 mutation, which is known to produce elevated levels of Aβ42.[21] Aβ42 was readily detected in the ER- and Golgi-enriched fractions, with most of the secreted Aβ1–42 in the Golgi-enriched fraction and most of the Aβx-42 in the ER-enriched fraction (Figure 3b). Aβ40 species were detected mainly in the Golgi-enriched fraction (Figure 3b).[9]

Discussion

Our immunohistochemical results support the concept that Aβ42 accumulation within neurons is an early pathological step in the cascade of events underlying AD neuropathology. Our immunohistochemical data cannot define the N-termini of the Aβ42 peptides, because our antibodies differentiate only the C-termini of Aβ. In addition to traditional Aβ1–40/42, various NH_2-terminal truncated Aβ species have been described and suggested to be pathologically important.[9,20,22,23] Similar to the earliest Aβ42 deposited in SPs, intraneuronally accumulating Aβ42 also appears to be N-terminally truncated, as evidenced by the relative paucity of Aβasp1 and 6E10 (directed at Aβ1–10 epitope) as compared with Aβ42 and 4G8 (directed at Aβ17–24 epitope) antibody immunoreactivities (G. K. Gouras, personal observations). The possibility of this Aβ42 staining being due to artifactually shared epitope(s) appears unlikely, because intraneuronal Aβ42 immunoreactivity was replicated by three sets of antibodies and was not found to be present either with the use of preimmune serum or after Aβ1–42 peptide competition. Because intraneuronal Aβ42 immunoreactivity becomes less noticeable with disease progression, it seems that Aβ42-containing neurons may be lost and/or replaced by "ghost" tangles and/or plaques. The abundance of Aβ within senile plaques may also compete for antibody with the less abundant intracellular Aβ. The apparent disappearance of this staining, early on in the process of dementia, may provide an explanation for why intraneuronal Aβ immunoreactivity has not been appreciated by earlier investigators.

The subcellular compartment(s) within which Aβ42 peptides accumulate remains to be identified. One interesting study reported disruption of the Golgi apparatus as an early event in AD neuropathology and postulated that this may even proceed NFT development.[24] Given the growing body of evidence that both Aβ40 and Aβ42 formation occurs in the Golgi,[4,9] it is conceivable that Aβ42 may begin accumulating abnormally within this organelle. However, more recent evidence indicates that Aβ42 cleavage can also occur earlier in the secretory pathway in the ER, with retention of the peptide within this compartment.[7–9]

Accumulating Aβ42 may cause disruption of the cytoskeleton and initiate the formation of aggregated intracellular tau. Our proposal that intracellular accumulation of Aβ42 disrupts the normal functioning of neurons is supported by increasing reports of cellular dysfunction within AD-susceptible neurons, such as the presence of markers of apoptosis[14] and oxidative injury,[25] even before senile plaque and NFT formation. This proposal is further supported by the recent report of intraneuronal Aβ42 accumulation and neural degeneration in FAD PS1 mutant transgenic mice in the absence of Aβ plaque deposition.[11] Neuronal dysfunction arising from aggregating intraneuronal Aβ42 may also explain recent studies reporting plaque-independent functional and structural disruption of neural circuits in βAPP transgenic mice.[26,27]

The role of apoE in AD remains incompletely understood. The decrease in plaque load of βAPP transgenic mice crossed to apoE knockouts suggests an important relationship between apoE and aggregated Aβ.[28] With

Aβ accumulation and neuronal dysfunction, neuronal or astrocyte-generated apoE may potentially bind to Aβ intraneuronally and/or extracellularly with subsequent neuronal internalization, explaining the observation of apparent increased apoE immunoreactivity in Aβ42 immunoreactive neurons.

Our observations of early intraneuronal accumulation ofAβ42 within those brain areas that are affected earliest by AD suggest a mechanism that may explain AD disease progression within the brain. Intraneuronal Aβ42 may act as a nidus for Aβ deposition, intraneuronally and extracellularly, at the soma and along processes and terminals of affected neurons. The resultant accumulation of Aβ in the parenchyma may hasten the pathological process, providing a potential mechanism for the "spread" of Aβ-related pathology.

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