Intraneuronal APP, Not Free A β Peptides in 3xTg-AD Mice: Implications for Tau versus A β -Mediated Alzheimer Neurodegeneration

Matthew J. Winton,¹ Edward B. Lee,¹ Eveline Sun,¹ Margaret M. Wong,¹ Susan Leight,¹ Bin Zhang,¹ John Q. Trojanowski,^{1,2} and Virginia M.-Y. Lee^{1,2}

¹Center for Neurodegenerative Disease Research, Department of Pathology and Laboratory Medicine and ²Institute on Aging, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

Alzheimer's disease (AD) is characterized by the accumulation of intraneuronal tau and extracellular amyloid- β (A β) peptide. A triple transgenic (Tg) mouse (3xTg-AD) was reported to develop A β plaques and tau inclusions as well as remarkable accumulations of intracellular A β that were suggested to be the initiators of AD pathogenesis. However, it was unclear whether the anti-A β antibodies were able to distinguish A β peptide from the same A β epitopes within the amyloid precursor protein (APP). To further elucidate the identity of the immunoreactive intraneuronal material in 3xTg-AD mice, we conducted immunohistochemical, biochemical, and ultrastructural studies using a well characterized panel of antibodies that distinguish A β within APP from cleaved A β peptides. We found that the intraneuronal material shared epitopes with full-length APP but not free A β . To demonstrate unequivocally that this intraneuronal material was not free A β peptide, we generated 3xTg-AD mice deficient for β -secretase (BACE), the protease required for A β generation from APP. In the absence of A β production, robust intraneuronal A β immunostaining was detected in the 3xTg-AD/BACE^{-/-} mice. Finally, we found that the formation of tau lesions was not different between 3xTg-AD versus 3xTg-AD/BACE^{-/-} mice, thereby demonstrating that tau pathology forms independently from A β peptide generation in this mouse model. Although we cannot corroborate the presence of intraneuronal A β peptide in 3xTg-AD mice, our findings warrant further study as to the role of aberrant APP accumulation in this unique model of AD.

Introduction

Alzheimer's disease (AD) pathology is characterized by senile plaques (SPs) and neurofibrillary tangles (NFTs) (Selkoe, 2001). SPs are extracellular deposits of amyloid- β (A β), a 3–4 kDa peptide derived from proteolytic cleavage of the amyloid precursor protein (APP) by β -site APP cleavage enzyme 1 (BACE) (Hussain et al., 1999; Sinha et al., 1999; Vassar et al., 1999; Yan et al., 1999) and the presenilin (PS)-containing γ -secretase complex (De Strooper et al., 1998; Wolfe et al., 1999). NFTs are intracellular accumulations of hyperphosphorylated tau (Lee et al., 2001). About 5% of AD cases are linked to pathogenic mutations in APP, PS1, or PS2 genes (Selkoe, 2001). Tau gene mutations are pathogenic for familial frontotemporal lobar degeneration

characterized by tau pathology without SPs, indicating that tau abnormalities alone cause neurodegenerative disease (Lee et al., 2001).

Multiple transgenic (Tg) AD mouse models expressing mutant forms of APP, PS, or tau have been developed (Duff and Suleman, 2004). Although these Tg models have been invaluable, they are limited by the absence of the full spectrum of AD neuropathology, including both NFTs and SPs. To overcome this, a triple Tg AD mouse model was generated (3xTg-AD) that over-express human tau and APP in a mutant PS1 knock-in background that results in AD-like SPs and NFTs (Oddo et al., 2003). These mice show early accumulations of intraneuronal A β peptide immunoreactivity that precede SPs, NFTs, synaptic dysfunction, and memory impairments. However, it is unclear whether the antibodies used in this study discriminated between free/cleaved A β peptides versus A β epitopes within the APP holoprotein. Indeed, although intraneuronal A β was first recovered from human neurons >15 years ago (Wertkin et al., 1993), their role in AD pathogenesis remains controversial (Gouras et al., 2010). Thus, certainty about whether intraneuronal A β immunoreactivity in 3xTg-AD mice represents free A β peptides or uncleaved A β within the APP holoprotein is critical for understanding if intraneuronal A β plays a mechanistic role in AD. For these reasons, we characterized the intraneuronal A β immunoreactivity in the 3xTg-AD mice using immunohistochemical (IHC), biochemical, and ultrastructural methods with a well characterized

Received Dec. 16, 2010; revised March 25, 2011; accepted April 15, 2011.

Author contributions: M.J.W., E.B.L., J.Q.T., and V.M.-Y.L. designed research; M.J.W., E.B.L., E.S., M.M.W., S.L., and B.Z. performed research; M.J.W., E.B.L., E.S., M.M.W., S.L., B.Z., J.Q.T., and V.M.-Y.L. analyzed data; M.J.W. and E.B.L. wrote the paper.

This work was funded by grants from the National Institutes of Health (AG17586, AG10124, AG00255). J.Q.T. is the William Maul Measey-Truman G. Schnabel Jr. M.D. Professor of Geriatric Medicine and Gerontology. V.M.-Y.L. is the John H. Ware third Chair of Alzheimer's Disease Research at the University of Pennsylvania. We thank Dr. L.K. Kwong for her comments and assistance on this manuscript, and Drs. J. C. Carroll and C. J. Pike for providing mouse tissues.

The authors declare no competing financial interests.

Correspondence should be addressed to Virginia M.-Y. Lee, Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, Maloney Building, Third Floor, Hospital of the University of Pennsylvania, 3600 Spruce Street, Philadelphia, PA 19104-4283; E-mail: vmylee@upenn.edu.

DOI:10.1523/JNEUROSCI.6637-10.2011

Copyright © 2011 the authors 0270-6474/11/317691-09\$15.00/0

Table 1. Panel of APP, A β , BACE, and tau antibodies

Protein	Antibody	Method	Reference/vendor
BACE	3D5 (mouse monoclonal antibody raised against the catalytic domain, residues 46–460)	IB	Zhao et al., 2007
Total APP	Karen (goat anti-N-terminal APP)	IB, IF, IHC	Turner et al., 1996
Full-length APP	22C11 (mouse monoclonal antibody recognizing N terminus of APP)	IF, IHC, immuno-EM	Weidemann et al., 1989
Full-length APP	5685 (rabbit polyclonal raised against last 40 C-terminal residues of APP)	IF, IB, IHC, IP	Lee et al., 2005
Phospho-APP	PhAT (rabbit polyclonal raised against residues 665–673 containing phospho-Thr668 of the C terminus of APP)	IP	Lee et al., 2005
APP/A β	4G8 (mouse monoclonal raised against A β _{17–24})	IF, IB, IHC	Kim et al., 1988
APP/A β	6E10 (mouse monoclonal recognizing A β _{1–10})	IF, IHC	Kim et al., 1988
APP/A β	Ban50 (mouse monoclonal recognizing A β _{1–10})	IB, IF, IHC, ELISA	Suzuki et al., 1994
A β /C99	82E1 (mouse monoclonal recognizing free N terminus of A β /C99)	IHC	Osawa et al., 2008
A β ₄₀	BA27 (mouse monoclonal recognizing A β peptides ending in residue 40)	IF, IHC, ELISA	Iwatsubo et al., 1994
A β ₄₀	JRF/c40 (mouse monoclonal recognizing A β ₄₀ peptides ending in residue 40)	IHC	Janus et al., 2000
A β ₄₂	BC05 (mouse monoclonal recognizing A β peptides ending in residue 42)	IF, IHC, ELISA	Iwatsubo et al., 1994
A β ₄₂	JRF/c42 (mouse monoclonal recognizing A β peptides ending in residue 42)	IHC	Janus et al., 2000
Tau	17025 (rabbit polyclonal raised against human recombinant tau)	IB, IHC	Ishihara et al., 2001
Phospho-tau	AT8 (mouse monoclonal specific for tau phosphorylated at Ser202 and Thr205)	IB, IHC	Mercken et al., 1992; Goedert et al., 1995
β -Tubulin	Tub2.1 (mouse monoclonal)	IB	Sigma-Aldrich

IB, Immunoblot; IF, immunofluorescence; IHC, immunohistochemistry; IP, immunoprecipitation; ELISA, enzyme-linked immunosorbent assay.

panel of end-specific A β antibodies that exclusively recognize A β peptides ending in A β ₄₀ and A β ₄₂, but not A β sequences within APP. Second, we adopted a genetic approach and generated a 3xTg-AD mouse deficient for BACE (3xTg-AD/BACE^{-/-}), thereby preventing A β peptide generation to determine whether intraneuronal A β immunoreactivity was still present. These combined strategies demonstrated the absence of cleaved A β and intraneuronal A β peptides in the 3xTg-AD mice. Thus, intraneuronal A β immunoreactivity in these Tg mice does not correspond to free A β peptides, but instead reflects the presence of uncleaved A β within the APP holoprotein. Moreover, BACE deficiency did not alter the temporal sequence or abundance of tau pathology in the 3xTg-AD mice, thereby showing that tau pathology in this mouse model develops independently of A β peptide generation. These studies further clarify the identity of intraneuronal A β immunoreactivity in the 3xTg-AD mice and document that the pathways leading to tau and A β amyloidosis in this mouse model are independent.

Materials and Methods

Generation of 3xTg-AD;BACE^{-/-} Tg mice. Homozygous 3xTg-AD mice were obtained from F. LaFerla, University of California, Irvine, CA (Oddo et al., 2003). Tg2576 mice were obtained from K. Ashe, University of Minnesota, Minneapolis, MN (Hsiao et al., 1996), and PSI_{P264L}/APP_{Swc} Tg mice and BACE^{-/-} mice (MGI:2389228) were obtained from The Jackson Laboratory (Cai et al., 2001). All mice were expanded according to University of Pennsylvania (Philadelphia, PA) Institutional Animal Care and Use Committee guidelines. To generate 3xTg-AD/BACE^{-/-} mice, 3xTg-AD homozygous mice were crossed with homozygous BACE^{-/-} mice to produce quadruple heterozygous Tg offspring, as determined by PCR analysis using mouse tail DNA. Heterozygous mice were subsequently bred to produce male and female mice homozygous for all four transgenes. Homozygous lineages were verified by backcrossing to non-Tg mice and by PCR.

Biochemical analysis of APP, tau, and BACE expression. Proteins were extracted by homogenizing wild type (WT) and Tg mouse brains in 1 ml of RIPA buffer (0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate, 5 mM EDTA, 150 mM NaCl, 50 mM Tris base, pH 8.0) per 150 mg of tissue. Protease inhibitors (1 μ g/ml pepstatin A, leupeptin, 1-L-tosylamido-2-

phenylethyl chloromethyl ketone, 1-chloro-3-tosylamido-7-amino-2-naphthanol, soybean trypsin inhibitor, and 0.5 mM PMSF) (Sigma-Aldrich) were added to buffers before use. Protein concentrations were determined by the bicinchoninic acid (BCA) method (Pierce), and proteins were resolved by SDS-PAGE (7.5%) or with 16% Tris-tricine gels for C-terminal APP fragments and transferred to nitrocellulose membranes for immunoblotting. Following transfer, nitrocellulose membranes were blocked in 5% powdered milk and incubated with primary antibody (for a complete list of all antibodies used in this study, see Table 1) overnight at 4°C. Primary antibodies were detected with horseradish peroxidase-conjugated IgG antibodies (Jackson ImmunoResearch), and blots were developed with Renaissance Enhanced Luminol Reagent (PerkinElmer). Digital images were acquired using a Fuji Film Intelligent Darkbox II (Fuji Systems). For enhanced sensitivity, C-terminal APP fragment blots were probed with IRDye-labeled secondary antibodies and visualized with the Odyssey Imager (Licor Biosciences).

Antibodies. The antibodies used in this study are described in Table 1.

Immunoprecipitation. Brain regions were homogenized in RIPA buffer and protein concentrations were determined by the BCA method (Pierce) for immunoprecipitation studies as reported earlier (Lee et al., 2005). Briefly, lysates were precleared with Protein A/G Agarose beads (Santa Cruz Biotechnology) and immunoprecipitated with 5685, a rabbit polyclonal antibody (PAb) raised to the last 40 aa of APP, or PhAT, a PAb raised to residues 665–673 containing phospho-Thr668 at the C terminus of APP (Lee et al., 2005), complexed with Protein A/G Agarose beads. Immunoprecipitated proteins were separated by SDS-PAGE (7.5%) and analyzed by immunoblot as described above.

Histochemical and IHC analysis. The mice were deeply anesthetized and transcardially perfused with PBS. Brains and spinal cords were removed, fixed in 10% neutral buffered formalin for 24 h, dehydrated through a series of graded ethanols to xylene, and infiltrated with paraffin. IHC analysis was conducted as described previously (Ishihara et al., 2001; Lee et al., 2005). Six-micrometer-thick tissue sections were rehydrated and endogenous peroxidases were blocked with methanol/hydrogen peroxide. Sections were blocked with 2% fetal bovine serum in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl and incubated with primary antibodies (see Table 1) overnight at 4°C. Sections were reacted with biotinylated-conjugated secondary antibodies and developed with diaminobenzidine using Vectastain avidin-biotin complex (ABC) kit (Vec-

tor Laboratories). Alternatively, 50 μ m vibratome sections were obtained from paraformaldehyde-fixed brains, and free floating sections were stained as described above using the ABC method. Double-labeling immunofluorescence (IF) studies were performed by coincubating sections with the primary antibodies indicated in Table 1 according to previously described procedures (Ishihara et al., 2001). Following intensive washes, sections were labeled with Alexa Fluor 488-conjugated (green) and 594-conjugated (red) secondary antibodies (Invitrogen) and coverslipped with Vectashield 4',6-diamidino-2-phenylindole mounting medium (Vector Laboratories). The sections were viewed using a Nikon TE-2000-E microscope (Nikon), and all images were captured using a CoolSnap-HQ camera (Photometrics).

Pre-embedding immuno-electron microscopy. Immuno-electron microscopy (immuno-EM) was performed as described previously (Yoshiyama et al., 2007) using brains from 5- to 7-month-old 3xTg-AD, Tg2576, or WT mice ($n = 3$ of each) that were anesthetized and perfused with 0.1 M cacodylate buffer, pH 7.5, followed by 2% paraformaldehyde and 0.5% glutaraldehyde. Cortical tissues were fixed for 6 h, washed in PBS, cut into 50- μ m-thick sections with a vibratome, and reacted with 0.1% sodium borohydride in PBS for 15 min. Following extensive blocking, sections were labeled with primary antibody, 22C11, and sequentially incubated with a biotinylated goat anti-mouse antibody and ABC reagents. The sections were silver enhanced as described previously (Rodriguez et al., 1984). The sections were dehydrated and embedded in epoxy resin. Ultrathin sections were cut and observed using a Joel 1010 electron microscope.

Sandwich ELISA analysis. To measure distinct species of A β peptides, cortices from WT, BACE^{-/-}, 3xTg-AD, and 3xTg-AD BACE^{-/-} mice were dissected and sonicated in RIPA buffer (1 ml/150 mg tissue) containing protease inhibitors and centrifuged at 100,000 $\times$ g for 30 min at 4°C. The supernatants were removed and the pellets were sonicated in 70% formic acid (FA) (2 μ l/mg tissue) and centrifuged at 100,000 $\times$ g for 30 min at 20°C. Both RIPA and FA lysates were assayed by sandwich ELISA as described previously (Lee et al., 2003a). Ban50 (anti-A β _{1–10}) was used as the human A β capturing antibody, whereas the A β end-specific BA27 and BC05 monoclonal antibodies were used to detect A β _{1–40} and A β _{1–42}, respectively.

Results

Intraneuronal APP, not free A β peptides, are detected in 3xTg-AD mice

We sought to clarify the identity of the intraneuronal A β immunoreactivity in the 3xTg-AD mice by performing IHC and biochemistry using an extensive panel of antibodies specific for the N-terminal ectodomain of APP, the extreme C terminus of APP, epitopes within the A β domain, and end-specific or cleavage-specific epitopes on the free C terminus of A β peptides ending at residue 40 or residue 42 (see Table 1 for details). No intracellular immunoreactivity was detected in either 5- to 7-month-old or 10- to 12-month-old 3xTg-AD mice by the A β _{x-40} or A β _{x-42} cleavage-specific antibody BA27 (Fig. 1*A,B*) or antibody BC05 (Fig. 1*C,D*), respectively. The absence of intraneuronal A β immunostaining was confirmed using the C-terminal cleavage-specific antibodies JRF/c40 or JRF/c42, which recognize A β , and the N-terminal cleavage-specific antibody 82E1, which recognize A β /C99 (data not shown). Despite the absence of intraneuronal immunoreactivity, these A β cleavage-specific antibodies (and antibodies recognizing epitopes within the A β domain) did detect extracellular A β plaques in 10- to 12-month-old 3xTg-AD mice, aged Tg2576 mice, aged APP/PS1 mice, and human AD brain sections, confirming their ability to detect A β peptides by IHC (Fig. 1*B,D,H,I*; data not shown). In contrast, robust intracellular APP immunoreactivity was observed with the N-terminal APP-specific antibodies 22C11 (Fig. 1*E,F*) and Karen (data not shown). Similar intracellular APP staining was also detected using the 6E10 (Fig. 1*G,H*), 4G8 (Fig. 1*I,J*), and

Figure 1. 3xTg-AD mice exhibit intraneuronal APP and not intraneuronal A β . Representative images of immunostained cerebral cortex (*A, C, E, G, I*) and hippocampus (*B, D, F, H, J*) from 5- to 7-month-old (*A, C, E, G, I*) and 10- to 12-month-old (*B, D, F, H, J*) 3xTgAD mice using a panel of antibodies specific to A β peptides ending in either residue 40 (BA27) (*A, B*) or 42 (BC05) (*C, D*), the N-terminal domain of APP (22C11) (*E, F*), and both APP/A β (6E10 or 4G8) (*G, H* or *I, J*, respectively). No intraneuronal A β immunoreactivity was detected with A β C-terminal cleavage-specific antibodies, although extracellular A β deposits were detected in 10- to 12-month-old 3xTg-AD.

Ban 50 (data not shown), antibodies that recognize an epitope within the A β domain and therefore recognize full-length APP and APP cleavage products containing the A β domain, including A β _{1–40/42}. Additionally, immunohistochemical staining of free-floating 50 μ m sections from 3xTg-AD mice similarly showed intracellular APP staining using N-terminal APP antibodies and no intraneuronal immunoreactivity using the C-terminal

cleavage-specific antibodies BC05 and BA27, indicating that the staining results presented here are not due to tissue processing artifacts (data not shown). Under the most sensitive conditions using both formic acid and citrate boiling antigen retrieval methods and high antibody concentrations, faint intraneuronal APP immunoreactivity was detected in the Tg2576 mice (data not shown), consistent with the known overexpression of APP in these mice. However, we observed no evidence of intraneuronal A β in 3xTg-AD or Tg2576 mice. Taken together, our results indicate that the observed intraneuronal immunostaining in 3xTg-AD mice reflects the presence of A β immunoreactivity in APP and not free A β peptides.

Intracellular accumulation of APP is independent of APP expression and phosphorylation

To assess whether the observed intraneuronal APP accumulations in 3xTg-AD mice are due to high levels of overexpressed APP, immunoblots directly comparing APP levels in 3xTg-AD and Tg2576 mice were conducted. Using a panel of antibodies specific for different APP species (see Table 1), significantly lower levels of APP transgene expression were detected in 3xTg-AD mice as compared to age-matched Tg2576 mice (Fig. 2A). C-terminal APP fragment levels were very low relative to Tg2576 mice and were barely increased over endogenous levels (Fig. 2A). Notably, Tg2576 mice develop robust extracellular amyloid pathology in the absence of intracellular APP accumulation (data not shown).

Phosphorylation of APP at Thr68 occurs after transit through the Golgi apparatus, just preceding its selective targeting out of neuronal perikarya into their axons (Suzuki et al., 1997). Thus, we asked whether the accumulation of intraneuronal APP in 3xTg-AD mice results in altered phosphorylation levels as a measure of aberrant perikaryal APP trafficking. To do this, we examined phospho-APP levels in both 3xTg-AD and Tg2576 mice using an anti-phospho-APP-threonine 688-specific antibody, PhAT (Lee et al., 2005) (Fig. 2B). Immunoprecipitation studies showed that similar proportions of total APP are phosphorylated in both 3xTg-AD and Tg2576 mice, suggesting that vesicular APP trafficking, at least to the relatively late compartment in which APP is phosphorylated, is similar between these two mouse models.

To better understand the localization of intraneuronal APP immunoreactivity,

Figure 2. Steady-state APP expression in Tg mice. **A**, Cortical lysates from Tg2576, 3xTg-AD, and non-Tg mice (nonTg) were immunoblotted for various APP species. Total APP, including full-length APP and all soluble APP (sAPP) species were probed with Karen (anti-N-terminal APP). Two exposures are shown for optimal visualization. Full-length APP was probed with 4G8 and 5685, and C-terminal APP fragments (CTF) were probed with 5685. β -Tubulin was used as a loading control. **B**, Cortical lysates from Tg2576, 3xTg-AD, and non-Tg mice were immunoprecipitated with 5685 or PhAT following immunoblotting with Karen (anti-N-terminal APP) to detect full-length APP or phosphorylated full length APP, respectively. **C–E**, Immuno-EM of cortex from 3xTg-AD mice using the N-terminus-specific APP antibody 22C11. **C**, **D**, Diffuse pools of APP are detected in the cytoplasm of cortical neurons. **E**, Some APP is localized to nerve terminals (arrowhead). Scale bars: **C**, **D**, 100 nm; **E**, 1 μ m.

Figure 3. Genetic removal of BACE in 3xTg-AD mice. **A**, RIPA lysates from the cortices of aged 3xTg-AD, nontransgenic (nTg), 3xTg-AD;BACE^{-/-}, and BACE^{-/-} mice were immunoblotted for tau (17025), APP (Ban50, a human-specific anti-A β 1–10 antibody), and Karen, anti-N-terminal APP that detects all forms of APP, BACE (3D5), and C-terminal APP fragments (5685). β -Tubulin was used as a loading control. Note the absence of BACE protein in the 3xTg-AD;BACE^{-/-} and BACE^{-/-} lanes. **B**, Steady-state A β levels from RIPA (soluble; gray bars) and FA (insoluble; black bars) cortical lysates ($n = 3$ per genotype 10–12 months old) were quantified by Ban50-BA27/BC05 sandwich ELISA for full-length A β ₄₀ (top) and A β ₄₂ (bottom).

immuno-EM analysis of 5- to 7-month-old 3xTg-AD mice was performed using an anti-APP antibody, 22C11, which detected abundant diffuse immunolabeling of APP of vesicular structures throughout neuronal perikarya (Fig. 2*C,D*) with some presynaptic terminal labeling (Fig. 2*E*) of cortical neurons. In contrast, no APP immunolabeling was seen in neuronal cytosol of any of the Tg2576 mice examined by immuno-EM with the same antibodies (data not shown). Finally, no EM evidence of degenerating neurons was seen in any of the mouse brains examined here as evidenced by electron-dense cytoplasm, pyknotic nuclei, etc.

Generation of 3xTg-AD;BACE^{-/-} Tg mice

BACE is the major β -secretase responsible for APP cleavage, and genetic knock-out of the BACE gene precludes A β production (Hussain et al., 1999; Sinha et al., 1999; Vassar et al., 1999; Yan et al., 1999). To unequivocally confirm the presence of intraneuronal APP and the absence of cleaved A β peptides in the 3xTg-AD mice, we generated 3xTg-AD mice on the BACE-null background. The genetic absence of BACE in 3xTg-AD/BACE^{-/-} animals was documented by PCR (data not shown), and the absence of BACE protein and activity was demonstrated biochemically by immunoblotting (Fig. 3*A*) and by sandwich ELISA (Fig. 3*B*). Immunoblots for BACE clearly show the absence of the ~70 kDa BACE protein in 3xTg-AD/BACE^{-/-} and BACE^{-/-} mice (Fig. 3*A*). The absence of BACE protein had no effect on the expression levels/accumulation of tau and APP transgenes in 3xTg-AD mice (Fig. 3*A*), but the lack of BACE resulted in increased levels of mature (i.e., N- and O-glycosylated) APP, consistent with the absence of BACE cleavage of APP and therefore increased flux through the Golgi apparatus (Fig. 3*A*). Although C-terminal APP fragments were difficult to detect given their low levels, we did observe a decrease in exogenous β -C-terminal fragment (C99) levels in 3xTg-AD; BACE^{-/-} mice and a decrease in endogenous β -C-terminal fragment (C89) levels in BACE^{-/-} mice relative to their BACE^{+/+} counterparts (Fig. 3*A*). In concert with the absence of A β generation in the BACE knock-out background, ELISA quantification of both soluble and insoluble cortical fractions from older 10- to 12-month-old 3xTg-AD; BACE^{-/-} showed that both A β ₁₋₄₀ (top) and A β ₁₋₄₂ (bottom) were undetectable in these 3xTg-AD/BACE^{-/-} animals (Fig. 3*B*).

Intracellular APP but not cleaved A β is detected in 3xTg-AD;BACE^{-/-} mice

At all time points examined (5–7 and 10–12 months), analysis of homozygous 3xTg-AD/BACE^{-/-} mice demonstrated a similar IHC profile to that of age-matched 3xTg-AD mice except for the presence A β plaques in 3xTg-AD mice and no plaques in the homozygous 3xTg-AD/BACE^{-/-} mice (Fig. 4, compare *B*, *D* to Fig. 1). Significantly, using the C-terminal cleavage-specific antibody panel, no intracellular or extracellular immunoreactivity was seen in 5- to 7-month-old or 10- to 12-month-old 3xTg-AD/BACE^{-/-} animals (Fig. 4*A–D*). This finding notwithstanding, robust intraneuronal APP immunoreactivity was seen as exemplified by staining with 22C11 (Fig. 4*E,F*) and Karen (data not shown). Similar intracellular staining was also detected using the antibodies that recognize the A β domain within APP such as 6E10 (Fig. 4*G,H*), 4G8 (Fig. 4*I,J*), and Ban 50 (data not shown).

To further confirm the presence of intraneuronal A β immunoreactivity in APP and not free A β peptides in 3xTg-AD mice, double-labeled IF staining was conducted using a variety of antibodies specific to APP (22C11 and 5685), the A β domain within APP (4G8 and Ban50), and cleaved A β peptides ending in either residue 40 or residue 42 (BA27 and BC05). 3xTg-AD mice at 5–7

Figure 4. 3xTg-AD;BACE^{-/-} mice exhibit intraneuronal APP but not intraneuronal A β . IHC was performed on cortical and hippocampal sections from 5- to 7-month-old (*A*, *C*, *E*, *G*, *I*) and 10- to 12-month-old (*B*, *D*, *F*, *H*, *J*) 3xTgAD;BACE^{-/-} mice using a panel of antibodies specific to cleaved A β peptides ending in either residue 40 (BA27) (*A*, *B*) or 42 (BC05) (*C*, *D*), the N-terminal domain of APP (22C11) (*E*, *F*), and both APP/A β (6E10 or 4G8) (*G*, *H* or *I*, *J*, respectively).

months of age showed complete colocalization between 5685, an antibody against the extreme C terminus of APP, and 22C11, an antibody specific for the N-terminal ectodomain of APP (Fig. 5). This colocalization of C-terminal and N-terminal APP epitopes was confirmed further using 5685 with the N terminus-specific goat PAb, Karen (data not shown). Colocalization was observed throughout the hippocampus (data not shown) and cortex (Fig. 5*A–C*).

Furthermore, IF of 3xTg-AD/*BACE*^{-/-} mice, in the absence of A β production, also showed complete colocalization between antibodies specific to the N terminus and C terminus of APP (22C11 and 5685, respectively), again throughout the hippocampus (data not shown) and cortex (Fig. 5*D–F*). A similar robust colocalization was detected using 5685 with 4G8 (Fig. 5*G–I*) and 5685 with Ban50 (data not shown) antibodies. Furthermore, IF analysis using the cleavage-specific A β antibodies BA27 (Fig. 6*A–F*) and BC05 (Fig. 6*G–L*) revealed no intraneuronal A β immunoreactivity in the cortex of either 3xTg-AD (Fig. 6*A–C, G–I*) or 3xTg-AD/*BACE*^{-/-} (Fig. 6*D–F, J–L*) mice. Cortical sections from Tg2576 mice and PS1_{P264L}/APP_{SWE} mice were used as controls for all IF studies (data not shown). Together, these results add additional confirmation that the intraneuronal A β immunoreactivity in the 3xTg-AD and 3xTg-AD/*BACE*^{-/-} mice corresponds to full-length intracellular APP and not free A β peptides.

The development of tau pathology is independent of A β production

The initial characterization of the 3xTg-AD mice demonstrated a temporal relationship between intraneuronal A β immunoreactivity and the subsequent development of A β plaques and intraneuronal tau pathologies (Oddo et al., 2003). To further understand the role of A β in the onset and progression of tau pathology, IHC was conducted on 3xTg-AD and 3xTg-AD/*BACE*^{-/-} mice. At all time points examined (5–7 and 10–12 months), IHC analysis of homozygous 3xTg-AD/*BACE*^{-/-} mice demonstrated a similar IHC tau immunoreactivity profile compared to homozygous 3xTg-AD mice. For example, using phospho-independent (affinity-purified 17025) (Fig. 7*A,B,D,E*) and phospho-dependent (AT8) (Fig. 7*C,F*) tau antibodies, we observed a similar extent of tau immunoreactivity in the perikarya of cortical and hippocampal neurons of 5- to 7-month-old 3xTg-AD (Fig. 7*A–C*) and 3xTg-AD/*BACE*^{-/-} mice (Fig. 7*D–F*). Tau immunoreactivity increased in older 10- to 12-month-old 3xTg-AD (Fig. 7*G–I*). However, the absence of BACE protein and therefore free A β peptides did not alter the distribution or intensity of tau pathology (Fig. 7*J–L*). Thus, these results demonstrate that tau pathology in 3xTg-AD mice develops independently of A β peptide generation.

Discussion

Extracellular A β is likely central to the pathogenesis of AD by contributing to neurotoxicity and the disruption of multiple physiological processes (Selkoe, 2001). In contrast, since our initial identification of intraneuronal A β in human neuronal NT2N cells (Wertkin et al., 1993; Turner et al., 1996; Skovronsky et al., 1998; Lee et al., 2003a), there has been much debate and controversy about the role of intracellular A β in the pathogenesis of AD

Figure 5. Double-labeling IF of full-length intraneuronal APP. *A–F*, Sections of cerebral cortex of 5–7-month-old 3xTg-AD (*A–C*) and 3xTg-AD/*BACE*^{-/-} (*D–F*) mice were double-labeled with 5685 (red), a PAb to the C terminus of APP, and 22C11 (green), a monoclonal antibody specific to the N-terminal domain of APP. Note that merge images (*C, F*) show strong colocalization. *G–I*, IF images of 3xTg-AD (*G–I*) and 3xTg-AD/*BACE*^{-/-} (*J–K*) mice double-labeled with 5685 (red) and 4G8 (green), a monoclonal antibody specific to residues 17–24 of A β . Merge images (*I, L*) show extensive colocalization. Scale bar 50 μ m.

(Gouras et al., 2010). Initial reports of intracellular A β in human tissue were later confounded by the realization that most antibodies that detect A β sequences or epitopes are unable to distinguish between A β epitopes in full-length APP versus cleaved A β peptides (Grundke-Iqbal et al., 1989) or cross reacted with components in neuronal lipofuscin (Bancher et al., 1989). To overcome this limitation, we used IHC and biochemical and genetic methods to show that the intraneuronal A β immunoreactivity in 3xTg-AD mice does not reflect the presence of cleaved A β peptides, but rather A β sequences within APP. Another strength of this study is the use of multiple well characterized antibodies that span the length of the APP molecule, in particular antibodies that recognize cleavage-specific epitopes. The use of cleavage-specific antibodies requires careful interpretation of results, as even the most widely accepted A β ₄₂-specific antibody, BC05, also recognizes A β ₄₃ and can cross-react with APP C-terminal fragments under certain conditions. However, we obtained remarkably consistent results using multiple N-terminal and C-terminal end-specific A β antibodies, which showed that the onset and progression of tau pathology in these mice does not require the generation of any species of A β peptides.

Several studies have detected intracellular A β peptide immunoreactivity in normal, AD, or Down syndrome brains (Gouras

Figure 6. Intraneuronal APP does not colocalize with cleaved A β . Images of 3xTg-AD (**A–C**, **G–I**) and 3xTg-AD;BACE $^{-/-}$ (**D–F**, **J–L**) mice double-labeled with 5685 (red) (**A–C**, **D–F**) and either A β_{40} (BA27) (green) (**A–F**) or A β_{42} (BC05) (green) (**G–I**) cleavage-specific monoclonal antibodies. Note that no intraneuronal A β staining was detected. Merge images (**C**, **F**, **I**, **L**) show no colocalization between APP and A β_{40} -specific (**C**, **F**) and A β_{42} -specific (**I**, **L**) antibodies. Scale bar 50 μ m.

et al., 2000; Nagele et al., 2002; Tabira et al., 2002; Takahashi et al., 2002; Wang et al., 2002; Wegiel et al., 2007). Thus, low levels of intracytoplasmic A β peptide immunoreactivity can be seen and may possibly play a role early in the biogenesis of amyloid plaques. Multiple groups have also attempted to develop experimental models to examine the effects of the accumulation or presence of intracellular A β peptides (Gouras et al., 2010). Although high levels of intracellular A β peptide appear to result in apoptotic cell death (LaFerla et al., 1995; Kienlen-Campard et al., 2002; Zhang et al., 2002), there is little evidence that a similar process occurs in human AD. Furthermore, intraneuronal A β has been reported in some mouse models overexpressing mutant APP transgenes (Gouras et al., 2010); but again the relevance of free intracellular A β peptides in neurons engineered to overexpress high levels of APP is unclear. Thus, further studies are still needed to understand the biological significance of intraneuronal A β peptides for AD pathology and neurodegeneration. With regard to the 3xTg-AD mouse model, others have suggested that A β peptides are not the main constituent of the observed intraneuronal immunoreactivity (Golde and Janus, 2005; McAlpine et al., 2009), and we extend these findings to further characterize the accumulation of intraneuronal APP using immunologic, biochemical, and genetic methods.

Mutant presenilin and tau are known to alter subcellular trafficking, in particular axonal transport. For example, FAD-linked

PS1 mutations alter subcellular vesicular transport and impair anterograde fast axonal transport of a variety of cargos (Cai et al., 2003; Pigino et al., 2003; Lazarov et al., 2007). Moreover, we have found that mice expressing WT tau or a pathogenic mutant form of tau found in patients with FTDP-17 (frontotemporal dementia and parkinsonism linked to chromosome 17) exhibit severe impairments in axonal transport that can be rescued with microtubule-stabilizing drugs (Ishihara et al., 2001; Zhang et al., 2004; Brunden et al., 2010). Since 3xTg-AD mice express both mutant presenilin and mutant tau, we conjecture that the accumulation of intraneuronal APP may represent an organelle transport defect. In support of this, our immuno-EM analysis demonstrated robust pools of predominantly cytoplasmic APP. Our analysis of APP glycosylation and phosphorylation did not reveal any reduction of mature or phosphorylated APP. Thus, if APP transport is impaired, this is likely occurring late in the secretory pathway after APP phosphorylation (Ando et al., 1999; Lee et al., 2003b). Multiple rodent models, in particular rat transgenic models expressing mutant forms of presenilin, have been reported to exhibit intraneuronal immunoreactivity similar to that described in the 3xTg-AD mouse model (Wirblich et al., 2002; Echeverria et al., 2004; Vercauteren et al., 2004; Golde and Janus, 2005; Flood et al., 2009). Therefore, we speculate that the perikaryal accumulation of APP may be due to an impairment of vesicular trafficking into the axonal compartment, perhaps caused by either the FTDP-17 tau mutation and/or the PS1 knock-in mutation, which are expressed in the 3xTg-AD mice.

In conclusion, our findings have significant implications for the analysis of 3xTg-AD mice in future studies of AD pathogenesis and drug development. More specifically, despite the absence of intracellular A β accumulation, the 3xTg-AD mouse remains a valuable tool in that it allows for the concurrent examination of both A β and tau pathologies; but our data clearly show that the onset and progression of tau pathology in these mice occurs independently of the accumulation of free or cleaved intraneuronal A β peptides. Finally, what role free A β peptides in the perikarya of neurons may play in mechanisms of AD neurodegeneration remains unclear.

References

- Ando K, Oishi M, Takeda S, Iijima K, Isohara T, Nairn AC, Kirino Y, Greenberg P, Suzuki T (1999) Role of phosphorylation of Alzheimer's amyloid precursor protein during neuronal differentiation. *J Neurosci* 19:4421–4427.
- Bancher C, Grundke-Iqbal I, Iqbal K, Kim KS, Wisniewski HM (1989) Immunoreactivity of neuronal lipofuscin with monoclonal antibodies to the amyloid beta-protein. *Neurobiol Aging* 10:125–132.
- Brunden KR, Zhang B, Carroll J, Yao Y, Potuzak JS, Hogan AM, Iba M, James MJ, Xie SX, Ballatore C, Smith AB 3rd, Lee VMY, Trojanowski JQ (2010) Epothilone D improves microtubule density, axonal integrity, and cognition in a transgenic mouse model of tauopathy. *J Neurosci* 30:13861–13866.

- Cai D, Leem JY, Greenfield JP, Wang P, Kim BS, Wang R, Lopes KO, Kim SH, Zheng H, Greengard P, Sisodia SS, Thinakaran G, Xu H (2003) Presenilin-1 regulates intracellular trafficking and cell surface delivery of beta-amyloid precursor protein. *J Biol Chem* 278:3446–3454.
- Cai H, Wang Y, McCarthy D, Wen H, Borchelt DR, Price DL, Wong PC (2001) BACE1 is the major beta-secretase for generation of Abeta peptides by neurons. *Nat Neurosci* 4:233–234.
- De Strooper B, Saftig P, Craessaerts K, Vanderschueren H, Guhde G, Annaert W, Von Figura K, Van Leuven F (1998) Deficiency of presenilin-1 inhibits the normal cleavage of amyloid precursor protein. *Nature* 391:387–390.
- Duff K, Suleman F (2004) Transgenic mouse models of Alzheimer's disease: how useful have they been for therapeutic development? *Brief Funct Genomic Proteomic* 3:47–59.
- Echeverria V, Ducatenzeiler A, Alhonen L, Janne J, Grant SM, Wandosell F, Muro A, Baralle F, Li H, Duff K, Szyf M, Cuervo AC (2004) Rat transgenic models with a phenotype of intracellular Abeta accumulation in hippocampus and cortex. *J Alzheimers Dis* 6:209–219.
- Flood DG, Lin YG, Lang DM, Trusko SP, Hirsch JD, Savage MJ, Scott RW, Howland DS (2009) A transgenic rat model of Alzheimer's disease with extracellular Abeta deposition. *Neurobiol Aging* 30:1078–1090.
- Goedert M, Jakes R, Vanmechelen E (1995) Monoclonal antibody AT8 recognizes tau protein phosphorylated at both serine 202 and threonine 205. *Neurosci Lett* 189:167–169.
- Golde TE, Janus C (2005) Homing in on intracellular Abeta? *Neuron* 45:639–642.
- Gouras GK, Tsai J, Naslund J, Vincent B, Edgar M, Checler F, Greenfield JP, Haroutunian V, Buxbaum JD, Xu H, Greengard P, Relkin NR (2000) Intraneuronal Abeta42 accumulation in human brain. *Am J Pathol* 156:15–20.
- Gouras GK, Tampellini D, Takahashi RH, Caporaso Z, Zarate E (2010) Intraneuronal beta-amyloid accumulation and synapse pathology in Alzheimer's disease. *Acta Neuropathol* 119:523–541.
- Grundke-Iqbal I, Iqbal K, George L, Tung YC, Kim KS, Wisniewski HM (1989) Amyloid protein and neurofibrillary tangles coexist in the same neuron in Alzheimer disease. *Proc Natl Acad Sci U S A* 86:2853–2857.
- Hsiao K, Chapman P, Nilsen S, Eckman C, Harigaya Y, Younkin S, Yang F, Cole G (1996) Correlative memory deficits, Abeta elevation, and amyloid plaques in transgenic mice. *Science* 274:99–102.
- Hussain I, Powell D, Howlett DR, Tew DG, Meek TD, Chapman C, Gloger IS, Murphy KE, Southern CD, Ryan DM, Smith TS, Simmons DL, Walsh FS, Dingwall C, Christie G (1999) Identification of a novel aspartic protease (Asp 2) as beta-secretase. *Mol Cell Neurosci* 14:419–427.
- Ishihara T, Zhang B, Higuchi M, Yoshiyama Y, Trojanowski JQ, Lee VMY (2001) Age-dependent induction of congophilic neurofibrillary tau inclusions in tau transgenic mice. *Am J Pathol* 158:555–562.
- Iwatsubo T, Odaka A, Suzuki N, Mizusawa H, Nukina N, Ihara Y (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.
- Janus C, Pearson J, McLaurin J, Mathews PM, Jiang Y, Schmidt SD, Chishti MA, Horne P, Heslin D, French J, Mount HT, Nixon RA, Mercken M, Bergeron C, Fraser PE, St George-Hyslop P, Westaway D (2000) A beta peptide immunization reduces behavioural impairment and plaques in a model of Alzheimer's disease. *Nature* 408:979–982.
- Kienlen-Campard P, Miolet S, Tasiaux B, Octave JN (2002) Intracellular

Figure 7. Tau pathology is unaffected by the absence of Aβ in 3xTg-AD;BACE^{-/-} mice. At 5–7 months perikaryal tau staining was detected in the cortex (Ctx) and hippocampus (Hipp) of both 3xTg-AD (A–C) and 3xTg-AD;BACE^{-/-} mice (D–F) using 17025 (A, B and D, E) and AT8 (C, F) antibodies. At 10–12 months, similar but more robust perikaryal tau staining is seen with the 17025 and AT8 antibodies in the cortex and hippocampus of both 3xTg-AD (G–I) and 3xTg-AD;BACE^{-/-} mice (J–L) using 17025 (G, H and J, K) and AT8 (I, L) antibodies. Scale bar, 50 μm.

- amyloid-beta 1–42, but not extracellular soluble amyloid-beta peptides, induces neuronal apoptosis. *J Biol Chem* 277:15666–15670.
- Kim KS, Miller DL, Sapienza VJ, Chen CM, Bai C, Gundke-Iqbal I, Currie JR, Wisniewski HM (1988) Production and characterization of monoclonal antibodies reactive to synthetic cerebrovascular amyloid peptide. *Neurosci Res Commun* 2:121–130.
- LaFerla FM, Tinkle BT, Bieberich CJ, Haudenschild CC, Jay G (1995) The Alzheimer's A beta peptide induces neurodegeneration and apoptotic cell death in transgenic mice. *Nat Genet* 9:21–30.
- Lazarov O, Morfini GA, Pigino G, Gadadhar A, Chen X, Robinson J, Ho H, Brady ST, Sisodia SS (2007) Impairments in fast axonal transport and motor neuron deficits in transgenic mice expressing familial Alzheimer's disease-linked mutant presenilin 1. *J Neurosci* 27:7011–7020.
- Lee EB, Skovronsky DM, Abtahian F, Doms RW, Lee VMY (2003a) Secretion and intracellular generation of truncated Abeta in beta-site amyloid-beta precursor protein-cleaving enzyme expressing human neurons. *J Biol Chem* 278:4458–4466.
- Lee EB, Zhang B, Liu K, Greenbaum EA, Doms RW, Trojanowski JQ, Lee VMY (2005) BACE overexpression alters the subcellular processing of APP and inhibits Abeta deposition in vivo. *J Cell Biol* 168:291–302.
- Lee MS, Kao SC, Lemere CA, Xia W, Tseng HC, Zhou Y, Neve R, Ahlgranian MK, Tsai LH (2003b) APP processing is regulated by cytoplasmic phosphorylation. *J Cell Biol* 163:83–95.
- Lee VMY, Goedert M, Trojanowski JQ (2001) Neurodegenerative tauopathies. *Annu Rev Neurosci* 24:1121–1159.
- McAlpine FE, Lee JK, Harms AS, Ruhn KA, Blurton-Jones M, Hong J, Das P,

- Golde TE, LaFerla FM, Oddo S, Blesch A, Tansey MG (2009) Inhibition of soluble TNF signaling in a mouse model of Alzheimer's disease prevents pre-plaque amyloid-associated neuropathology. *Neurobiol Dis* 34:163–177.
- Mercken M, Vandermeeren M, Lübke U, Six J, Boons J, Van de Voorde A, Martin JJ, Gheuens J (1992) Monoclonal antibodies with selective specificity for Alzheimer Tau are directed against phosphatase-sensitive epitopes. *Acta Neuropathol* 84:265–272.
- Nagele RG, D'Andrea MR, Anderson WJ, Wang HY (2002) Intracellular accumulation of beta-amyloid(1–42) in neurons is facilitated by the alpha 7 nicotinic acetylcholine receptor in Alzheimer's disease. *Neuroscience* 110:199–211.
- Oddo S, Caccamo A, Shepherd JD, Murphy MP, Golde TE, Kaye R, Metherate R, Mattson MP, Akbari Y, LaFerla FM (2003) Triple-transgenic model of Alzheimer's disease with plaques and tangles: intracellular Abeta and synaptic dysfunction. *Neuron* 39:409–421.
- Osawa S, Funamoto S, Nobuhara M, Wada-Kakuda S, Shimojo M, Yagishita S, Ihara Y (2008) Phosphoinositides suppress gamma-secretase in both the detergent-soluble and -insoluble states. *J Biol Chem* 283:19283–19292.
- Pigino G, Morfini G, Pelsman A, Mattson MP, Brady ST, Busciglio J (2003) Alzheimer's presenilin 1 mutations impair kinesin-based axonal transport. *J Neurosci* 23:4499–4508.
- Rodríguez EM, Yulis R, Peruzzo B, Alvial G, Andrade R (1984) Standardization of various applications of methacrylate embedding and silver methenamine for light and electron microscopy immunocytochemistry. *Histochemistry* 81:253–263.
- Selkoe DJ (2001) Alzheimer's disease: genes, proteins, and therapy. *Physiol Rev* 81:741–766.
- Sinha S, Anderson JP, Barbour R, Basi GS, Caccavello R, Davis D, Doan M, Dovey HF, Frigon N, Hong J, Jacobson-Croak K, Jewett N, Keim P, Knops J, Lieberburg I, Power M, Tan H, Tatsuno G, Tung J, Schenk D, et al. (1999) Purification and cloning of amyloid precursor protein beta-secretase from human brain. *Nature* 402:537–540.
- Skovronsky DM, Doms RW, Lee VMY (1998) Detection of a novel intraneuronal pool of insoluble amyloid beta protein that accumulates with time in culture. *J Cell Biol* 141:1031–1039.
- Suzuki N, Cheung TT, Cai XD, Odaoka A, Otvos L Jr, Eckman CS, Golde TE, Younkin SG (1994) An increased percentage of long amyloid beta protein secreted by familial amyloid beta protein precursor (betaAPP717) mutants. *Science* 264:1336–1340.
- Suzuki T, Ando K, Isohara T, Oishi M, Lim GS, Saitoh Y, Wasco W, Tanzi RE, Nairn AC, Greengard P, Gandy SE, Kirino T (1997) Phosphorylation of Alzheimer beta-amyloid precursor-like proteins. *Biochemistry* 36:4643–4649.
- Tabira T, Chui DH, Kuroda S (2002) Significance of intracellular Abeta42 accumulation in Alzheimer's disease. *Front Biosci* 7:a44–a49.
- Takahashi RH, Milner TA, Li F, Nam EE, Edgar MA, Yamaguchi H, Beal MF, Xu H, Greengard P, Gouras GK (2002) Intraneuronal Alzheimer abeta42 accumulates in multivesicular bodies and is associated with synaptic pathology. *Am J Pathol* 161:1869–1879.
- Turner RS, Suzuki N, Chyung AS, Younkin SG, Lee VMY (1996) Amyloids beta40 and beta42 are generated intracellularly in cultured human neurons and their secretion increases with maturation. *J Biol Chem* 271:8966–8970.
- Vassar R, Bennett BD, Babu-Khan S, Kahn S, Mendiaz EA, Denis P, Teplow DB, Ross S, Amarante P, Loeloff R, Luo Y, Fisher S, Fuller J, Edenson S, Lile J, Jarosinski MA, Biere AL, Curran E, Burgess T, Louis JC, et al. (1999) Beta-secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE. *Science* 286:735–741.
- Vercauteren FG, Clerens S, Roy L, Hamel N, Arckens L, Vandesande F, Alhonen L, Janne J, Szyf M, Cuello AC (2004) Early dysregulation of hippocampal proteins in transgenic rats with Alzheimer's disease-linked mutations in amyloid precursor protein and presenilin 1. *Brain Res Mol Brain Res* 132:241–259.
- Wang HY, D'Andrea MR, Nagele RG (2002) Cerebellar diffuse amyloid plaques are derived from dendritic Abeta42 accumulations in Purkinje cells. *Neurobiol Aging* 23:213–223.
- Wegiel J, Kuchna I, Nowicki K, Frackowiak J, Mazur-Kolecka B, Imaki H, Wegiel J, Mehta PD, Silverman WP, Reisberg B, DeLeon M, Wisniewski T, Pirttilä T, Frey H, Lehtimäki T, Kivimäki T, Visser FE, Kamphorst W, Potempska A, Bolton D, et al. (2007) Intraneuronal Abeta immunoreactivity is not a predictor of brain amyloidosis-beta or neurofibrillary degeneration. *Acta Neuropathol* 113:389–402.
- Weidemann A, König G, Bunke D, Fischer P, Salbaum JM, Masters CL, Beyreuther K (1989) Identification, biogenesis, and localization of precursors of Alzheimer's disease A4 amyloid protein. *Cell* 57:115–126.
- Wertkin AM, Turner RS, Pleasure RJ, Golde TE, Younkin SG, Trojanowski JQ, Lee VMY (1993) Human neurons derived from a teratocarcinoma cell line express solely the 95-amino acid amyloid precursor protein and produce intracellular beta-amyloid or A4 peptides. *Proc Natl Acad Sci U S A* 90:5115–5117.
- Wirths O, Multhaup G, Czech C, Feldmann N, Blanchard V, Tremp G, Beyreuther K, Pradier L, Bayer TA (2002) Intraneuronal APP/Abeta trafficking and plaque formation in beta-amyloid precursor protein and presenilin-1 transgenic mice. *Brain Pathol* 12:275–286.
- Volfe MS, Xia W, Ostaszewski BL, Diehl TS, Kimberly WT, Selkoe DJ (1999) Two transmembrane aspartates in presenilin-1 required for presenilin endoproteolysis and gamma-secretase activity. *Nature* 398:513–517.
- R, Bienkowski MJ, Shuck ME, Miao H, Tory MC, Pauley AM, Brashier JR, Stratman NC, Mathews WR, Buhl AE, Carter DB, Tomasselli AG, Parodi LA, Heinrichson RL, Gurney ME (1999) Membrane-anchored aspartyl protease with Alzheimer's disease beta-secretase activity. *Nature* 402:533–537.
- Yoshiyama Y, Higuchi M, Zhang B, Huang SM, Iwata N, Saido TC, Maeda J, Suhara T, Trojanowski JQ, Lee VMY (2007) Synapse loss and microglial activation precede tangles in a P301S tauopathy mouse model. *Neuron* 53:337–351.
- Zhang B, Higuchi M, Yoshiyama Y, Ishihara T, Forman MS, Martinez D, Joyce S, Trojanowski JQ, Lee VMY (2004) Retarded axonal transport of R406W mutant tau in transgenic mice with a neurodegenerative tauopathy. *J Neurosci* 24:4657–4667.
- Zhang Y, McLaughlin R, Goodyer C, LeBlanc A (2002) Selective cytotoxicity of intracellular amyloid beta peptide1–42 through p53 and Bax in cultured primary human neurons. *J Cell Biol* 156:519–529.
- Zhao J, Fu Y, Yasvoina M, Shao P, Hitt B, O'Connor T, Logan S, Maus E, Citron M, Berry R, Binder L, Vassar R (2007) Beta-site amyloid precursor protein cleaving enzyme 1 levels become elevated in neurons around amyloid plaques: implications for Alzheimer's disease pathogenesis. *J Neurosci* 27:3639–3649.