APP<sub>sw</sub> Transgenic Mice Develop Age-related Aβ Deposits and Neuropil Abnormalities, but no Neuronal Loss in CA1

MICHAEL C. IRIZARRY, MD, MEGAN McNAMARA, BS, KERRI FEDORCHAK, BS, KAREN HSIAO, MD, PhD, AND BRADLEY T. HYMAN, MD, PhD

Abstract. The recent availability of transgenic mouse models of Alzheimer disease has allowed direct in vivo assessment of the molecular and neuropathological effects of cerebral amyloid deposition. We examined 16-month-old Tg(HuAPP695.K670N-M671L)2576 mice expressing human APP K670N-M671L (APP<sub>sw</sub>), which have amyloid deposition and behavioral deficits by 11 months of age. Transgene expression is predominantly neuronal, and results in amyloid deposits, comparable to human senile plaques, at terminal zones of transgene positive neurons in cortical and limbic regions. Amyloid deposits were associated with prominent gliosis and neuritic dystrophy, without neuronal loss in CA1, loss of synaptophysin immunoreactivity in the hippocampal dentate gyrus, or loss of messenger RNA for neuronal synaptic, cytoskeletal, or metabolic proteins. We conclude that Aβ is not acutely neurotoxic, but can disrupt neuronal processes and provoke an inflammatory response.

Key Words: Alzheimer disease; Amyloid β protein; Amyloid precursor protein; Hippocampus; mRNA; Synaptophysin; Transgenic models.

INTRODUCTION

The "amyloid hypothesis" of Alzheimer disease (AD) asserts that amyloid β protein (Aβ) induces neuronal loss with resultant cognitive impairment (1). Although Aβ neurotoxicity has been demonstrated in vitro (2–6), confirming Aβ neurotoxicity in vivo has been more problematic (7–9). Moreover, the amount of Aβ deposited in AD brain does not correlate with the degree of clinical symptoms (10–12) or the amount of neuronal loss (13, 14).

The development of a transgenic mouse that exhibits age-related Aβ deposition and behavioral impairment (15) allows us to test the hypothesis that Aβ deposits are directly neurotoxic in vivo. The Tg(HuAPP695.K670N-M671L)2576 transgenic mouse (Tg2576) contains the 695 amino acid splice form of the human amyloid precursor protein, hAPP695, modified by the Swedish familial AD double mutation K670N-M671L, driven by the hamster prion protein promoter. The transgene is expressed in C57B6/SJL F1 mice backcrossed to C57B6 breeders at levels 5 times that of endogenous mouse APP; Aβ deposition occurs by 11 to 13 months of age, and behavioral deficits in Y-maze and water-maze are evident by 10 months of age in transgene-positive offspring of the founder (15). We investigated regional transgene expression, amyloid deposition, neuronal mRNA expression, plaque morphology, synaptophysin immunoreactivity and neuronal counts in 16-month-old Tg2576 transgenic mice and nontransgenic littermates. Surprisingly, despite prominent amyloid deposition, neuritic dystrophy, and gliosis, we do not find loss of neurons in the hippocampal CA1 subfield, loss of synaptophysin immunoreactivity in the molecular layer of the dentate gyrus, or loss of neuronal mRNAs.

MATERIALS AND METHODS

Tissue

Tg2576 mice were bred from lines produced and described previously (15). The transgene is expressed in C57B6/SJL F1 mice backcrossed to C57B6 breeders. N2 generation mice were studied. Nontransgenic littermates served as controls. Four heterozygote transgenic and 5 nontransgenic mice were studied at 16 months of age.

Tissue Preparation

Mice were sacrificed under ether anesthesia and brains were removed and snap frozen in isopentane chilled with dry ice. For in situ hybridization studies and cresyl violet staining, 14-µm coronal cryostat sections from one hemisphere were mounted onto Probe-On (Fisher) slides coated with sterile 0.01% poly-L-lysine and fixed for 5 minutes (min) in ice-cold 4% paraformaldehyde in phosphate buffered saline (16) and maintained in 95% ethanol at 5°C. For immunohistochemistry, 40-µm coronal sections from a freezing sledge microtome were fixed in 4% paraformaldehyde for 6 hours (h) and maintained in 90% glycerol at −20°C.

In Situ Hybridization

In situ hybridization was performed according to previously published instructions (16, 17). Forty-five-mer oligonucleotide

From the Department of Neurology, Massachusetts General Hospital, Boston, Massachusetts (MCI, MM, KF, BTH) and Department of Neurology, University of Minnesota, Minneapolis, Minnesota (KH).

Correspondence to: Bradley T. Hyman, MD, PhD, Alzheimer's Disease Research Unit, Massachusetts General Hospital-Bast, 149 13th St., Charlestown, MA 02129.

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probes were synthesized on an Applied Biosystems DNA synthesizer (17) or purchased commercially (Gibco). The probes used for hAPP isoforms are detailed in (18); additional probes used were (probe, Genbank accession no., bases) rat synaptophysin, X06177, 1143-1187; mouse microtubule associated protein 2 (MAP-2), M21041, 5131-5175; mouse cytochrome oxidase subunit 2 (COX-2), V00711, 7015-7059; mouse cytochrome oxidase subunit 4 (COX-4), S57870, 538-582 and mouse glial fibrillary acidic protein (GFAP), K01347, 1784-1828. Fourteen-µm coronal fixed cryostat sections were hybridized overnight with [35S]-adenosine (DuPont/NEN) end-labeled 45-mer oligonucleotide probes (10,000 cpm/µl) at 42°C in sealed chambers humidified with 50% formamide/0.1% diethylpyrocarboxylate water, then washed in 1× standard sodium citrate (1× SSC) at 55°C. Slides were exposed to Amersham b-max autoradiography film for 1 to 14 days.

 Autoradiographic images of coronal sections were captured using a Bio-Rad GS-700 Imaging Densitometer under maximum resolution (1200 dpi, pixel depth 12) for relative optical density (ROD) measurement using Molecular Analyst software (Bio-Rad). For the measurements of ROD across multiple CNS loci, fixed boxes were outlined as accurately as the resolution of the autoradiograms would allow and RODs were measured directly within the superimposed measurement frames. The ROD measurements were corrected for background and were expressed as the mean ROD within the measurement frame. For each animal, the mean ROD for each region in 3 sections was averaged. The data were analyzed using STAT-View by ANOVA analysis for genotype and region with Fisher exact post-hoc analysis. The power of our study was sufficient to have an 80% chance of detecting a 20% loss of neurons or mRNA signal at a confidence level of 0.05.

 Sense- and antisense-hybridized slides were dipped in Amersham LM-1 emulsion, stored desiccated at 4°C for 1 to 3 weeks and developed in Kodak D-19. After fixation, sections were counterstained in thionin before mounting and viewing through a Leitz Aristophan microscope.

 Immunohistochemistry
 Fourteen-µm coronal mounted sections or 40-µm free floating sections were sequentially incubated in primary antibody (rabbit anti-GFAP 1:500, Sigma; R1282 1:500, D. Selkoe, Boston, MA [19]; mouse anti-synaptophysin SVP-38 1:200, Sigma; mouse anti-APP 22C11 1:500, Boehringer Mannheim [20]; mouse anti-phosphorylated medium/high molecular weight neurofilaments SMI-312 1:1000, Sternberger Monoclonals, Baltimore, MD) overnight and secondary antibody (horseradish peroxidase [hrp] conjugated-anti-rabbit 1:200, Jackson Immunobiocemicals, West Grove, PA; cy3-anti-rabbit 1:200, Jackson; BODIPY-flourescein anti-mouse 1:200, Molecular Probes, Eugene, OR; biotinylated anti-mouse IgG1 1:500, Jackson; cy3-streptavidin 1:500, Jackson). Confocal images were obtained on the Bio-Rad laser confocal imaging system at an excitation wavelength of 586 nm and an emission wavelength of 605 nm for Cy3, and 488 nm and 522 nm for BODIPY. The chromagen for horseradish peroxidase was 3,3′-diaminobenzidine.

 Quantitative Confocal Microscopy
 Forty-µm free floating sections from 4 transgenic animals and 4 nontransgenic littermates were simultaneously immunostained for synaptophysin (rabbit anti-synaptophysin 1:120, Dako; cy3-anti-rabbit 1:200, Jackson) and mounted onto 0.1% poly-L-lysine-coated slides under glass coverslips. The sections were viewed under a 100× objective (numerical aperture 1.4) on a Leica DMRB microscope with an attached laser confocal scanning system MRC 1024 (Bio-Rad, Watford, UK) (after [21]). The cy3 signal (excitation wavelength 568 nm, emission wavelength 505 nm) was collected under linear conditions, accumulating 6 images under the photon counting mode (iris 2.9, gain 1500, black level –4). From each case, 3 fields from the outer molecular layer, middle molecular layer, and inner molecular layer of the dentate gyrus were collected from 3 sections. The images were transferred to Photoshop 4.0, where mean pixel intensity for each field was determined and adjusted for background (cy3-anti-rabbit staining of tissue alone).

 Amyloid Burden
 Amyloid deposition was quantified using Aβ immunostaining (polyclonal anti-Aβ R1282, hmr-anti-rabbit) and a Biocuant image analysis system (12). Video images of 3 to 5 forty-µm sections through each anatomic region of interest were captured and a threshold optical density was obtained that discriminated staining from background. Manual editing of each field eliminated artifacts. The “amyloid burden,” defined as the total percentage of cortical surface area covered by Aβ immunoreactivity, was calculated for CA1, cingulate, dentate gyrus, and entorhinal cortex of each mouse.

 Neuron Counts
 Neuron counts were performed using the optical dissector technique (22) in 14-µm cresyl violet stained coronal sections spaced 420 µm apart. The entire volume of CA1 was estimated according to the principle of Cavalieri (23), using the Bioquant Image Analysis System (Nashville, TN). Neuronal counts were obtained from a systematically random sampling scheme from sections spanning the entire CA1. The number of neurons in the entire CA1 was estimated using approximately 25 optical dissectors in each case. Each optical dissector was a 25 × 50 µm sampling box with extended exclusion lines. Using a 100× oil immersion lens, neurons with a visible nucleolus were counted if they were not present in the initial plane of focus, but came into focus as the optical plane moved through the tissue. The estimation of total neurons was calculated by multiplying the volume density of the neurons by the volume of CA1. All the counts were performed by a single examiner (M.C.I.) blinded as to transgenic status.

 The appropriateness of the sampling scheme was evaluated by calculating the precision of the estimates made in each mouse, expressed as the coefficient of error (CE). The average coefficient of error from the counting technique was 0.04, suggesting that a minimal amount of variance observed in the counts is due to variance from the technique, and instead reflects biological variance.

 RESULTS
 Transgene Expression
 We examined the expression of transgene mRNA by in situ hybridization with a junctional probe specific for hAPP695 (Fig. 1). As expected, no message for human
APP was detected in 5 nontransgenic littermates, confirming the specificity of the probe for the human sequence. Analysis of 4 transgenic mice demonstrated expression of the transgene most prominently in the neuronal layers of the hippocampal formation, followed by homogeneous expression in cortical regions, including the entorhinal cortex, and the amygdala. Subcortical regions showed low transgene expression, and white matter transgene expression was nonexistent or below the limits of detection. Emulsion-dipped sections confirm the predominantly neuronal expression of hAPP695 suggested by the pattern of signal in the in situ film, and suggest that there is no or undetectable glial or vascular expression of the gene (Fig. 1C). As further controls, in situ hybridization with mRNA probes specific for the longer human APP splice variants—hAPP751 and hAPP770—in the transgenic mice and in nontransgenic littermates were used and were found to be blank.

Amyloid Deposits

Aβ immunostaining using the polyclonal antibody R1282 in 14- and 40-μm-thick sections extending from the anterior hippocampus to the posterior pole of 4 transgenic animals demonstrated amyloid deposits in the molecular layer of hippocampal subfields, the cortex, and the amygdala (Fig. 2A, B). We focused on the entorhinal cortex, entorhinal cortex, molecular layer of the dentate gyrus, and the CA1 hippocampal subfield because these are areas of early and prominent involvement in both the transgenic mice and in human AD, and represent neural structures likely implicated as dysfunctional by tests of memory impairment. Amyloid burden in CA1, dentate gyrus, cingulate, and entorhinal cortex in 40-μm sections ranged from 3.6 to 8.5% (Fig. 2C). There was rare subcortical amyloid deposition in the internal capsule and basal ganglia. Five nontransgenic littermates did not demonstrate any Aβ immunoreactivity.
Fig. 2. Amyloid burden in 16-month-old Tg2576 mice. Immunostaining demonstrates Aβ deposits in cingulate, dentate gyrus (especially outer molecular layer), CA1 (A) and entorhinal cortex (B), quantitated by % amyloid burden (± SD) in 40-μm sections (C). There is little amyloid deposition in the thalamus and other subcortical regions.

Double immunostaining and confocal scanning microscopy was performed in 4 transgenic animals to assess pathological structures associated with amyloid deposition in the neuropil. Amyloid plaques labeled with R1282 were decorated with APP immunoreactivity (22C11), and contained SMI-312-immunoreactive structures consistent with dystrophic neurites (Fig. 3A, B). These were enlarged processes found surrounding and within the Aβ deposits, and are most likely axonal in origin based on the presence of synaptophysin and SMI-312 immunoreactivity. Reactive astrocytes stained with GFAP immunocytochemistry were also markedly associated with neuritic plaques (Fig. 3C, D).

CA1 Neuronal Counts

To assess Aβ neurotoxicity, we concentrated on the CA1 region of the hippocampus for neuronal counts because; (1) this region undergoes early amyloid deposition in both the transgenic mouse and human AD; (2) impaired function in Y-maze and Morris Water maze in mice have been associated with hippocampal pathology (24); and (3) CA1 undergoes marked neuronal loss in humans with AD (25). Using the optical dissector technique in the CA1 region of the hippocampus, neuronal density and CA1 volume were determined, and total neurons in CA1 were calculated in 4 transgenic mice and 5 nontransgenic littermates (Fig. 4). There was no significant difference in neuronal counts in the CA1 region of the hippocampus in 16-month-old transgenic mice with substantial Aβ deposits compared with 16-month-old nontransgenic littermates.

mRNA Expression

Although neuronal loss was not evident in CA1, the stereological procedures would not reveal functional alterations in neurons. As a screen for transcriptional deficits, in situ hybridization was performed on brain sections from 4 transgenic mice and 5 nontransgenic
littermates for neuronal metabolic, synaptic and cytoskeletal proteins (Fig. 5A–D). Specific decrements of gene expression for synaptic, cytoskeletal and metabolic neuronal proteins by in situ hybridization or northern blot have been reported in Alzheimer disease, inversely related to increasing neurofibrillary tangles; however, gene expression was also reduced in intact neurons (26–30). Furthermore, cytochrome oxidase mRNA levels reflect functional activity of metabolic pathways (31, 32). The specific regions analyzed were chosen to reflect the varying levels of hAPP695 expression and Aβ deposition: dentate gyrus granule cell layer and CA1 neuronal layer (strong transgene expression and early amyloid deposition), cingulate and entorhinal cortex (moderate transgene expression and early Aβ deposition), and thalamus (low transgene expression and no amyloid deposition). MAP-2 and synaptophysin mRNA demonstrated a similar pattern of expression, with prominent expression in hippocampal and cortical regions, lower expression in subcortical regions, and minimal expression in white matter. COX-2 and COX-4 had similar uniform expression in these regions, with very low expression in the white matter. The pattern of mRNA expression of these proteins did not differ between transgenic animals and nontransgenic littermates. There was no demonstrable loss of neuronal metabolic (COX-2, COX-4), synaptic (synaptophysin), or cytoskeletal (MAP-2) mRNA expression in the transgenic mice compared with nontransgenic littermates, even in subregions that develop Aβ deposits.

GFAP mRNA expression was greatest in the CA1, dentate granule cell layer, and dentate molecular layer, with lower expression in cortex and subcortical regions of both transgenic and nontransgenic mice (Fig. 5E). Focal areas of increased GFAP mRNA were present in the transgenic mice, but were rare in nontransgenic littermates. When GFAP mRNA signal was quantitated excluding these focal areas, there was no difference in average GFAP mRNA expression between the transgenic and nontransgenic mice; thus, rather than demonstrating a global glial response to transgene expression, the transgenic mice exhibit focal regions of gliosis with elevated GFAP mRNA by in situ hybridization and collections of activated astrocytes by GFAP/Aβ immunostaining.

**Synaptophysin Immunoreactivity**

Since synaptophysin message in the entorhinal cortex may not reflect synaptophysin protein in the terminal fields of these neurons in the dentate gyrus, we evaluated synaptophysin immunoreactivity in the outer (oml), middle (mml), and inner (iml) molecular layer of the dentate gyrus of 4 transgenic animals and 4 nontransgenic littermates. In human AD, amyloid deposition is associated with disruption of synaptophysin immunoreactivity in the
dentate gyrus (33), and loss of synaptophysin immunoactivity in the outer molecular layer of the dentate gyrus is detected even in early stages of AD (34). There was no loss of synaptophysin immunoreactivity in the heterozygote transgenic mice: the mean synaptophysin pixel intensities for the transgenic mice were oml 124.6±21.6, mml 130.4±29.2, and iml 127.1±18.5, while those for the nontransgenic littermates were oml 124.3±18.6, mml 123.5±32.5, and iml 128.4±18.4.

**DISCUSSION**

Transgenic mouse models of Alzheimer disease are novel resources for assessing the neuropathological consequences of Aβ deposition. Compelling genetic evidence for a key role of Aβ in the pathogenesis of AD is suggested by mutations in the hAPP gene on chromosome 21 in some families with autosomal dominant inherited forms of early onset-AD (35, 36). In vitro studies have demonstrated that aggregated amyloid is toxic to neurons in culture (4, 6) and that Aβ can initiate apoptotic mechanisms (5) and predispose to excitotoxic insult (2, 3). Despite these in vitro studies of Aβ neurotoxicity, there is no clear relationship between the amount of amyloid deposition and the amount of neuronal loss in AD brain (13). Even the source of cerebral Aβ has been debated, with arguments for a neuronal or circulating blood source. Our data examine several points of controversy in AD that are difficult to address without an animal model: (a) What are potential sources of Aβ deposited in the brain? (b) What underlies the regional specificity of Aβ?

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Fig. 5. mRNA expression in Tg2576 mice. There is no loss of mRNA for neuronal synaptic (synaptophysin, A), cytoskeletal (MAP-2, B), or metabolic (COX-2, C; COX-4, D) proteins in 16-month-old heterozygote transgenic mice compared with non-transgenic littermates. GFAP mRNA (E) is increased focally in the transgenic mice, but there is not a generalized increase in GFAP mRNA when measured outside these discrete areas. Scale bar, 1 mm. Error bars reflect SD.

deposition? (c) What is the response of the adjacent neuropil to Aβ deposition? (d) Is Aβ neurotoxic in vivo? Our data in the Tg2576 mouse only touch on the first two questions, but more directly address the last two.

The Tg2576 mouse demonstrates that Aβ deposition can occur with an exclusively neuronal expression of hAPP. The PrP promoter expresses the transgene only in the brain (37), and our data demonstrate that this is a reflection of neuronal expression. However, despite the fact that neurons express hAPP in widespread areas of the central nervous system, Aβ deposits are limited to specific regions in a pattern quite similar to that seen in sporadic AD in the human. This suggests that regional metabolic or architectural features present in both the mouse brain and the human brain must also be important in determining the exact anatomy of the deposits. For example, there is a propensity for Aβ deposition in terminal zones of neurons expressing the hAPP transgene,
such as the molecular layer of the dentate gyrus, which receives projections from the entorhinal cortex. This is the exact locale of the most prominent deposition in the PDAPP transgenic mouse model of cerebral amyloid deposition (38, 39), and it is a frequent site of Aβ deposits in human AD (33). Disruption of the perforant pathway terminal zone in the outer molecular layer of the dentate gyrus potentially impairs a major projection in memory-related systems, with concomitant behavioral changes. However, it is important to note that this effect may be fairly subtle, in that markers of deafferentation (loss of COX-2 mRNA, synaptophysin mRNA, and synaptophysin protein) have not occurred. Yet the local effects of amyloid deposition are evident from immunostaining and in situ hybridization, which show neuritic changes and astrogliosis.

Although the 4 to 8% amyloid burden in these 16-month-old mice is comparable to that seen in human AD, the latter is also associated with profound loss of CA1 neurons (25). Despite plaque-associated axonal changes and glial response, Aβ deposits do not cause detectable neuronal loss in area CA1 at least 5 months after initial amyloid deposition and development of behavioral deficits in the transgenic mice. Thus, Aβ-induced neuronal loss does not appear to be responsible for the observed behavioral changes, although we cannot rule out the possibility that loss of a small subpopulation of vulnerable neurons escaped detection. Nonetheless, these data are consistent with preliminary data (39) from the 18-month-old PDAPP mice (38), which show a preservation of neuronal number in that model in the face of even greater amounts of Aβ deposits; together, the two sets of data suggest that Aβ deposits are not acutely neurotoxic in the mouse brain.

In summary, we find cortical and limbic Aβ deposits, neuropil changes, and gliosis, but not neuronal loss in the CA1 hippocampal subfield, in aged Tg2576 mice known to have memory impairment. We hypothesize that alterations in the function or structure of the terminal zones of critical neural circuits, such as the perforant pathway where we observe dystrophic, swollen neurites around Aβ deposits, are responsible for the behavioral impairments. Additional inflammatory changes, including prominent plaque-associated microgliosis, have also been characterized in these transgenic mice (15). Electrophysiological changes consequent to APP overexpression must also be considered. By extension, we speculate that Aβ deposits do not cause direct neurotoxicity in AD, but instead disrupt local neuronal processes.

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