Conditional Neuronal Simian Virus 40 T Antigen Expression Induces Alzheimer-Like Tau and Amyloid Pathology in Mice

Kevin H. J. Park,¹ Janice L. Hallows,¹ Paramita Chakrabarty,² Peter Davies,³ and Inez Vincent¹

¹Centre for Molecular Medicine and Therapeutics, Department of Pediatrics, Child and Family Research Institute, University of British Columbia, Vancouver, British Columbia, Canada, V5Z 4H4, ²Department of Neuroscience, Mayo Clinic, Mayo Clinic College of Medicine, Jacksonville, Florida 32224, and ³Department of Pathology, Albert Einstein College of Medicine, Bronx, New York 10461

A large body of evidence has shown the activation of a cohort of cell cycle regulators and the duplication of DNA in degenerating neurons of Alzheimer's disease (AD) brain. Activation of these regulators and duplication of chromosomes precede neurodegeneration and formation of neurofibrillary tangles (NFTs), one of the diagnostic lesions of AD. These findings, in combination with evidence for cell cycle regulation of amyloid precursor protein and tau, has led to the hypothesis that reentry into the cell cycle underlies AD pathogenesis. To test this hypothesis directly, we have created transgenic mice with forced cell cycle activation in postmitotic neurons via conditional expression of the simian virus 40 large T antigen (TAG) oncogene. We show that TAG mice recapitulate the cell cycle changes seen in AD and display a neurodegenerative phenotype accompanied by tau pathology and NFT-like profiles. Moreover, plaque-like amyloid deposits, similar to those seen in AD, are also observed in the brains of TAG mice. These data provide support for an essential role of ectopic cell cycle activation in the generation of the characteristic pathological hallmarks of AD. Furthermore, our TAG mice are the first model to develop NFTs and amyloid pathology simultaneously and in the absence of any human transgenes. These mice will be useful for further defining the nongenetic mechanisms in AD pathogenesis and for the development of cell cycle-based therapies for AD.

Key words: Alzheimer's disease; cell cycle; SV40 T antigen; amyloid plaque; neurofibrillary tangle; transgenic mice

Introduction

The majority of all Alzheimer's disease (AD) cases arises sporadically and is characterized as late onset (>65 years), whereas early onset or familial AD (FAD) is caused by mutations in any one of the *amyloid precursor protein* (APP) or *presenilin 1* and 2 genes. Neurofibrillary tangles (NFTs), amyloid plaques, and a similar selective loss of brain neurons characterize both sporadic and familial AD.

Amyloid plaques are produced in transgenic mice overexpressing inherited APP mutations (Games et al., 1995; Hsiao et al., 1996; Sturchler-Pierrat et al., 1997). Transgenic expression of mutant presenilins does not by itself cause AD pathology but exacerbates plaque formation in mutant APP-expressing mice (Borchelt et al., 1997; Holcomb et al., 1998). Surprisingly, none of these plaque producing transgenic models display NFTs or AD-type neurodegenerative changes (Van Leuven, 2000), suggesting that additional mechanisms underlie AD. Although NFTs are seen in independent transgenic models overexpressing mutant

tau isoforms, which are linked to tauopathies in humans (Lewis et al., 2000; Gotz et al., 2001; Tanemura et al., 2001; Tatebayashi et al., 2002), there are no known tau mutations associated with AD.

Another potential mechanism underlying AD pathology is aberrant cell cycle activation in neurons (Herrup et al., 2004; Neve and McPhie, 2006). Various cell cycle regulators are activated in affected vulnerable neurons in familial and sporadic AD (Vincent et al., 1996; McShea et al., 1997; Nagy et al., 1997; Busser et al., 1998), and duplicated chromosomes are detected in such neurons before tau pathology (Yang et al., 2001). Compelling *in vitro* and *in vivo* models have also supported a connection between the cell cycle and APP processing (Suzuki et al., 1994; Chen et al., 2000; McPhie et al., 2003), the cell cycle and tau regulation (Vincent et al., 1996; Illenberger et al., 1998; Wen et al., 2004; Delobel et al., 2006), and the cell cycle and neuronal death induced by different agents (Giovanni et al., 2000; Wu et al., 2000; Copani et al., 2001; Khurana et al., 2006). Collectively, these findings support the notion that cell cycle changes contribute to pathogenic mechanisms in AD.

Here, we directly tested this hypothesis by generating conditional transgenic mice with neuronally targeted expression of simian virus 40 large T antigen (TAG) using the tet-off system driven by the CaM kinase II α promoter (Mansuy et al., 1998). In its classical role as cell cycle inducer, TAG inhibits the retinoblastoma (Rb) and p53 tumor suppressor proteins that are the major gatekeepers of proliferation and terminal differentiation (Ahuja et al., 2005). A previous study showed that constitutive TAG ex-

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Correspondence should be addressed to Dr. Inez Vincent, Department of Pediatrics, University of British Columbia, Vancouver, British Columbia, Canada, V5Z 4H4. E-mail: ivincent@cmmt.ubc.ca.

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Table 1. Antibodies

Group	Antibody	Epitope	Dilution		Source
			IHC	IB	
Simian virus 40 TAg	TAg (pAb 101)	TAg C terminus	1:50		Santa Cruz Biotechnology (Santa Cruz, CA)
Cell cycle markers	PCNA (PC10)	PCNA p36	1:500	1:1000	Santa Cruz Biotechnology
	BrdU (Bu20a)	BrdU	1:50		DakoCytomation
	cdc2 (06–141)	cdc2 C terminus	1:200		Upstate Biotechnology (Lake Placid, NY)
	cdk4 (C-22)	cdk4 C terminus	1:200		Santa Cruz Biotechnology
	cdk4 (H303)	Full-length cdk4		1:200	Santa Cruz Biotechnology
	Cyclin B1 (GNS1)	Full-length cyclin B1		1:200	Santa Cruz Biotechnology
	Cyclin B1 (CB169)	Cyclin B1 C terminus	1:100		Upstate Biotechnology
	Cyclin D1 (DCS-6)	Cyclin D1	1:100	1:500	Santa Cruz Biotechnology
	Rb P-ser795	Rb P-Ser795	1:500	1:500	New England Biolabs (Beverly, MA)
	MPM-2	P-Ser/Thr-Pro	1:500	1:500	Upstate Biotechnology
	H5	P-RNA polymerase II		1:2	D. Bregman (Albert Einstein College of Medicine, Bronx, NY)
	Cleaved caspase-3	Cleaved region of caspase-3	1:250		Promega (Madison, WI)
	Tau				
	CP13	tau P-Ser202	1:25	1:25	P. Davies
	CP10	tau P-Thr-231	1:10	1:10	P. Davies
	TG3	tau P-Thr-231	1:10	1:10	I. Vincent
APP/A β	MC6	tau P-Ser235	1:10	1:10	P. Davies
	PHF-1	tau P-Ser396/404	1:25	1:25	P. Davies
	MC1	tau 5–15, 312–322	1:10	1:10	P. Davies
	Alz50	tau 5–15, 312–322	1:10	1:10	P. Davies
	CP22	tau P-ser 175	1:10	1:10	P. Davies
	PG5	tau P-ser 409	1:10	1:10	P. Davies
	tau C3	Caspase-cleaved tau	1:1000	1:1000	L. Binder (Northwestern University, Chicago, IL)
	TG5	Total tau	1:10	1:10	I. Vincent
	22C11	APP 66–81	1:1000	1:3000	Chemicon
	6E10	A β _{1–17}	1:250	1:250	Signet Laboratories (Dedham, MA)
	4G8	A β _{17–24}	1:250	1:500	Signet Laboratories
	82E1	β -BACE cleavage site		1:500	IBL (Gunma, Japan)
	A β _{42.2}	A β _{35–42}	1:250		T. Golde (Mayo Clinic College of Medicine, Jacksonville, FL)
	A β _{40.1}	A β _{35–40}	1:250		T. Golde
	CT15	APP 691–695	1:500	1:3000	E. Koo (University of California at San Diego, La Jolla, CA)
β -Actin	GF7	APP P-Thr668	1:20	1:50	P. Davies
	Anti-I	APP 649–664	1:2000		D. Selkoe (Harvard University, Boston, MA)
	β -Actin (600–501)	β -Actin N terminus		1:4000	Novus Biologicals (Littleton, CO)

IB, Immunoblotting; cdk4, cyclin-dependent kinase 4.

pression in cerebellar Purkinje neurons in mice led to neurodegeneration (Feddersen et al., 1992). We report that TAg expression in our model effectively induced cell cycle reentry in brain neurons of mice and resulted in neurodegeneration. Moreover, TAg expression led to NFT-like profiles and amyloid deposits resembling the two major neuropathological hallmarks of AD. Our findings suggest that aberrant cell cycle activation via TAg expression induces AD-like pathology. Furthermore, this pathology develops in the absence of any FAD mutant genes, indicating the TAg model will be valuable for studying nongenetic mechanisms underlying AD.

Materials and Methods

Mice. TAg/tet-off (TAg) mice were generated by microinjection of the TAg plasmid flanked by the tet operator sequence (S. Efrat, Tel Aviv University, Tel Aviv, Israel) into embryos from tet-off mice (E. Kandel, Columbia University, New York, NY) in C57BL/6 background. The TAg mice were maintained in this pure C57BL/6 background for all of the present studies. Mice were maintained in our colony on a 12 h light/dark cycle with access to food and water *ad libitum*. Breeder mice were maintained on Dox Diet (200 mg/kg; Bio-Serv, Frenchtown, NJ) to ensure maintenance of offsprings on Dox Diet *in utero* and after birth until weaning. Mice were weaned at 3 weeks of age and maintained on Dox Diet through 4–6 weeks of age and then switched to normal diet. In some mice, bromodeoxyuridine (BrdU) (Sigma, St. Louis, MO) was administered intraperitoneally at 300 mg/kg twice daily for 2 weeks at the onset of normal diet to assess the extent of DNA synthesis resulting from TAg expression. All animal protocols were approved by the Animal Care and Use Committee at the University of Washington (where the mice were

generated and initial experiments performed) and the Animal Care Committee at the University of British Columbia.

Genomic DNA was isolated from tail biopsies obtained at the time of weaning. Identification of the five TAg lines was performed by Southern blotting using *Bam*HI-digested genomic DNA and ³²P-labeled *Bam*HI-digested TAg plasmid DNA as a probe using standard techniques. TAg genotyping was performed using genomic DNA as reported previously (Berkovich and Efrat, 2001). Tet-off genotyping was performed similarly using the following primers: forward, 5-GTCCACTTAGACGGC-GAGGA-3'; reverse, 5'-AGGGCATCGGTAAACATCTGC-3'.

Initially, mice from all five transgenic lines were analyzed, and the phenotype was found to be similar. More detailed analyses were focused on three of the most prolific lines (Fig. 1A, lanes 1, 4, 5). Mice from six successive generations were analyzed and found to display similar changes. Data from males and females were pooled because no gender differences were observed in any of the analyses.

Mice were killed by decapitation at various weeks after the switch to normal diet (weeks off-Dox), and brains were quickly dissected and halved sagittally. One hemibrain was flash frozen for biochemical analyses, and the other hemibrain was fixed for histological analyses. In total, TAg mice at <9 weeks off-Dox ($n = 13$), 9–14 weeks off-Dox ($n = 17$), 15–30 weeks off-Dox ($n = 20$), and >30 weeks off-Dox ($n = 27$) as well as appropriately matched wild-type (WT) mice ($n = 18$) were studied.

Antibodies. Table 1 lists all of the antibodies used for immunohistochemistry (IHC) and immunoblotting. The specific epitopes recognized by APP/amyloid β (A β) antibodies are described in Table 1. Subtype specific secondary antibodies conjugated to HRP or biotin and streptavidin–HRP were from Southern Biotechnology (Birmingham, AL), and streptavidin–Alexa 594 (used for fluorescent TAg IHC) was from In-

Figure 1. Neuronal expression of TAG in transgenic mice. **A**, Southern blot showing TAG DNA incorporation in five distinct transgenic lines (arrow). **B**, Immunohistochemical staining of hippocampal sections from TAG and WT brains showing TAG immunoreactivity only in neuronal nuclei (arrowheads) of TAG mice. Immunoreactivity is absent in smaller glial nuclei (arrows). Scale bar, 25 μ m.

vitrogen (Carlsbad, CA). The 10 nm polygold goat anti-mouse IgG for electron microscopy (EM) was from Polysciences (Warrington, PA).

Histological analyses. Immunohistochemical staining was performed using 4% paraformaldehyde-fixed, paraffin-embedded sections as reported previously (Hallows et al., 2003), with the addition of heat-induced epitope retrieval (sections were boiled in 10 mM sodium citrate solution, pH 6.0, for 20 min) using a pressure cooker (DakoCytomation, Carpinteria, CA) before antibody incubations.

Thioflavin-S (thio-S), Congo red, 4',6'-diamidino-2-phenylindole (DAPI), and propidium iodide (PI) (all from Sigma) stainings were performed according to standard protocols. FluoroJade B staining was performed according to the directions of the manufacturer (Chemicon, Temecula, CA).

Karnovsky's fix was used for vibratome sectioning and immunostaining followed by EM using a Hitachi (Tokyo, Japan) H7600 transmission electron microscope operating at 80 kV.

Immunoblotting/immunoprecipitation. Western blotting was performed as reported previously (Hallows et al., 2003) using supernatants from hemibrain homogenates. In all experiments, protein levels were normalized to levels of β actin. For immunoprecipitations (IPs), supernatants from hemibrain homogenates (200 μ g for 4G8 or 100 μ g for GF7 and CT15) were incubated with antibody (3 μ g of 4G8, 2 μ g of GF7, or 3 μ l of CT15) overnight at 4°C and were then incubated with protein A-Sepharose (4G8 and CT15) or protein G-Sepharose (GF7) at 4°C for 1 h. After removing the supernatants and washing three times with TBS, samples were electrophoresed, transferred to nitrocellulose (Protran; Schleicher and Schuell, Dassel, Germany), and subjected to immunoblotting.

For detecting endogenous mouse $A\beta$, large-scale immunoprecipitation of $A\beta$ was performed on 4 mg of hemibrain lysate supernatants from TAG and WT samples using 4G8 antibody covalently coupled to protein G support using Seize X protein G immunoprecipitation kit (Pierce, Rockford, IL). Eluted antigen was concentrated using microcon centrifugal filter devices (30 and 3 kDa; Millipore, Bedford, MA). The samples were electrophoresed and transferred to nitrocellulose membrane. Supernatants from Tg2576 homogenates were used as positive controls for $A\beta$.

Sarcosyl insoluble tau preparations. Sarcosyl extraction of tau, negative staining of filaments with uranyl acetate, and immunogold EM were performed as reported previously (Andorfer et al., 2003). Samples for sarcosyl extraction were ~2 mg of hemibrain homogenates.

Sandwich ELISA. $A\beta$ sandwich ELISAs were performed as reported previously (Jin et al., 2004) using 0.5 μ g of 4G8 as the capture antibody and 0.05 μ g of 6E10 antibody for detection. Samples for ELISA were 1 mg of hemibrain homogenates that were rehomogenized in 60% formic acid.

Statistical analyses. One-way ANOVA with Tukey's *post hoc* test was performed using GraphPad InStat (version 3.00 for Windows 95; GraphPad Software, San Diego, CA). Unpaired Student's *t* test was performed using Microsoft (Redmond, WA) Excel.

Results

TAG expression in postmitotic neurons activates the cell cycle and causes neuronal death

Five distinct lines expressing TAG were confirmed via Southern blot analysis (Fig. 1A). Switch to normal diet (off-Dox) starting at 4–6 weeks of age led to neuron-specific TAG protein detection in the hippocampus (Fig. 1B) and cortex (data not shown) within 2–3 weeks off-Dox and throughout the brain (data not shown) in all lines. Consistent with CaM kinase II α promoter activity (Ochiishi et al., 1994; Mansuy et al., 1998; Ochiishi et al., 1998; Yamauchi, 2005), the distribution and morphology of the Tag-positive cells indicated specific expression of TAG in neurons (Fig. 1B). The TAG-induced phenotype was similar in all five lines studied,

with minor variations in timing and severity but no gender differences. These results suggest that the phenotype is caused by TAG expression and not by an artifact of random gene disruption. The data presented here are from three lines (Fig. 1A, lanes 1, 4, 5). The histological changes observed were quite widespread, but, for simplicity, most of the data shown are restricted to the hippocampus or cortex.

As expected, TAG expression induced cyclin D1 and cyclin-dependent kinase 4 (data not shown) expression and activation, with resulting phosphorylation of Rb at Ser795 and accumulation of proliferating cell nuclear antigen (PCNA) in neuronal nuclei (Fig. 2A,B). To assess *de novo* synthesis of DNA, BrdU incorporation studies were performed. A striking distribution of BrdU-positive nuclei was detected in the brains of TAG mice. BrdU labeling was first observed ~6 weeks off-Dox, peaked between 9 and 12 weeks off-Dox, and proceeded at a lower rate until ~40 weeks off-Dox. In contrast, WT mice had no BrdU labeling except in restricted areas, such as the dentate granule layer of the hippocampus, in which neurogenesis normally occurs (Kaplan and Hinds, 1977) (Fig. 2A). TAG mice that were not exposed to BrdU had no labeling, vouching for the specificity of the BrdU antibody. DNA synthesis in TAG mice was accompanied by activation of cell division cycle 2 protein kinase (cdc2) (Fig. 2A) and its coactivator cyclin B1 (Fig. 2B), and appearance of classic mitotic phosphoepitopes (Hussemann et al., 2000) recognized by the TG3, MPM-2, and H5 antibodies (Fig. 2B). No other histological abnormality was evident at these early time points.

Starting at ~10–15 weeks off-Dox, these cell cycle events were followed by progressive and variable extents of neurodegeneration. FluoroJade immunofluorescence showed the presence of shrunken degenerating neurons in TAG mice, with condensed chromatin visualized with DAPI and hematoxylin (Fig. 3A,C). Compromised integrity of these neurons was supported by their permeability to PI without previous detergent treatment (Fig. 3A). Such shrunken degenerating neurons were positive for activated (cleaved) caspase-3 (Fig. 3B,C). Neuronal loss, as evidenced by the presence of empty spaces or holes, was observed in cortex (Fig. 3B) and in hippocampus (Fig. 3C) starting from 12–15 weeks off-Dox. Some animals were exposed to BrdU between 0 and 2 weeks off-Dox and were allowed to live until 36 weeks off-Dox, at which time they were analyzed for BrdU incorporation. Many shrunken neurons in these older animals dis-

played BrdU labeling (Fig. 3C), suggesting that some neurons remain in a degenerative state for extended durations after DNA synthesis.

Activation of TAg and resulting cell cycle changes did not alter the survival rate of young animals. No gross behavioral or anatomical abnormalities were noted in these animals compared with wild-type littermates. Conversely, ~50% of TAg mice die at ~1 year of age. Postmortem necropsies on these mice did not reveal evidence of tumors or any other gross abnormality that would explain their increased mortality. The cause of premature death of TAg mice is therefore yet unclear, and more detailed studies will be needed for understanding their lethality. Although functional deficits stemming from neuronal degeneration in the nervous system may cause premature death, it was reasoned that the overall behavioral picture is likely to depart from that of AD, because of the widespread expression of TAg and subsequent neurodegenerative changes in the TAg mice. For instance, many brain regions, such as the brainstem, cerebellum, the dentate granule layers of the hippocampus, as well as the cortical layers II and IV that are not normally affected in AD (West et al., 1994; Braak et al., 2000), display degenerative pathology in TAg mice. Therefore, we chose to focus the present study on neuropathological rather than behavioral characteristics of the TAg mice.

TAg expression leads to NFT pathology in brain

Recent studies in a *Drosophila* model showed that cell cycle activation leads to neurodegeneration with accompanying tau hyperphosphorylation (Khurana et al., 2006). Immunoblot analysis of hemibrains from TAg mice also revealed a significant increase in tau phosphorylation at the CP13, PHF-1, CP10 (108%, $p = 0.003$; 43.2%, $p = 0.0005$; 32.5%, $p = 0.049$, respectively) (Fig. 4A), TG3 (59.6%, $p = 0.028$; data not shown), and MC6 (71.9%, $p = 0.05$; data not shown) epitopes. Accordingly, an increase in apparent molecular weight of tau was detected in the TAg mice (Fig. 4A). These changes were first evident at 9–12 weeks off-Dox and worsened progressively. In contrast to the above tau epitopes, CP22, PG5, and tau C3 immunoreactivity with tau was not statistically significant in the TAg mice (data not shown).

Immunohistochemical analysis with CP13, PHF-1, CP10, and MC6 (data not shown) confirmed the accumulation of hyperphosphorylated tau epitopes in numerous neurons with pyknotic appearance throughout the cortex and hippocampus (Fig. 4B), as well as in forebrain areas, cerebellum, and brainstem (data not shown). Curiously, no consistent immunohistochemical changes were observed using MC1, Alz-50, CP22, PG5, TG3, TG5, or tau C3 antibodies (data not shown). Whereas immunoblotting

Figure 2. Cell cycle activation in TAg mice. **A**, Immunohistochemical staining of hippocampal sections from TAg and WT mice. An antibody specific for PCNA distinctly labeled larger neuronal nuclei (arrowheads) in TAg mice but not WT. A few smaller glial nuclei (arrows) were also immunoreactive for PCNA in both TAg and WT mice. In TAg mice not exposed to BrdU, no immunostaining with BrdU antibody was observed. In the WT mice, DNA synthesis was observed in some glial cells (black arrows) as well as in neurons of the dentate gyrus (white arrows). BrdU immunoreactivity was detected in many neuronal nuclei in TAg mice exposed to BrdU (arrowheads). Cell cycle progression to M phase was evidenced by expression of cdc2 in hippocampal neurons of TAg mice (arrowheads) but not in WT. Scale bar: PCNA, BrdU, 80 μ m; cdc2, 40 μ m. **B**, Immunoblots demonstrating quantitative increases in G1/S phase markers (PCNA, cyclin D1, and phospho-Rb Ser795) and G2/M phase markers (cyclin B1, TG3, MPM-2, and H5) in TAg mice compared with WT. β -Actin is shown as a protein loading control. Lane 1, 6 weeks off-Dox; lane 2, 12 weeks off-Dox; lane 3, 18 weeks off-Dox.

showed an increase in tau phosphorylation starting from 9–12 weeks off-Dox, tau histopathology with CP13, PHF-1, and CP-10 became evident in the hippocampus and cortex from ~22 weeks off-Dox and increased in an age-dependent manner (Fig. 4B). An obvious shift from axonal to somatic compartments, resembling the “pre-tangle” state observed in AD, occurred concomitantly (Fig. 4B, CP10). A few tau inclusions were thio-S-positive (data not shown). Neurons immunostained with CP13 and PHF-1 sometimes had an NFT-like appearance. Electron microscopic examination revealed the presence of filaments resembling those of AD brain, in thickness and in their positive immunoreactivity with the tau antibodies. They were contrasted from the more slender, normal microfilaments of neurons that are not decorated with tau antibodies (Fig. 4C). Such NFT-like profiles were also observed in the brainstem of TAg mice. In addition, tau pathology was seen in the forebrain, midbrain, and the Purkinje layer of the cerebellum of TAg mice (data not shown). Sarcosyl extraction verified an increase in insoluble tau in TAg mouse

Figure 3. Neurodegenerative changes in TAg mice. **A**, Hippocampal sections from TAg mice demonstrating TAg-expressing neurons (arrows) also show degenerative changes preceding neuronal death at 15 weeks off-Dox: condensed chromatin visualized with DAPI staining, FluoroJade-positive cells indicating degeneration, and compromised cell membrane integrity visualized with PI staining without detergent treatment. FluoroJade staining was absent in WT mice, whereas nuclei were stained with PI only after detergent treatment. Scale bar, 25 μ m. **B**, Cortical sections from TAg and WT mice stained with the phospho-tau antibody CP13 or cleaved caspase-3 antibody. Many large holes left behind by dying neurons (arrowheads, top and bottom) were seen in TAg mice but not in age-matched WT mice. Several degenerating neurons immunoreactive for CP13 or cleaved caspase-3 (arrow, top and bottom) were interspersed with the dead neurons in TAg mice. Several neurons were intensely labeled with cleaved caspase-3 in TAg mice, suggesting that the holes likely resulted from apoptosis. Scale bars: top, 50 μ m; bottom, 25 μ m. **C**, Hippocampal sections from TAg and WT mice stained with hematoxylin, cleaved caspase-3, or BrdU. Hematoxylin staining demonstrated numerous neurons with a shrunken or pyknotic appearance (arrows) in TAg mice interspersed with large holes left behind by dying neurons (arrowheads). Similar neurons were also immunoreactive for cleaved caspase-3 and BrdU (arrows). Mice were exposed to BrdU during 6–10 weeks off-Dox and were subsequently allowed to live until 36 weeks off-Dox. Scale bar, 25 μ m.

brains relative to controls (Fig. 4D). During electron microscopic analysis, uranyl acetate-stained preparations exhibited straight filaments that were smaller than and lacked the definite paired helical property of AD-paired helical filament (Fig. 4E). Presence of phosphorylated tau in the filaments was confirmed via immunogold labeling with CP13 and PHF-1 (Fig. 4F). The smaller phospho-tau aggregates suggest an early or premature state of tau aggregation in TAg mice.

A comparison between the neurodegenerative changes described in Figure 3 and tau pathology showed some dissociation of these two phenomena. Cortical and hippocampal neurodegeneration was observed in TAg mice starting ~10 weeks off-Dox using FluoroJade as a histological marker for neurodegeneration, and neuronal loss (holes left by dead neurons) was detected

~12 weeks off-Dox (Fig. 3). Although an increase in tau phosphorylation is detected ~9 weeks off-Dox by immunoblotting (Fig. 4A), tau pathology is not visible in TAg mice until 22 weeks off-Dox (Fig. 4B). The presence of neuronal loss before and concomitant with tauopathy suggests two types of death, a more rapid (before 22 weeks), tau-independent type and a more protracted (after 22 weeks), tau-dependent type. NFT- and non-NFT-associated neuronal loss is well established in AD as well (Gomez-Isla et al., 1997).

TAg expression leads to amyloid pathology in brain

Because APP processing is influenced by the cell cycle (Suzuki et al., 1994), hallmarks of amyloid pathology in the TAg mice were explored using antibodies against various epitopes of APP and its

degradation products (Table 1). Several diffuse deposits were detected with the 4G8 and 6E10 antibodies in all brain regions, including brainstem and cerebellum, from ~30 weeks off-Dox (shown for cortex in Fig. 5A). These deposits resembled diffuse plaques of AD and the Tg2576 mouse model that overexpresses mutant APP (Hsiao et al., 1996) but were much fewer in number (~2–15 per sagittal section) and generally smaller in size (Fig. 5A, 4G8 staining). The amyloid specific nature of the deposits was confirmed by the absence of similar immunostaining in APP knock-out (APP KO) mice (only shown for 6E10 staining in Fig. 5A), which were used as negative controls for all of the APP antibodies used in our study. The 4G8- and 6E10-positive deposits of TAg mice were also immunoreactive with $A\beta_{42,2}$, which specifically recognizes $A\beta$ peptides terminating at residue 42, and with $A\beta_{40,1}$, which is specific for $A\beta$ terminating at residue 40 (Fig. 5B), indicating that $A\beta_{40}$ - and $A\beta_{42}$ -like entities are deposited in TAg mice. A small number of these deposits appeared more fibrillar, were thio-S positive, and exhibited Congo red birefringence (Fig. 5C). A quantitative increase in soluble and insoluble 6E10 immunoreactivity in animals maintained 21–30 weeks and longer off-Dox was observed by sandwich ELISA using 4G8 as the capture antibody (Fig. 5D).

Intense immunoreactivity of neuronal nuclei in TAg mice was also observed with 6E10, $A\beta_{42,2}$ (Fig. 5A,B), and Anti-I (data not shown). Similar nuclear staining was also observed with these antibodies in AD and in Tg2576 mice but not in APP KO mice (Fig. 5A, shown for 6E10), supporting the specificity of staining. The similar nuclear staining pattern among TAg, Tg2576, and AD cases suggests that TAg expression may lead to similar pathological APP processing observed in AD.

To examine the increase in immunoreactivity to $A\beta$ antibodies in TAg mice in more detail, IPs were performed with 4G8 as capture antibody and 6E10 or 82E1, an antibody recognizing the N-terminal neopeptide generated by β -secretase cleavage of APP, as detection antibody. Both antibodies detected the 4 kDa $A\beta$ peptide in AD but not in TAg mice (Fig. 6A), which may be explained by the smaller number of amyloid deposits in TAg mice relative to AD and Tg2576 mice (Fig. 5A, 4G8 and 6E10). Alternatively, because 6E10 was raised against the N-terminal region of human $A\beta$, which differs from mouse by three amino acids, this negative result may be attributable to a weaker affinity of 6E10

Figure 4. NFT pathology in TAg mice. **A**, Tau immunoblots from TAg and WT mice. TG5 demonstrated a size shift in total tau levels in TAg mice. Tau was hyperphosphorylated at serine 202 (CP13), serine 396/404 (PHF-1), and threonine 231 (CP10) in TAg mice starting ~9 weeks after Dox withdrawal and onward compared with WT. Lanes 1 and 2 are representative of 9–14 and 15–30 weeks after Dox withdrawal, respectively. β -Actin was used as a protein loading control. **B**, Hippocampal sections from TAg and WT mice stained with the NFT markers CP13 and PHF-1 and cortical sections stained with CP10 showed robust labeling of degenerating neurons in TAg mice only. CP10, a tau antibody recognizing phospho-serine 231, showed somatic accumulation of tau reminiscent of AD NFT in TAg mice. Scale bar, 25 μ m. **C**, High-magnification images of neurons stained with CP13 and PHF-1 (left column) demonstrating tangle-like tau accumulation in TAg mice. EM of neurons immunostained with CP13 and PHF-1 *in situ* revealed filamentous aggregates (middle column). These filamentous aggregates were similar to those in AD (bottom right) but were clearly distinguished from those lacking tau immunoreactivity (top right) in non-tangle-bearing neurons. Scale bars: IHC, 10 μ m; EM, 500 nm. **D**, Immunoblots of sarcosyl-extracted hemibrain homogenates (sark) verified an increase in insoluble hyperphosphorylated tau in TAg mice similar to AD. Sup, Supernatant. **E**, Sarcosyl extracts negatively stained with uranyl acetate were subjected to EM and revealed formation of straight filaments that lacked the helical periodicity seen in AD. Scale bar, 100 nm. **F**, Immunogold labeling of sarcosyl extracts with CP13 and PHF-1 confirmed the presence of tau in the filaments from AD (CP13) and TAg mice. Scale bar, 100 nm.

Figure 5. Amyloid plaque pathology in TAg mice. **A**, Cortical sections from AD, Tg2576 mice, TAg mice, and control (Ctrl; WT for 4G8 and APP KO for 6E10) mice stained with amyloid antibodies. Using the 4G8 and 6E10 antibodies, diffuse amyloid plaque-like deposits similar to those of AD and the Tg2576 amyloid model were detected in TAg mice but not in WT (4G8) or APP KO (6E10) mice. Neuronal nuclear staining was also observed in AD, Tg2576, and TAg sections with 6E10 (arrows). Scale bar, 50 μ m. **B**, The $A\beta_{40.1}$ and $A\beta_{42.2}$ antibodies showed that $A\beta_{1-40}$ and $A\beta_{1-42}$ are present in the same diffuse plaque in adjacent sections (landmark, large arrow). Neuronal nuclear staining was also seen with $A\beta_{42.2}$ antibody in AD, Tg2576, and TAg mice but not in WT (arrows). Scale bar, 25 μ m. **C**, A few fibrillar plaques in TAg mice were labeled with thio-S and exhibited Congo red birefringence similar to Tg2576 mice. Scale bar, 20 μ m. **D**, Using sandwich ELISA (4G8 capture, 6E10 detection), increased 6E10 immunoreactivity was observed in soluble (one-way ANOVA, $F_{(3,25)} = 11.136$; $p = 0.0001$) and insoluble (one-way ANOVA, $F_{(3,25)} = 27.813$; $p < 0.0001$) fractions from ~21–30 weeks off-Dox and onward, but levels were much lower than in AD or Tg2576 mice.

for mouse APP/ $A\beta$. Endogenous levels of $A\beta$ in mice are extremely low, and this peptide is not normally detected by Western blot analyses (Dr. Y. Matsuoka, personal communication) (Fig. 6B). However, $A\beta$ was detected in TAg but not wild-type mouse brain, by performing a large-scale immunoprecipitation using 4G8 antibody covalently coupled to protein G-Sepharose beads and 4G8 for detection (Fig. 6B). Based on the similar band intensity between the control lane (straight supernatant from Tg2576 homogenate, 200 μ g in supernatant) and TAg lane (immunoprecipitate using 4 mg of supernatant), the $A\beta$ level in TAg mice is ~20-fold less than in Tg2576. Accordingly, when 6E10 antibody was used

for detection, $A\beta$ was observed in the Tg2576 mouse and very faintly in the 4G8 immunoprecipitate from TAg mouse (Fig. 6B), reaffirming the weaker affinity of 6E10 for mouse $A\beta$. Another factor that may contribute to the difficulty in detecting $A\beta$ in the TAg mice is heterogeneity of the $A\beta$ species generated. It has been reported that the N-terminal region containing the 6E10 and 82E1 epitopes is typically lacking in nonhuman species (Tekirian, 2001). Additional studies will be required to clarify the precise nature of the species $A\beta$ in this model.

In addition to $A\beta$, an increase in the 13 kDa, amyloidogenic C-terminal fragments (CTFs) was observed in TAg mice and in AD, whereas a similar band was absent in the APP KO mice (Fig. 6A). APP and its CTF are thought to have a role in transcriptional regulation and cytotoxicity (Cao and Südhof, 2001; Suh and Chang, 2005). To verify that CTFs were increased in TAg mice, direct immunoblot analysis of brain lysates with CT15, a C-terminal APP antibody was performed. A noticeable increase in CTFs was observed in TAg mice starting from 21–30 weeks after Dox withdrawal onward, and no similar band was seen in the APP KO mice, confirming that the CTFs observed were indeed APP derived (Fig. 6C). CT15 also displayed a robust increase in labeling of cytoplasmic vesicles in neurons of TAg mice (Fig. 6D). A broader investigation of APP revealed similar vesicular enrichment of APP epitopes in TAg mice, AD, and Tg2576 mice (Fig. 6E). Such staining was absent in the APP KO mice. The increase in vesicular staining further supports the interpretation of increased APP processing in the TAg mice. Immunoblots showed an increase in immunoreactivity with 22C11 and GF7, an antibody against the phosphorylated Thr668 epitope, in TAg mice relative to WT (Fig. 6F). GF7 immunoprecipitation/22C11 blotting showed increased phosphorylation of the immature form of APP (Fig. 6G). Additionally, CT15 immunoprecipitation/GF7 blotting indicated an increase in phosphorylation of CTFs in TAg mice (Fig. 6G). These data are consistent with enhanced β -secretase cleavage of APP after phosphorylation at Thr668 (Lee et al., 2003).

Discussion

In this study, we show that transgenic mice conditionally expressing TAg in postmitotic neurons recapitulate three of the defining neuropathological features resembling that of AD (NFT, plaques, and neurodegeneration) after cell cycle reentry. Moreover, this is the first model to generate NFT-like profiles and amyloid depos-

its from endogenous mouse tau and APP, without dependence on mutated human *tau* or *APP* genes. TAg is an established cell cycle activator that is used extensively in cancer research (Ahuja et al., 2005). Its demonstrated ability in inducing diagnostic pathology of AD in mice lends credence to the concept that the origin of AD resembles that of cancer (Herrup and Yang, 2001). Evidence of cell cycle reentry and DNA synthesis in neurons preceding tau and amyloid pathology in our study as well as *in vivo* data showing the early appearance of cell cycle markers in individuals with AD and mild cognitive impairment (Yang et al., 2003) lends support to the essential role of cell cycle activation in AD pathogenesis. Conversely, a preponderance of genetic evidence favors A β as the prime instigator of AD pathology (LaFerla and Oddo, 2006). However, the lack of NFT and neurodegenerative pathology in the A β -based models (Borchelt et al., 1997; Holcomb et al., 1998), together with the simultaneous display of NFT- and plaque-like profiles in the TAg mice, suggests that cell cycle dysregulation lies upstream rather than downstream of A β production in the AD pathological cascade *in vivo*. This sequence is supported by the activation of cell cycle markers well before plaque deposition in several FAD mutant APP transgenic mice (Yang et al., 2006). Additionally, mitotic activation via knock-out of the prolyl-isomerase Pin1 leads to tau and APP changes in mice (Lu et al., 2003). In our TAg model, tau hyperphosphorylation and NFT-like profiles precede amyloid deposition, resembling neuropathological evidence for a similar sequence in humans (Braak and Braak, 1997). Collectively with evidence for presenilin 1 function in cell proliferation (Chevallier et al., 2005) and tumorigenesis (Xia et al., 2001), it appears that cell cycle activation constitutes an early mechanism that may be influenced by genetic and insidious etiologies in AD.

Ectopic cell cycle activation has been linked to other neurodegenerative conditions, particularly those involving tau or neurofilament inclusions (Vincent et al., 2003). In particular, cell cycle changes have been found in disorders, such as amyotrophic lateral sclerosis (Ranganathan et al., 2001; Nguyen et al., 2003; Ranganathan and Bowser, 2003) and Niemann-Pick disease type C (Hussemann et al., 2000), that are characterized by axonal spheroids. A cursory screen of TAg mice did not reveal the presence of spheroids or any other type of inclusion, although axonal dystrophy was evident

Figure 6. Increased APP processing in TAg mice brain. **A**, Immunoprecipitation with 4G8 followed by immunoblotting with 6E10 or 82E1 demonstrating elevated CTF (arrow) in TAg mice and AD but not APP KO or WT brains. Light and dark exposures are shown for AD (82E1 blot). A β band is clearly present in AD lane of both blots, although absent for other lanes. **B**, A β was detected in TAg but not in WT brain sample after a large-scale immunoprecipitation of A β in 4 mg of TAg and WT brain homogenate supernatant using 4G8 covalently coupled to protein G-Sepharose beads and 4G8 for detection. A total of 200 μ g of brain homogenate supernatant from Tg2576 brain was used as a positive control. Endogenous mouse A β was also detected using 6E10, but very faintly, indicating weaker affinity of 6E10 for mouse A β . **C**, Immunoblot using the CT15 antibody showing increased CTF in brain supernatants from TAg mice. Absence of CTF band in APP KO confirms the APP-specific origin. β -Actin was used as a loading control. **D**, Cortical sections of TAg and WT mice stained with the CT15 antibody showing an increase in CT15 immunolabeling of

(data not shown). These findings indicate that the populations of neurons targeted in our model may have a greater propensity for tau and amyloid pathology or that the timing or extent of cell cycle activation dictates the type of lesion and disease. Despite this unexpected “selectivity,” degenerative changes in the TAg mice have a wider distribution than typically seen in AD, which complicates the study of AD-specific behavioral changes. For instance, NFT-like profiles are prominent in the brainstem, and increased immunoreactivity with some phospho-tau antibodies is detected in forebrain, midbrain, and cerebellum, as well as in certain cortical neurons that are more resistant to AD (Braak et al., 2000). Nevertheless, the simultaneous generation of NFT and amyloid pathology evoked via a single manipulation represents a significant step in the field of AD research. Because TAg is expressed exclusively in neurons in the TAg model, it seems that neuronal dysfunction alone is sufficient in generating the seminal features of AD pathology.

The presence of diffuse and some fibrillar amyloid deposits suggest that amyloid pathology is relatively primitive in the TAg mice. The paucity of mature plaque-like amyloid deposits with dystrophic neurites and glial activation, thio-S-positive NFTs, and neuritic pathology implies that additional factors may be required or that the premature mortality of TAg mice precludes progression to a more fulminate disease stage. It has been found that the predominant A β species produced naturally in rodents is A β _{1–40/42}, an N-terminally truncated version of A β _{1–40/42} (Tekirian, 2001). Similar truncated A β species are also detected early in AD and in Down’s syndrome brains and are thought to contribute significantly to the pathogenesis of both diseases (Liu et al., 2006). Additional studies will be required to clarify the identity of the A β species initiating amyloid deposition in the TAg mice.

Although TAg is a seemingly nonphysiological entity in humans, its ability to induce concomitant development of tau and amyloid pathology presents a unique opportunity for elucidating a unified mechanism leading to NFT and plaque production. TAg is a classical inducer of cell division and the most extensively used oncogene for transforming many cell types (Ahuja et al., 2005), but it may have other physiological effects (Ali and DeCaprio, 2001). Thus, it still needs to be determined whether the AD-associated pathology described in this model is a result of cell cycle reentry or an independent effect of TAg.

The generation of a more complete spectrum of AD pathologies without requiring mutations in APP, presenilin, or tau simulates the development of AD pathology in the majority of cases that are typified by a sporadic origin of disease. Thus, our model may facilitate the identification of additional genetic and nongenetic factors required for progression to a more advanced or severe AD pathological state. Furthermore, the TAg mice will also be useful for testing and developing cell cycle-inhibiting cancer drugs into treatment modalities for AD.

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cytoplasmic vesicles in TAg neurons, suggesting an increased APP processing in TAg mice. Scale bar, 10 μ m. **E**, Cortical sections of AD, Tg2576, TAg, WT, and APP KO mice stained with the 22C11 antibody also showed increase in vesicular staining in AD, Tg2576, and TAg brain sections compared with WT, and immunoreactivity was absent from APP KO brains. Scale bars: 25 μ m; inset, 10 μ m. **F**, Immunoblotting showed increased 22C11 and GF7 immunoreactivity in TAg mice. **G**, Immunoprecipitation/blotting confirmed hyperphosphorylation of immature form of the full-length APP and CTF in TAg mice. Lanes 1, 2, and 3 represent <20, 21–30, and >30 weeks post-Dox withdrawal, respectively, for all of the blots.

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