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Cholinergic Forebrain Degeneration in the APPswe/PS1ΔE9 Transgenic Mouse

Sylvia E. Perez¹, Saleem Dar¹, Milos D. Ikonomic², Steven T. DeKosky², and Elliott J. Mufson¹

¹Department of Neurological Sciences, Rush University Medical Center, Chicago, IL 60612

²Departments of Neurology and Psychiatry, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261

Abstract

The impact of Aβ deposition upon cholinergic intrinsic cortical and striatal, as well as basal forebrain long projection neuronal systems was qualitatively and quantitatively evaluated in young (2-6 months) and middle-aged (10-16 months) APPswe/PS1ΔE9 transgenic (tg) mice. Cholinergic neuritic swellings occurred as early as 2-3 months of age in the cortex and hippocampus and 5-6 months in the striatum of tg mice. However, cholinergic neuron number or choline acetyltransferase (ChAT) optical density measurements remained unchanged in the forebrain structures with age in APPswe/PS1ΔE9 tg mice. ChAT enzyme activity decreased significantly in the cortex and hippocampus of middle-aged tg mice. These results suggest that Aβ deposition has age-dependent effects on cortical and hippocampal ChAT fiber networks and enzyme activity, but does not impact the survival of cholinergic intrinsic or long projection forebrain neurons in APPswe/PS1ΔE9 tg mice.

Keywords

Alzheimer's disease; amyloid; forebrain; transgenics; acetylcholine; ChAT activity; AChE; stereology; interneurons; degeneration

INTRODUCTION

Alzheimer's disease (AD) is characterized by cognitive decline, which is accompanied by beta-amyloid (Aβ) plaque and neurofibrillary tangle formation in the diseased brain (Arnold et al., 1991; Braak and Braak, 1991). In early onset familial forms of AD (FAD) and in sporadic AD Aβ-deposits are composed of ~4 kDa Aβ-peptides derived from the amyloid precursor proteins (APP). FAD are autosomal dominant forms of AD caused by the expression of mutant human genes encoding APP, presenilin 1 (PS1) or presenilin 2 (PS2), that lead to increased production of highly pathogenic 42-amino-acid β-amyloid (Aβ₁₋₄₂) peptides (Price and Sisodia, 1998). In addition to plaque and tangle formation there is a reduction in subcortical cholinergic nucleus basalis neurons as well as cortical acetylcholine in AD (Davies and Maloney, 1976; Davies, 1979; Richter et al., 1980; Whitehouse et al., 1981, 1985; Mufson et al., 1989; DeKosky et al., 2002). Likewise, cholinergic interneurons and metabolic markers are reduced in the striatum

Address correspondence to: Elliott J. Mufson, Ph.D., Professor of Neurological Sciences, Alla V. and Solomon Jesmer Chair in Aging, Rush University Medical Center, 1735 W. Harrison Street, Suite 300, Chicago, IL 60612, 312-563-3558 tel., 312-563-3571 fax., e-mail: emufson@rush.edu.

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in presence of A β and neurofibrillary tangles in AD (Davies, 1979; Oyanagi et al., 1989; Braak and Braak, 1990; Selden et al., 1994a,b; Scott et al., 1995; Boissiere et al., 1997; Klunk et al., 2004). Although A β aggregates/deposits are thought to be neurotoxic (Hartley et al., 1999; Selkoe, 2001), perhaps by spreading from the cortex to subcortical nuclei (Saper et al., 1987; Mufson et al., 1995; Yanker et al., 1996), the impact A β upon central cholinergic neuron degeneration in AD remains unclear.

Various types of transgenic mice have been engineered to mimic different aspects of AD neurodegeneration (Suh and Checler, 2002; Oddo et al., 2003; Götz et al., 2004). In terms of cholinergic pathology, most mice over-expressing mutant APP and/or PS1 genes display an age-dependent A β deposition, cortical and hippocampal cholinergic fiber degeneration (Wong et al., 1999; Hernandez et al., 2001; Jaffar et al., 2001; Boncristiano et al., 2002; Buttini et al., 2002; German et al., 2003; Aucoin et al., 2005) which is accompanied by memory deficits (see Suh and Checler, 2002). However, these changes are not accompanied by a loss of cholinergic basal forebrain neurons (Hernandez et al., 2001; Jaffar et al., 2001; Boncristiano et al., 2002; German et al., 2003). Therefore, none of these mutant mice fully recapitulates the robust cholinergic neurodegeneration, seen as one of the hallmarks of human AD. It is interesting to note that the majority of studies reporting cholinergic pathologies were performed in single APP or PS1 mutant mice, which have limited or no A β deposition (Hernandez et al., 2001; Jaffar et al., 2001; Boncristiano et al., 2002; Buttini et al., 2002; German et al., 2003; Aucoin et al., 2005; Bales et al., 2006) compared to APP/PS1 double transgenic mice (Wong et al., 1999; Hernandez et al., 2001; Jaffar et al., 2001). Moreover, A β ₁₋₄₂ disrupts normal cholinergic neurotransmission (Wang et al. 2000; Nagele et al., 2002; Chen et al., 2006), further suggesting that A β may precipitate cholinergic system dysfunction in AD.

The APP^{swe}/PS1 Δ E9 transgenic mouse displays an early and aggressive onset of neuritic A β deposition in the cortex, hippocampus and striatum (Lazarov et al., 2002; Perez et al., 2005) together with memory impairment (Savonenko et al., 2005). Despite the report of alterations in cortical acetylcholinesterase and ChAT activity seen in 19-month-old APP^{swe}/PS1 Δ E9 transgenic mice (Savonenko et al., 2005), there are no detailed investigations of the impact of A β deposition upon cholinergic local and long projection systems in this transgenic mouse model of AD. Therefore, we performed quantitative morphologic and biochemical analyses of cortical, striatal, hippocampal, and nucleus basalis cholinergic systems in young (2-6 months) and middle-aged (10-16 months) amyloid over-expressing APP^{swe}/PS1 Δ E9 transgenic compared to age-matched non-transgenic mice.

MATERIAL AND METHODS

The present study used a total of 40 animals (both genders) consisting of young (2-6 months of age) and old (10-16 months of age) heterozygous transgenic (tg) mice harboring FAD-linked mutant APP^{swe}/PS1 Δ E9 [co-expressing presenilin 1 (PS1) and a chimeric mouse-human amyloid precursor protein (APP) 695 with mutations (K595N, M596L) linked to Swedish FAD pedigrees (APP^{swe}) via the mouse prion protein promoter (Borchelt et al., 1996a,b, 1997; Lesuisse et al., 2001)] and age-matched non-transgenic (ntg) littermate mice. At least two female mice were included in each group examined. These mice were obtained by crossing single APP^{swe} line C3-3 and PS1 Δ E9 line S-9 tg mice and PS1 Δ E9 line S-9 tg with ntg littermate mice. The background strains for APP^{swe} are {C3H/HeJ x C57BL/6J F3} x C57BL/6J n1, and PS1 Δ E9 are C3H/HeJ x C57BL/6J F3. Animal care and procedures were conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All mice were anesthetized with an injection of ketamine/xylazine (285mg/kg/9.5mg/kg) and perfused transcardially with ice-cold 0.9% sodium chloride (NaCl) solution. Brains were rapidly removed from the skull and hemisected in a frozen stainless steel brain blocker. One hemisphere was sectioned into 1 mm coronal slabs on wet ice in the brain blocker. Tissue

pieces were dissected from the cortex, hippocampus and striatum using fiduciary landmarks and frozen at -80 °C until processed for enzymatic assay. The other hemisphere was immersion-fixed in 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer for 12 hours at 4 °C and cryoprotected in 30% sucrose at 4 °C until the brain sank, and then cut on a freezing sliding knife microtome in the coronal plane at a thickness of 40 µm. The sections were stored at -4 °C in a cryoprotectant solution (30% glycerol, 30% ethylene glycol in 0.1 M phosphate buffered saline) prior to use.

Immunohistochemistry, Immunofluorescence and Histochemistry

Fixed tissue was processed as free-floating sections and singly labeled with a polyclonal antiserum raised against choline acetyltransferase (ChAT) from human placenta (1:1000 dilution, Chemicon, CA, USA). The specificity of the antibody has been described extensively (Mesulam et al., 1983; Mufson et al., 1989). Sections were washed several times in Tris-buffered saline (TBS) before incubation with 0.1 M sodium periodate (to inhibit endogenous peroxidase activity) in a TBS solution for 20 minutes. After several rinses in a solution containing 0.25% Triton X-100 in TBS, the tissue was placed in a blocking solution containing TBS with 0.25% Triton X-100 and 3% horse serum for 1 hour. Sections were subsequently incubated in goat anti-human ChAT for 48 hours in a solution containing TBS, 1% Triton X-100 and 1% horse normal serum. Following washes with 1% horse normal serum in TBS, sections were incubated with biotinylated horse anti-goat secondary antibodies (Vector Laboratories, CA, USA) for 1 hour. After several washes in TBS the tissue was incubated for 60 minutes with an avidin-biotin complex (1:500; 'Elite Kit', Vector Laboratories, CA, USA). Tissue was rinsed in 0.2 M sodium acetate, 1.0 M imidazol buffer (pH 7.4), and developed in an acetate-imidazol buffer containing 2.5% nickel sulfate, 0.05% 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich, St. Louis, MO, USA) and 0.0015% H₂O₂. The reaction was terminated using an acetate-imidazol buffer solution. Finally, sections were mounted on glass slides, dehydrated in graded alcohols, cleared in xylenes and cover slipped with DPX (Biochemica Fluka, Switzerland). All sections were processed at the same time using the same chemical reagents to avoid batch-to-batch variation during immunostaining. Additional sections were dual-labeled for ChAT and Aβ using the 10D5 monoclonal antibody raised against amino acids 1-16 of the human beta-amyloid protein (1:10,000 dilution, gift of Elan Pharmaceuticals, San Francisco, CA, USA). For double immunostaining, ChAT was developed using a nickel chromagen followed by incubation with the monoclonal 10D5 antibody and visualized using a nova red substrate kit (Vector Laboratories, CA, USA). This dual-staining method results in a two-colored profile: dark-blue ChAT positive profiles, and red Aβ containing plaques. Immunofluorescence was also used for Aβ visualization: these sections were stained with 10D5 antiserum (1:1000 dilution) and developed with Cy3-conjugated donkey anti-mouse antibodies (1:300; Jackson ImmunoResearch Labs, West Grove, PA, USA). In addition sections were also double stained with 10D5 (nova red substrate kit) and/or histochemically reacted for acetylcholinesterase (AChE), the degrading enzyme for acetylcholine, using the Karnovsky and Roots method (1964) followed by silver intensification (Emre et al., 1993).

Radioenzymatic assay

Brain regions including cortex, hippocampus and striatum from 2-6 months old and 10-16 months old APP^{swe}/PS1^{ΔE9} tg and age-matched ntg littermate mice were processed for determination of ChAT enzyme activity using a modification of the Fonnum method (Fonnum, 1975; DeKosky et al., 1985). Frozen tissue was homogenized using high frequency sonication in a solution containing 0.5% Triton X-100 and 10 mM EDTA. Briefly, 5 µl of tissue homogenate was combined with C-14 labeled acetyl Co-A (New England Nuclear, Boston, MA, USA), incubation buffer (100 mM sodium phosphate, 600 mM NaCl, 20 mM choline chloride, 10 mM disodium EDTA, pH7.4), and physostigmine (20 mM, Sigma-Aldrich, St.

Louis, MO, USA). After 30 minutes of incubation at 37°C in a water bath, the reaction was stopped by the addition of 4 ml of 10 mM phosphate buffer (pH 7.4). Subsequently, 1.6 ml of acetonitrile/tetraphenylboron mixture and 8 ml of scintillation fluid were added to cause phase separation. After the samples stabilized for 24 hours, they were counted in a scintillation counter. Protein content of the samples was determined using a BCA protein assay kits (Pierce, Rockford, IL, USA). ChAT activity was expressed as $\mu\text{mol/hr/g}$ protein. Samples were coded, and all assays performed in triplicate by a technician blinded to experimental groups.

Stereologic analysis

The optical dissector method was used to determine the number of ChAT immunoreactive (-ir) neurons in the cerebral cortex (motor, cingulate and sensory cortices), striatum and nucleus basalis in 3- to 6-month-old and 10- to 16-month-old APPswe/PS1 ΔE9 tg as well as age-matched ntg mice as previously described (Jaffar et al., 2001; Perez et al., 2005). The regions were manually outlined under low magnification and systematically analyzed using a random sampling design. The estimated numbers of ChAT-ir neurons were performed using MicroBrightField stereological software (Williston, VT) in an Olympus BX-60 microscope coupled with LEP MAC5000 (BioVision Technologies; Exton, PA, USA). The coefficients of error were calculated according to Gundersen et al. (1988) and values <0.10 were accepted (West, 1993). The ChAT-ir neurons in the nucleus basalis were counted immediately caudal to decussation of the anterior commissure to the end of the globus pallidus (Fig. 1B-D). These neurons were located ventral to and within the globus pallidus as well as intermingled within the internal capsule tracts. The ChAT-ir neurons in the sensory cortex were counted until the appearance of cholinergic immunoreactivity in the interpeduncular nucleus (Fig. 1B-E), whereas in the cingulate and motor cortex as well as in the striatum they were counted throughout their entire extension (Fig. 1). Since hemi-secting a brain can result in asymmetrical midline cuts, quantification of ChAT-ir neurons in the medial septum and vertical limb of the diagonal band of Broca was not performed. All of the ChAT-ir neuron counts were performed by an observer blinded to age and genotype.

Optical density and area measurements

Quantification of the relative optical density (OD) of neuronal ChAT immunoreactivity in the cingulate, motor and sensory cortices, striatum and nucleus basalis from young (3-6 months) and middle-aged (10-16 months) APPswe/PS1 ΔE9 tg and ntg mice was performed using a densitometry software program (Image 1.60, Scion 1.6) as previously described (Mufson et al., 1997; Ma et al., 1999; Jaffar et al., 2001; Perez et al., 2005). The ChAT-ir neurons of the different regions were outlined manually, and the OD and area measurements were automatically analyzed in gray-scale images. Background tissue levels were measured and the average subtracted from the OD measurements of ChAT-ir. These measurements were performed by an observed blinded to age and genotype.

Statistical analysis

Data obtained from the ChAT enzymatic assay, stereologic and OD measurements were evaluated using the Mann-Whitney rank-sum test, a non-parametric test (SigmaStat 3.0; Aspire Software International, Leesburg, VA, USA). This test is more powerful evaluating small samples and minimizes the effect of the outliers than the *t*-test (Siegel, 1956). The level of statistical significance was set at 0.05 (two-sided).

RESULTS

General characteristics of cholinergic pathology in the cerebral cortex, hippocampus, striatum and nucleus basalis in APPswe/PS1ΔE9 tg mice

To evaluate the effects of the Aβ deposition on the cholinergic systems in APPswe/PS1ΔE9 tg mice, tissue sections were stained for the cholinergic markers, ChAT and AChE, as well as Aβ. In the cortex, hippocampus and striatum Aβ-plaque burden increased in an age-dependent fashion (Fig. 2). All plaques were thioflavine-S-positive indicating the compact/neuritic nature of these deposits in APPswe/PS1ΔE9 tg mice, as previously described (Lazarov et al., 2002; Perez et al., 2005). Qualitative analysis revealed ChAT- and AChE-positive dystrophic neurites in the cerebral cortex (motor, cingulate and sensory cortices) and hippocampus as early as 2-3 months of age (Fig. 3A,B,F), whereas the striatum displayed this type of pathology at 5-6 months of age (Fig. 6B), consistent with the onset of Aβ-ir plaques in these structures in the APPswe/PS1ΔE9 tg mice (Lazarov et al., 2002; Perez et al., 2005). Cholinergic dystrophic neurites displayed swollen processes organized mostly in rosette or grape-like clusters (Fig. 3C,E, F,J) in close proximity to Aβ-ir plaques. Although the number and size of the cholinergic dystrophic neurites were variable, they increased in parallel with the increase in plaque load seen in the cortex, hippocampus and striatum (Fig. 3A,C,F,J). However, within the region containing the cholinergic nucleus basalis neurons occasional Aβ-ir plaques and cholinergic dystrophic neurites were seen mainly in the oldest APPswe/PS1ΔE9 tg mice (Fig. 6J,K). Furthermore, cholinergic processes arising from the neurons of nucleus basalis in 16 months old tg mice showed larger axonal varicosities compared with age-matched ntg mice. ChAT and AChE staining revealed similar alterations in the laminar organization of cholinergic fibers in the cortex and the hippocampus of young and middle-aged APPswe/PS1ΔE9 tg mice (Fig. 4B,D,F,H,J,L). In young APPswe/PS1ΔE9 tg mice, there was virtually no disruption of cortical cholinergic fiber innervation compared to the dramatic disturbance of these fibers, which occurs in association with the presence of amyloid plaques in older mutant mice (Figs. 4D,H,L,Q and 5). However the older transgenic animals did not display a layer-specific alteration in cortical cholinergic fiber innervation (Fig. 5D). Aged tg mice (16 months) also showed a striking reduction in the density of cholinergic fibers staining revealed by ChAT immuno- or AChE histochemical staining (Figs. 4D,H,O,Q and 5D). Cholinergic fiber trajectory was altered near as well beyond the borders of the Aβ plaque in mutant, but was more pronounced in aged tg mice (Figs. 3A,D and 4B,D,L,Q).

Cholinergic neuronal number in the cerebral cortex, striatum and nucleus basalis of APPswe/PS1ΔE9 tg mice

To examine the effects of Aβ-plaques upon local and long projection cholinergic neurons in young and middle-aged APPswe/PS1ΔE9 tg mice (Table 1), we quantified the number of ChAT-ir cortical, striatal and nucleus basalis neurons using an unbiased stereological counting method. Cortical and striatal ChAT-ir neurons are implicated in the modulation of local neuronal circuitry, whereas ChAT-ir neurons in the nucleus basalis have long projections and are the main source of acetylcholine in the cerebral cortex (Wainer et al., 1984). Cortical small and mainly bipolar ChAT-ir neurons were counted in the motor, cingulate and sensory cortices (Figs. 4A-L and 5). ChAT-ir neurons in the cingulate and motor cortex were found mainly in layers II-III, whereas in the sensory cortex they were seen in layers II to VI. Despite the presence of Aβ-ir plaques in these cortical areas, as well as the striatum there was not a significant difference in the number of ChAT-ir interneurons in these structures, except in the motor cortex (Fig. 7A). In this cortical area the number of ChAT-ir neurons were significantly lower in the APPswe/PS1ΔE9 tg compared to ntg mice, independent of the age-group examined (Fig. 7A; Mann-Whitney rank-sum test; $p=0.033$). No differences were detected in the number of ChAT-ir neurons in the nucleus basalis between the young and middle-aged double mutant compared to the age-matched ntg mice (Mann-Whitney rank-sum test; $p>0.05$). These data

suggest that A β deposition may not lead to frank degeneration of the cholinergic local and long projection neurons in the cingulate and sensory cortices, striatum and nucleus basalis.

Optical density measurements of cortical, striatal and nucleus basalis neurons in APPswe/PS1 Δ E9 tg mice

Levels of neuronal ChAT-ir were evaluated in neurons of the motor, cingulate and sensory cortices, striatum as well as in the nucleus basalis in young and middle-aged tg mice (Table 1), using OD measurements (Scion image system). OD measurements of the ChAT-ir neurons in the motor, cingulate and sensory cortices as well as the striatum and nucleus basalis did not show significant differences between young and middle-aged tg mice in comparison with ntg controls. In addition, no changes in average cell size, measured as area of the soma (μm^2), were detected for cortical and striatal cholinergic neurons in mutant compared to the ntg mice. However, the ChAT-ir neurons in the nucleus basalis of the oldest APPswe/PS1 Δ E9 tg mice (12-16 months) were significantly enlarged compared to the age-matched ntg mice (Fig. 7B; Mann-Whitney rank-sum test; $p=0.029$). Therefore, except for the nucleus basalis, the cholinergic neurons in the cortex and striatum failed to show a neurodegenerative phenotype in these double mutant mice.

ChAT-enzyme activity decreases with age in the cerebral cortex and the hippocampus, but not in the striatum of APPswe/PS1 Δ E9 transgenic mice

To evaluate possible alterations of the cholinergic metabolic markers, ChAT enzyme activity was analyzed in the cortex, hippocampus and striatum of young (2-6 months) and middle-aged (10-16 months) APPswe/PS1 Δ E9 tg mice as well as in age-matched ntg mice (Table 2). ChAT enzyme activity in the cerebral cortex and hippocampus of middle-aged APPswe/PS1 Δ E9 was significantly decreased compared to young tg mice (Fig. 8A,B; Mann-Whitney rank-sum test; cerebral cortex $p=0.048$, hippocampus $p=0.035$). A comparison of ChAT enzyme activity between young and middle-aged tg mice revealed a greater reduction in the hippocampus (~30%) than in the cerebral cortex (~15%). Cortical ChAT enzyme activity in middle-aged tg mice was significantly reduced compared to the middle-aged ntg mice, but not in the hippocampus (Fig. 8A; Mann-Whitney rank-sum test; $p=0.018$). ChAT enzyme activity in the cerebral cortex and hippocampus was not statistically different between young and middle-aged ntg mice (Fig. 8A,B). Striatal ChAT enzyme activity did not differ between the young and the middle-aged APPswe/PS1 Δ E9 tg mice in comparison with ntg mice (Fig. 8C; Mann-Whitney rank-sum test, $p>0.05$).

DISCUSSION

The present study evaluated the impact of A β deposition upon cholinergic local and long projection forebrain neuronal systems in APPswe/PS1 Δ E9 tg mice. Swollen dystrophic cholinergic neurites were seen in close association with A β plaques throughout the cortex and hippocampus at 2-3 months and in the striatum at 5-6 months in these mutant tg mice. In addition, we also observed that the trajectory of non-dystrophic cholinergic fibers was displaced beyond plaque borders in young and middle-aged mutant mice (D'Amore et al., 2003; Knowles et al., 1998, 1999). ChAT positive neurite size and number increased in parallel with plaque load and was more conspicuous in older mutant tg mice. These findings support and expand other investigations showing that the formation of cholinergic neurites in the cortex, hippocampus and in the striatum are associated with compact (thioflavine-S-positive) rather than diffuse plaques described in other over-expressing APP mice (Hernandez et al., 2001; Boncristiano et al., 2002; Brendza et al., 2003; D'Amore et al., 2003; German et al., 2003; Hu et al., 2003). These observations suggest that the toxic properties of A β proteins are dictated by its conformational features as reported for AD (Probst et al., 1983; Masliah et al., 1990; Knowles et al., 1998). Recently, it was shown that cortical cholinergic neurites are the first to

form in APP over-expressed mice, followed by glutamatergic and GABA-ergic neurites (Hu et al., 2003; Bell et al., 2003, 2005). Stokin et al. (2005) showed that cholinergic axonal swellings arising from nucleus basalis neurons precede plaque formation in single APP^{swe} tg mice leading to the suggestion that their presence is related to axonal transport deficits (Pigino et al., 2003). Other studies suggest that A β deposition has neurotrophic effects (Jaffar et al., 2001), which induce aberrant neuritic sprouting in both, animal models of AD as well as in the disease itself (Masliah et al., 1991, 2003; Koo et al., 1993; Phinney et al., 1999; Hernandez et al., 2001). Studies also demonstrate that dystrophic neurites are secondary to the presence of A β and are only partially reversed by anti-A β antibody treatments (Lombardo et al., 2003, Brendza et al., 2005). These observations lend support to the concept that the very early morphological changes seen in cholinergic projections in APP^{swe}/PS1 Δ E9 tg mice parallels early changes in cholinergic transmission in these same tg mice assessed by neuropharmacological techniques (Machová et al., 2006). Ultrastructural analysis of dystrophic swellings in APP^{swe}/PS1 Δ E9 (Perez et al., 2004) and other over-expressing APP tg mice reveal the presence of vesicular organelles (Masliah et al., 1996; Phinney et al., 1999; Luth et al., 2003), which are part of lysosomal systems implicated in endocytic (Cataldo et al., 2000, 2004; Mathews et al., 2002) and/or autophagic (Wang et al., 2006) mechanisms. Autophagia may be an early remodeling response to aberrant neuritic processes (Wang et al., 2006), and therefore, an attempt by the brain at self-repair.

In addition to the cholinergic dystrophic pathology, we found a significant decrease in ChAT enzyme activity in the cerebral cortex and hippocampus of 10-16 months old compared with 2-6 months old APP^{swe}/PS1 Δ E9 tg mice, but not in the striatum. Similar to previous studies in aged APP^{swe}/PS1 Δ E9 tg mice (19 months; Savonenko et al., 2005) and homozygous PDAPP tg mice (24 months; German et al., 2003), we found significant differences in ChAT enzyme activity in the cerebral cortex between middle-aged tg and ntg mice, but not in the hippocampus. This discrepancy may be due to the pattern of reduction depicted for hippocampal ChAT enzyme activity in tg mice, and in the fact that young double mutant mice showed higher levels of ChAT enzyme activity than the age-matched control mice. These findings are similar to those reported in end-stage of AD, where there is marked decrease of ChAT enzyme activity in the cortex, but not in the striatum (Davies, 1979; Araujo et al., 1988; DeKosky et al., 2002). Interestingly, ChAT activity was reported to be increased in cortical areas in middle-aged APP and PS1 single tg mice (Hernandez et al., 2001), perhaps resembling the up-regulation of cortical and hippocampal ChAT enzyme activity seen in prodromal AD (DeKosky et al., 2002), which is not due to an increase in cholinergic axonal innervation (Ikonomic et al., 2007). We also observed a marked disruption and reduction in the density of cholinergic fibers in these regions in older tg mice, suggesting that the down-regulation of ChAT enzyme activity may, in part, reflect the cholinergic fiber degeneration in these tg mice. Although we did not perform cholinergic fiber counts, previous studies have shown an age-dependent loss of hippocampal and cortical cholinergic fibers in other over-expressing APP tg mice (Boncristiano et al., 2002; Aucoin et al., 2005; German et al., 2003), which is reminiscent of cortical cholinergic fiber depletion seen in AD (Geula and Mesulam, 1996). Functionally, the loss of cortical and hippocampal ChAT activity correlates with the severity of dementia (Perry et al., 1978; Wilcock et al., 1982; Dekosky et al., 1992) and the loss of cortical innervation is more severe and occurs earlier than other cortical neurotransmitter systems in AD (see Geula and Mesulam, 1996). However, cognitive impairment is better correlated with A β concentrations than cholinergic deficits in cortex and hippocampus in 19 months APP^{swe}/PS1 Δ E9 tg mice (Savonenko et al., 2005). By contrast, we found a significant decrease in cholinergic cortical activity in 10-16 months APP^{swe}/PS1 Δ E9 tg mice. Therefore, future studies are required to more fully understand the relationship between amyloid deposition, cholinergic degeneration and cognitive impairment in this animal model of AD.

Despite the A β burden and cholinergic cortical and subcortical neuritic pathology, except for the motor cortex, there were no differences in the number of ChAT-positive neurons in the cholinergic forebrain systems examined in APPswe/PS1 Δ E9 tg mice. The motor cortex displayed a lower number of ChAT-ir neurons in APPswe/PS1 Δ E9 tg versus control littermate mice independent of age, suggesting a trans-gene developmental effect or the presence of toxic A β oligomers, rather than a direct effect of A β plaque formation. ChAT-ir neuron shrinkage was also not detected in any region including the motor cortex. In rodents cholinergic cortical bipolar interneurons express vasoactive intestinal peptide (VIP) and/or GABA which mediate neurovascular control of the microcirculation in cortical and subcortical areas (Bayraktar et al., 1997; Cauli et al., 2004). A recent study revealed a substantial early loss of somatostatin and/or neuropeptide Y hippocampal interneurons in another double APP/PS1 tg mouse (Ramos et al., 2005) suggesting that amyloid and/or other AD related processes selectively affect particular cellular phenotypes. Interestingly, these cholinergic cortical and hippocampal neurons are neither AChE- nor NGF receptor (p75^{NTR} or TrkA, personal observations) positive, whereas the cholinergic striatal neurons contain the former as well as TrkA in adult rodents (Levey et al., 1984; Mufson and Cunningham, 1988; Fagan et al., 1997). These findings indicate differences in cholinergic phenotype between cortical, hippocampal, striatal as opposed to nucleus basalis neurons which display AChE and both NGF receptors (Sobrevela et al., 1994). It is important to note that cortical or hippocampal cholinergic neurons have not been reported in the primate brain (Wainer et al., 1984) suggesting a species and perhaps functional difference between rodents and non-human primates which needs to be considered when modeling AD.

Although the present study failed to reveal a loss of cholinergic striatal neurons in APPswe/PS1 Δ E9 tg mice, it should be noted that the prior protein promoter drives the highest trans-gene expression in the cerebral cortex and hippocampus. This may possibly be a factor underlying the lower amyloid load and cholinergic pathology seen in the striatum (Borchelt et al., 1996a). Although dystrophic neurites and plaques are found in the AD striatum (Rudelli et al., 1984; Suenaga et al., 1990; Brilliant et al., 1997), it remains controversial whether there is a loss of the cholinergic neurons in the neostriatum (Oyanagi et al., 1989; Shelden et al., 1994b) as compared to the reduction seen in the ventral striatum in AD (Lehericy et al., 1989; Shelden et al., 1994b). These observations suggest that the current model of AD is not a complete replication of the disease induced pathology. Similarly, the present and other studies have failed to show cholinergic neuronal loss in the nucleus basalis (Jaffar et al., 2001; Hernandez et al., 2001; Boncristiano et al., 2002) or in the medial septum/vertical and horizontal limb of the band of Broca in animal models of amyloidosis (Boncristiano et al., 2002; German et al., 2003) as compared to the dramatic loss of these neurons in advanced AD (Whitehouse et al., 1981). Interestingly, we reported an increase in p75^{NTR}/cholinergic neuron number in the medial septum in APPswe mice (Jaffar et al., 2001). In the present study, we found a significant increase in nucleus basalis cholinergic neuron cell size in aged APPswe/PS1 Δ E9 tg mice unlike that seen in APP23 mice (Boncristiano et al., 2002). The increase in nucleus basalis cell size may be indicative of cytoskeletal changes and/or axonal transport defects, which have been implicated in cellular neurodegeneration in AD and in transgenic animal models of cholinergic neuronal dysfunction (Gajdusek, 1985; Morfini et al., 2002; Pigino et al., 2003; Stokin et al., 2005). The differential effect of amyloid upon different cholinergic neurons may also be related to a particular cholinergic phenotype and hodology, which confers resistance to A β toxicity or to trans-gene expression.

In summary, the present findings indicate that A β plaques do not directly impact the survival of cholinergic neurons in APPswe/PS1 Δ E9 tg mice. It is possible that if our APP over-expressing mice had lived longer or expressed tangle bearing material (Oddo et al., 2003), a necessary precondition for neuronal death in AD, they would have displayed frank neuronal loss. However, the increase of cholinergic dystrophic neuritic and A β pathology with age in

the cortex, striatum and hippocampus, together with the reduction of cortical ChAT enzyme activity in the absence of neuronal death in the APP^{swe}/PS1 Δ E9 tg mouse suggest that A β may affect cholinergic neuronal transport processes without impacting upon cortical or subcortical cholinergic neuronal viability.

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Figure 1.

A-E, Rostral to caudal diagrams showing transverse sections delimitating the level and borders (dotted-lines) outlining the regions of the motor, cingulate and sensory cortices as well as the striatum and nucleus basalis processed for stereology in APP^{swe}/PS1 Δ E9 tg and ntg mice. A, amygdala complex; B, nucleus basalis; BST, bed nucleus of the stria terminalis; Cg, cingulate cortex; DG, dentate gyrus; Ect, ectorhinal cortex; Ent, entorhinal cortex; fi, fimbria; G, geniculate nucleus; GP, globus pallidus; HB, horizontal limb of the diagonal band of Broca; HC, hippocampus; I, insular cortex; ic, internal capsule; IP, interpeduncular nucleus; LH, lateral hypothalamic area; lo, lateral olfactory tract; LV, lateral ventricle; M, motor cortex; ml, medial lemniscus; opt, optic tract; St, striatum; Pir, piriform cortex; R, red nucleus; RS, retrosplenial cortex; S, sensory cortex; SC, superior colliculus; SI/B, substantia innominata/nucleus basalis; sm, stria medullaris thalami; SN, substantia nigra; Th, thalamus.

Figure 2.

Fluorescence images showing the age-related A β deposition in the cortex and hippocampus in an APPswe/PS1 Δ E9 tg mouse. **A-D**, coronal sections showing a few A β -ir plaques in the motor (**A**), cingulate (**A**) and parietal (**B**) cortices of a 3-month-old APPswe/PS1 Δ E9 tg mouse (white arrowheads), whereas at 10 and 16 months of age extensive A β -ir deposition is seen in these areas (**B,C,E,F**) including in the hippocampal formation (**E,F**) in tg mice. Note the intense accumulation of A β -ir plaques in the dentate gyrus (**E,F**; white arrows). Au, Auditory cortex; Cg, cingulate cortex; DG, dentate gyrus; M, motor cortex; PP, posterior parietal association area; St, striatum; Vi, visual cortex. mos, months. Scale bars: **A,D**=50 μ m; **B,C,E,F**=200 μ m.

Figure 3.

Bright-field photos showing examples of swollen ChAT-ir and AChE (**B,H**) positive dystrophic neurites forming rosette (**A,C,E,F,K**) and grape (**E**; arrow) -like patterns, other dystrophic morphologies (**D,G,I,J,L,K**) in the cerebral cortex, hippocampus and striatum of 3-16-month-old double mutant mice. Note that the voluptuousness appearance and number of ChAT-ir dystrophic neurites increase with age (**A,C,F,G**), as well as the alterations in non-dystrophic ChAT-ir fiber trajectories in close proximities to dystrophic clusters (**A,D,I**; arrowheads) probably due to the presence of A β -plaque deposition. Scale bars=20 μ m.

Figure 4.

Photographs of coronal sections of the cingulate (A-D), motor (E-H) and sensory (visual (I, J) and parietal (K, L) cortices showing the healthy morphology of ChAT-ir interneurons and the disruption of the ChAT-ir fiber network in 3-16-month-old APPswe/PS1ΔE9 tg and ntg mice. Note the presence of few ChAT-ir dystrophic neurite-clusters in a 3-month-old tg mouse (B,F,J), whereas many more are seen at 16 months of age (D,H,L), as well as the dramatic disturbance in cholinergic fiber trajectory (D, L) and decrease in ChAT-ir fiber density in these cortices in a 16 months of age tg mouse compared to ntg mouse (C,K,D,L). H, Section of the motor cortex of a 16-month-old APPswe/PS1ΔE9 tg mouse showing Aβ-ir plaques (red) and ChAT-ir fibers and neuron (blue). Note that cholinergic neuronal morphology (white arrowhead) appears intact despite close proximity to Aβ-ir plaques. M-P, Coronal sections illustrating the decrease in the ChAT-ir fibers in the hippocampus and the presence the dystrophic neurites (arrowheads) in a 16-month-old tg mouse. N, P, Insets showing detail of the ChAT-ir innervation in the dentate gyrus from M and O, respectively. Q, Coronal section of the hippocampus of a 16-months-old tg mouse showing numerous Aβ-ir plaques (red) mainly in the dentate gyrus and/or associated swollen ChAT-ir dystrophic neurites (blue; arrowheads) and ChAT-ir fiber distribution (blue) compared to a ntg mouse (M). No ChAT-ir dystrophic neurites or decrease in cholinergic fiber density was observed in the cortex and hippocampus of ntg mice at any age (A,C,E,G,I,K,M). ntg, non-transgenic mice; tg, APPswe/PS1ΔE9 transgenic mice; mos, months. Scale bars: A-D, F-L, N,P=20μm; E,M,O,Q=50μm.

Figure 5.

Photographs showing lamina disturbances of cholinergic fiber innervation of the somatosensory cortex in 3- and 16-month-old tg mice (**B,D**) compared to ntg mice (**A,C**). There was no age-related alteration in the laminar distribution of cholinergic fibers in the somatosensory cortex between young and aged ntg mice. By contrast, there was an extensive disruption of the cholinergic fiber network and the presence of dystrophic neurites (arrowheads) in the somatosensory cortex between young and aged mutant mice. Cortical layers are indicated by the roman numerals I-VI; ntg, non-transgenic mice; tg, APP^{swe}/PS1 Δ E9 transgenic mice. Scale bar=150 μ m.

Figure 6.

Photographs of the striatum and nucleus basalis showing the normal morphology of the ChAT-ir neurons and ChAT-ir dystrophic neurites in 3-, 6- and 16-month-old APP^{swe}/PS1 Δ E9 tg and ntg mice. **A-F**, Coronal sections of the striatum illustrating the presence of ChAT-ir dystrophic neurites at 6- and 16-month-old tg mice (arrowheads) compared with their absence in age-matched ntg mice (**A**, **D**). **C,F**, Insets showing detail of ChAT-ir dystrophic neurites from **B** and **E**, respectively. **G-I**, Coronal sections of the nucleus basalis showing the absence of dystrophic neurites in a 3-month-old double mutant mouse (**H**) as well as in 3- and 16-month-old ntg mice (**G,I**). **J**, Coronal section of the nucleus basalis showing a few A β -ir plaques (red; arrows) surrounded by ChAT-ir dystrophic neurites (dark blue) in a 16-month-old double mutant mice. **K**, Inset illustrating detail of swollen ChAT-ir dystrophic neurites (dark blue) in the vicinities of A β -ir plaques (red) in the nucleus basalis of a 16-month-old double tg mouse. ntg, non-transgenic mice; tg, APP^{swe}/PS1 Δ E9 transgenic mice; mos, months. Scale bars: **A,B,D,E,G-J**=50 μ m; **C,E,K**=20 μ m.

Figure 7.

A. Histogram showing significant differences in the mean values of ChAT-ir neuronal number (asterisk) in the motor cortex of 3- to 16-month-old tg and age-matched ntg littermate mice (Mann-Whitney rank-sum test; $p=0.033$). **B.** Histogram showing the mean area of ChAT-ir neurons within the nucleus basalis of 12- to 16-month-old tg and ntg mice. Mann-Whitney rank-sum test revealed significant increase (asterisk) in the mean area of cholinergic neurons in the nucleus basalis of the oldest APPswe/PS1 Δ E9 tg compared to ntg mice ($p=0.029$). ntg, non-transgenic mice; tg, APPswe/PS1 Δ E9 transgenic mice; error bars=standard deviation of the mean; mos, months.

Figure 8.

Histograms illustrating the mean values of the ChAT enzyme activity ($\mu\text{mol/hr/g}$ protein) in the cerebral cortex, hippocampus and striatum of 2- to 6-month-old and 10- to 16-month-old tg mice and ntg. **A, B**, Statistical analysis revealed a significant decrease (asterisks) in cortical and hippocampal ChAT enzyme activity between 2-6- and 10-16-month-old APPswe/PS1 ΔE9 tg mice (Mann-Whitney rank-sum test; cerebral cortex $p=0.048$, hippocampus $p=0.035$). Statistical evaluation also revealed significant differences (diamonds) in cortical ChAT enzyme activity at 10-16 months between APPswe/PS1 ΔE9 tg and ntg mice, but not in the hippocampus ($p=0.018$). **C**, No significant differences in ChAT enzyme activity was detected in the striatum of the examined groups (Mann-Whitney rank-sum test; $p<0.05$). ntg,

non-transgenic mice; tg, APP^{swe}/PS1 Δ E9 transgenic mice; error bars=standard deviation of the mean; mos, months.

Table 1

Morphological measurements in the cortex, striatum and nucleus basalis of APPswe/PS1ΔE9 tg and ntg mice

Number of ChAT-ir neurons	Cingulate Cortex	Motor Cortex	Sensory Cortex	Striatum	Nucleus basalis
ntg 3-6 mos	1188.53 ±319.233* n=4	7111.6 ±976.934 n=6	14143.824 ±4002.317 n=7	11341.189 ±4061.925 n=7	6632.297 ±1105.372 n=6
ntg 10-16 mos	1643.2 ±151.225 n=3	6669.681 ±453.385 n=7	13675.644 ±4318.949 n=8	11430.594 ±2931.402 n=8	4649.027 ±1404.253 n=6
tg 3-6 mos	1618.54 ±425.624 n=4	5913.215 ±1314.729 n=8	14699.1 ±4583.494 n=9	14496.199 ±4227.56 n=9	6449.839 ±1972.913 n=8
tg 10-16 mos	1647.85 ±531.763 n=6	5595.065 ±1530.432 n=8	12604.269 ±3466.744 n=8	11652.364 ±3731.705 n=9	5138.078 ±918.175 n=5
ntg (3-16 mos) n=13 tg (3-16 mos) n=16		6873.644 ±1227.358 5754.14 ±1388.044			
OD of ChAT-ir neurons					
ntg 3-6 mos	43.915 ±10.155 n=6	40.647 ±13.06 n=7	38.873 ±11.821 n=7	84.016 ±19.227 n=7	91.726 ±15.253 n=7
ntg 10-16 mos	43.426 ±17.118 n=5	48.002 ±20.837 n=7	49.255 ±18.793 n=8	85.531 ±10.364 n=8	102.111 ±10.738 n=8
tg 3-6 mos	52.24 ±13.855 n=6	43.801 ±17.593 n=8	45.141 ±19.834 n=8	87.112 ±17.742 n=9	101.695 ±19.965 n=9
tg 10-16 mos	58.329 ±15.615 n=7	56.255 ±18.574 n=8	54.762 ±18.359 n=7	91.774 ±18.74 n=9	108.14 ±18.801 n=8
Area of ChAT-ir neurons (μm²)					
ntg 3-6 mos	72.24 ±17.934 n=6	70.388 ±18.691 n=7	65.446 ±12.919 n=7	216.258 ±40.026 n=7	256.739 ±38.411 n=7
ntg 10-16 mos	63.058 ±7.323 n=5	61.649 ±7.497 n=7	63.137 ±8.782 n=8	193.445 ±10.802 n=8	238.384 ±14.986 n=8
tg 3-6 mos	71.722 ±15.707 n=7	71.919 ±18.682 n=8	64.864 ±15.834 n=9	219.413 ±48.371 n=9	273.189 ±47.002 n=9
tg 10-16 mos	67.492 ±9.244 n=8	67.235 ±8.681 n=9	63.633 ±8.11 n=8	190.854 ±12.16 n=9	251.7 ±22.907 n=8
ntg 12-16 mos (n=4) tg 12-16 mos (n=4)					250.885 ±6.122 269.59 ±3.283

* mean±standart deviation of the mean

ntg, non-transgenic littermate mouse; tg, APPswe/PS1ΔE9 transgenic mouse; mos, months

Table 2
ChAT enzyme activity in APPswe/PS1ΔE9 tg and ntg mice

Groups	Cerebral Cortex	Hippocampus	Striatum
ntg 2-6 mos	34.907 ±7.147* n=7	35.783 ±7.906 n=9	121.885 ±22.725 n=6
ntg 10-16 mos	45.102 ±8.228 n=5	45.015 ±11.432 n=6	129.198 ±29.741 n=6
tg 2-6 mos	40.658 ±5.804 n=5	41.903 ±9.007 n=7	127.566 ±38.766 n=7
tg 10-16 mos	34.109 ±3.492 n=7	31.893 ±7.736 n=6	138.166 ±12.668 n=5

* mean (μmol/hr/g protein) ±standart deviation of the mean

ntg, non-transgenic littermate mouse; tg, APPswe/PS1ΔE9 transgenic mouse; mos, months