SELECTIVE CHOLINERGIC DENERVATION, INDEPENDENT FROM OXIDATIVE STRESS, IN A MOUSE MODEL OF ALZHEIMER’S DISEASE

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Abstract—Alzheimer’s disease (AD) is characterized by increases in amyloid-β (Aβ) peptides, neurofibrillary tangles, oxidative stress and cholinergic deficits. However, the selectivity of these deficits and their relation with the Aβ pathology or oxidative stress remain unclear. We therefore investigated amyloidosis-related changes in acetylcholine (ACh) and serotonin (5-HT) innervations of hippocampus and parietal cortex by quantitative choline acetyltransferase (ChAT) and 5-HT immunocytochemistry, in 6, 12/14 and 18 month-old transgenic mice carrying familial AD-linked mutations (hAPPsw,Ind). Further, using manganese superoxide dismutase (MnSOD) and nitrotyrosine immunoreactivity as markers, we evaluated the relationship between oxidative stress and the ACh deficit in 18 month-old mice. Thioflavin-positive Aβ plaques were seen in both regions at all ages; they were more numerous in hippocampus and increased in number ( > 15-fold) and size as a function of age. A majority of plaques exhibited or were surrounded by increased MnSOD immunoreactivity, and dystrophic ACh or 5-HT axons were seen in their immediate vicinity. Counts of immunoreactive axon varicosities revealed significant decreases in ACh innervation, with a sparing of the 5-HT, even in aged mice. First apparent in hippocampus, the loss of ACh terminals was in the order of 20% at 12/14 months, and not significantly greater (28%) at 18 months. In parietal cortex, the ACh denervation was significant at 18 months only, averaging 24% across the different layers. Despite increased perivascular MnSOD immunoreactivity, there was no evidence of dystrophic ACh varicosities or their accentuated loss in the perivascular area. Moreover, there was virtually no sign of tyrosine nitration in ChAT nerve terminals or neuronal cell bodies. These data suggest that aggregated Aβ exerts an early, non-selective and focal neurotoxic effect on both ACh and 5-HT axons, but that a selective, plaque- and oxidative stress-independent diffuse cholinotoxicity, most likely caused by soluble Aβ assemblies, is responsible for the hippocampal and cortical ACh denervation. © 2005 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: amyloid precursor protein (APP), acetylcholine, inflammation, amyloid, manganese SOD, neurodegeneration.

Increased brain levels of amyloid-β (Aβ) peptides and their deposition in neuritic plaques and cerebral blood vessels (cerebral amyloid angiopathy, CAA), neurofibrillary tangles, oxidative stress and neuronal deficits are prominent neuropathological features of Alzheimer’s disease (AD; Kalaria, 1997; Auld et al., 2002; Hardy and Selkoe, 2002; Huang et al., 2004). It is not yet clear, however, whether the extensive loss of neurons and pre-synaptic terminals observed in AD is one of the primary features of this disease or a consequence of the Aβ pathology. The acetylcholine (ACh) neurons in basal forebrain, which provide major inputs to the hippocampus and neocortex, are among the most precociously and severely affected in AD (Davies and Maloney, 1976; Whitehouse et al., 1982; Tong and Hamel, 1999; Beach et al., 2000; Mufson et al., 2002), and the resulting ACh deficits in these regions have been correlated with memory and cognitive impairments (Bierer et al., 1995; Shinotoh et al., 2000; Auld et al., 2002). There are indications that the serotonin (5-hydroxytryptamine, 5-HT) system, which is implicated in various aspects of cognition (Steckler and Sahgal, 1995; Buhot et al., 2000), could also be affected at relatively early stages in AD (Porter et al., 2000). At later stages, several neurotransmitter systems appear to be disturbed (Chan-Palay et al., 1986; Mufson et al., 1993). Interestingly, recent evidence suggests that the CAA may contribute more than Aβ plaques to cognitive loss (Pfeifer et al., 2002), and oxidative stress has been identified as an important accompanying factor in the Aβ pathology (Pappolla et al., 1998; Smith et al., 1998).

Most identified familial forms of AD (FAD) are caused by dominant mutations in genes encoding the amyloid precursor protein (APP) or the presenelin 1 or 2 (PS1 or PS2), all of which resulting in increased production of APP-derived Aβ peptides (Hardy, 1997; Selkoe, 1997; Price and Sisodia, 1998). In transgenic mouse models of AD that overexpress mutated forms of the human APP (hAPP), neuritic plaques, reactive gliosis, loss of synaptophysin-immunoreactive pre-synaptic terminals, and alterations in the innervation of various CNS regions have been reported (Games et al., 1995; Hsiao et al., 1996; Sturchler-Pierrat et al., 1997; Hsia et al., 2003-65220S$30.00 © 2005 IBRO. Published by Elsevier Ltd. All rights reserved. doi:10.1016/j.neuroscience.2004.11.047
1999; Mucke et al., 2000; Lewis et al., 2001). Some mice also exhibit synaptic transmission deficits and major behavioral/cognitive impairments (Hsiao et al., 1996; Chapman et al., 1999; Hsia et al., 1999; Larson et al., 1999; Chen et al., 2000; Palop et al., 2003). Cholinergic alterations of varying severity and magnitude have been documented in hAPP transgenic mice depending on age, mutations (hAPPΔ PS mutations) and brain areas (cortex, hippocampus) investigated (Wong et al., 1999; Bronfman et al., 2000; Apelt et al., 2002; Boncristiano et al., 2002; Buttini et al., 2002; German et al., 2003; Klingner et al., 2003). However, the selectivity of these changes, as well as their regional progression and relation with the amyloidosis or oxidative stress, remains unclear.

In the present study, we investigated the selectivity and severity of an eventual loss of ACh and 5-HT innervation in the hippocampus and parietal cortex of hAPPSw,Ind transgenic mice at different ages, its temporal relationship with the Aβ pathology, and its association with oxidative stress. Quantitative immunocytochemical and cytochemical data were obtained on the density of choline acetyltransferase (ChAT) and 5-HT-immunostained innervations and number of neuritic plaques in the hippocampus and parietal cortex of wild type and transgenic mice aged 6, 12/14 and 18 months. Further, to gain insights into the pathological mechanisms leading to neuronal degeneration, we achieved simultaneous visualization of ChAT or 5-HT terminals and Aβ plaques, or the oxidative stress markers manganese superoxide dismutase (MnSOD) and nitrotyrosine which are both upregulated in AD brains (Furuta et al., 1995; Pappolla et al., 1992; Smith et al., 1997). Some of these results have been reported in abstract form (Descarries et al., 2002; Aznavour et al., 2003).

**EXPERIMENTAL PROCEDURES**

**Animals**

Experiments were approved by the Animal Ethics Committee of the respective institutions, and abided by the guidelines of the Canadian or American Council for Animal Care aimed at minimizing the number of animals used and their suffering. We used hAPP transgenic mice overexpressing the FAD-linked mutations hAPPSw,Ind carrying the 670/671KM–NL (Sw) double mutation and the 717VΔ F mutation (Ind; line J20; Mucke et al., 2000). Mice were screened for transgene expression by Touchdown PCR with specific sense (5'-GGTGAGTTGTAAGTGCACC) and antisense (5’-TCTTTCTTCCACCTCCAGC) hAPP primers, using tail extracted DNA (Games et al., 1995). Heterozygous transgenic founders were backcrossed with nontransgenic C57BL/6 mice. The study was performed on transgenic mice and their littermate controls (body weight: 40 ± 10 g) aged 6, 12/14 and 18 months.

**Tissues and cyto/immunocytochemical labelings**

Mice (16 wild-type and 16 transgenic) were anesthetized with sodium pentobarbital (65–80 mg/kg; i.p.) and perfused through the heart with 25 ml of ice-cold saline, followed by 250–300 ml of 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (50 mM, pH 7.4). Brains were rapidly removed, post-fixed overnight in PFA (4 °C) and either immediately cut on a vibratome (Leica, Montréal, QC, Canada) and sections stored at −20 °C in cryoprotectant solution (Vaucher and Hamel, 1995) until immunostaining, or cryoprotected (30% sucrose), frozen and stored at −80 °C until freezing microtome sectioning. The dorsal hippocampus and primary somatosensory (Par1) cortex were sampled in 20 μm-thick transverse sections between stereotaxic planes interaural A 1.5 and 1.98 mm (Franklin and Paxinos, 1997; Fig. 1). To confirm the specificity of primary antibodies not previously used by us (MnSOD, Aβ42 and nitrotyrosine), some sections were processed without these before incubation with secondary antibodies.

ChAT, 5-HT, MnSOD and/or vascular (PECAM-1) immunostainings. In initial experiments, free-floating frozen sections from 12/14 month-old wild-type and hAPPSw,Ind mice (n=6 per group) were processed for ChAT or 5-HT immunocytochemistry, as previously described (Tong and Hamel, 1999). Sections were incubated (24 h, room temperature) either with goat anti-ChAT (1:500; Chemicon, Temecula, CA, USA) or rabbit anti-5-HT antibody (1:10,000; ImmunoStar, Hudson, WI, USA), then with biotinylated rabbit anti-goat or goat anti-rabbit secondary antibody (1.5 h; 1:200; Vector, Burlingame, CA, USA), and the reaction revealed with the ABC method (ABC Kit; Vectastain Elite; Vector; 1.5 h and detected (1.5 min) with a 0.05% solution of 3,3’-diaminobenzidine (DAB; Sigma) activated with 0.005% H2O2. One of triplicate sections was mounted on slides, while the CA3 region of hippocampus and parietal cortex of the other two sections were resin-embedded and cut on an ultramicrotome in 2 μm-thick semithin sections (Tong and Hamel, 2000) for quantitative analysis of axon varicosities (see below).

To further assess the onset, evolution and selectivity of the ACh denervation, a method allowing for quantification of immunostained axon length and varicosity number (Mechawar et al., 2000; Aznavour et al., 2002) was used. Vibratome-cut sections from 6 month-old and 18 month-old wild-type mice and an equivalent number of hAPPSw,Ind mice (n=4) were processed for ChAT immunocytochemistry (Aznavour et al., 2002), or for 5-HT immunostaining after antigen retrieval (Shi et al., 1991). Sections were incubated overnight with 2 μg/ml of monoclonal anti-ChAT antibody (B. K. Hartman, University of Minneapolis, USA) or rabbit 5-HT antibody (as above). Immunostaining was detected with the ABC method by incubation (2 h) with biotinylated horse antimouse or goat anti-rabbit secondary antibody (Vector; 1:200), the ABC kit (1:2 h), and detection (1:5 min) with H2O2-activated DAB (5-HT) or H2O2-activated DAB supplemented with 0.01% CoCl2, 0.01% NISO4, 0.01% (NH4)2SO4 (ChAT).

Other vibratome-cut sections from 18 month-old wild type and hAPPSw,Ind mice (n=3–6 per group) were immunostained for MnSOD (a marker of increased production of the free radical superoxide; Pappolla et al., 1992), or double-immunolabeled for ChAT and MnSOD, ChAT and nitrotyrosine (a marker of peroxynitrite-mediated oxidative stress; Crow and Ischiropoulos, 1996; Smith et al., 1997), or for ChAT and the endothelial cell marker PECAM-1. For MnSOD, following overnight incubation with a rabbit anti-MnSOD antibody (1:400; Stressgen, BC, Canada), sections were incubated (1.5 h) with a goat anti-rabbit IgG (Vector; the ABComplex (1:25 h), and the reaction visualized with the slate gray reagent (gray precipitate, SG kit; Vector). For ChAT and MnSOD, ChAT was detected in first position with goat anti-ChAT (1:500; Chemicon) and DAB (brown precipitate), prior to MnSOD immunodetection with the SG reagent. For ChAT and PECAM-1 double-immunostaining, ChAT was detected in the first position prior to overnight incubation with a rat anti-PECAM-1 antibody (CD31, 1:200; BD Biosciences, San Diego, CA, USA) followed (2 h) by anti-rat IgG (1:200; Vector). Both reactions were detected with DAB as there is no overlap between neuronal ChAT and vascular CD-31 immunoreactivities, the latter being used to highlight blood vessel contours. Sections were then resin-embedded for semithin sectioning. For ChAT and nitrotyrosine double-immunofluorescence, sections were incubated overnight with goat anti-ChAT (1:500; Chemicon) and mouse anti-nitrotyrosine (1:1000; Upstate, Lake Placid, NY, USA) primary antibodies, respectively detected with Cy3- and Cy2-conjugated affiniPure species-specific secondary antibodies (1:200; Jackson ImmunoResearch, Montréal, QC, Canada).
West Grove, PA, USA). Sections were observed under confocal microscopy (Zeiss LSM 510; Zeiss, Jena, Germany) using simultaneous double channel visualization with emission intensities of 488 nm (Cy2) and 543 nm (Cy3).

β plaques staining combined or not with ChAT or 5-HT immunofluorescence. Sections from all hAPPsw,Ind and wildtype mice were stained (8 min) for amyloid with Thioflavin-S (Sigma; Wyss-Coray et al., 1997). The number of β plaques in dorsal hippocampus and parietal cortex (up to the rhinal sulcus) was counted on a Leitz Aristoplan microscope equipped for epifluorescence with an FITC filter. Counts (two to three sections/mouse) were obtained from both regions in both hemispheres, averaged and expressed as number of plaques per region. Additionally, sections from two to three transgenic mice in each age group were used to examine the relationships between neuritic plaques and MnSOD, ChAT or 5-HT immunoreactivities by fluorescence immunocytochemistry (as above, but detected with Cy3-labeled secondary antibodies) followed by Thioflavin-S staining of β plaques on immunostained sections already mounted on slides. In some 18 month-old hAPPsw,Ind mice (n=3), β plaques were simultaneously visualized with a specific antibody against Aβ1–42 (rabbit anti-Aβ42, 1:200; Biosource, Camarillo, CA, USA) detected with a Cy3-labeled secondary antibody, followed by co-staining for Thioflavin-S. Other sections were immunostained for ChAT and Aβ42, by detecting ChAT (Chemicon) in first position with Cy3-labeled secondary antibody, followed by Aβ42 immunodetection with Cy2-labeled secondary antibody. All immunofluorescence-labeled sections were examined under confocal microscopy as described above.

Quantification of ACh and 5-HT innervations. The number of ChAT or 5-HT-immunostained varicosities were counted from three or more semithin sections per region in each 12/14 month-old wild-type or hAPPsw,Ind mouse. Immunostained varicosities, defined as small punctate dilations 0.5 μm or more in transverse diameter (Umbriaco et al., 1994), were counted by two independent observers directly on photomicrographs from the CA3 region of dorsal hippocampus or the parietal cortex (layers IV and V), printed at a final magnification of 410x. In view of their respective distribution, hippocampal ChAT terminals were counted in the stratum pyramidale and 5-HT terminals in the stratum radiatum, using a transparent grid overlay (Geula and Mesulam, 1989; Tong and Hamel, 2000). Counts of varicosities (obtained from areas of approximately 60,000 μm² in hippocampus and 125,000 μm² in the cortex) from all sections in each region, and from each observer, were averaged and expressed as percent change from control mice. The size of ChAT varicosities in hippocampus was also measured on these photomicrographs, using a video camera, a frame grabber and the appropriate software (MCID/M4-Image Analysis System; Imaging Research, ON, Canada).

In 6 and 18 month-old mice, digitized images of ChAT- and/or 5-HT-immunostained axons were obtained at the selected levels (Fig. 1) with a RT Color camera (Diagnostic Instruments, Inc., Sterling Heights, MI, USA) mounted on a Leitz Diaplan microscope equipped with SPOT RT Software (v3.1; Diagnostic Instruments installed on a PC computer). Length measurements of these respective immunostained axon networks were determined in both regions, using a semi-computerized method as previously described (Mechawar et al., 2000; Aznavour et al., 2002). A total hippocampal area of 90,000 μm² and cortical area of 37,500 μm² was sampled in each mouse. Using a stereological formula (Soghomonian et al., 1987), the data were expressed as average density of axons (m/mm³) for each layer and region. For ACh innervation, a further step was carried out to express its density in number of ChAT-immunostained axon varicosities per mm³ of tissue. The average number of varicosities (defined as above) per unit length of immunostained axon was counted directly at the light microscope (×1000), on 50 axon segments from the different layers of the three hippocampal sectors and parietal cortex of wild-type and hAPPsw,Ind mice (total of 800 counts). As the aver-
age number of varicosities per unit length of axon was fixed (four per 10 μm of axon), the density of axons could be arithmetically converted to density of varicosities.

To ensure that tissue volume changes would not bias the density measurements, the surface area of dorsal hippocampus and thickness of parietal cortex were determined by means of the same image analysis system in ChAT-immunostained sections from six wild-type and six transgenic 18-month-old mice.

Cortical perivascular ChAT varicosities (touching blood vessel walls in layers II–IV immunodetected with CD31 antibody; Tong and Hamel, 2000) were counted in double-immunostained semi-thin sections (two to three sections/mouse) from 18 month-old mice (n=6 wild-type and n=6 transgenic mice). Digitalized color pictures (Nikon Coolpix 4500 camera mounted on the Leitz Aristoplan microscope) covering a tissue area of approximately 3000 μm² were used for the analysis (Metamorph software, version 6.13; Universal Imaging Corp., Downingtown, PA, USA). Perivascular terminals were expressed per μm of vessel perimeter in the corresponding tissue sections.

**ChAT enzyme activity in the medial septum**

ChAT activity was measured in the medial septum of 12/14 month-old hAPPsw,Ind (n=15) and wildtype (n=12) mice as described (Buttini et al., 2002). Tissues extracts were obtained by sonication (diluted 1:20 wet/wet in 50 mM Tris/0.2% Triton buffer), followed by centrifugation. Supernatants were collected, protein levels determined (bicinchoninic acid assay; Pierce, Rockford, IL, USA), and diluted to 50 μl (50 mM Tris/0.5 M sodium-phosphate buffer, pH 7.0) before incubation (37 °C, 30 min) with 50 μl reaction buffer (0.4 M NaCl, 80 μg/ml eserine, 12 mM choline chloride, 10 μg/ml albumin, and 0.05 μCi 14C-labeled acetyl-CoA in Na-phosphate buffer). Re-actions were stopped with ice-cold H2O (500 μl), samples loaded onto columns of Dowex 1X-8 resin (Bio-Rad, Hercules, CA, USA), washed (2× with 600 μlH2O), and effluents collected for counting on a Beckman scintillation counter. ChAT activity over that determined in boiled (5 min) tissue extracts was calculated and expressed as nanomoles/mg protein/h.

**Statistical analysis**

Statistical comparisons of the laminal or regional density of ACh and 5-HT innervations in the cortex and hippocampus were performed by one-way ANOVA followed by two-tailed Student’s t-test. Changes in innervation densities (ACh and 5-HT) or ChAT activity in 12/14 month-old mice, and perivascular ChAT terminals in 18 month-old mice were assessed by Student’s t-test. A P<0.05 was considered significant.

**RESULTS**

**ACh and 5-HT innervations in wild-type mouse hippocampus and parietal cortex**

In keeping with earlier reports in mouse (Wong et al., 1999; Aznavour et al., 2002), fine varicose ChAT-immunostained fibers pervaded all layers of the dorsal hippocampus and parietal cortex, as illustrated here in an 18 month-old wild-type mouse (Fig. 2A, C). ChAT-positive cell bodies were rare in hippocampus (not shown), and the overall regional density of the ACh innervation was comparable (15–20 m of axons per mm³ of tissue) between CA1, CA3 and DG (Fig. 2A; see Figs. 4–6). In parietal cortex, ChAT-positive neurons were present (Fig. 2C), and the mean density of innervation was comparable across all layers and similar to that in the most densely innervated hippocampal layers (Fig. 2B, Figs. 4–6). The density of ACh innervation did not decrease in hippocampus or parietal cortex between 6 and 18 months of age (quantified in Figs. 4 and 6).

5-HT-immunoreactive axons were also distributed as previously reported, in both hippocampus and parietal cortex (Fujimiya et al., 1986; Donovan et al., 2002; data not shown). In hippocampus, the highest laminar densities of 5-HT innervation were in the stratum lacunosum moleculare of CA1 and CA3, and the lowest (8–9 vs approximately 4 m of axons per mm³ of tissue, respectively) in the strata pyramidalae and the stratum granulare of DG. In parietal cortex, the density of 5-HT innervation was highest
in layer I, at almost 10 m of axons per mm³ of tissue. In both regions, the density of 5-HT axons and varicosities was clearly less than that of the ACh innervation (see Figs. 4 and 6).

**Aβ plaques, oxidative stress, and ChAT or 5-HT innervation in hAPP<sub>Sw,Ind</sub> mouse**

All transgenic mice showed Thioflavin-S positive Aβ plaques in hippocampus, corpus callosum and cerebral cortex (Fig. 3, top panels), as well as in several pial and intracortical blood vessels (not shown). The plaques varied in number and size depending on mouse and age, being small and sparse in 6 month-old mice, and increasing in size, but mainly in number, with age. They were consistently more numerous in hippocampus than parietal cortex (approximately 50% at 6 and 12/14 months and approximately 20% at 18 months). They were also abundant in posterior cingulate and frontoparietal areas (Fig. 3, top panels). Compared with 6 months, their number increased almost seven-fold in both hippocampus and parietal cortex at 12/14 months, and more than 20-fold at 18 months (histogram in Fig. 3). There were no Thioflavin-S positive amyloid deposits in the brain or cerebrovascular tissue of wild-type littermate control mice at any age.

In hAPP<sub>Sw,Ind</sub> mice of all age groups, dystrophic, swollen and distorted ChAT- and 5-HT-immunofluorescent neurites were present in the immediate vicinity or within the core of Thioflavin-S stained Aβ plaques (Fig. 3, middle panels). Strikingly, even in the younger mice when rare and mainly small punctuate plaques could be detected, a proportion of these were surrounded or penetrated by abnormal ChAT- and 5-HT-immunoreactive varicose fibers (Fig. 3, 6 month panels). With increasing age, the increased number and size of plaques translated into a more frequent appearance of associated dystrophic terminals. Except for a minority of plaques, Thioflavin-S and Aβ42-positive Aβ plaques were equivalent in location (>90%), but could vary in size and extent depending on marker; Thioflavin-S positive plaques were easier to locate and exhibited a larger surface area (Fig. 3, bottom panel). A vast majority of Aβ42-immunostained plaques were invaded by dystrophic terminals as shown here for ChAT (Fig. 3, bottom panel). Thioflavin-S positive Aβ plaques either exhibited MnSOD immunostaining or were unstained in their core but surrounded by MnSOD immunoreactive material (Fig. 3; see also Fig. 7A, B insets). MnSOD-positive plaques were similarly invaded or surrounded by dystrophic ChAT varicosities (Fig. 3). Normal looking nerve terminals coursing through the tissue could be seen to become abnormally large and dysmorphic when approaching or penetrating Thioflavin-S, Aβ42- or MnSOD-stained Aβ plaques (Fig. 3).

**Selective, age-dependent loss of ChAT innervation in hAPP<sub>Sw,Ind</sub> mice**

The quantitative analysis of ChAT- and 5-HT-immunostained axon density in hAPP<sub>Sw,Ind</sub> mice of the three age groups revealed a loss of ACh innervation that progressed regionally with age. In both hippocampus and parietal cortex, the density of ChAT-immunostained axon varicosities was unaltered at 6 months (Fig. 4). At 12/14 months, there was a significant decrease in the relative number of ChAT varicosities in the stratum pyramidale of CA3 (19.5±5.6%, P<0.05), but not in the parietal cortex (Fig. 5A, B and 5E, F, and left bottom panels). As also shown in Fig. 5 (right bottom graphs), the average size of hippocampal ChAT varicosities remained unchanged, but ChAT activity in the medial septum was significantly decreased by comparison to wild-type littermate controls (23.4±3.6%, P<0.01).

At 18 months, comparable decreases in ACh innervation density (24–26%, P<0.05–0.001) were measured in both hippocampus and parietal cortex (compare Fig. 2B, D with 2A, C, and see Fig. 6). More specifically, the mean number of ChAT varicosities was reduced to 5.8 million/mm³ in hippocampus and 6.3 million/mm³ in parietal cortex, compared with corresponding values of 7.8 and 8.3 million/mm³ in littermate controls (Fig. 6). In both regions, all layers appeared to be similarly affected (Fig. 6). This decrease in ChAT axons and varicosities occurred in the absence of any significant change in either the overall surface area of hippocampus (2.09±0.13 vs 2.01±0.22 mm² in wild-type and transgenic, respectively) or thickness of parietal cortex (0.91±0.06 vs 9.02±0.05 mm in wild-type and transgenic, respectively). Also, once established, there was no apparent progression in the ACh deficit, as illustrated by the comparable severity of denervation at 12/14 and 18 months in the CA3 region (stratum radiatum) of hippocampus (19.5±5.6% vs 28±5.9%, P=0.34).

In contrast to this progressive ACh denervation in hippocampus and then parietal cortex, there were no significant differences in density of 5-HT-immunostained varicose fibers between wild-type and transgenic mice in either the stratum radiatum of CA3 and layers IV–V of the parietal cortex at 12/14 months (Fig. 5), or across the different layers of the three hippocampal sectors and of parietal cortex at 18 months (Fig. 6).

**Oxidative stress and loss of ChAT-immunoreactive varicose fibers**

Another striking finding of the present study was an upregulation of MnSOD immunoreactivity in both hippocampus and parietal cortex of 18 month-old hAPP<sub>Sw,Ind</sub> mice (Fig. 7). In addition to the granular neuronal labeling seen in wild-type littermates, consistent with mitochondrial localization of this antioxidant enzyme (Lindenau et al., 2000), 18 month-old hAPP<sub>Sw,Ind</sub> mice displayed plaque-associated MnSOD immunoreactivity (Fig. 7A, inset; see also Fig. 3) and, more notably, increased perivascular MnSOD-immunostained small punctate structures around some penetrating, intracortical and intrahippocampal blood vessels. In some mice, perivascular rims and cuffs of MnSOD immunoreactivity were salient (Fig. 7A). ChAT-immunostained varicosities within these perivascular areas were intermingled with MnSOD positive elements, but were not dystrophic or abnormal in appearance (Fig. 7B–D) except when located in the immediate vicinity of MnSOD immunoreactive plaques (Fig. 7B, inset).
Despite such apparent increase in perivascular oxidative stress, quantitative analysis on semithin sections (Fig. 7E, F) showed that the loss of perivascular ChAT varicosities (3.22 ± 0.46 vs 2.53 ± 0.28 terminals/μm of vascular perimeter, ↓ 21%, n.s.) was comparable to that in the corresponding parenchyma (477.3 ± 21.7 vs 409.3 ± 12.0 terminals/3000 μm², ↓ 14%, P<0.05). Additionally, peroxynitrite-mediated protein nitration was evidenced in hAPPsw,Ind mice by increased intensity in nitrotyrosine immunostaining as compared with wild-type controls (not shown). Nitrotyrosine immunoreactivity could be seen in some neurons, but was prominent in glial cells distributed in plaque-like structures often associated with dystrophic ChAT-positive varicosities (Fig. 7G) or diffusely throughout the neuropil (Fig. 7H). As such, the distribution pattern of nitrotyrosine immunoreactivity was totally distinct from that of ChAT-immunostained neuronal structures (Fig. 7H, I). Virtually no ChAT neuronal cell bodies or axonal varicos-
DISCUSSION

Early and non-selective neurotoxicity of Aβ plaques in the hAPPSw,Ind mouse

A first finding of this study was that neuritic plaques, as soon as they appeared, were locally neurotoxic to both ACh and 5-HT axons in hAPPSw,Ind mouse brain. This was evidenced by the presence of swollen and distorted ChAT- and 5-HT-immunostained axons in the vicinity or within the core of plaques in 6 month-old mice, an age at which the plaques were still rare and minuscule, and when there was no measurable denervation. This clearly indicates that aggregated Aβ species exert a local, non-selective toxicity on neighboring neuronal elements. Another original finding was that, despite increased oxidative stress in brain of hAPPSw,Ind mice, substantiated by upregulation of MnSOD immunoreactivity in the vicinity of both Aβ plaques and blood vessels, and of nitrotyrosine immunostaining in glial cells around plaques and in the neuropil, ChAT terminals were dystrophic only when associated with plaques, not preferentially affected around blood vessels, and did not, nor did ChAT neuronal perikarya in cortex, show sign of peroxynitrite-mediated oxidative damage. These findings foster the neurotoxic role of Aβ deposits and, further, suggest that oxidative stress per se is not cholinotoxic. Finally, the Aβ-associated local neuronal degeneration did not appear to be related to the subsequent widespread ACh denervation, since only ACh axons, and not the 5-HT, were eventually lost with age.

Such lack of selectivity in the neurotoxicity of Aβ deposits in vivo agrees with previous reports albeit in aged (12–23 months) transgenic mouse models of AD, showing swollen and distorted neurites around Aβ plaques that could be labeled not only with ACh markers (ACHE; Sturchler-Pierrat et al., 1997; Bronfman et al., 2000; Boncristiano et al., 2002); vAChT (Wong et al., 1999); p75 (Jaffar et al., 2001); ChAT (Hernandez et al., 2001; German et al., 2003; Luth et al., 2003), but also for tyrosine hydroxylase or for different neuropeptides (Diez et al., 2000; Tomidokoro et al., 2000). As the electrophysiological properties of distorted neurites crossing through Aβ plaques are altered in AD (Knowles et al., 1999), our finding imply that there could be focal impaired neuronal functions resulting from these abnormal neurites at an early stage of the disease process. An important contributor to aggregated Aβ neurotoxicity is probably the highly fibrillogenic Aβ1–42, as it is the form primarily but not exclusively increased in neuritic plaques of transgenic hAPP mice (Sturchler-Pierrat et al., 1997; Hsia et al., 1999; Mucke et al., 2000; Klein et al., 2001; Klingner et al., 2003), and in those of AD brains (Roher et al., 1993; Selkoe, 1997; Hardy and Selkoe, 2002). This is in accord with the robust overlap seen here between Thioflavin-S and Aβ42-positive plaques. Several mechanisms responsible for the neurotoxicity of aggregated Aβ have been proposed: [Ca2+] dysregulation (Mattson et al.
1993; Palop et al., 2003), increased neurofibrillary tangles formation (Gotz et al., 2001) and tau phosphorylation (Zheng et al., 2002), electrostatic membrane interaction or persistent binding and activation of cell surface receptors (Yankner, 1996; Hertel et al., 1997) and, more recently, apoptosis (Estus et al., 1997) and, more recently, enhanced local inflammation (Luth et al., 2003) or oxidative stress (Pappolla et al., 1998; Smith et al., 1998; Huang et al., 2004).

In this respect, we found increased MnSOD and nitrotyrosine immunoreactivities and hence, oxidative stress, within and around Aβ plaques, in proximity of brain vessels or in microglial cells in hAPPsw,Ind mice. Such findings were in keeping with earlier reports of plaque-associated MnSOD-positive material in AD brains (Pappolla et al., 1992; Furuta et al., 1995). They corroborate observations in other APP transgenic mice of increased vascular (Paris et al., 2000; Park et al., 2004) or Aβ deposits-associated (Smith et al., 1998) oxidative stress, and protein nitration in glial cells-attributed to excessive NO production by activated glia (Rodrigo et al., 2004). The upregulated MnSOD perivascular cuffs seen here in hAPPsw,Ind mice were also remarkably similar to those in rats with lipopolysaccharide-induced endothelial cell activation, and found to encompass endothelial, neuronal and glial elements (Ruetzler et al., 2001). However, despite such signs of increased oxidative stress in aged hAPPsw,Ind mice, we found no evi-

![Fig. 5. The top panels (A–H) depict the ChAT- and 5-HT-immunostained innervations in CA3 of hippocampus and layers IV–V of the parietal cortex, as visualized in semi-thin sections from 12/14 month-old wild-type controls (A, C, E, G) and their littermate hAPPsw,Ind mice (B, D, F, H). Note the decreased density of axon varicosities in the stratum pyramidale (Pyr) of CA3 of the transgenic mouse (compare A to B), but lack of change in the neocortex (E, F). In contrast, there was no significant change in either region (Rad, stratum radiatum) in sections immunostained for 5-HT (C, D, G, H). Scale bar=50 μm. The graphs at bottom represent the relative number of ChAT- and 5-HT-immunostained axon varicosities in both regions, the size of ChAT-immunostained axon varicosities in hippocampus, and measurements of ChAT activity in the medial septum of the same mice. Means±S.E.M. from four to six mice in each group, except for ChAT activity which was measured in 12 and 15 wild type and hAPPsw,Ind mice, respectively. * P<0.05 and ** P<0.01 by Student’s t-test.](image)
DORSAL HIPPOCAMPUS

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ACh VARICOSITIES (10⁶/mm³)

PARIETAL CORTEX

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ACh VARICOSITIES (10⁶/mm³)

Fig. 6. Laminar and regional density of ChAT-immunostained axons and varicosities (left), and of 5-HT-immunostained axons (right), in dorsal hippocampus (CA1, CA3 and DG) and parietal cortex (layers I–VI) of 18 month-old wild-type and hAPPsw,Ind mice. Data (means ± S.E.M.) are from four mice in each group, and are expressed in meters of axons and/or millions of axon varicosities per mm³. Statistically significant differences between wild-type and hAPPsw,Ind mice are designated by asterisks. * P<0.05, ** P<0.01, and *** P<0.001 by one-way ANOVA followed by Student’s t-test.

dence for enhanced ACh denervation in perivascular areas or for peroxynitrite-mediated oxidative damage in ChAT varicosities or neuronal perikarya. Indeed, dystrophic ChAT fibers were strictly found in association with Thioflavin-, Aβ42-, MnSOD- or nitrotyrosine-positive plaques. Together, these findings support a role for oxidative stress in the neurotoxicity of aggregated Aβ1–42 (Butterfield and Bush, 2004), and further indicate that oxidative stress per se—independent
Fig. 7. MnSOD and nitrotyrosine immunoreactivities in 18 month-old hAPPSw,Ind mice. In hippocampus and cerebral cortex (A), strong MnSOD-immunoreactivity is distributed in rims around penetrating and intraparenchymal blood vessels, as well as in association with plaques (bottom inset). Scale bar=250 μm. In MnSOD and ChAT double-immunostained thick (B, C) and semithin (D) sections, MnSOD immunoreactivity (gray/black) is seen in neurons (arrows in B and C) and in small punctuate structures around blood vessels through which normal ChAT varicose fibers (brown) are coursing. ChAT varicosities are dystrophic when approaching MnSOD-positive plaques (inset in B), but normal in size and appearance within perivascular rims where they are intermingled with MnSOD-positive elements (D), as better appreciated at a higher magnification (inset in D). Semithin sections from wild-type (E) and hAPPSw,Ind (F) mice double-immunostained for ChAT and CD31 showing that perivascular ChAT varicosities (small
from aggregates of Aβ—is not implicated in the selective and widespread ACh denervation occurring uniformly throughout the neocortex and hippocampus of hAPP<sub>sw,Ind</sub> mice.

**Selective and progressive cholinergic denervation in the hAPP<sub>sw,Ind</sub> mouse**

The most striking finding of the present study was the selective and progressive decrease in ACh innervation density, which first involved the hippocampus at 12/14 months (20%), and then the parietal cortex at 18 months (25% in both regions). There was no direct relationship between the age-dependent increase in the number of plaques and the decrease in the density of ChAT positive axonal varicosities in hippocampus and parietal cortex. Only a small increase (20% to 26%, n.s.) in the ACh denervation was measured in hippocampus between 12/14 and 18 months, while there was a 2.6-fold increase in the number of plaques. Moreover, the ACh denervation was selective in that, even in the oldest hAPP<sub>sw,Ind</sub> mice, the density of 5-HT axonal varicosities in hippocampus and parietal cortex.

The most striking finding of the present study was the selective and progressive cholinergic denervation in the hAPP<sub>sw,Ind</sub> mouse. This would be compatible with the reported stabilization (or even slight decrease) in soluble Aβ species levels in brain and cerebrospinal fluid of these mice due to increased sequestration into plaques between 9 and 23 months (Kawarabayashi et al., 2001).

In keeping with the hypothesis of a cholinotoxicity of soluble Aβ (Lue et al., 1999; McLean et al., 1999; Hardy and Selkoe, 2002; Klein et al., 2001), an important cortical ACh deficit has recently been shown to occur in homozygous 4 month-old hAPP<sub>Ind</sub> mice, at a time when these mice exhibit high levels of Aβ but minimal plaque load (German et al., 2003). Our findings together with these previous observations strongly suggest that soluble Aβ, including its amorphous, non-congophilic protofibrils and small oligomers not readily detected here with either Thioflavin-S or Aβ42 antibody, might be causing the cholinotoxicity. Among possible mechanisms, soluble Aβ has been shown to increase choline fluxes in PC12 cells (Allen et al., 1997), to bind with high affinity to nicotinic ACh receptors (Wang et al., 2000), and to affect the high affinity choline uptake and the rate of ACh synthesis and release (Kar et al., 1998; for review, Dolezal and Kasparova, 2003). The latter would be consistent with the decrease (23%) in ChAT activity measured here in the medial septum of 12/14 month-old hAPP<sub>sw,Ind</sub> mice, or in the medial septum, hippocampus or neocortex of other APP transgenic mouse models (Bronfman et al., 2000; Boncristiano et al., 2002; German et al., 2003).

**CONCLUSIONS**

Our study shows that the plaque-associated, non-selective neurodegeneration and the diffuse, selective ACh denervation detected here in hAPP<sub>sw,Ind</sub> transgenic mice are independent events, most likely secondary to increased levels of aggregated and soluble forms of Aβ peptides.
respectively. Our results also indicate that oxidative stress is not a primary factor in the diffuse ACh deficit. In view of the selectivity and regional progression of this ACh degeneration in hippocampus, and later appearance in parietal cortex, this animal model could prove particularly useful to investigate the biological mechanisms of Aβ amyloid cholinotoxicity, as well as the efficacy of vaccines or other therapeutic measures aimed at protecting ACh neurons.

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