

Behavioral Disturbances without Amyloid Deposits in Mice Overexpressing Human Amyloid Precursor Protein with Flemish (A692G) or Dutch (E693Q) Mutation

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The contribution of mutations in the amyloid precursor protein (APP) gene known as Flemish (APP/A692G) and Dutch (APP/E693Q) to the pathogenesis of Alzheimer's disease and hereditary cerebral hemorrhage with amyloidosis of the Dutch type, respectively, was studied in transgenic mice that overexpress the mutant APP in brain. These transgenic mice showed the same early behavioral disturbances and defects and increased premature death as the APP/London (APP V717I), APP/Swedish (K670N, M671L), and other APP transgenic mice described previously. Pathological changes included intense glial reaction, extensive microspangiosis in the white matter, and apoptotic neurons in select areas of the brain, while amyloid deposits were absent, even in mice over 18 months of age. This contrasts with extensive amyloid deposition in APP/London transgenic mice and less pronounced amyloid deposition in APP/Swedish transgenic mice generated identically. It demonstrated, however, that the behavioral deficiencies and the pathological changes in brain resulting from an impaired neuronal function are caused directly by APP or its proteolytic derivative(s). These accelerate or impinge on the normal process of aging and amyloid deposits per se are not essential for this phenotype. © 2000 Academic Press

Key Words: Alzheimer's disease; transgenic mice; amyloid precursor protein; Flemish APP mutation; Dutch APP mutation.

INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disorder of the central nervous system and the most frequent form of dementia afflicting the aging population. The main characteristics of AD brain

pathology are senile plaques and neurofibrillary tangles composed mainly of ~4-kDa amyloid- β peptide (A β) and hyperphosphorylated microtubule-associated protein τ , respectively, and associated with dystrophic neurites and neuronal cell loss (Price & Sisodia, 1994; Selkoe, 1994). A primary pathogenic role in AD for amyloid- β precursor protein (APP) and its proteolytically derived amyloid peptides (A β 40/42) is suggested by specific mutations in APP flanking the A β

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sequence that are tightly linked to autosomal dominant forms of the presenile AD (Van Broeckhoven, 1994). Concurrently *in vivo* mouse models overexpressing mutant forms of APP produce variable AD pathologies ranging from amyloid plaque deposition (Quon *et al.*, 1991; Games *et al.*, 1995; Moechars *et al.*, 1998a) to vascular amyloid (Walker, 1997), some form of τ pathology (Sturchler-Pierrat *et al.*, 1997), cognitive deficits (Hsiao *et al.*, 1996; Moechars *et al.*, 1998a), neurodegeneration, and neuronal loss (Calhoun *et al.*, 1998; Moechars *et al.*, 1999b). Two mutants of APP have been most frequently used in reproducing the AD pathology in transgenic mice, i.e., the Swedish mutation (APP/Sw) located near the β -secretase site (Mullan *et al.*, 1992) and the London (APP/Lo) mutation(s) distal to the γ -secretase cleavage site(s) (Goate *et al.*, 1991). APP/Sw causes more of total A β production while APP/Lo is subject to altered cleavage by γ -secretase, thereby producing more of the amyloidogenic A β 42 peptide (Jarrett & Lansbury-PT, 1993; Citron *et al.*, 1996). While the β - and γ -secretases are essential for the production of A β , a third secretase (α -secretase) is known and its activity precludes the formation of A β due to cleavage within the A β sequence (Anderson *et al.*, 1991).

Patients presenting with mutations in APP residing in proximity of the α cleavage site present with different phenotypes (Hendriks *et al.*, 1995). The Dutch APP mutation (APP/Du) at codon 693 of APP (E693Q; numbering according to the largest APP isoform, APP770) causes hereditary cerebral hemorrhage with amyloidosis of the Dutch type (HCHWA-D) and segregates in an autosomal dominant pattern in a subset of patients with cerebral amyloid angiopathy (CAA) (Van Broeckhoven *et al.*, 1990; Levy *et al.*, 1990). It is characterized by extensive amyloid deposits in the small leptomeningeal and cortical arterioles, which leads to an early death of those afflicted in their fifth or sixth decade (Bornebroek *et al.*, 1996). No such predilection for blood vessels, however, is noted for the Flemish APP mutation at the preceding codon (A692G) (APP/FI) with which patients present with a combined pathology of CAA and presenile AD, with the presence of both large-cored parenchymal senile plaques and extensive amyloid deposits in blood vessels (Hendriks *et al.*, 1992; Cras *et al.*, 1998; van Harskamp *et al.*, 1999). Moreover, on the basis of clinical features, HCHWA-D can be considered to represent a mixed form of AD and multi-infarct dementia (Roos *et al.*, 1991). These results suggest that the clinically distinct entities, presenile AD and CAA, can be caused by mutations in the APP gene (Van Broeckhoven *et al.*, 1990), causing, unlike the β - and γ -secretase-related presenile AD mutations, a

changed specificity for α -secretase. To test this and to study the processing of mutated human APP *in vivo*, we have generated transgenic mouse strains expressing human APP/FI and APP/Du from the neuron-specific mouse thy-1 gene promoter (Moechars *et al.*, 1996). Human APP was expressed in brains of all transgenic mouse strains analyzed. We also demonstrated an increased processing of human APP at the β -secretase cleavage site causing increased formation of N-terminal fragments of A β protein. However, the mice did not develop amyloid plaques or τ pathology, even by 22 months of age, nor was there any evidence found in vasculature for circulatory A β peptides. Nevertheless, behavioral, pharmacological, and pathological observations in these mice disclosed subtle aspects of early and late onset AD similar to those observed in APP/Lo and other mutant and wild-type APP (APP/Wt) transgenic mice (Moechars *et al.*, 1999a). These defects comprised differential glutamatergic responses, increased aggression, some form of neurodegeneration, occasional spontaneous seizures, and variable premature death. These signs were largely independent of the actual mutant APP that was expressed and were not directly correlated with a single APP metabolite (Moechars *et al.*, 1996, 1999a). The current models could shed more light on various other aspects of APP/FI and APP/Du processing in rodent brain, but require additional investigation to understand the pathobiological pathways leading to two distinct clinical entities.

MATERIALS AND METHODS

Generation of Transgenic Mice

The Flemish (A692G) and Dutch (E693Q) APP mutations were introduced in the human APP cDNA using *in vitro* mutagenesis (Hendriks *et al.*, 1995; De Jonghe *et al.*, 1998). APP cDNA encoding these mutants were placed under the transcriptional control of the mouse thy-1 promoter (Moechars *et al.*, 1996). From the 8.1-kb *EcoRI* fragment comprising the mouse thy-1 gene, a 1.5-kb *BanI*-*XhoI* fragment was replaced by the respective cDNAs employing blunt-end ligation. The replaced fragment included the thymus-specific regulatory elements, present in intron 3 of the thy-1 gene. The brain-specific regulatory sequences in intron 2 were retained (Moechars *et al.*, 1996). Linearized constructs, devoid of all plasmid sequences, were purified (Sephaglass; Pharmacia) and microinjected into 1.5-day-old pre-nuclear embryos isolated from superovulated FVB/N females according to standard procedures

(Hogan *et al.*, 1994). The injected eggs were allowed overnight to reach the two-cell stage and subsequently transferred to pseudopregnant fosters (CD1). Offspring were genotyped by Southern blotting of tail-biopsy DNA restricted with *KpnI* and probed with a radiolabeled 490-bp PCR amplicon of human APP cDNA with primers 5'-CCGATGGGTAGTGAAG-CAATGGTT and 5'-TGTGCCAGCCAACACAGA-AAAC, which were also used for subsequent routine genotyping of offspring by PCR. Stable transgenic lines were established by breeding founders to FVB/N mice.

Analysis of APP Transgene RNA and Protein

Mouse brain RNA was analyzed by Northern blotting (Moechars *et al.*, 1996) and relative mRNA levels quantified densitometrically after autoradiography or by phosphorimaging. APP was analyzed by Western blotting and immunoprecipitation with antibodies indicated. Brain was homogenized in 15 vol of 50 mM Tris pH 7.4, 150 mM NaCl, and proteinase inhibitors (Boehringer Mannheim, Germany) and cleared by centrifugation (100,000g, 1 h, 4°C). The pellet was reextracted with the same buffer containing 2% Triton X-100, 2% Nonidet-P40 and centrifuged as before. Samples were denatured and reduced (2% SDS, 1% 2-mercaptoethanol, 95°C, 5 min), separated on polyacrylamide gels (4–20% Tris-glycine), and transferred to nitrocellulose filters (Hybond-C; Amersham, UK) for Western blotting.

Antibodies

The monoclonal antibody (mAb) 1G5 (Athena Neurosciences, San Francisco, CA) is directed against amino acid residues 444–592 of human APP. Rabbit antisera B11/4 and B12/4 were generated against a synthetic peptide representing the 20 C-terminal residues of APP, synthesized on Tentagel-MAP branched resin (ABI, Foster City, CA). The antiserum reacts equally well with human and mouse C-terminal stubs (De Strooper *et al.*, 1995). Rabbit antiserum R1736 is directed against a synthetic peptide representing part of the A β peptide located between the α - and β -secretase cleavage sites (residues 595–611) (Haass *et al.*, 1992). C-terminal fragments were immunoprecipitated with rabbit antiserum B11/4 and separated on 10–20% Tris-tricine gels and fragments detected via Western blotting by ECL chemiluminescence using B12/4 (1/1000) as primary antibody. For immunohistochemistry, the following antibodies were used: mAb 4G8 for

β -amyloid (18–24 amino acid residues of A β ; Senetek), rabbit antisera FCA3340 and FCA3542 for A β residues ending at 40 and 42 amino acids (Barelli *et al.*, 1997), mAb 22C11 directed against N-term APP (Boehringer Mannheim), abnormally phosphorylated PHF- τ (AT8; Innogenetics), and glial fibrillary acidic protein (GFAP; Dako). Blocking sera (rabbit and goat sera), link antibodies (biotinylated rabbit anti-mouse and goat anti-rabbit), and horseradish avidin-biotin complex were used in recommended dilutions (Dako).

A β Peptide Levels

Mouse brains were homogenized in 6.5 vol of 20 mM Tris-HCl, pH 8.5, 5 mM EDTA, 2 mM PMSF, 0.5 μ g/ml leupeptin, 0.7 μ g/ml pepstatin, 0.1 mg/ml phenanthroline, and 0.1 mg/ml benzamidine. The homogenate was cleared (220,000g, 2 h, 4°C) and concentrated on Sepak C18 cartridges (Waters, Milford, MA). Sandwich ELISA was used to measure A β 40, using mAb 1101.1 as capturing and BA#1 as detecting antibody (Higgins *et al.*, 1994).

Resident Intruder Test

All mice were identically housed in a temperature- and humidity-controlled vivarium, kept on a 12-h dark-light cycle (light on 7:00 AM EST), with free access to food and water, except during the test. In the isolation-induced aggression test “resident” male nontransgenic or transgenic mice were 10–12 weeks of age at the time of testing and were solitarily housed for 4 weeks prior to the test. Bedding was changed once a week without touching or handling the animal. “Intruders” were nontransgenic male FVB/N mice, 8–10 weeks of age at the time of testing, housed in groups of four since weaning. The test consisted of placing a randomly chosen intruder in the resident cage and aggression of the resident was scored by measuring (i) attack latency, i.e., the time in seconds between introduction into the cage and the first attack, and (ii) the number of attacks during a 3-min session, with “attack” defined as biting the intruder. One week following the first test, the same resident mice were tested a second time with a different set of intruder mice. Statistical analysis of means was performed by Student’s *t* test.

Glutamate Analogs

N-Methyl-D-aspartate (NMDA) and kainic acid (KA), dissolved in pyrogen-free 0.9% sodium chloride solu-

tion, were injected intraperitoneally (ip) into mice of 3 to 4 months. Reactivity of the mice was scored by determining the LD50.

Histopathology

Mice were anesthetized with ip injection of sodium pentobarbital (5 mg) and Ketamine (4 mg). Transcardial perfusion was performed in some animals with ice-cold 0.01 M PBS (pH 7.4) followed by perfusion with 4% paraformaldehyde in PBS. The brains were dissected free from the skull by incising the cranial nerves and spinal cord and placing them in saline. For each specimen, half the brain, cut midsagittally or transversely, was fixed in methacarn overnight at 4°C and further processed for paraffin embedding, and the rest was either processed for electron microscopy or snap frozen until further use.

Serial 4- μ m parasagittal and/or transverse sections were made from embedded brain tissue specimens for histological and immunohistochemical stainings with the exception for Congo red staining (10 μ m). Classical histological staining included hematoxylin–eosin, cresyl violet, and Bodian and Congo red. For immunohistochemistry, sections were immersed in 0.03% hydrogen peroxide in methanol and incubated for ½ h to block endogenous peroxidase followed by preincubation with normal sera diluted 1:5. Sections were incubated overnight (16 h) at 4°C with the primary antibodies, followed by ½-h incubations with first biotinylated secondary antibodies and then horseradish peroxidase-conjugated avidin–biotin diluted 1:1:100. Sections were finally treated with peroxidase substrate solution containing 0.01% hydrogen peroxide and 0.05% diaminobenzidine tetrahydrochloride and counterstained with Harris hematoxylin and coverslipped in DPX. All dilutions were made in 0.1 M PBS containing 0.1% bovine serum albumin. *In situ* detection of apoptotic cells by terminal transferase incorporation of dUTP nick end labeling (TUNEL) was performed on sections retrieved in citrate buffer (pH 6), microwaved, and stained according to the guidelines provided by the supplier (Boehringer Mannheim).

Transmission Electron Microscopy

To examine any fibrillar deposits in or around blood vessels and to rule out minute amounts of diffuse A β , tissue was processed for electron microscopy and immunoelectron microscopy. For the former, brain

hemiblocks were fixed with 4% glutaraldehyde solution in phosphate buffer (PB; pH 7.2; 0.2 M) for 4 h at 4°C, washed in PB and water, and dehydrated in a graded ethanol series (30, 50, 75, 90, and 2 \times 100%), 15 min each, at –20°C. Specimens for Unicryl embedding were further processed according to guidelines provided by the manufacturer (British Biocel International Ltd, Cardiff, UK). In short, tissue was brought immediately from 100% ethanol to pure resin for an infiltration period of 60 min and one infiltration overnight in fresh pure resin, both at –20°C. Tissue blocks were polymerized using Philips LTD 15W/05 lamps for a period of 4 days, with a final curing period of 7 days at 4°C. Ultrathin sections were cut and collected on nickel grids. For immunoelectron microscopy, tissues were processed as described above, except that they were fixed with 2% paraformaldehyde solution in PB containing 0.05% glutaraldehyde. Ultrathin sections were collected on Formvar-coated nickel grids. The grids were preincubated for 30 min on 20- μ l drops of 20% normal serum in Tris-buffer saline (0.05 M, pH 7.6) followed by incubation with primary antibodies and detection with a 10-nm gold-labeled secondary antibody (AuroProbe EM; Amersham International plc, Slough, UK). After staining, the sample grids were postfixed in 2% glutaraldehyde for 5 min. Sections were thereafter stained with uranyl acetate and Reynolds' lead citrate (5 min each) and examined using a Jeol 100 C electron microscope (Japan).

RESULTS

Transgene Expression

Wild-type APP and Flemish and Dutch mutant APP were expressed in the brain of transgenic FVB/N mice by the mouse thy-1 gene promoter (Moechars *et al.*, 1996). Founders and offspring in FVB/N background were genotyped by Southern blotting over four to six generations, confirming stability of integration and Mendelian transmission of the gene. The 13 independent transgenic lines expressed transgene mRNA at moderate to high levels (Table 1, Fig. 1).

Biochemical Analysis of APP Intermediates

In brain of heterozygous transgenic mice, ages 6 to 8 weeks, full-length cell-associated APP and its catabolites were analyzed and quantified, comprising total secreted and α -cleaved secreted APP, the residual

TABLE 1
Overview of Different Transgenic APP Mouse Lines

Minigene	Founders	Line	RNA level	Mortality (%)
APP/FI	7	APP/FI/1	14	31.4
		APP/FI/3	2	7.7
APP/Du	6	APP/Du/4	5	31.6
		APP/Du/5	3	13.7

Note. Tabulation of transgenic founders generated from the two minigenes and of some transgenic lines analyzed for RNA expression levels (relative to wt APP) and mortality in the different APP transgenic lines after 180 days.

C-terminal fragments (C-stubs), and the A β (1–40) peptide (Fig. 2, Table 2). Expression levels of mutant APP in brains of mice of lines APP/FI/1 and APP/Du/4 were 47 and 60%, respectively, of the expression level in APP/Wt/4 transgenic mice. Effects of the Flemish and Dutch APP mutations on APP processing were analyzed by quantification of APP-processing products, normalized for expression level for human APP/Wt transgenic mice. This revealed a significant relative increase in β -carboxyl fragments in transgenic APP/FI and APP/Du mouse brains (Fig. 2). Furthermore, standardization of α -secreted APP to total secreted APP revealed a consistent and significant decrease in α secretion of APP in APP/FI and APP/Du transgenic mice (Fig. 2). The same protocols and methods revealed a marked increase in β -carboxyl APP fragments and decrease in α -cleaved secreted APP for transgenic mice bearing the Swedish APP (Moechars *et al.*, 1998a).

Phenotypic Characterization

Premature Death

FVB/N mice live in our conventional animal house for more than 2 years, while APP transgenic mice in this genetic background die variably but prematurely (Moechars *et al.*, 1996), a phenomenon that was also observed by others (Hsiao *et al.*, 1995; LaFerla *et al.*, 1995). Monitoring of 150 heterozygous mice from two APP/FI and two APP/Du transgenic strains, housed with 46 nontransgenic littermates for 6 months, revealed that only 3% of nontransgenic mice died, as opposed to between 8 and 16% of the APP transgenic mice (Table 1). Early mortality correlated with higher levels of mutant APP expression as reported for murine APP/RK transgenic mice (Moechars *et al.*, 1996). Premature death was absent in a large number of other transgenic mouse strains, housed under identical conditions in the animal house, but overexpressing other transgenes unrelated to APP using the same mouse thy-1 gene promoter and in the same genetic background (results not shown). Premature death is thus specific for APP transgenic mice and occurs most of the time without overt signs of deteriorating health and very rarely with decreased bodyweight or muscle wasting.

Spontaneous Behavior and Seizures

Typical for the APP transgenic mice were alternating episodes of hyperactivity, anxiety, and aggression in the home cage and in a minority of mice progressing into seizures as reported before for other APP trans-

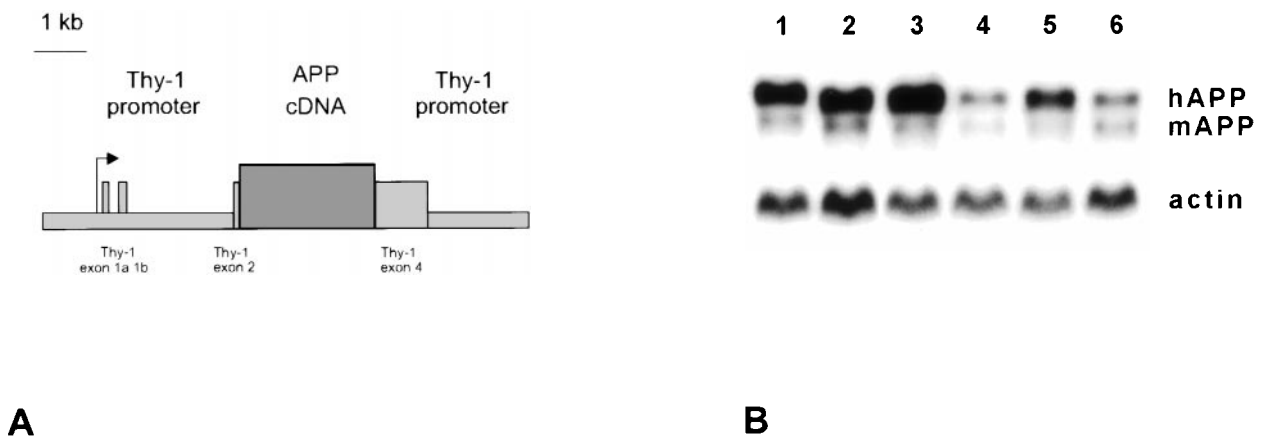


FIG. 1. Generation of APP transgenic mice. (A) Schematic representation of the mouse thy-1 gene promoter construct. (B) Northern blot of total brain RNA from the following transgenic mice: lane 1, APP/Wt/4; lane 2, APP/Wt/2; lane 3, APP/FI/1; lane 4, APP/FI/3; lane 5, APP/Du/4; and lane 6, APP/Du/5. The 3.5-kb endogenous mouse APP transcript (mAPP) and the larger transgenic mRNA (hAPP) are indicated.

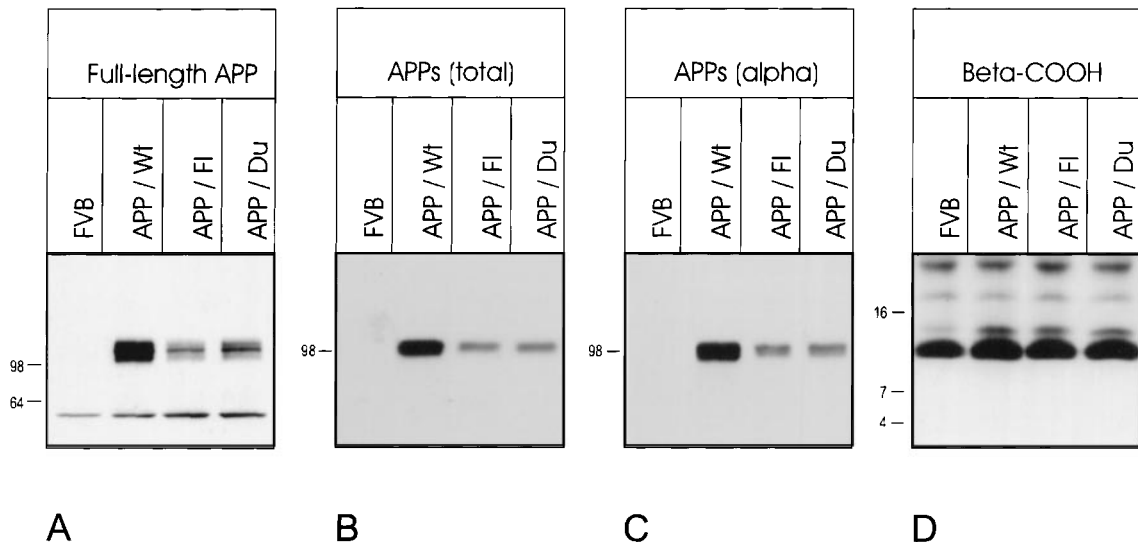


FIG. 2. Analysis of APP-processing products. (A) Western blot of membrane extracts of brains of nontransgenic and heterozygous transgenic mice of lines APP/Wt/4, APP/FI/1, and APP/Du/4 ($n = 8$). Full-length transgenic APP was detected by 1G5. Semiquantitative analysis (Table 2) was done by densitometric scanning in a linear range of the assay (determined by measurement of a dilution curve, not shown). (B and C) Western blots of soluble fractions of brains of nontransgenic and heterozygous transgenic mice of lines APP/Wt/4, APP/FI/1, and APP/Du/4 ($n = 4$). Total secreted APP was detected by 1G5, α -secretase-cleaved APP by R1736. Blots were semiquantitatively analyzed (Results, see Table 2). (D) Detection of C-terminal fragments was done by immunoprecipitation with B11/4, followed by Western blotting with B12/4. Membrane fractions of brains of nontransgenic and heterozygous transgenic mice of lines APP/Wt/4, APP/FI/1, and APP/Du/4 ($n = 8$) were analyzed. Blots were semiquantitatively analyzed (Results, see Table 2).

genic mice (Moechars *et al.*, 1996, 1999a). Offspring of the APP/FI and APP/Du transgenic strains developed and behaved normally for 6–8 weeks after birth, judged by weight gain, feeding, drinking, locomotion, and climbing in the home cage. Progressively, more mice of both sexes in the different APP transgenic strains displayed signs of increased agitation and bouts of wild running with an age of onset of 8 weeks or more, depending on the expression level of the transgene. Subsequently seizures, a neurologic sign of

abnormal brain activity, became apparent in a subset of the transgenic mice. In about 15% of surviving mice older than 6 months, seizures that progressed from mild to severe tonic-clonic were observed and were independent of the APP transgene. Mild seizures consisted of facial movements, forelimb clonus, and whole-body clonus lasting less than 10 s while severe tonic-clonic seizures lasted 30 to 60 s. Mice gasped and produced tonic seizures, stretching their limbs with the tail upright, followed by whole-body clonic sei-

TABLE 2
Biochemical Analysis of APP Metabolites

	Expression level	β -COOH	β -COOH normalized for expression level	β -Amyloid (A β 40) (ng/g brain)	β -Amyloid (A β 40) normalized for expression level	APP α normalized for total APPs
APP/Wt	100 \pm 0 ($n = 8$)	100 \pm 0 ($n = 8$)	100 \pm 0 ($n = 8$)	1.8, 2.6 ($n = 2$)	1.8, 2.6 ($n = 2$)	100 \pm 0 ($n = 4$)
APP/FI	47 \pm 6 ($n = 8$)	102 \pm 23 ($n = 8$)	215 \pm 49 ($n = 8$)	1.2 \pm 0.2 ($n = 3$)	2.6 \pm 0.4 ($n = 3$)	77 \pm 10 ($n = 4$)
APP/Du	60 \pm 8 ($n = 8$)	108 \pm 27 ($n = 8$)	180 \pm 45 ($n = 8$)	0.8, 0.8 ($n = 2$)	1.3, 1.3 ($n = 2$)	83 \pm 11 ($n = 4$)

Note. Tabulation of the levels of APP and APP processing intermediates determined by semiquantitative Western blotting and ELISA (for A β 40). Values represent mean values and standard errors of the mean of the indicated analyzed numbers of samples.

zures. Subsequently, mice became lethargic for varying periods.

Male Aggression

Transgenic mice expressing human APP displayed an intense aggressive behavior not observed in nontransgenic littermates handled and housed under identical conditions (Moechars *et al.*, 1996, 1998a, 1999a). Major indications were wounds and bald patches in the fur resulting from male–male but also male–female fighting. This behavior was typical only for APP transgenic mice housed in rooms with many other transgenic mice expressing unrelated minigenes from the same or other promoters, but was independent of the actual isoform or mutant of APP expressed. Augmented aggressive behavior in male mice of lines APP/FI/1 and APP/Du/4 was tested, under strictly controlled conditions, in comparison to nontransgenic mice, employing isolation-induced aggression. “Resident” males were housed in isolation for 4 weeks before being confronted with male nontransgenic “intruders” who were reared “socially” in groups. In each test, aggression was scored over a 3-min observation period by (i) measuring the latency of the first attack and by (ii) recording the number of attacks.

Transgenic residents expressing mutant APP attacked the intruder significantly sooner than nontransgenic FVB/N resident males. Furthermore, the number of attacks by all APP transgenic mice was significantly higher than in nontransgenic mice and was also experienced by the observer to be “more fierce.” A second test of the same residents with different intruders, 1 week after the first test, demonstrated a considerable increased intensity of aggression, both for nontransgenic and for transgenic residents (Fig. 3). This is consistent with the fact that aggressiveness increases with fighting experience. Quantitatively, in the first test 78 and 86% of the APP/FI and APP/Du transgenic residents, respectively, attacked the intruder in the 3-min test period as opposed to only 11% of nontransgenic male mice. Between 33 and 44% of the transgenic residents of the different transgenic strains attacked within the first minute, which was never observed for nontransgenic residents in the first test. In the second test, the percentage of transgenic mice with short-latency attack was increased to between 71 and 84% in APP/FI and APP/Du residents compared to only 16% in nontransgenic residents.

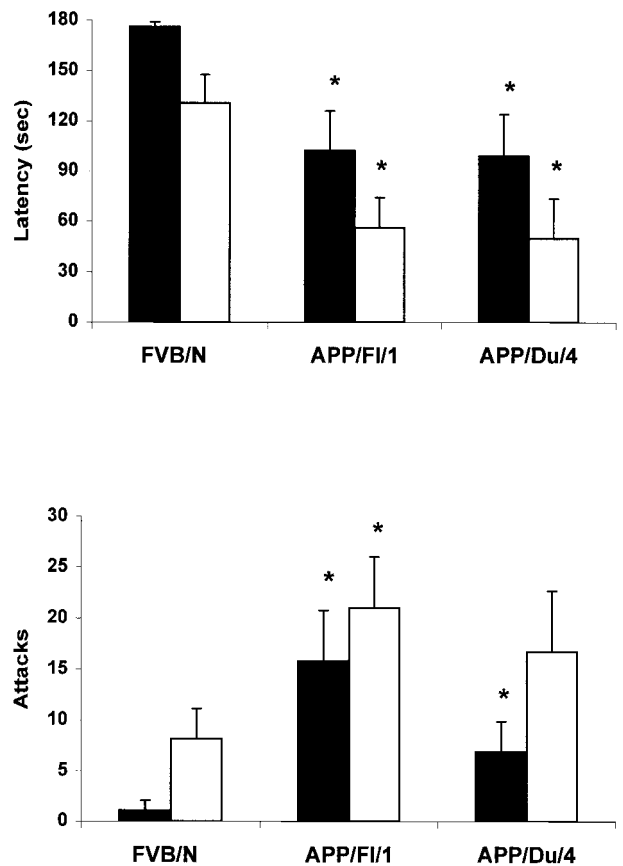
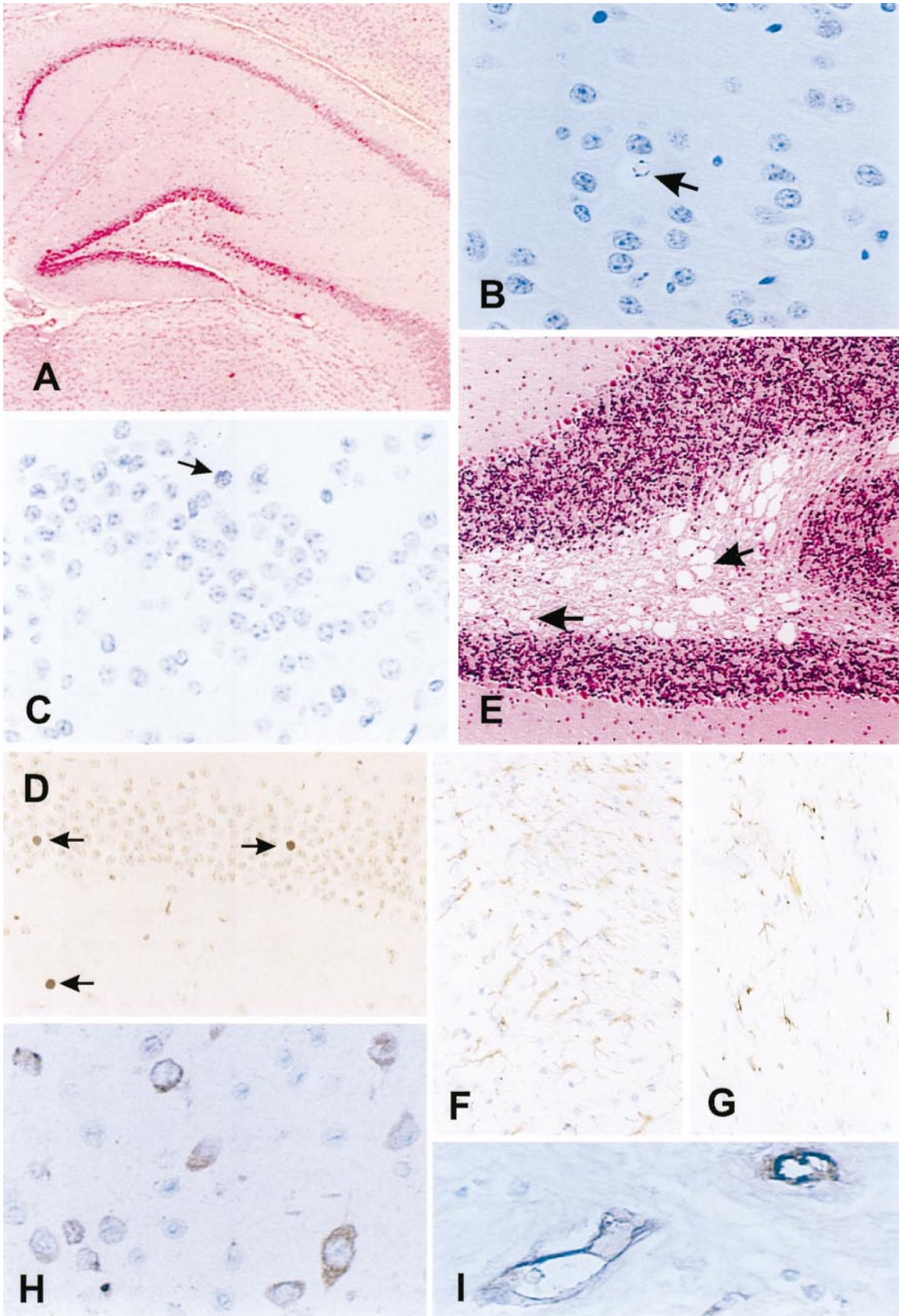


FIG. 3. Male aggressive behavior. Resident–intruder test of aggression with nontransgenic FVB/N mice ($n = 15$) and heterozygous transgenic mice from lines APP/FI/1 ($n = 9$) and APP/Du/4 ($n = 7$). Attack latency (top) and number of attacks (bottom) were scored in two consecutive tests (black bar, test 1; open bar, test 2) over 3 min and results are means with standard errors. Two-tailed Student's *t* test indicated most differences with nontransgenic mice to be significant (at least $P < 0.05$).

Histopathology and Ultrastructural Examination

Brains of APP/FI ($n = 18$) and APP/Du ($n = 18$) transgenic mice, including those of two mice that died prematurely from both groups, along with those of wt FVB/N mice ($n = 5$) were subjected to histological and immunohistochemical examinations (Fig. 4). On gross examination, brains of transgenic mice were not different from age-matched wt mice nor was any gross pathology of any other organ evident. In the majority of the APP/FI and APP/Du animals, a characteristic loosening or spongiosis of the neuropil was noticed in the frontal cortical regions and parahippocampal and cerebellar areas (Fig. 4E). Associated with it was an intense glial response, demonstrated by a dense stain-



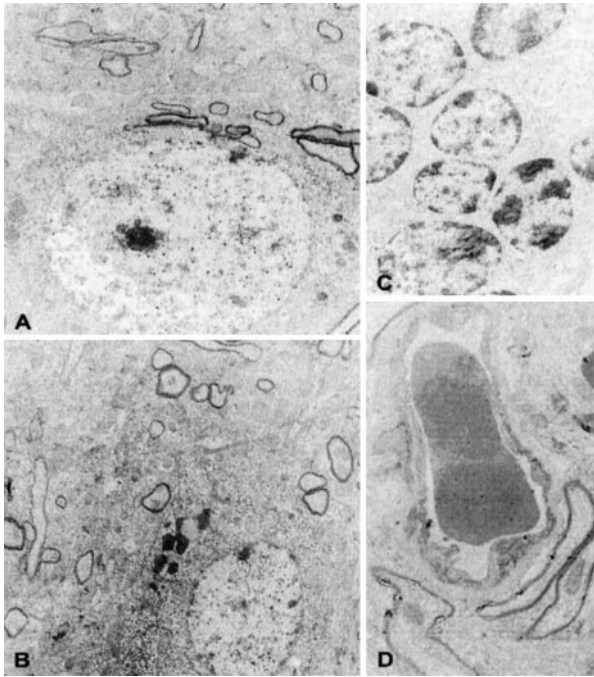


FIG. 5. Electron microscopy. Nuclear changes seen on light microscopy were also confirmed ultrastructurally in which nuclear ballooning was noticed in APP/FI (A, original magnification $\times 40,000$) as well as in APP/Du mice section (B, original magnification $\times 40,000$) with occasional deposition of lipofuscin consequent to aging (20 months). Granular cell layer of the dentate gyrus was normal in this section of APP/Du mouse brain (C, original magnification $\times 40,000$) and no amyloid-like fibrils were noticed in or around blood vessels in a nonperfused specimen of APP/Du mouse brain specimen (D, original magnification $\times 80,000$).

ing pattern with a GFAP antibody, present in cerebral cortical areas and cerebellar white matter, as well as in hippocampus and hilus of dentate gyrus, and extending to the granular cell layer. In animals experiencing seizures and premature death a more intense reactive astrogliosis was demonstrated by GFAP immunostaining (Figs. 4F and 4G). Reactive gliosis was present in the brain of all APP transgenic mice independent of the mutation, while absent in transgenic mice overexpressing unrelated genes under control of the thy-1 pro-

motor, in the same genetic background. Reactive glial cells were also observed ultrastructurally. No viral-like particles were noted in these areas, and axons were appropriately myelinated (Fig. 5).

Both APP/FI and APP/Du transgenic mice were noticed to harbor occasional abnormal neurons with dense chromatin and condensed nucleus in the cerebral cortex especially in layers II, IV, and V and in the dentate gyrus. Abnormally condensed neurons were also evident in similar areas in mice that died prematurely. Although rare, parenchymal lacunas associated with disintegrating neurons were sometimes noticed, especially in the mice with premature death (Figs. 4A to 4C). Ultrastructurally, ballooning of nucleus was also noticed occasionally (Fig. 5A). TUNEL staining could demonstrate a considerable number of positive cells in these areas (Fig. 4D). However, only a minority of these neurons were shown to have a typical morphology of an apoptotic cell ultrastructurally (Figs. 5A to 5C).

No parenchymal or vascular amyloid deposits were noticed on Congo red staining or on immunohistochemistry with mAb 4G8 and rabbit sera FCA3340 and FCA3542. Although some amyloid staining existed, especially with 4G8 and for hyperphosphorylated τ (AT8) (Fig. 4I), neither amyloid fibrils nor paired helical filaments were noted within neurons ultrastructurally (Fig. 5) nor was any reactivity on immunoelectron microscopy (data not shown). Some blood vessels were dilated, perhaps due to the perfusion performed prior to fixation. Nonetheless both APP/FI and APP/Du transgenic mice were shown to express elevated amounts of APP compared to the age-matched controls. Using mAb 22C11, recognizing both mouse and human APP, expression of APP was noticed not only in neurons but also in glial cells, ependymal cells, epithelium of the choroid plexus, and occasionally in leptomeninx. Neuronally, expression was strongest in cerebral cortical layers II, IV, and V; pyramidal layer of hippocampus; olfactory bulbs; nucleus basis pontis; cranial nerve nuclei; and weakly in Purkinje and granular cell layers of the cerebellum (Fig. 4F).

FIG. 4. Histology and immunohistochemistry of the mouse brain. HE staining of APP/FI mice (A) overlooking hippocampal region, highlighting occasional condensed nuclei in the granular layer of the dentate gyrus and pyramidal layer of hippocampus, which are detailed further in (B). Note here a neuron with karyorrhexis (arrow). Similarly loss of neurons, consequent to possible apoptosis, was also occasionally noticed like here in a section of APP/Du mouse brain (C, arrow). TUNEL staining recognizing apoptotic neurons in a section of APP/FI mouse brain in the region of the granular layer and polymorphic layer of the dentate gyrus (D). Cerebellar white matter spongiosis in APP/FI mouse brain (E). Reactive gliosis in cerebellar cortex illustrated by GFAP immunostaining in APP/FI (F) compared with FVB/Wt (G). APP (22C11) immunostaining in APP/Du brain specimen in the region of cerebral cortex (H) and staining of blood vessels for A β (4G8) in APP/FI mice as a result of background (I).

TABLE 3
Mortality Resulting from Administration of KA and NMDA

KA		24 mg/kg	28 mg/kg	32 mg/kg		
FVB/N		0% (<i>n</i> = 5)	0% (<i>n</i> = 8)	0% (<i>n</i> = 10)		
APP/Du/4		0% (<i>n</i> = 2)	33% (<i>n</i> = 6)	87% (<i>n</i> = 8)		
APP/Fl/1		25% (<i>n</i> = 4)	37% (<i>n</i> = 8)	75% (<i>n</i> = 8)		
NMDA		40 mg/kg	80 mg/kg	120 mg/kg	160 mg/kg	200 mg/kg
FVB/N	0% (<i>n</i> = 15)	67% (<i>n</i> = 21)	100% (<i>n</i> = 16)	100% (<i>n</i> = 2)	100% (<i>n</i> = 2)	
APP/Du/4	0% (<i>n</i> = 2)	0% (<i>n</i> = 2)	0% (<i>n</i> = 8)	29% (<i>n</i> = 7)	100% (<i>n</i> = 5)	
APP/Fl/1	0% (<i>n</i> = 2)	0% (<i>n</i> = 2)	0% (<i>n</i> = 7)	43% (<i>n</i> = 7)	100% (<i>n</i> = 5)	

Mortality resulting from intraperitoneal administration of KA and NMDA, to non-transgenic, heterozygous APP/Du/4 and APP/Fl/1 mice. *n* equals the number of mice tested.

Reactivity to Glutamate Analogs

To address neuropathological changes at the molecular level, we studied reactivity of the glutamate neurotransmitter system, known to be involved in mechanisms of learning and memory as well as in neurodegeneration and excitotoxicity. The present human APP mutant transgenic mice behaved very similarly to the mutant APP/RK and other previously reported human APP transgenic mice in response to the glutamate analogs NMDA and KA (Moechars *et al.*, 1996, 1999a). Dose-response curves for KA-induced mortality clearly indicated that all APP transgenic mice were more sensitive to KA than nontransgenic FVB/N mice: the LD50 for the APP/Fl/1 and APP/Du/4 mice was situated between 28 and 32 mg/kg body weight, while all nontransgenic FVB/N mice survived the dosage of 32 mg/kg (Table 3). In contrast to the hypersensitivity to KA the present APP transgenic mice were hyposensitive toward NMDA as were the previously reported APP transgenic mice (Moechars *et al.*, 1996, 1999a). The LD50 in nontransgenic FVB/N mice of 3 to 4 months was about 70 mg NMDA/kg. All age-matched APP transgenic mice were less sensitive with LD50 values that ranged between 160 and 200 mg NMDA/kg (Table 3B).

DISCUSSION

So far, transgenic mouse models have not yet succeeded in fully recapitulating all aspects of AD pathology, a problem that cannot be judged yet to be impossible to reach. The ultimate development of the phenotype in any mouse model will depend on many factors, i.e., the transgene copy number, the specific

promoter used to drive the transgene, the genetic background of the mouse strain, the relative preponderance of the A β subspecies, etc. (reviewed in Price & Sisodia, 1994). Even additional genetic or environmental factors in humans affect the development of AD (van Duijn *et al.*, 1994; Finch & Tanzi, 1997). Therefore, a direct extrapolation of a human phenotype into a mouse model should be carefully considered. Development of amyloid plaques has now been demonstrated in several different models of transgenic mice and the problem is now evidently shifted to determining what their relevance and precise contribution is, if any, to the phenotype and the pathology.

Despite the early behavioral disturbances and defects, increased premature death and subtle pathological changes like astrogliosis and microspangiosis with indications for limited apoptosis, neither APP/Fl nor APP/Du transgenic mice produce appreciable amyloid plaques. We hypothesize that APP is not efficiently directed into the amyloidogenic pathway by these mutants as it is by other clinical mutants, APP/Lo and APP/Sw, similarly expressed by the same promoter in the same mouse strain that do produce A β deposits (Moechars *et al.*, 1999a). Mutations in APP/Du and APP/Fl transgenic mice do, however, significantly decrease α -secreted APP while increasing β -secretase-cleaved C-terminal fragments in mouse brain, relative to wild type and normalized for total secreted APP. This effect was similar, although less pronounced than in APP/Sw transgenic mice and conforms to the known metabolic effects of mutations in APP on its metabolism *in vitro* in which the mutations located near the α -secretase cleavage site also favor β cleavage of APP (Haass *et al.*, 1994, Mc Phie *et al.*, 1997). Despite this shift from α to β cleavage, there was no increase in levels of A β peptides in APP/Fl transgenic mouse

brain, whereas in the APP/Du transgenic mice, relative β -amyloid levels were even lower. Interestingly, this parallels our earlier experiments with transfected CHO-K1 cells, HEK-293 cells, and human neuroglioma H4 cells in which APP/Fl increased the production of A β while A β levels in the supernatants of APP/Du-transfected cells were slightly lower than in APP/Wt-transfected cells (De Jonghe *et al.*, 1998).

Absence of any vascular A β deposits in the brains of APP/Du and APP/Fl transgenic mice could not be due to the use of a specific neuronal (thy-1) promoter, as APP mice using the same promoter have been shown to develop vascular amyloid deposits (Calhoun *et al.*, 1998). Moreover, the only other study with APP/Du transgenic mice (Howland *et al.*, 1995) also failed to demonstrate any amyloid deposition in brain. It was suggested that E693Q and A692G substitutions in the APP/Du and APP/Fl mutants, respectively, could alter the secondary structure and aggregating properties of the A β peptide, thereby contributing differently to the final phenomenon. In the APP/Du isoform, an accelerated amyloid fibril formation relative to the native sequence was noticed (Wisniewski *et al.*, 1991; Clements *et al.*, 1993). For APP/Fl mutation, however, no significant change in the rate of *in vitro* amyloid aggregation was detected. In fact, the aggregation of mutant A β amyloid fibrils bearing the A692G mutation, was slightly slower than that of wild-type A β (Clements *et al.*, 1993). The clinical cases of APP/Du and APP/Fl mutations could thus result either from nonelevated but highly fibrillogenic A β in the former or from mild to moderately elevated A β levels in the latter, but extended over a much more prolonged period, which is obviously not possible in rodents. Alternatively or in addition, the mutations might cause a reduced clearance of the A β in humans while mice might remain equally efficient in clearing these mutant A β peptides.

Occurrence of behavioral disturbances in these transgenic mice is not surprising as neuropsychiatric abnormalities in the form of increased irritability and aggressive behavior are present in a large percentage of AD patients (Hope *et al.*, 1999) and also remain the major underlying cause for caregivers to place these patients in an institution (Stoppe *et al.*, 1999). Moreover, seizures in both sporadic and familial AD patients as well as in patients with HCHWA-D has been frequently recorded, especially in the later stages of the disease (Romanelli *et al.*, 1990; Martin *et al.*, 1991; Kennedy *et al.*, 1993; Mullan *et al.*, 1993; Lampe *et al.*, 1994; Silbert *et al.*, 1995). Due to the poor visual performance of FVB, measurement of memory and learning abilities in the

Morris Water Maze is not possible on this mouse strain (Smith *et al.*, 1997). However, in the resident-intruder test, APP transgenic mice displayed increased aggressiveness compared to the nontransgenic mice. Also, seizures were one of the major symptoms in both APP/Fl and APP/Du mice. Earlier, excitotoxicity mediated by the glutamate neurotransmitter system was implicated in the physiology of hyperactivity, seizures, neurodegeneration, and neuronal cell death in AD (Greenamyre & Young, 1989; Koh *et al.*, 1990; Albin & Greenamyre, 1992; Rothman & Olney, 1995), as well as in more general phenomena like learning and memory (Malenka, 1994; Collingridge & Bliss, 1995). Moreover, APP was also recently shown to participate in the regulation of extracellular levels of excitotoxic neurotransmitters like glutamate, by controlling their uptake into astrocytes (Masliah *et al.*, 1998). Thus we tested sensitivity toward KA and NMDA, as these are the major glutamate agonists in the CNS. LD50 was taken as a pharmacological end point in these experiments, as both KA and NMDA are known to induce a wide range of reactions, which are difficult to monitor. Using this criterion, increased sensitivity against excitotoxic insults was apparent by hypersensitivity of APP transgenic mice toward KA and of hypofunctional NMDA-signaling pathways. Interestingly, NMDA binding is decreased in specific brain regions in AD cases (Greenamyre *et al.*, 1987; Jansen *et al.*, 1990; Rothman & Olney, 1995) while KA binding is increased, at least in the outer molecular layer of the dentate gyrus (Geddes *et al.*, 1992). However, absence of changes in NMDA receptor density or distribution in APP/RK transgenic mice eliciting similar glutamatergic drug response (Moechars *et al.*, 1996) points toward subtle functional disturbance in the glutamate neurotransmitter system (Moechars *et al.*, 1998b).

Nevertheless, the fact that transgenic mouse strains overexpressing APP display neuronal degeneration as well as behavioral disturbances in the absence of amyloid deposits or neurofibrillary pathology, as demonstrated here, in previous studies (Moechars *et al.*, 1996, 1999a), and by others (Hsiao *et al.*, 1995; LaFerla *et al.*, 1995; Moran *et al.*, 1995), suggests a neurotoxic effect of either APP or its C-stubs, which is independent of the amyloid plaque or its major constituent A β . Part of this phenomenon is also explained by an increased sensitivity of certain mouse strains to overexpression of APP in brain (Hsiao *et al.*, 1995; Moechars *et al.*, 1999a). However, since the consistent and common early defects shown here in APP/Du and APP/Fl mice were never observed in other transgenic mouse strains overexpressing proteins unrelated to APP and driven

by the same recombinant thy-1 promoter construct, and such defects were also essentially similar to those documented previously in at least two different mouse genetic backgrounds (Moechars *et al.*, 1996), the specificity for and by APP is guaranteed. A direct role of APP or its intermediates in the development of the present phenotype is supported by recent data on APP/Lo transgenic mice in which a decrease in the density of presynaptic terminals and neurons, and a prominent deficit in hippocampal synaptic transmission on electrophysiology, occurs well before these mice develop amyloid plaques (Moechars *et al.*, 1999a; Hsia *et al.*, 1999).

In summary, the results presented here on human APP/FI and APP/Du transgenic mice demonstrate a shift from α to β cleavage of APP. A β levels did, however, remain low, not exceeding threshold levels for amyloid precipitation and formation of amyloid deposits. In concordance with earlier reports, overexpression of APP and/or its proteolytic products causes an AD-related neuronal pathology. Further studies are needed to address the distinct and common mechanisms that underlie AD as well as HCHWA-D pathophysiology.

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