

Early Phenotypic Changes in Transgenic Mice That Overexpress Different Mutants of Amyloid Precursor Protein in Brain*

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Transgenic mice overexpressing different forms of amyloid precursor protein (APP), i.e. wild type or clinical mutants, displayed an essentially comparable early phenotype in terms of behavior, differential glutamatergic responses, deficits in maintenance of long term potentiation, and premature death. The cognitive impairment, demonstrated in F1 hybrids of the different APP transgenic lines, was significantly different from non-transgenic littermates as early as 3 months of age. Biochemical analysis of secreted and membrane-bound APP, C-terminal “stubs,” and A β (40) and A β (42) peptides in brain indicated that no single intermediate can be responsible for the complex of phenotypic dysfunctions. As expected, the A β (42) levels were most prominent in APP/London transgenic mice and correlated directly with the formation of amyloid plaques in older mice of this line. Plaques were associated with immunoreactivity for hyperphosphorylated tau, eventually signaling some form of tau pathology. In conclusion, the different APP transgenic mouse lines studied display cognitive deficits and phenotypic traits early in life that dissociated in time from the formation of amyloid plaques and will be good models for both early and late neuropathological and clinical aspects of Alzheimer’s disease.

The central role of the amyloid precursor protein (APP)¹ in the pathogenesis of Alzheimer’s disease (AD) was identified by distinct mutations in the APP gene that cause early onset familial AD (for review see Ref. 1). The Swedish (APP/Sw) (2) and London mutation(s) (3) alter APP processing, causing increased production of the A β peptide of 42 amino acids (4), hypothesized to be pivotal in AD pathology (1, 5). Early onset

familial AD caused by mutations in the presenilin genes supports this hypothesis, because they increase production of A β (42) peptide (6, 7) due to the gain of an unknown function (8). The extensive cell biological definition of the metabolic effects of the different mutations in APP *in vitro* requires matching analysis of their physiological impact *in vivo*. Transgenic mice with wild type and different mutant forms of APP have been generated and the original, most wanted end point, i.e. AD-like amyloid plaques in mouse brain, was obtained (9, 10), accompanied by cognitive deficits (11) and by hyperphosphorylation of protein tau (12). In other transgenic mouse strains overexpression of APP caused behavioral, synaptotrophic, and neurodegenerative effects, accelerated senescence, and premature death, in the absence of amyloid deposits (13–16). Intracellular expression of the A β peptide yielded mice with extensive neuronal loss but no amyloidosis (17). Overexpression of the C-terminal domain of APP caused neuronal degeneration (18), whereas in another model, pre-amyloid deposits, hippocampal cell loss, and cognitive deficits were documented (19).

We have generated additional transgenic mouse strains, expressing human APP, either wild type or the London or Swedish clinical mutations, from the neuron-specific mouse *thy-1* gene promoter. Their phenotype was analyzed by biochemical, histochemical, behavioral, electrophysiological, and pharmacological methods. Measurements of different APP metabolites in brain demonstrated that increased A β (42) levels correlated with the formation of amyloid plaques in the brain of old APP/London transgenic mice. The plaques were extensively characterized immunohistochemically and displayed many aspects typically observed in the brain of AD patients. As opposed to plaques that developed only after at least 12 months of age, other deficits were observed from 3 months onwards and included cognitive impairment, decreased long term potentiation, differential glutamatergic responses, aggression, and neophobia, among others. These signs were largely independent of the actual isoform or mutant of APP that was expressed, were not correlated with a single APP metabolite, and are dissociated in time from plaque formation. These mice will be good models to study both early and late, neuropathological, and clinical aspects related to Alzheimer’s disease.

EXPERIMENTAL PROCEDURES

Generation of Transgenic Mice—cDNA coding for human wild type APP (695 isoform), the Swedish (K670N,M671L) mutant (770 isoform), and the London (V642I) mutant (695 isoform) were cloned in the pTSC vector in the mouse *thy-1* gene (16). The purified, linearized minigenes were microinjected into pre-nuclear embryos from superovulated FVB/N females.

Antibodies—Rabbit antisera B11/4 and B12/4, generated against a

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¹ The abbreviations used are: APP, amyloid precursor protein; AD, Alzheimer’s disease; mAb, monoclonal antibody; NMDA, *N*-methyl-D-aspartic acid; KA, kainic acid.

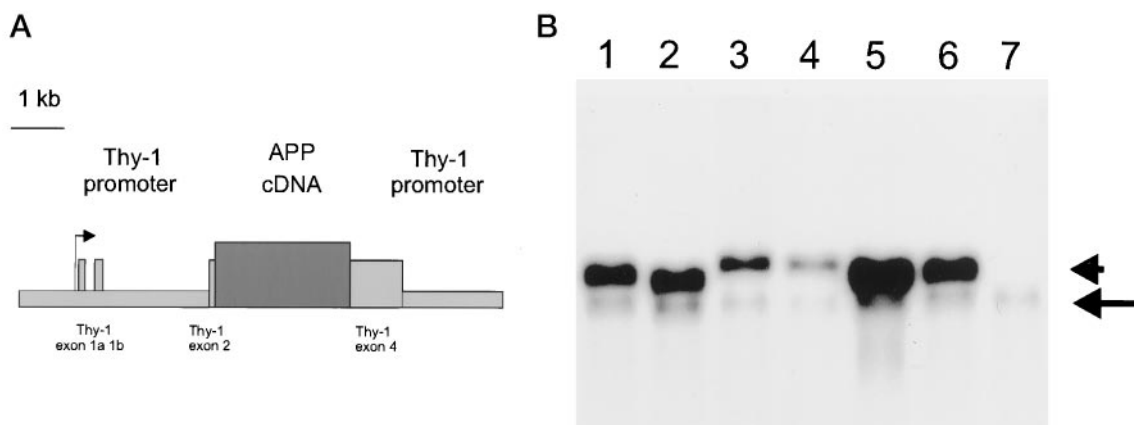


FIG. 1. Generation of APP transgenic mice. *A*, schematic representation of the mouse *thy-1* gene promoter construct. *B*, Northern blot of brain total RNA. Lane 1, APP/Wt/4; lane 2, APP/Wt/2; lane 3, APP/Sw/1; lane 4, APP/Sw/3; lane 5, APP/Ld/2; lane 6, APP/Ld/6; lane 7, nontransgenic FVB/N. The 3.5-kilobase endogenous APP transcript (arrow) and the larger transgenic mRNA (arrowhead).

synthetic peptide representing the 20 C-terminal residues of APP, react equally well with human and mouse APP C-terminal stubs (20). Antibodies FCA18, FCA3340, and FCA3542 were generated against synthetic peptides (21). The following commercially available antibodies were used: SMI31 (Affiniti); anti-GFAP, anti-synaptophysin, and anti-ubiquitin (Dakopatts, Glostrup, Denmark); mAb 22C11 and anti-MAP-2 (Boehringer Mannheim); and mAb 1G5 (Athena Neurosciences, San Francisco, CA).

Analysis of APP Transgene RNA and Protein—Mouse brain RNA was analyzed by Northern blotting (16). Relative mRNA levels of transgene versus endogenous APP were quantified densitometrically after autoradiography. APP protein was analyzed by Western blotting and immunoprecipitation with the antibodies indicated. Total brain was homogenized in 15 volumes of 50 mM Tris, pH 7.4, 150 mM NaCl, and a mixture of proteinase inhibitors (Boehringer Mannheim) and cleared by centrifugation ($100,000 \times g$, 1 h, 4 °C). The pellet was re-extracted with the same buffer containing 2% Triton X-100, 2% Nonidet P40 and centrifuged as before. Proteins 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) for Western blotting (16). C-terminal fragments were immunoprecipitated with rabbit antiserum B11/4, separated on 10–20% Tris-tricine gels, and detected by Western blotting with B12/4 (1/1000) and ECL detection.

Soluble A β Peptides—Mouse brain was homogenized in 6.5 volumes of 20 mM Tris-HCl, pH 8.5, with 5 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 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 ($135,000 \times g$, 1 h, 4 °C and $220,000 \times g$, 2 h, 4 °C) and concentrated on Sepak C18 cartridges (Waters, Milford, MA). Sandwich enzyme-linked immunosorbent assay for A β (40) and A β (42) peptides used mAb 1101.1 to capture and rabbit antiserum BA#1 and mAb 108.1, respectively, as detecting antibodies (22).

Behavioral Tests—Behavioral tests were performed conforming with the Society of Animal Experimentation policy. Corner crossing and aggression tests were described (16). Statistical analysis was by Student's *t* test ($\alpha = 0.05$) and two-way analysis of variance.

Morris Water Maze Test—F1 offspring of matings of transgenic FVB/N males with C57/B16 females were tested. The pool (a white, circular vessel 1 m in diameter) contained water at 20 °C with titanium-oxide as an odorless, nontoxic additive to hide the escape platform. Swimming of each mouse was videotaped and analyzed (Poly-Track, San Diego Instruments, CA). Prior to place navigation tests all mice were allowed a forced swim test for a 120 s period. For place navigation tests, mice were trained to locate the hidden platform in seven blocks of three trials over 4 consecutive days. For different subjects the location of the platform was changed between the four quadrants. 24 h after the last training, each animal was tested (probe trial) with the platform removed. Mice were allowed to search for 60 s and quadrant search time and crossings of the original platform position were measured. The same mice were tested 18 days later for cue navigation tests (platform marked with visible cylinder), and platform location was changed over the four quadrants. Statistical analysis of variance was by one- and two-way analysis of variance with repeated measures and post-hoc analysis for variable escape latency by Student's *t* test.

Electrophysiology—Hippocampal slices were studied in an artificial

cerebrospinal fluid containing 124 mM NaCl, 5 mM KCl, 26 mM NaHCO₃, 1.24 mM KH₂PO₄, 2.4 mM CaCl₂, 1.3 mM MgSO₄, and 10 mM glucose and bubbled with 95% O₂ and 5% CO₂. Slices were cut transversally (400 μ m) in cold artificial cerebrospinal fluid and kept at room temperature till placed in the interface recording chamber at 30 °C. Experiments started 3 h 30 min after dissection to allow recovery of the slice, and the chamber was perfused with artificial cerebrospinal fluid (1 ml/min). A monopolar platinum microelectrode was used to stimulate Schaffer collaterals, and evoked field excitatory postsynaptic potentials were recorded in the stratum radiatum of the CA1 region with low resistance (2 megohm) glass microelectrodes filled with 2 M NaCl. Test stimuli were biphasic pulses of 0.1 ms every 30 s. Tetanus-induced long term potentiation was by two pulse trains of 1 s at 100-Hz, separated by 20 s with each pulse of 0.2 ms. Intensity was adjusted to obtain responses with amplitudes of about 30% of maximal response. The slope of the excitatory postsynaptic potential (mV/ms) was measured from the average wave form from four consecutive responses.

Glutamate Analogs—NMDA and KA, dissolved in pyrogen free 0.9% sodium chloride solution, were injected intraperitoneal in 3–4-month-old mice. The mice were observed for 2 h to determine LD₅₀ by NMDA, while seizures induced by KA were scored in seven stages: lethargy, rigid posture, head bobbing or circling, clonic seizure, rearing alone or with falling, tonic-clonic seizures, and death (16).

Histology and Immunohistochemistry—Mice were perfused transcardially with 4% paraformaldehyde. Brains were fixed overnight at 4 °C, rinsed in phosphate-buffered saline, dehydrated, embedded in paraffin, and sectioned. Histological staining by hematoxylin-eosin, cresyl-violet, or silver impregnation were by routine procedures (24). For immunohistochemistry, sections were dewaxed, rehydrated, and endogenous peroxidase quenched with hydrogen peroxide (1% in 50% methanol). After 1 h in blocking buffer (10% goat serum in 10 mM Tris, pH 7.4, 0.15 M NaCl, 0.1% Triton X-100), appropriately diluted primary antibody was applied (overnight at room temperature). Following rinsing, peroxidase or biotin-conjugated secondary antibody was applied (1:300, 1 h), and the latter was followed by streptavidin-peroxidase. Immunoprecipitates were stained with diaminobenzidine/H₂O₂. For immunostaining for APP and synaptophysin, silanated slides were microwaved in 10 mM sodium citrate, pH 6 (8 min, 450 W). AT-8 staining was performed on free floating vibratome sections. Microglia staining was performed by incubation with biotinylated tomato lectin, followed by streptavidin-peroxidase and staining with diaminobenzidine/H₂O₂.

RESULTS

Transgene Expression—Wild type APP (APP/Wt) and London and Swedish mutant APP (APP/Ld and APP/Sw, respectively) were expressed in the brain of transgenic FVB/N mice by the mouse *thy-1* gene promoter (16). The 13 independent transgenic lines expressed transgene mRNA at moderate to high levels (Fig. 1). In all lines the human APP protein was expressed in hippocampus and in parietal and frontal cortex (levels two to five times higher than endogenous APP), with lower levels in olfactory bulb and thalamus (about 65 and 30%) and very low in cerebellum (results not shown).

Biochemical Analysis of APP Protein Intermediates—APP

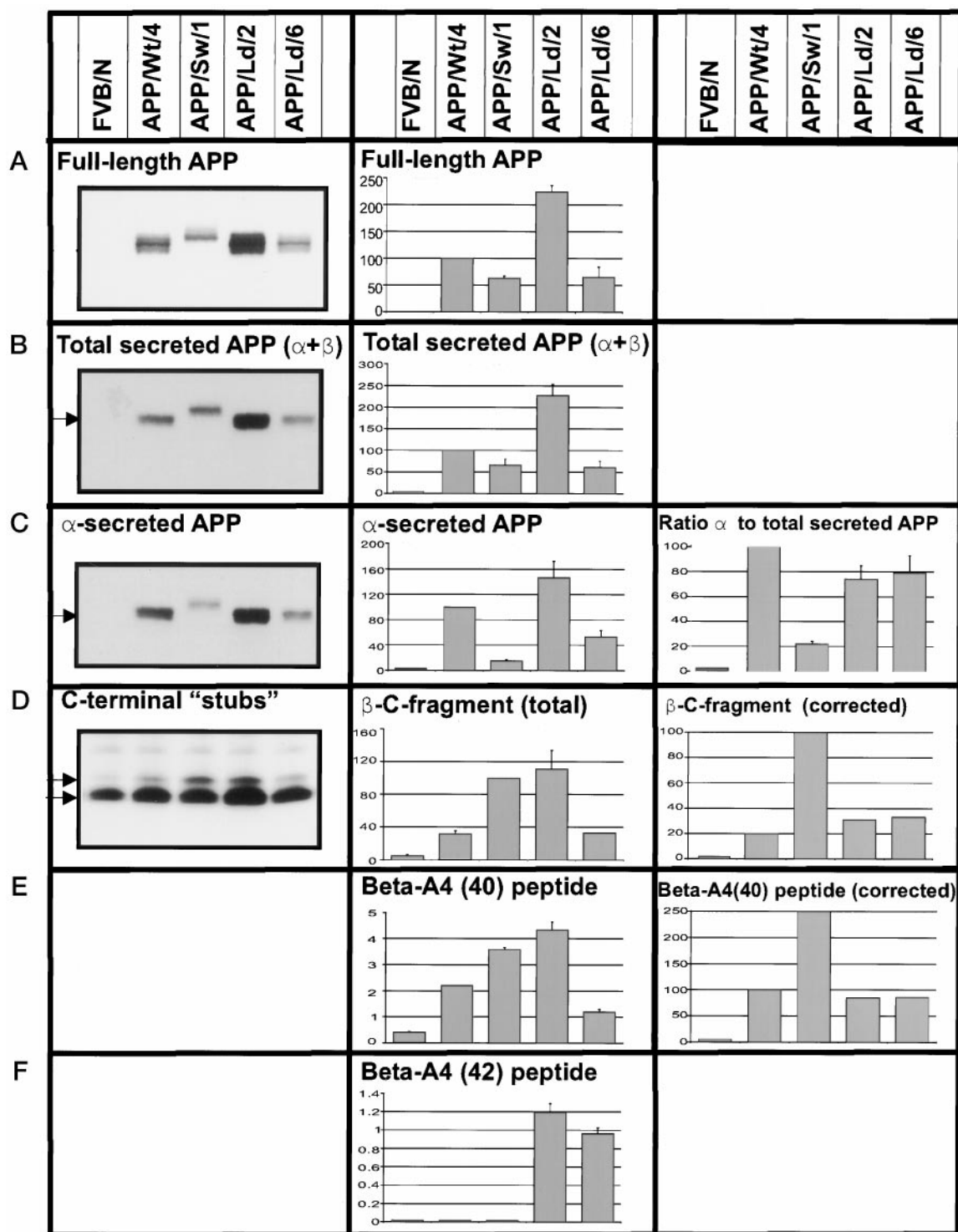


FIG. 2. Analysis of APP and its processing metabolites. *A*, Western blotting with mAb 1G5 of membrane associated human APP. *B*, Western blotting with mAb 1G5 of soluble human α - plus β -cleaved APP. *C*, Western blotting with antiserum R1736 of α -cleaved soluble APP. *D*, immunoprecipitation of α - and the larger β -cleaved C-terminal APP stubs with antiserum B11/4 followed by Western blotting with antiserum B12/4. *E* and *F*, levels of $A\beta(40)$ and $A\beta(42)$ peptides in brain determined by enzyme-linked immunosorbent assay. Quantitative analysis of Western blots was normalized (*middle panels*) and corrected for expression levels per transgenic line (*right panels*). Western blots shown are representative examples, whereas histograms represent the means \pm S.E. of four to six mice per transgenic strain.

intermediates analyzed in the brain of heterozygous transgenic mice, aged between 2 and 4 months, included the integral membrane-bound APP, the secreted α -cleaved and total secreted APP, the residual C-terminal fragments (stubs), and the soluble $A\beta(40)$ and $A\beta(42)$ peptides. In APP/Sw transgenic mice, α -cleaved APP was 5-fold lower than in APP/Wt mice (Fig. 2). The β -cleaved C-terminal fragments were most pro-

nounced in APP/Sw and APP/Ld transgenic mice (Fig. 2). When normalized for expression level, a 3–5-fold overproduction of β -cleaved C-terminal fragments was evident in line APP/Sw/1 relative to all other transgenic lines (Fig. 2, *right column*). Absolute levels of soluble $A\beta(40)$ peptide correlated most closely with levels of β -cleaved C-terminal stubs (Fig. 2), whereas normalization highlighted their preponderant forma-

TABLE I
Overview of different transgenic APP mouse lines with some characteristics

Tabulation of transgenic founders generated from different mini-genes and transgenic lines analyzed in detail for relative expression levels (RNA levels) and mortality in the different APP transgenic lines after a 180- or 360-day period. ND, not determined.

Mini-gene	Founders	Lines	RNA levels	Mortality	
				180	360
APP/Wt	4	APP/Wt/2	6	23.5	53.3
		APP/Wt/4	9	59.1	68.4
APP/Sw	3	APP/Sw/1	7	47.1	70.6
		APP/Sw/3	4	5.4	21.6
APP/Ld	6	APP/Ld/2	25	72.2	ND
		APP/Ld/6	10	36.0	ND

tion in APP/Sw mice. Measurements by specific enzyme-linked immunosorbent assay indicated that A β (42) peptide levels were high only in the brain of APP/Ld mice (Fig. 2). These data essentially confirm the known metabolic effects of mutations in APP, *i.e.* the Swedish mutation specifically favored β -cleavage, resulting in increased concentrations of β -cleaved C-terminal fragments and of A β (40) peptide, whereas the London mutation not only produced β -cleaved C-terminal stubs but produced most conspicuously A β (42) peptides.

Premature Death—The APP transgenic mice died prematurely (16) as reported by others (17, 14). We monitored 136 heterozygous mice from six APP transgenic strains and 46 nontransgenic littermates for 1 year. This revealed that only 4.3% of nontransgenic mice died, as opposed to between 21 and 72% of APP transgenic mice (Table I). Mortality increased with increased APP expression as reported before (16). Premature death was absent in other transgenic strains housed in identical conditions and overexpressing transgenes unrelated to APP at comparable protein levels using the same promoter.

Spontaneous Behavior—Typical for all APP transgenic mice were alternating episodes of hyperactivity, anxiety, and aggression in the home cage. Offspring developed and behaved normally for 6–8 weeks after birth. APP/Ld transgenic mice with the highest expression levels began to show increased agitation from 8 weeks onwards. Progressively, more mice of both sexes in this and other APP transgenic strains displayed this behavior, which was absent in nontransgenic littermates housed and handled in identical conditions. Indications for increased incidence of spontaneous seizures were also noted in the current human APP transgenic mice (16). Spontaneous seizures are clearly not an early phenomenon and were observed in less than 15% of APP transgenic mice older than 6 months. Systematic observation and attempts to obtain encephalographic recordings of epileptic activity proved the rare nature of this phenomenon.²

Open Field-Corner Crossing Test—Many APP transgenic mice showed transient cessation of locomotion upon routine transfer to a new cage. This behavior was systematically approached by measuring motor activity in a corner-crossing variant of the open field test (14, 16, 25) at three different ages. In nontransgenic mice, independent of age, corner crossing behavior was distributed in a gaussian fashion around a mean of about seven crossings in 30 s (Fig. 3 and Table II). All APP transgenic mice were significantly reduced in ambulation, which became progressively more evident with age. Statistical analysis of the most extensively studied APP/Sw/1 mice (Fig. 3 and Table II) was typical for the other transgenic strains; the decrease in ambulation is related to genetic status and age, with a significant interaction of both independent variables

(see legend to Fig. 3). Age was determinant in all transgenic strains, except in the highest expressing APP/Ld/2 transgenic mice, which showed already minimal ambulation even before 3 months old (Table II). In these young APP/Ld/2 transgenic mice, also the second index of motor activity measured, *i.e.* posture freezing induced by the new environment, was already maximal at 3 months, as opposed to all other APP transgenic mice, in which posture freezing frequency increased with age (Table II). Reduced ambulation reflected a neophobic response and not a motor impairment or lack of exploratory motivation, because this behavior was transient and the mice were normally active in the cued variant of the water maze paradigm and the forced swim test (see below).

Morris Water Maze Test—The functional repercussion of expression of the APP transgenes on cognitive processes was measured in the Morris water maze paradigm. Because FVB/N mice are not optimal for this test (Ref. 23 and references therein), we generated F1 hybrid mice from male transgenic mice and female C57/Bl6 mice. This offered the important additional advantage that the genotyped nontransgenic and transgenic littermates constitute perfectly matched experimental groups. Data for heterozygous transgenic mice, aged between 3 and 6 months, of lines APP/Wt/4 and APP/Ld/2 were most intensely studied and are presented, because both of these transgenic lines presented similar early phenotypic traits but differed most prominently in amyloid plaque formation later in life (see “Brain Histology and Immunohistochemistry”). Both in place and in cue navigation tests, nontransgenic F1 mice learned to rapidly locate and escape upon the platform with stable terminal acquisition latency of less than 12 s (place navigation: F(6,497) = 48.3, $p < 0.001$; cue navigation: F(6,497) = 14.18, $p < 0.001$). Transgenic littermates of lines APP/Wt/4 and APP/Ld/2 were less apt, and although they improved with training (APP/Wt/4: F(6,392) = 20.0, $p < 0.001$; APP/Ld/2: F(6,476) = 20.0, $p < 0.001$), they remained significantly impaired in the place navigation test. The overall escape latency, an estimate of spatial learning and memory capacity, was significantly longer for transgenic as opposed to nontransgenic littermates (Fig. 4A). Statistical comparison by two-way analysis of variance of the performances of APP/Wt/4 and of APP/Ld/2 *versus* their respective nontransgenic F1 littermates explained the difference in escape latency by genetic status (APP/Wt/4: F(1,127) = 26.7, $p < 0.001$; APP/Ld/2: F(1,139) = 87.21, $p < 0.001$) and by blocks (F(6,123) = 42.9, $p < 0.001$ and F(6,135) = 22.4, $p < 0.001$) with a significant interactive effect of these independent variables (APP/Wt/4: F(6,123) = 2.5, $p < 0.05$; APP/Ld/2: F(6,135) = 3.4, $p < 0.01$). The impairment in escape latency was not observed when the same transgenic mice were tested to locate the platform visually (cued) rather than from spatial memory (Fig. 4A). This important demonstration of normal motivation to escape and of swimming ability was independently confirmed by a forced swim test (Fig. 4B). The magnitude of the spatial deficit was assessed in a probe test, conducted 24 h after the place navigation test. Nontransgenic F1 mice (F(1,94) = 104.4, $p < 0.001$) and transgenic APP/Wt/4 mice (F(1,69) = 14.5, $p < 0.001$) and APP/Ld/2 mice (F(1,90) = 23.9, $p < 0.001$) searched the correct quadrant (Fig. 4C), confirming that all mice exhibited spatial learning capabilities. The spatial deficit was confirmed by the significant decreased time spent in the correct quadrant by the transgenic APP/Wt/4 mice (F(1,39) = 3.0, $p < 0.01$) and APP/Ld/2 mice (F(1,45) = 3.5, $p < 0.05$) relative to their nontransgenic F1 littermates. A more refined parameter for the spatial bias in the place navigation test is the number of crossings over the exact former location of the platform (26). Nontransgenic F1 mice crossed the correct site significantly more often than their transgenic littermates of line

² J. Noebels, personal communication.

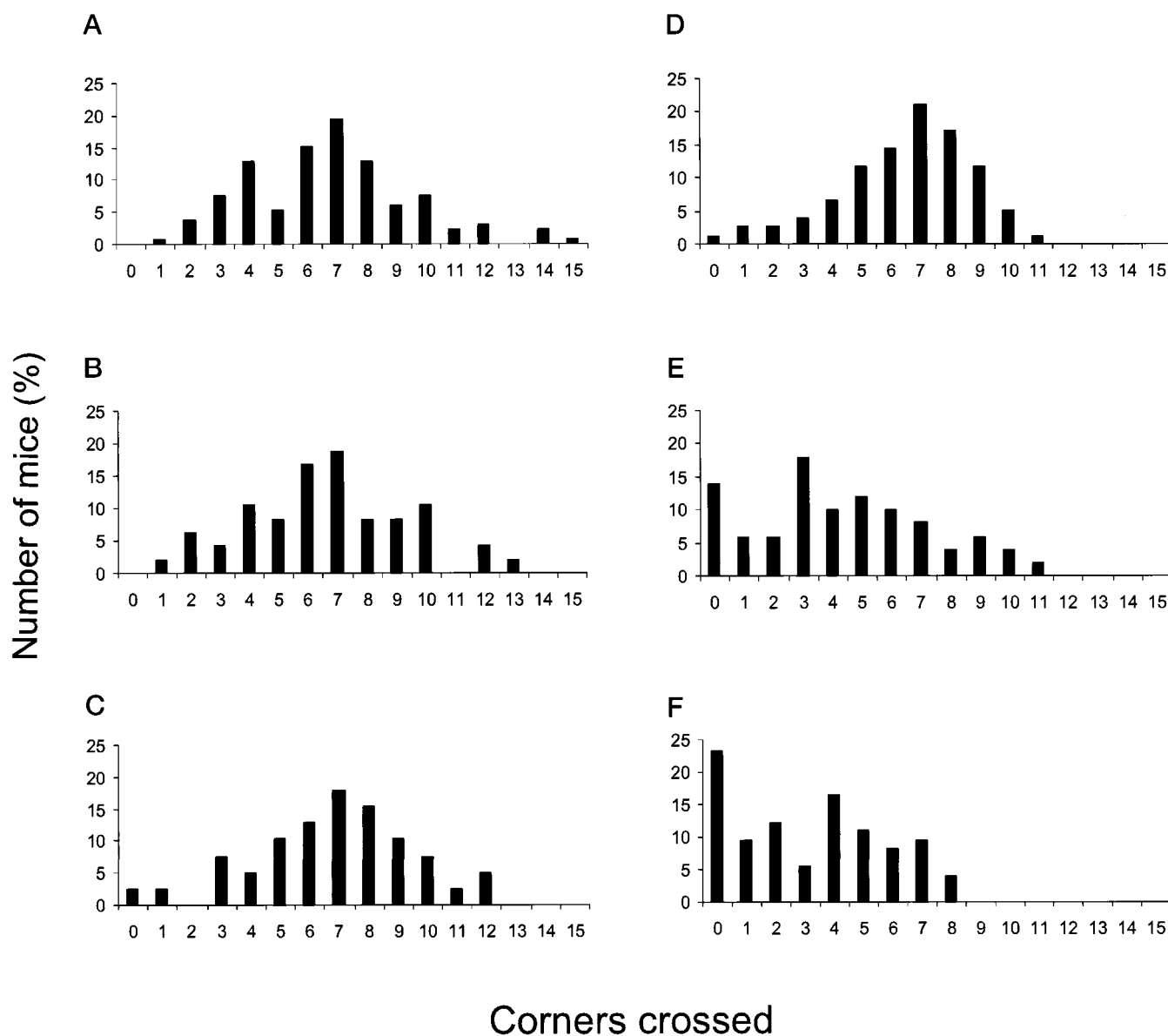


FIG. 3. **Corner crossing test.** Shown is the relative number of mice (*abscissa*) that crossed a given number of corners (*ordinate*) in 30 s. Nontransgenic FVB/N mice of the different age groups reacted similarly: A, 4–9 weeks ($n = 133$); B, 12–17 weeks ($n = 48$); and C, 20–52 weeks ($n = 39$). Three age groups of APP/Sw1 transgenic mice reacted differently with age: D, 4–9 weeks ($n = 76$); E, 12–17 weeks ($n = 50$); and F, 20–52 weeks ($n = 73$). Two-way analysis of variance (genetic status and age) analysis of corner crossing revealed significant effects of genetic status ($F(1,413) = 27.3, p < 0.001$), age ($F(2,413) = 15.1, p < 0.001$) and genetic status combined with age ($F(2,413) = 10.1, p < 0.001$) indicative of a neophobic reaction that progresses with age.

TABLE II
Corner crossing test

Tabulation of the locomotor activity of transgenic APP mice as an index for the neophobic reaction is shown as the average number of corners crossed (mean \pm S.E.) and posture freezing expressed as a percentage of all mice. ND, not done. Statistical significant differences (two-tailed Student's t test) are denoted.

	4–9 weeks			12–17 weeks			20–52 weeks		
	<i>n</i>	Corners \pm S.E.	Freezing	<i>n</i>	Corners \pm S.E.	Freezing	<i>n</i>	Corners \pm S.E.	Freezing
Nontransgenic	133	6.8 \pm 0.2	0.0	48	6.7 \pm 0.4	0.0	39	6.9 \pm 0.4	2.6
APP/Wt/4	18	5.2 \pm 0.7 ^a	11.1	8	4.3 \pm 1.0 ^a	12.5	16	2.0 \pm 0.9 ^b	33.3
APP/Sw1	76	6.5 \pm 0.3	0.0	50	4.6 \pm 0.5 ^b	14.0	73	3.3 \pm 0.3 ^b	23.3
APP/Ld/2	42	1.4 \pm 0.3 ^b	47.6	ND	ND	ND	12	1.3 \pm 0.5 ^b	50.0
APP/Ld/6	14	4.4 \pm 0.8 ^b	7.1	19	2.3 \pm 0.6 ^b	26.3	14	0.9 \pm 0.4 ^b	57.1

^a $P < 0.05$.

^b $P < 0.01$.

APP/Wt/4 ($F(1,41) = 15.75, p < 0.001$) and APP/Ld/2 ($F(1,45) = 18.52, p < 0.001$) (Fig. 4D).

In conclusion, these combined results obtained in the Morris

water maze paradigm demonstrated unequivocally that the APP transgenic mice were markedly cognitively impaired already early in life.

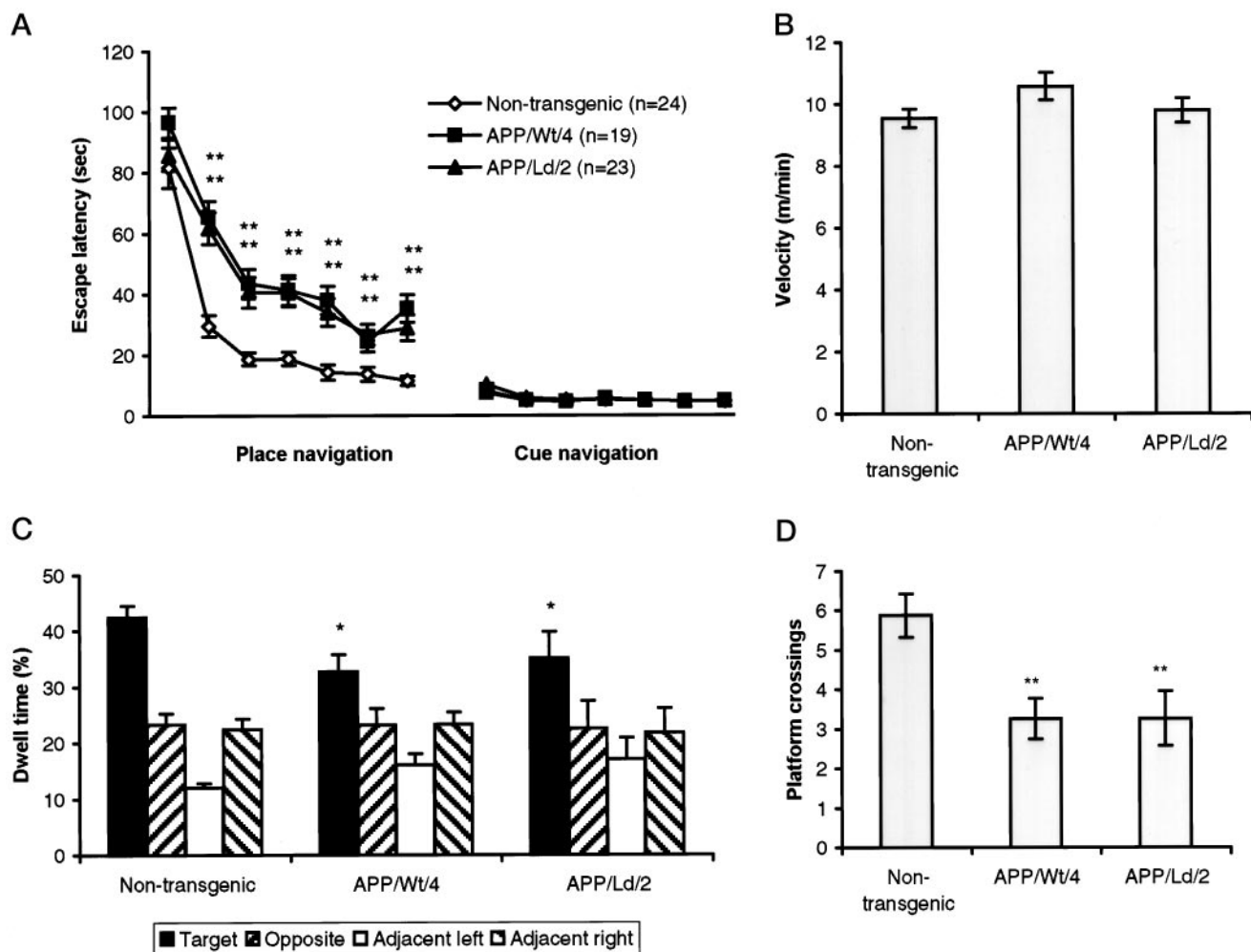


FIG. 4. Morris water maze of transgenic and nontransgenic F1 (FVB/N \times C57/B16) littermates. *A*, mean escape latency in the different blocks of the place and cue navigation tests. Asterisks denote significant differences of transgenic with nontransgenic littermates (** for $p < 0.001$). Student *t* testing of nontransgenic versus transgenic mice showed significant differences in all blocks of the place navigation test apart from the first block but not in cue navigation tests. *B*, average swim velocity in a forced swim test. Student *t* testing revealed no significant differences between nontransgenic and transgenic mice. *C*, average time spent in each quadrant in probe test. Dwelling in the target quadrant is significantly lower for transgenic mice relative to nontransgenic littermates (* for $p < 0.05$). *D*, number of platform crossings is significantly lower for transgenic mice relative to nontransgenic littermates (** for $p < 0.001$).

Electrophysiology—Memory and learning is dependent on changes in the efficiency of synaptic transmission. An excellent and widely used model for this type of plasticity is long term potentiation. Synaptic potentiation was induced in the CA1 in hippocampal slices of 5–7-month-old APP/Ld/2 mice and age-matched nontransgenic littermates, by applying a tetanic stimulus, activating the Schaffer collaterals. There was no significant modification of the degree of short term potentiation in the first 2 min following the stimulation in transgenic mice (207.7 ± 26.5 S.E.) compared with nontransgenic littermates (236.1 ± 20.3). However, with time there was a progressive decay of long term potentiation in the transgenic mice. In nontransgenic mice potentiation was 172 ± 9.6 , 161.1 ± 11.4 , 156.5 ± 5.8 , and 150.9 ± 4.8 after 30, 60, 90, and 120 min, respectively. In transgenic mice potentiation was significantly decreased to 133.8 ± 6.8 ($p < 0.01$, Student's *t* test), 118.2 ± 6.9 ($p < 0.01$), 116.2 ± 5.7 ($p < 0.001$), and 109.9 ± 3.3 ($p < 0.001$) 30, 60, 90, and 120 min after stimulation (Fig. 5C). The degree of paired pulse facilitation measured in transgenic APP/Ld/2 mice was not significantly altered compared with nontransgenic mice (Fig. 6), indicating that presynaptic function is not altered in transgenic mice.

Reactivity to Glutamate Analogs—The present human APP transgenic mice behaved very similar to the previously re-

ported mutant mouse APP (APP/RK) transgenic mice that reacted differentially to glutamate analogs NMDA and KA (16). In nontransgenic FVB/N mice of 3–4 months, the LD₅₀ of NMDA was about 60–70 mg NMDA/kg. All age-matched APP transgenic mice were less sensitive, with LD₅₀ values that ranged between 100 and 140 mg NMDA/kg (results not shown). Sensitivity to KA (cumulative score of seven stages of seizures; see under “Experimental Procedures”) was maximal in nontransgenic mice at 32 mg/kg (mortality of 15%). All APP transgenic mice were more sensitive to KA; maximal cumulative scores were obtained for APP/Wt/4 and APP/Ld/6 mice following doses of 28 and 24 mg KA/kg, respectively, resulting in a mortality of 43 and 29%, respectively (results not shown).

Brain Histology and Immunohistochemistry—Histological analysis was performed on the brain of 60 representative heterozygous transgenic mice, *i.e.* 20 APP/Ld/2 mice (2–18 months), 4 APP/Ld/6 mice (4–14 months), 20 APP/Sw/1 mice (3–25 months), and 6 APP/Wt/4 mice (2–19 months). All were sacrificed when healthy and of normal body weight. Hematoxylin-eosin and cresyl-violet staining did not indicate overt neuronal loss or degeneration, with the exception of one APP/Ld/2 mouse (6 months) and two APP/Sw/1 mice (aged 12 and 25 months), in which some neurons were detected with a condensed nucleus surrounded by an halo of clear cytoplasm. This

FIG. 5. Long term potentiation in hippocampal slices. Samples of traces obtained from a nontransgenic (A) and an APP/Ld/2 mouse (B). Either field excitatory postsynaptic potential recorded 10 min after (left panels) or 1 h 30 after (right panels) tetanic stimulation are superimposed on a same control trace obtained before stimulation. C, field excitatory postsynaptic potential slopes recorded before and after tetanic stimulation in slices from APP/Ld/2 mice (open circles) or nontransgenic mice (filled circles). Each point shown is the mean \pm S.D. ($n = 6$ animals, 6 slices for APP/Ld/2 and nontransgenic mice).

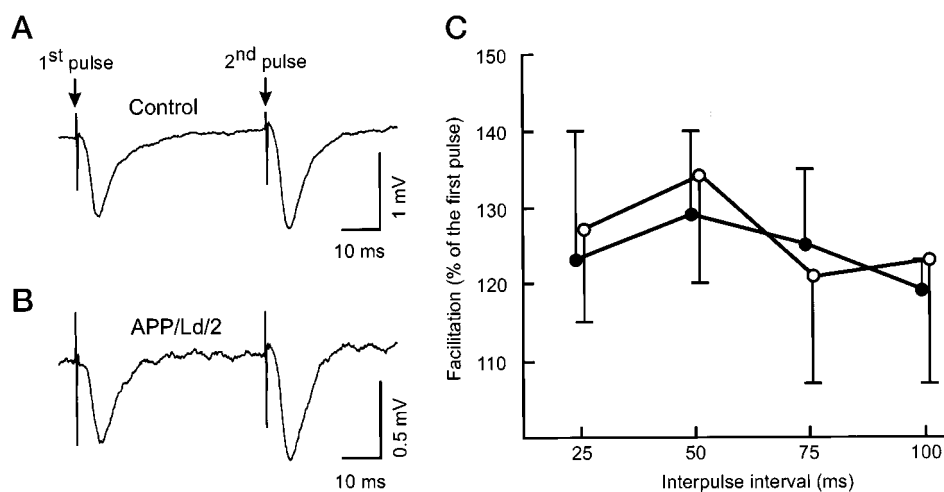
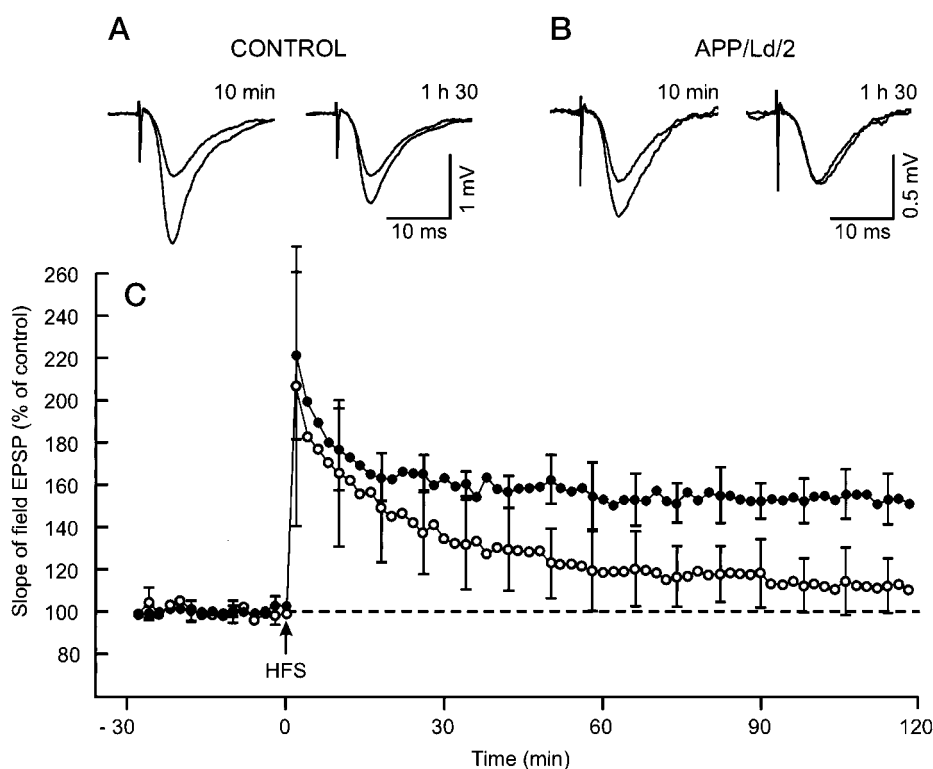


FIG. 6. Paired pulse facilitation in hippocampal slices. Paired pulse facilitation is not altered in APP/Ld/2 mice. Traces showing typical paired pulse facilitation (50-ms interval) in an APP/Ld/2 (A) and in a nontransgenic mouse (B). C, paired pulse facilitation as a function of the interpulse interval. Points are the means \pm S.D. ($n = 6$ animals, 6 slices).

morphology and location at the border of the granular layer and the hilus of the dentate gyrus is reminiscent of transgenic mice that overexpress the amyloid peptide (17) or APP/RK (16, 25, 27). Immunohistological staining for human APP demonstrated that the mouse *thy-1* gene construct steered expression of the transgene predominantly to neurons, resulting in robust staining of large pyramidal neurons in all cortical layers, in hippocampus, and in amygdala. Diffuse amyloid deposits and compact neuritic plaques were detected by silver staining and by immunohistochemistry in all old APP/Ld/2 mice (13–18 months) and in six of ten old APP/Sw mice (18–25 months) (Fig. 7) and were, particularly in the old APP/Ld/2 mice, most abundant in hippocampus and cortex. Occasional deposits were observed in thalamus and fimbria, external capsule, pontine nuclei, and white matter. In the APP/Sw transgenic mice, the amyloid deposits were less frequent but much larger (up to 130 μ m) and were located in the primary olfactory cortex and in the

amygdala. All amyloid deposits reacted with six different antibodies specific for the amyloid peptide (results not shown). Differential staining of adjacent sections with antibodies specific for A β (40) or A β (42) (21) showed preferential staining for A β (42) of the plaques in APP/Ld mice, whereas plaques of APP/Sw mice reacted prevalently with A β (40) antibodies (Fig. 7). Amyloid deposits were absent in the brains of APP/Wt mice and in all mice analyzed when younger than 12 months. The deposits were also readily detected by silver and thioflavin-S staining, resulting in patterns that were reminiscent of AD brain with wisps of fibers radiating from a central mass (Fig. 7). In addition, amyloid deposits were immunoreactive for the ectodomain of APP, for ubiquitin, and for cathepsin D, and they were surrounded by reactive astrocytes and microglia (Fig. 8). Astroglia was further evident in the hippocampus and cortex of many APP transgenic mice from all lines, being more widespread and intense in older mice. Neuritic pathology, *i.e.* dys-

FIG. 7. Immunohistochemical and histological characterization of amyloid plaques. Overview (A) and detail (B) of an immunostaining for A β with FCA18 in the brain of an APP/Ld/2 mouse (18 months). Extensive plaque formation is visible in the hippocampus and entorhinal cortex. C, Garvey silver impregnation of the piriform cortex of an APP/Sw/1 mouse (20 months). D, thioflavin S staining of an amyloid deposit in the thalamus of an APP/Ld/2 mouse (16 months). Also shown is immunostaining of adjacent sections of an APP/Ld/2 mouse (E and F) (16 months) and of an APP/Sw/1 mouse (G and H) (18 months) with FCA3340 antibodies specific for A β (40) (E and G) and with FCA3542 specific for A β (42) (F and H). The plaques in APP/Ld mice show preferential staining for A β (42), in contrast with prevalent A β (40) immunoreactivity in APP/Sw mice. Scale bars: A, 1 mm; B–D, = 20 μ m; E–H, 10 μ m.

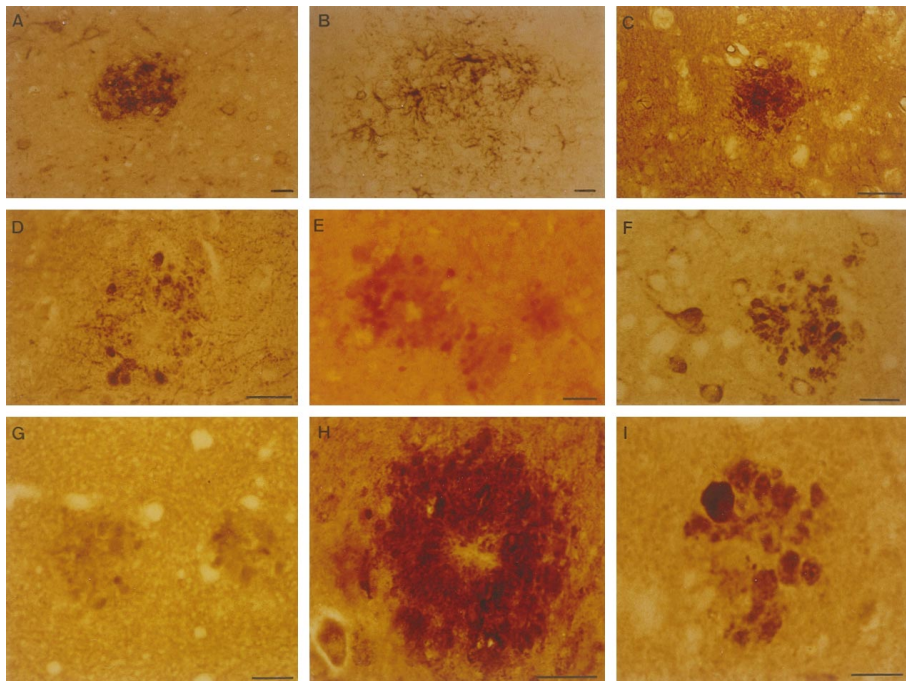
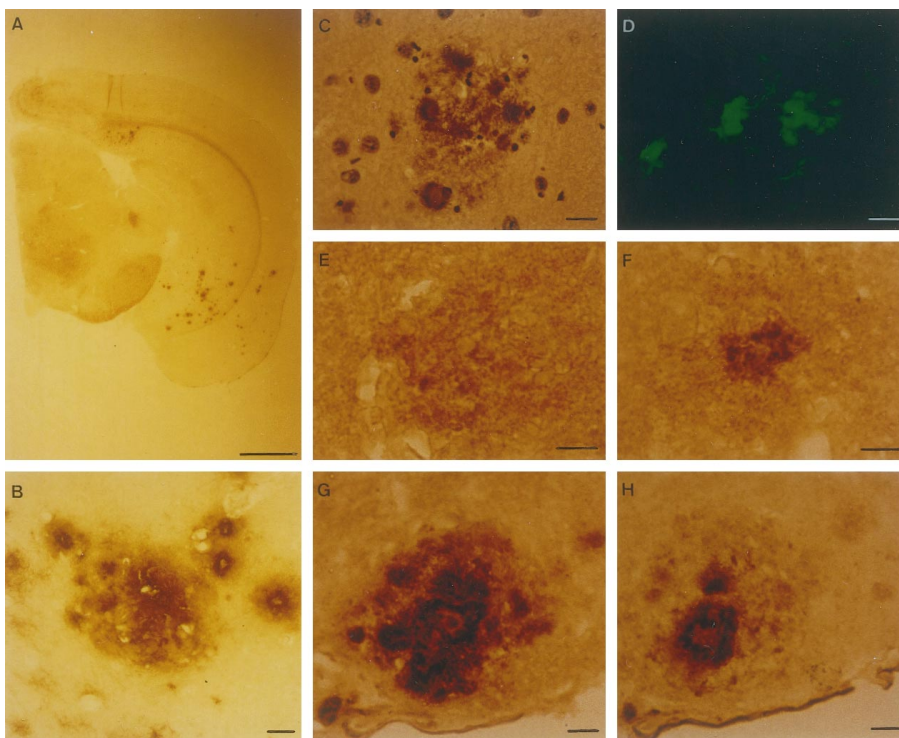


FIG. 8. Immunostaining for different plaque-associated proteins. Immunostaining of adjacent sections of an APP/Ld/2 mouse (16 months) for A β with FCA 18 (A) and anti-GFAP (B) demonstrated reactive astrocytes infiltrating and surrounding the plaques. Microglial staining with tomato lectin also showed positive reaction around plaques (C). Immunostaining with antibody SMI31 (D), AT8 (E), and APP (mAb 1G5) (F) in APP/Ld/2 transgenic mice revealing dystrophic neurites and associated hyperphosphorylated tau. Antibodies against synaptophysin (G), cathepsin D (H), and ubiquitin (I) also reacted with plaques in APP/Ld/2 mice (13–18 months). Scale bars: A–H, 20 μ m; I, 10 μ m.

trophic neurites associated with thioflavine-S-positive amyloid deposits were visualized by immunostaining for APP, ubiquitin, and synaptophysin. Staining with the monoclonal antibody AT-8 recognizing hyperphosphorylated tau and with antibody SMI31, revealing an epitope shared by phosphorylated tau, neurofilament proteins, and MAP-1 detected structures resembling distorted neurites surrounding amyloid plaques, but comparing adjacent sections, less plaques were immunoreactive for AT-8 than for A β .

DISCUSSION

Transgenic mice have been reported that overexpress wild type or mutant APP (9–14, 16), the A β peptide (17), or the C-terminal domain of APP (18, 19). Amyloid plaques, regarded

as the first or ultimate goal, are an acquired phenotypic trait in transgenic mice that overexpress human APP mutants (Refs. 10–12 and this study). These models allow us to test the long standing question in AD, *i.e.* if and how amyloid plaques play a role in cognitive decline and dementia. We have experimentally addressed part of this problem and observed that formation of typical amyloid plaques is a late process, correlated with age and increased levels of A β (42) peptide. Conversely, the earlier phenomena of behavioral and cognitive defects that we have observed and extensively documented as shared by the different APP transgenic mouse lines occur before and independent of amyloid plaques.

The senile plaques in the old APP/Ld transgenic mice were

authenticated by silver and thioflavin-S staining, immunoreactivity for A β (40) and A β (42) peptides, APP, ubiquitin, cathepsin D, and many additional other antigens. The plaques appear very similar to plaques in AD brain, but no indications for neurofibrillary tangles were observed. An epitope typical for hyperphosphorylated tau was, however, detected in dystrophic neurites associated with amyloid plaques, which might be related to similar early neurofibrillary changes in AD (28). Additional comprehensive quantitative analysis of all these parameters in a large number of transgenic mice at different ages is ongoing. The preliminary results already confirm that amyloid plaques in the brain of transgenic mice are a late phenomenon in the overall phenotype, whereas the data presented here clearly dissociate plaque formation in brain from other phenotypic traits, in time but not necessary in mechanism.

The observation of amyloid plaques in transgenic mice older than 12 months especially when producing measurable A β (42) in brain confirms previous reports (11, 29). The documented lesions were not paralleled by neuronal loss in mice from either strain (30, 31). In a third model, plaque formation was observed without reference to A β levels or behavioral or cognitive defects (12). In the present study, the disturbed behavior and cognitive defects occurred before plaque formation, and the reduced water maze performance and deficits were common to all transgenic lines that overexpressed wild type or mutant APP, including those that did not develop amyloid plaques. The defects were never observed in other transgenic mouse strains overexpressing proteins unrelated to APP from a similar recombinant *thy-1* promoter construct. The consistent common early defects in all APP transgenic mouse lines, presented here and previously documented in two different mouse genetic backgrounds (16), are the strongest evidence for the direct role of APP, although it leaves open questions as to which of its intermediates are involved. Differences among the APP transgenic mouse strains in intensity or age of onset of phenotypic characteristics appeared related to the level of the APP transgene. In addition, many of the symptoms intensified and progressively worsened with age, as revealed by the results presented and by research in progress, but signs were already evident in young animals.

Correlation of these observations to precise biochemical intermediates or end products of APP remained elusive, with the exception of amyloid plaque formation. Besides confirming the convincing role of A β (42) in amyloid plaque formation at late age, the data further suggest important molecular pathological aspects of APP in brain. The functional neuronal disturbances can be mediated by the amyloid peptides but in either a molecular form or conformation that is different from that needed for plaque formation (32). In addition, they could exert their effects in combination with the amyloidogenic C-terminal fragments (19, 33). Whereas high levels of A β (42) peptide were confined to APP/Ld transgenic mice, production of A β (40) peptide was general in all transgenic lines and closely linked to levels of the β -cleaved C-terminal stubs of APP, their obligate immediate precursor. The early increased levels of both these APP intermediates correlate with the early occurrence of phenotypic traits common to all APP transgenic lines.

To explain the additional phenotypic traits, the glutamate neurotransmitter system was advanced as a prime candidate in accord with previous reports (16, 19). Glutamate is implicated in phenomena of learning and memory, as well as in hyperactivity, seizures, neophobia, and many other phenomena, underlined by many reports and beyond the present discussion. The impaired learning and memory convincingly demonstrated in the APP transgenic mice and underlined by the electrophysiological data presented might be relevant for the central pathol-

ogy in AD. Possible dysfunction of NMDA signaling pathways, as long term potentiation, is likely to affect memory directly (34–36). Seizures of variable intensity are a clinical feature of advanced AD, although the reported incidence varies widely, *i.e.* from 6 to 64% in late onset AD patients but as high as 70% in early onset familial AD caused by Presenilin-1 mutations, as discussed before (Ref. 16 and references therein).

The presented data are evidence that the phenotype results from overexpression of APP and that it is a functional physiological disturbance and not the consequence of a developmental problem, because expression of APP controlled by the *thy-1* gene promoter construct is immediately after birth about 2 orders of magnitude lower than in adults (results not shown). As already indicated, aspects of the phenotype have been studied and reported by others in different transgenic mouse strains overexpressing APP (9–12, 14, 16). Differences among these studies include the APP isoform or mutant, the expression levels, and the mouse host strain. The current study demonstrates that with the exception of amyloid plaques, the qualitative differences between transgenic lines were minimal. The recipient mouse genetic background continues to remain an issue (37). The very similar phenotype of transgenic mice that overexpress either the A β peptide intracellularly (17), wild type or mutant APP (14), our mouse mutant APP/RK (16), or the human APP transgenic mice presented here indicate that the FVB/N strain presents the lowest threshold to physiological effects of APP and its metabolites. Nevertheless, it remains a fact and very important to note that this is not unique for the FVB/N background because the previously generated APP/RK transgenic mice in the C57/Bl6 and the FVB/N genetic background presented a phenotype that overlapped almost entirely (Ref. 16 and results not shown). Behavioral abnormalities have been reported in other APP overexpressing mice generated on very different genetic backgrounds (15, 38). Moreover, there is an intriguing phenotypic overlap (impairment of learning and long term potentiation) between the presented APP transgenic mice and the transgenic mice expressing a C-terminal 104-amino acid fragment of APP, generated on an outbred background (B6x3H) (19). Insight into the genetic aspects of the different mouse strains is likely to bring forward important information on fundamental issues of AD. In this respect, the F1 hybrid transgenic mice presented in this study, constitute a major advantage in studying the cognitive early deficits in comparison to perfectly matched littermates arising from the same matings. This consideration is very important for behavioral and cognitive studies, and the APP transgenic mice offer models allowing studies of the early and the late phase of APP induced AD-like pathology.

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