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19. Immunophenotypes of patients with RAG mutations were obtained from routine clinical specimens at the admission of the patients. B cells (CD20) and T cells (CD3) are expressed as a percentage of total peripheral blood mononuclear cells (PBMCs): P1: 0% CD20, 0% CD3; P2: 0% CD20, 15% CD3; P3: 0% CD20, 59% CD3; P4: 0% CD20, 15% CD3; P5: 0% CD20, 0% CD3; and P6: 1% CD20, 70% CD3. The CD3-positive cells in P2, P4, and P6 were identified as maternal T cells by HLA typing. The CD3-positive cells in P3 were of patient origin as assessed by HLA classification and minisatellite analysis. The CD3 cells were not revertants because no wild-type RAG signal was detected in PCR or SSCP in MNCs of the patient. V_{α} and V_{β} repertoires were addressed by reverse transcriptase-PCR in PBMCs and exhibited an oligoclonal pattern. Thus, patient P3 was considered leaky.
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22. RAG-1 and RAG-2 expression vectors used for all recombination assays were identical except for the single mutation introduced; thus, promoter and 5' and 3' untranslated region influences on expression are excluded. Cells transfected with RAG expression vectors were boiled in SDS lysis buffer. Equal amounts of total protein (100 μ g) were fractionated by 10% SDS-polyacrylamide gel electrophoresis. Protein was transferred to nitrocellulose and detected by immunoblotting with affinity-purified antibodies to RAG as described (20).
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Correlative Memory Deficits, A β Elevation, and Amyloid Plaques in Transgenic Mice

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Transgenic mice overexpressing the 695-amino acid isoform of human Alzheimer β -amyloid (A β) precursor protein containing a Lys⁶⁷⁰ \rightarrow Asn, Met⁶⁷¹ \rightarrow Leu mutation had normal learning and memory in spatial reference and alternation tasks at 3 months of age but showed impairment by 9 to 10 months of age. A fivefold increase in A β (1–40) and a 14-fold increase in A β (1–42/43) accompanied the appearance of these behavioral deficits. Numerous A β plaques that stained with Congo red dye were present in cortical and limbic structures of mice with elevated amounts of A β . The correlative appearance of behavioral, biochemical, and pathological abnormalities reminiscent of Alzheimer's disease in these transgenic mice suggests new opportunities for exploring the pathophysiology and neurobiology of this disease.

Alzheimer's disease (AD), the most common cause of dementia in aged humans, is a disease of unknown etiology. Amyloid plaques are routinely used for diagnosing AD in brain tissue (1), even though other histologic changes such as neurofibrillary tangles, synaptic and neuronal loss, and dystrophic neurites are also usually present and sometimes correlate better with dementia (2, 3). The amyloid in senile plaques is composed of A β , a 39- to 43-amino acid protein derived from the larger amyloid precursor protein (APP). Small numbers of

classic senile plaques develop in the brain with age, but large numbers of senile plaques are found almost exclusively in patients with Alzheimer's type dementia. A diagnosis of AD is made only if both cognitive deterioration and senile plaques are present (4). APP isoforms resulting from alternative splicing form a set of polypeptides ranging from 563 to 770 residues in length. The most abundant of these, APP₆₉₅, is predominantly expressed in neurons (5) and lacks a Kunitz-protease inhibitor (KPI) domain present in the APP₇₅₁ and APP₇₇₀ isoforms. Five mutations in APP, all located in or near the A β domain, have been identified in families with early-onset AD (6–10).

Transgenic mice (Swiss Webster \times C57B6/DBA2) expressing three isoforms of mutant APP (Val⁷¹⁷ \rightarrow Phe) with an overrepresentation of KPI-containing isoforms showed Alzheimer-type neuropathology, including abundant thioflavin S-positive A β deposits, neuritic plaques, synaptic loss, as-

trocytosis, and microgliosis (11), but deficits in memory and learning have not yet been reported. Transgenic mice (JU) expressing human wild-type APP₇₅₁ showed deficits in spatial reference and alternation tasks by 12 months of age (12). However, only 4% of aged (\geq 12 months) transgenic mice exhibited A β deposits, and these were rare and diffuse and did not stain with Congo red dye (13). Transgenic mice (FVB/N) overexpressing wild-type and variant human or mouse APP₆₉₅ developed a central nervous system disorder that involved most of the corticolimbic regions of the brain (except the somatosensorimotor area) and resembled an accelerated naturally occurring senescent disorder of FVB/N mice (14). Parameters that influence the phenotype of transgenic mice expressing APP include host strain, APP primary structure, and extent of APP expression (14). We investigated the effects of APP overexpression in C57B6/SJL F₂ mice backcrossed to C57B6 breeders because of their greater longevity compared with FVB/N mice expressing identical transgenes.

Human APP₆₉₅ containing the double mutation Lys⁶⁷⁰ \rightarrow Asn, Met⁶⁷¹ \rightarrow Leu (K670N,M671L; APP₇₇₀ numbering), which was found in a large Swedish family with early-onset AD (10), was inserted into a hamster prion protein (PrP) cosmid vector (15) in which the PrP open reading frame (ORF) was replaced with the variant APP ORF [see (14)]. The resulting mice, Tg(HuAPP695.K670N-M671L)2576, produced 5.56 ± 0.33 units (mean \pm SEM; 73-day-old mice) to 5.76 ± 0.74 units (430-day-old mice) of transgenic brain APP expression, where a unit of expression is equivalent to the amount of endogenous mouse APP in nontransgenic (control) littermates (Fig. 1). Transgenic APP expres-

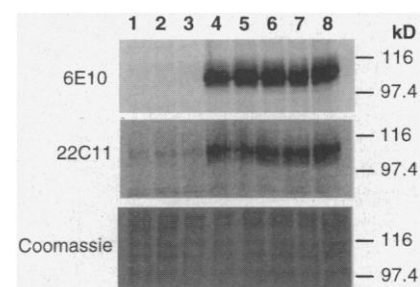


Fig. 1. Brain APP immunoblot of young and old Tg⁺ mice and nontransgenic control mice with 6E10 (24), which recognizes human but not mouse APP, and 22C11 (Boehringer Mannheim), which recognizes both human and mouse APP. Lanes 1 to 3, nontransgenic mice; lanes 4 to 6, 73-day-old mice; lanes 7 and 8, 430-day-old mice. Detailed methods for APP quantitation were described previously (14); antibody binding was revealed with ³⁵S-labeled protein A instead of ¹²⁵I-labeled protein A.

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sion appeared to remain unchanged between 2 and 14 months of age.

Two groups of 7 to 9 transgene-positive (Tg^+) mice and 10 to 11 transgene-negative (Tg^-) control littermates underwent spatial alternation testing in a Y-maze at 3 and 10 months of age. Three groups of 9 to 13 Tg^+ mice and 10 to 14 Tg^- littermates underwent spatial reference learning and memory testing in the Morris water maze (16) at 2, 6, and 9 to 10 months of age. The test experience for each set of mice was novel, and all mice were tested in a coded manner. The 9- to 10-month-old mice were N_1 -generation mice ($C57B6 \times C57B6/SJL F_2$); the 2- and 6-month-old mice were N_2 -generation mice ($C57B6 \times C57B6 \times C57B6/SJL F_2$). A subset of the N_2 -generation mice (8 transgenic and 10 control mice) were retested at 12 to 15 months of age.

When transgenic and control mice were

given a choice of entering either of two arms in a Y-maze, they tended to alternate their choices spontaneously. Ten-month-old transgenic mice, however, showed significantly less tendency ($P < 0.03$) than did age-matched control mice to alternate the arms on successive choices (Fig. 2F). The behavior of the older transgenic mice on the spatial alternation task was characteristic of animals with damage to the hippocampal formation (17).

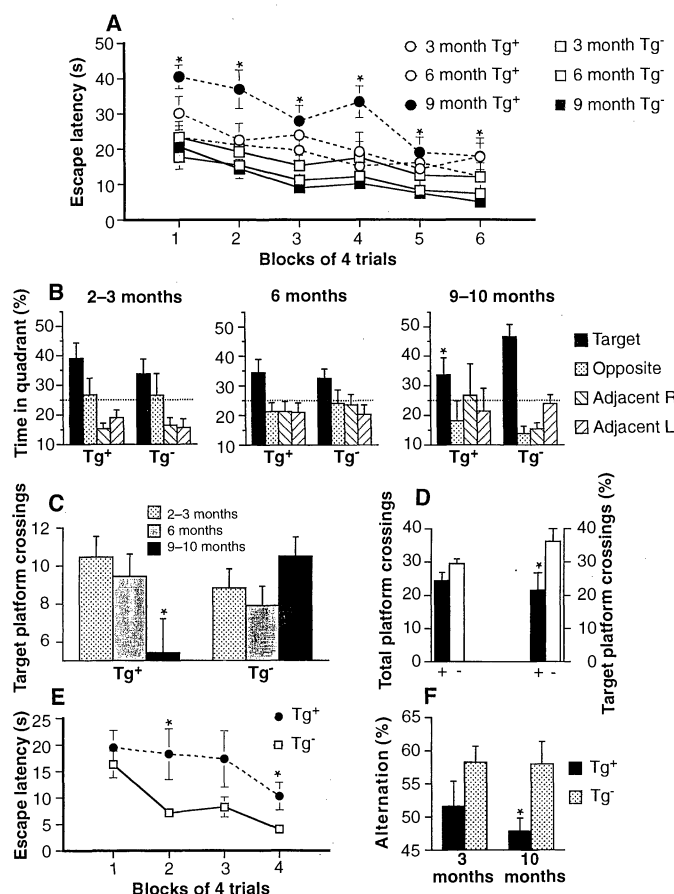
Nine- to 10-month-old transgenic mice were also impaired in their performance in the water maze relative to age-matched controls (18) (Fig. 2). The performance of transgenic mice trained and tested at 2 or 6 months of age was not significantly different from that of age-matched control mice on most measures. The amount of time taken by the mice to reach the hidden platform (the escape latency) did not differ

between 2-month-old transgenic and control mice at any point during training, whereas the latency was significantly different on every day for 9- to 10-month-old mice (19). Six-month-old transgenic mice differed from controls in escape latency only on the last day of training. After the last training day (day 6), all mice were given a probe trial, in which they swam in the pool for 60 s with the platform removed (20). One measure of the animals' knowledge of platform location is the percentage of the 60-s swim spent in the target quadrant (the quadrant that held the platform during training; Fig. 2B). Because the platform is placed in the center of the target quadrant during training, an additional measure that has proven especially useful for mice involves recording the number of times they cross the center of each quadrant. The number of times each mouse crossed the center of the target quadrant (platform crossings; Fig. 2C) and the percentage of total quadrant center crossings that were in the target quadrant were both significantly different [$21.5 \pm 5.2\%$ for transgenic mice versus $36.1 \pm 3.9\%$ for control mice ($P < 0.05$), where 25% is performance at the level of chance] (Fig. 2D) for 9- to 10-month-old transgenic mice compared with age-matched controls.

When 12- to 15-month-old N_2 -generation transgenic mice were retested in the water maze (after rearranging the extramaze cues), they showed significantly impaired performance ($P < 0.05$) compared with control littermates on escape latencies after the fifth trial block and on probe trials given after the sixth and ninth trial blocks. These data suggest that the age-related learning impairment seen in N_1 -generation Tg^+ mice can occur despite further genetic dilution of the SJL strain. Although the escape latencies of the transgenic N_2 -generation mice were significantly longer than those of their control littermates, they were also shorter than those of naïve Tg^+ mice of comparable age. Thus, deficits in escape latency in aged transgenic mice are unlikely to result from difficulty in swimming, as aged mice given sufficient practice can swim as well as younger mice.

Because it is possible that the performance of older transgenic mice was attributable to sensory or motor impairments, we also tested 9- to 10-month-old mice on the visible-platform version of the water maze (Fig. 2E). Although differences in escape latency were evident on the second and fourth of four training days, there were no differences on day 1. These data suggest that although older transgenic mice may show generalized cognitive impairment, they are capable of performing as well as controls when both are relatively naïve. We

Fig. 2. Learning and memory tests of transgenic and control mice. Asterisks indicate measures in which transgenic mice differed significantly from controls ($P < 0.05$). (A) The latency to escape to the hidden platform in the water maze is impaired in Tg^+ mice relative to age-matched nontransgenic controls (19). Although the impairment increases with age, Tg^+ mice showed a consistent trend toward longer escape latencies than those of Tg^- controls. (B) After 24 trials (over 6 days) with the platform in its fixed location, mice were given a probe trial in which they swam for 60 s with the platform removed. Two- and 6-month-old Tg^- and Tg^+ mice spent significantly more than 25% of their time in the target quadrant, indicating that they had learned its location. Although 9- to 10-month-old control mice still searched selectively for the platform, older transgenic mice spent no more time in the target quadrant than in the other three quadrants, suggesting that they had not learned the platform's location (20). (C) The implications of (B) are supported by the observation that on probe trials, 9- to 10-month-old Tg^+ mice crossed what had been the exact location of the platform significantly less frequently than did age-matched Tg^- mice. (D) The bars on the left indicate that transgenic (+) mice did not differ from control (-) mice in the total number of platform crossings (that is, the centers of all four quadrants); the bars on the right show the significant difference between 9- to 10-month-old transgenic mice and controls on the percentage of total platform crossings that were over the target. (E) Nine- to 10-month-old Tg^+ mice were also impaired in swimming to a visible platform, although escape latencies did not differ significantly on the first visible-platform training trial. (F) Aged Tg^+ mice were impaired in their tendency to spontaneously alternate arm-entry in a Y-maze, another behavioral task sensitive to hippocampal damage.



also compared motor performance of the transgenic and control 9-month-old mice by scoring the total number of times during the probe trial that each mouse crossed imaginary platforms located in each of the four quadrants. If impaired mice swim normally but in a random pattern during probe trials, they should cross the center of all four quadrants combined as many times as would unimpaired mice; they will simply cross the target platform fewer times. If, on the other hand, they are impaired on probe trials simply because they are not swimming, there will be fewer total platform crossings. In fact, the total numbers of platform crossings for transgenic mice (24.4 ± 8.7 , mean \pm SEM) and control mice (29.5 ± 1.4) were not significantly different, which indicated that motor impairment was not a cause of poor performance in the water maze (Fig. 2D).

After behavioral testing, a subset of each group of mice was killed painlessly. One hemisphere was frozen for cerebral cortical A β measurements, and the other hemisphere was immersion-fixed for histopathological analysis. All brains were analyzed in a coded fashion. Measurements of A β (1–40) and of A β (1–42/43) were done with the use of

either the Ban-50/Ba-27 or Ban-50/Bc-05 enzyme-linked immunosorbent assay (ELISA) systems (21, 22). These measurements showed a fivefold increase in the concentration of A β (1–40) ($P = 0.03$, rank sum test) and a 14-fold increase in that of A β (1–42/43) ($P = 0.03$, rank sum test) between the youngest (2 to 8 months) and oldest (11 to 13 months) Tg⁺ mice (Table 1). Thus, there was an association between significantly elevated amounts of A β and the appearance of memory and learning deficits in the oldest group of transgenic mice.

Classic senile plaques (with dense amyloid cores) and diffuse deposits were both present in all three mice with elevated A β , as determined by ELISA. The A β deposits were immunoreactive with antibodies recognizing A β (1–5) (23), A β (1–17) (24), A β (17–24) (25), A β (34–40) (26), A β (42/43) (27), and free A β 42 (28). The same plaques were readily identified with multiple antibodies on adjacent sections and were not seen with preimmune or nonspecific ascites, and the immunoreactivity was eliminated by preabsorption with the relevant peptides (Fig. 3). Deposits could not be found in the older or younger controls or in

the younger transgenic mice examined. The deposits were found in frontal, temporal, and entorhinal cortex, hippocampus, pre-subiculum, subiculum, and cerebellum, in a pattern similar to that reported by Games *et al.* (11). Dense amyloid plaques were most frequent in cortex, subiculum, and presubiculum. The dense amyloid deposits were readily detected with thioflavin S fluorescence and typically could also be labeled with Congo red to give the characteristic apple-green birefringence of classical amyloid (29). Some small deposits had the “Maltese cross” signature pattern of the amyloid cores found in AD brains. Under high magnification, the thioflavin S- and Congo red-positive amyloid plaques usually exhibited wisps or fibers radiating from the central mass, which was often ringed by glial nuclei with both astrocytic and microglial morphology. Glial fibrillary acidic protein-immunoreactive astrocytes were associated with amyloid deposition. Staining by the Gallyas silver method revealed dystrophic neurites surrounding dense core plaques.

In contrast to plaques from patients with sporadic AD, antibodies to β 1 and to both free A β (42) and A β (34–40) (which preferentially recognizes x-40) labeled the ma-

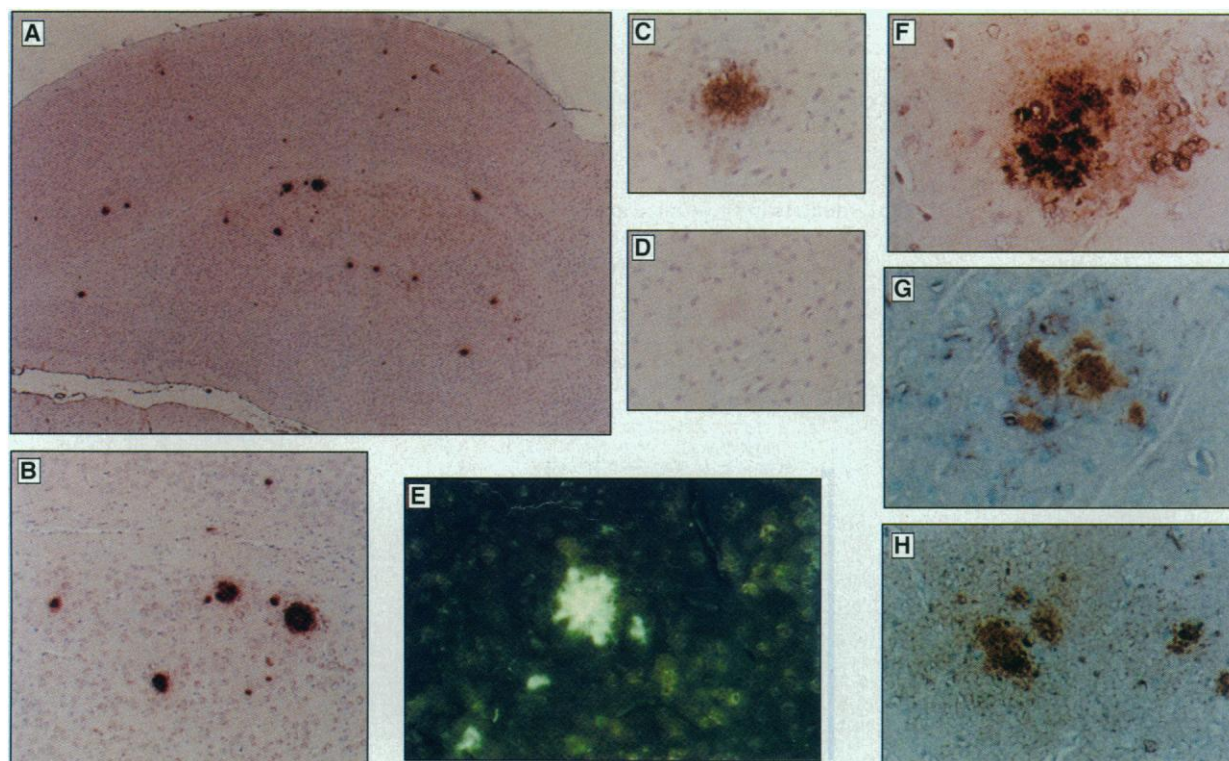


Fig. 3. Extracellular amyloid deposits in transgenic mice A01493 (age, 368 days) and A01488 (354 days) overexpressing human APP₆₉₅ with the K670N,M671L mutation. (A) A01493, multiple plaques in the cerebral cortex and subiculum staining with 4G8 mAb. (B) A01493, inset from (A). (C) A01488, plaque in subiculum staining with 4G8 mAb. (D) A01488, plaque in section adjacent to (C) fails to stain with 4G8 mAb preabsorbed with A β (14–24). (E) A01488, plaques staining with thioflavin S. (F)

A01488, plaque staining with A β (1) affinity-purified antiserum specifically recognizing the NH₂-terminus of A β . (G) A01488, plaque staining with A β (42) affinity-purified antiserum specifically recognizing the COOH-terminus of A β (1–42). (H) A01488, plaque staining with α 40 affinity-purified antiserum specifically recognizing the COOH-terminus of A β (1–40). Magnifications: $\times 100$ (A), $\times 250$ (B), $\times 1000$ (C, D, F, and G), $\times 640$ (E), and $\times 500$ (H).

Table 1. Concentrations of A β in transgenic and control mouse brains. Brain tissue was stained with monoclonal antibody (mAb) 4G8 (25), which recognizes both mouse and human A β . All amyloid deposits stained with 6E10 (24), which specifically recognizes human A β . No extracellular 6E10 staining was detected in three 105- to 106-day-old Tg⁺ mice or one 155-day-old Tg⁺ mouse (A01480, A01547, A01548, and Tg2576 founder). ++, 2 to 5 plaques per section; +++, 6 to 10 plaques per section; + + + +, >10 plaques per section; -, no staining. Because all the pathological specimens were analyzed in a coded fashion, some nonspecific, equivocal staining that could not be blocked by preabsorption of the antibody with specific peptides was observed in some sections (indicated by \pm).

Mouse number	Trans-gene	Age when killed (days)	A β (1-40) (pmol/g)	A β (1-42/43) (pmol/g)	Amyloid plaques
<i>Mice killed at 11 to 13 months of age</i>					
A01484	+	361	325	219	+++
A01488	+	354	192	129	++
A01489	-	354	<2	<2	\pm
A01492	-	371	<2	<2	-
A01493	+	368	273	177	+++
A01495	-	354	<2	<2	-
A01496	-	354	<2	<2	\pm
Mean (\pm SEM) A β concentration in Tg ⁺ mice:			264 \pm 38	175 \pm 26	
<i>Mice killed at 6 to 8 months of age</i>					
A01984	-	233	<2	<2	\pm
A01987	-	219	<2	<2	-
A01989	+	219	45	18	-
A02561	-	214	<2	<2	-
A02595	-	207	<2	<2	-
<i>Mice killed at 2 to 5 months of age</i>					
A02428	-	139	<2	<2	-
A02429	-	139	<2	<2	-
A02430	-	139	<2	<2	-
A02565	+	118	71	21	-
A02900	-	85	<2	<2	-
A03103	+	67	32	2	-
A03107	+	67	45	10	-
Mean (\pm SEM) A β concentration in Tg ⁺ mice:			48 \pm 8	13 \pm 4	

jority of deposits. This may reflect the APP₆₇₀₋₆₇₁ mutations, which greatly increase cleavage at the β 1 site, leading to large concentrations of all fragments beginning with the β 1 epitope. In contrast, the Val⁷¹⁷ \rightarrow Phe mutations increase the percentage of x-42 (21, 30).

Our results demonstrate the feasibility of creating transgenic mice with robust behavioral and pathological features resembling those found in AD. Impairment in learning and memory became apparent in mice 9 months of age and older; this impairment was correlated with markedly increased amounts of A β and was accompanied by numerous amyloid plaques and A β deposits. We have demonstrated that an APP transgene lacking the KPI domain is also capable of engendering amyloid plaques in mice. The increase in the concentration of A β cannot be explained by a rise in transgenic APP expression, which appeared to remain unchanged with age. Concentrations of A β (1-42/43) rose more markedly than did those of A β (1-40). This result parallels the finding in humans with presenilin 1 and presenilin 2 mutations showing more significant elevations of A β (1-42/43) than of A β (1-40) in serum and cultured fibroblasts (31). Studies correlating individual performance in learning and memory tests with

concentration of A β and extent of amyloid deposition may help to ascertain the contribution of each parameter to behavioral deficits. Whether the learning and memory deficits in these mice are caused by or merely correlate with a rise in brain A β levels and amyloid deposition remains unresolved.

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- The water maze was a circular pool (diameter 1 m) filled with water maintained at 29°C and made opaque by the addition of powdered milk. Mice were pretrained by swimming to a 12.7 cm by 12.7

cm Plexiglas platform that was submerged 1.5 cm beneath the surface of the water and placed at random locations within the pool. During pretraining, heavy curtains were drawn around the pool so that mice were unfamiliar with the extramaze room cues on the first day of spatial training. Spatial training consisted of four trials per day, each trial lasting until the mouse reached the platform or 60 s, whichever came first. After each trial, mice remained on the platform for 30 s. Twenty-four hours after the 12th and 24th trials, all mice were subjected to a probe trial in which they swam for 60 s in the pool with the platform removed. Mice were monitored by a camera mounted in the ceiling directly above the pool, and all trials were stored on videotape for subsequent analysis of platform crossings and percent time spent in each quadrant during probe trials. Visible-platform training—in the same pool but with a platform that was black, slightly larger (14.2 cm by 14.2 cm), and raised above the surface of the water—was given at least 24 hours after the second probe trial. The platform location was varied randomly from trial to trial to eliminate the potentially confounding contribution of extramaze spatial cues. In both visible-platform and hidden-platform versions, mice were placed in the pool facing toward the wall of the pool in one of seven randomly selected locations. The numbers of mice tested in the water maze were 12 transgenic and 12 controls at 2 months, 13 transgenic and 14 controls at 6 months, and 9 transgenic and 10 controls at 9 to 10 months of age.

- The escape latency data were examined with a multifactor analysis of variance (ANOVA) including genotype (transgenic vs. control), age (2 months, 6 months, or 9 to 10 months), and training day (four trials per day). The ANOVA revealed significant main effects of genotype [$F(1, 384) = 65.19, P < 0.0001$], age [$F(2, 384) = 7.64, P < 0.001$], and trial block [$F(5, 384) = 12.20, P < 0.0001$]. Moreover, there was a significant interaction between genotype and age [$F(2, 384) = 10.13, P < 0.0001$], indicating that the transgene-induced impairment of escape latency increases with age.
- All mice were also given a probe trial after 12 training trials (3 days at four trials per day). However, neither the transgenic nor the control mice had learned to search selectively after only 12 trials. The early probe trial was necessary because of the possibility of transient differences manifested only early in training, and because of the likelihood that we would have missed these differences because all behavioral tests were conducted blind to genotype. As none of the mice learned the task, there were no differences among any groups; for the sake of clarity, these data have not been presented graphically.
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