Accelerated Plaque Accumulation, Associative Learning Deficits, and Up-regulation of α7 Nicotinic Receptor Protein in Transgenic Mice Co-expressing Mutant Human Presenilin 1 and Amyloid Precursor Proteins

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Familial Alzheimer’s disease-associated mutations in presenilin 1 or 2 or amyloid precursor protein result in elevated β-amyloid, β-amyloid accumulation, and plaque formation in the brains of affected individuals. By crossing presenilin 1 transgenic mice carrying the A246E mutation with plaque-producing amyloid precursor protein K670N/M671L transgenic mice (Tg2576), we show that co-expression of both mutant transgenes results in acceleration of amyloid accumulation and associative learning deficits. At 5 months of age with no detectable plaque pathology, amyloid precursor protein transgenic animals are impaired in contextual fear learning following two pairings of conditioned and unconditioned stimuli but appear normal following a more robust five-pairing training. At 9 months of age when β-amyloid deposition is evident, these mice are impaired following both two-pairing and five-pairing protocols. Mice carrying both transgenes are impaired in contextual fear conditioning at either age. All transgenic animal groups performed as well as controls in cued fear conditioning, indicating that the contextual fear learning deficits are hippocampus-specific. The associative learning impairments are coincident with elevated α7 nicotinic acetylcholine receptor protein in the dentate gyrus. These findings provide two robust and rapid assays for β-amyloid-associated effects that can be performed on young animals: impaired contextual fear learning and up-regulation of α7 nicotinic receptors.

Early on, Alzheimer’s disease (AD) presents clinically as impaired memory formation, yet despite intensive study the mechanisms underlying AD-related memory dysfunction remain mysterious. Familial AD (FAD) is associated with several risk factors, the best-correlated being age and the inheritance of specific genes (mutations or allele type) that result in increased β-amyloid (Aβ) production. The discovery that soluble Aβ is elevated in the brains of AD patients raises the issue whether these molecules play a causative role in AD (1). Aβ is generated from the amyloid precursor protein (APP) through endoproteolytic cleavage by β- and γ-secretases (2). In normal individuals, Aβ40 comprises the majority of the Aβ population; a far smaller fraction is made up of Aβ42 (1). Aβ42 is highly fibrilligenic and exhibits toxic effects on neurons (3–5).

Utilizing acute and organotypic hippocampal slice preparations, we have recently shown that Aβ42 activates the mitogen-activated protein kinase (MAPK) cascade through a7 nicotinic acetylcholine receptors (a7 nAChRs) (6). We also demonstrated that elevation of Aβ in vivo using an animal model for AD (Tg2576) (7), leads to the up-regulation of hippocampal a7 nAChR protein. a7 nAChR up-regulation in the hippocampus of Tg2576 animals negatively correlates with their performance in the Morris water maze, a hippocampus-dependent spatial learning task, and a7 nAChR up-regulation occurs concomitantly with dysregulation of the 42-kDa isoform of extrasynaptic signal-regulated kinase (ERK2) MAPK (6). Considering that ERK MAPK activity is necessary for robust spatial learning, a7 nAChR up-regulation in hippocampus may serve as a biochemical marker for the synaptic plasticity impairments and learning and memory deficits in Tg2576 animals (8–10). Our working model posits that hippocampus-dependent learning and memory impairments in AD arise because of increased Aβ burden and chronic activation of the ERK MAPK cascade in hippocampus through α7 nAChRs.

We tested the hypothesis that transgenic animals in which Aβ is elevated to varying degrees will exhibit hippocampus-dependent behavioral impairments. We also tested whether the cognitive deficits might precede plaque deposition and coincide with increased a7 nAChR protein in the hippocampus. In this study, we performed a histopathological, biochemical, and behavioral characterization of three transgenic mouse lines expressing mutant human PS-1 (A246E) (11), mutant human APP (K670N/M671L) (7), or both the mutated human genes that are linked to FAD. We found that plaque appearance is accelerated in the brains of PS-1/APP transgenic animals. In addition, the three groups of transgenic animals and non-trans-
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75-W bulb. The open field was divided into 225 equally sized squares by 15 photoreceptor beams on each side of the arena. Locomotor activity was quantified using a Digiscan optical animal activity system (RX2ZCM, Aecusan Electronics) (16). Activity measures represent the number of photoreceptor beam breaks in both the horizontal and vertical planes collected in 2-min intervals over a 30-min test period. Additional activity data include the total distance traveled in the horizontal plane (in cm) and the ratio of the time spent in the 16 most centrally located squares compared with the total distance traveled (center:distance ratio): 5 months, n = 11 control animals, n = 6 PS-1, n = 19 APP, and n = 12 PS-1/APP transgenic animals; 9 months, n = 11 control animals, n = 9 PS-1, n = 15 APP, and n = 9 PS-1/APP transgenic animals.

Rotarod Test—Motor coordination, balance, and motor learning were evaluated with the accelerating rotarot test. In this test, mice were assessed for their ability to maintain their balance on a rotating bar that accelerates 7.2 rpm/min. Over the course of each 5-min trial, the speed of the rotarot accelerated from 4 to 40 rpm. The amount of time before the subject fell from the rod was measured. Mice underwent four rotarot trials per day for two consecutive days with an intertrial interval of no less than 30 min: 5 months, n = 10 control animals, n = 6 PS-1, n = 18 APP, and n = 11 PS-1/APP transgenic animals; 9 months, n = 11 control animals, n = 9 PS-1, n = 14 APP, and n = 7 PS-1/APP transgenic animals.

Shock Threshold—Sensory perception of the shock used in fear conditioning was determined through shock threshold assessment. A sequence of single foot shocks was delivered to animals placed on the same electrified grid used for fear conditioning. Initially, a 0.1-mV shock was delivered for 1 s, and the animals’ behavior was evaluated for flinching, jumping, and vocalization. At 30-s intervals the shock intensity was increase by 0.1 mV up to 0.7 mV and then returned to 0 mV in 0.1-mV increments at 30-s intervals. Threshold to vocalization, flinching, and then jumping was quantified for each animal by averaging the shock intensity at which each animal manifested a behavioral response to the foot shock: 9 months, n = 11 control animals, n = 6 PS-1, n = 4 APP, and n = 5 PS-1/APP transgenic animals.

Fear Conditioning and Assessing Conditioned Fear-tested Associative Learning—First, with a two-pairing paradigm of cue and mild foot shock pairing, fear conditioning was initiated by conditioning mice for a total of 7 min. Animals were left free to explore for 3 min, and then a 30-s acoustic-conditioned stimulus (CS; white noise, 80 dB) was delivered. At the end of the CS, a 2-s shock unconditioned stimulus (US; 0.5 mA) was applied to the grid floor. The CS-US pairing was delivered again at the 5-min mark. To evaluate contextual fear learning, the animals were returned to the training context 24 h posttraining, and freezing behavior was scored for 5 min. To evaluate cued fear learning, the animals were placed in a different context (novel odor, lighting, cage floor, and visual cues) following contextual testing. Baseline behavior was scored for 3 min, and then the CS was presented for a period of 3 min. Freezing behavior was scored as follows: each animal was observed for ~1 s every 5 s and judged to be freezing or not by postural criteria. Data is expressed as percent freezing in 30- or 60-s epochs, each epoch divided into 6 or 12 5-s bins. The experimenter was blind to the genotype of the subjects. No less than six animals of each genotype were tested per group.

For the five-pairing paradigm, 20-s acoustic CS-US pairings were given five times. There was a 40-s interval between each CS-US pairing. All other aspects of this protocol were the same as in the two-pairing paradigm.

RESULTS

To produce mice of the desired genotypes, APP K670N/M671L transgenic mice (7) were crossed with PS-1 transgenic mice containing the A246E FAD mutation (11). Nomenclature for the transgenic animal lines is as follows: PS-1 refers to animals heterozygous for the PS-1 A246E transgene; APP refers to animals heterozygous for the APP (A246E) PS-1 (50% C57B6, 50% SJL) (11) transgenic mice to generate nontransgenic controls were evaluated for behavioral and associative learning performance at 5 and 9 months of age. Behavioral analyses were followed by biochemical measurement of α7 nAChR levels in CA1 and DG of the hippocampus. We found an age-dependent decrement in hippocampus-dependent associative learning. This cognitive defect coincides with elevated α7 nAChR protein in DG. Our data provide evidence that the age-of-onset for behavioral manifestation of hippocampal dysfunction precedes gross plaque deposition, and α7 nAChR protein level in the hippocampus serves as a biochemical marker for this defect.

MATERIALS AND METHODS

Mouse Lines—To generate the four mouse genotypes used in this study, heterozygous mutant (K670N/M671L) APP (50% C57B6, 50% SJL) (7) transgenic mice were crossed with heterozygous mutant (A246E) PS-1 (50% C57B6, 50% SJL) (11) transgenic mice to generate heterozygous PS-1/APP transgenic mice as well as heterozygously singly transgenic and wild-type animals. Nomenclature for the transgenic animal lines is as follows: PS-1 refers to animals heterozygous for the PS-1 A246E transgene; APP refers to animals heterozygous for the APP K670N/M671L transgene; PS-1/APP refers to animals heterozygous for both mutant transgenes. Nontransgenic control animals were littermates generated in the breeding for PS-1/APP transgenic mice. APP and PS-1/APP refer to animals heterozygous for both human wild-type PS-1 and mutant PS-1 transgenes were generated. Mouse genotype was determined by PCR (11). The genetic backgrounds of the mice analyzed were 50% C57B6, 50% SJL. Male and female mice were used. All animal experiments were performed in accordance with the Bayler College of Medicine Institutional Animal Care and Use Committee and with national regulations and policies.

Immunohistochemistry—Mice were cardiac-perfused with phosphate buffered saline (10 mM NaH2PO4, 150 mM NaCl, pH 7.2) and fixed with 4% paraformaldehyde. Frozen brain sections were sectioned coronally at a 12-μm thickness using a cryostat, mounted on ProbeOn Plus microscope slides (Fisher Scientific), and air-dried. Immediately before staining, the brain sections were fixed with acetone. Tissue sections were incubated for 30 min in 0.5% H2O2 and 0.3% normal goat serum, washed in phosphate-buffered saline, and incubated with 1.5% normal goat serum in phosphate-buffered saline for 30 min. Brain sections were then incubated with anti-Aβ antibody 6E10 (1:1000, Senetik) stained with biotinylated anti-mouse IgG (1:200, Vector Laboratories), and immunodetected with Vectastain ABC Reagent (1:100, Vector Laboratories). Sections were counter-stained with hematoxylin.

Hippocampus Dissection—Animals were decapitated, and both hippocampi removed and placed into ice-cold cutting solution (1.25 mM NaH2PO4, 28 mM NaHCO3, 60 mM NaCl, 3 mM KCl, 110 mM sucrose, 0.5 mM CaCl2, 7 mM MgCl2, 5 mM glucose, 0.6 mM ascorbate). Area CA1 and DG were subdissected from each hippocampus by trimming away the alveus and fimbria of cross-sections of ventral hippocampus. CA1 and DG were separated from CA3 by slicing cross-wise at the lateral aspect of the hippocampal fissure. The slice was then sectioned through the hippocampal fissure to separate CA1 and DG. Isolated hippocampal regions were prepared for quantitative immunoblotting as described below.

Quantitative Immunoblotting—Quantitative immunoblotting was previously described in Dineley et al. (6). Briefly, harvested brain tissue was sonicated in sonication buffer (10 mM HEPES, pH7.4, 150 mM NaCl, 50 mM NaF, 1 mM EDTA, 10 mM Na2PO4, 200 mM calcium A, 10 μg/ml leupeptin, 2 μg/ml aprotinin, 1 μM microcystin-LR, 1 mM NaVO3), and protein concentration was determined with BCA (Pierce). Samples were subjected to SDS-PAGE, transferred to Immobilon-P (Millipore), and followed by immunoblot with the appropriate primary and secondary antibodies and chemiluminescence (ECL, Amersham Biosciences). Band intensity was quantified with Scion Image software (NIH Image) from film exposures (BioMax, Kodak) in the linear range for each antibody and normalized to control level. Normalized control values were determined for each immunoblot by averaging control values, dividing each control and transgenic sample density by the average of the control set, and then determining the average and standard error of the mean (S.E.) for control and transgenic samples: 5 months, n = 9 control animals, n = 6 PS-1, n = 15 APP, and n = 10 PS-1/APP transgenic animals; 9 months, n = 14 control animals, n = 5 PS-1, n = 11 APP, and n = 11 PS-1/APP transgenic animals.

Open Field Test—Each subject was placed into the center of an open field chamber, a clear arena 43 x 43 cm in dimension illuminated by a
6E10 to visualize plaques (Fig. 1). We estimate that, with our methodology, we can detect plaques >5 μm in diameter. At all ages examined, both the density and the size of plaques in PS-1/APP mice exceeded that of APP mice at comparable ages. Plaque deposition pattern for PS-1/APP mice was similar to that reported for the APP mice from which they were generated (7, 12, 13). Plaques were first observed in cortical regions, followed by hippocampus.

Plaques were not detected in any group at five months of age (data not shown). β-amyloid plaques were consistently detected in the neocortex of 7-month-old PS-1/APP transgenic mice when APP transgenic mice were free of plaques (Fig. 1). At nine months of age, although APP transgenic mice exhibit occasional Aβ plaques in the cortex, positive staining cannot be detected in the hippocampus. At this age, however, PS-1/APP transgenic mice show numerous Aβ deposits in various regions of the brain, including neocortex and hippocampus (Fig. 1). Plaque deposits were further enhanced in 13-month-old PS-1/APP mice; multiple brain areas were covered with plaques (Fig. 1). Littermates that express PS-1 A246E alone do not develop detectable amyloid plaques up to 24 months of age, and the wild-type human PS-1 transgene does not facilitate plaque deposition in mice when co-expressed with the mutant APP transgene (data not shown).

Having seen differences in Aβ load and deposition rates between the various transgenic animals, we wanted to determine whether there were any cognitive correlates of plaque deposition. Behavioral characterization of non-transgenic control mice and APP, PS-1, or PS-1/APP transgenic mouse lines included the open field test, rotarod test, and an associative learning test, fear conditioning. In addition, each group of mice was subjected to a general health assessment and evaluated for normal sensory processing. As reported in previous studies, the APP and PS-1 animals exhibited no apparent differences in general health from control animals (7, 11, 14). Similarly, differences in general health between PS-1/APP mice and singly transgenic or control animals were undetectable.

Open Field—PS-1, APP, PS-1/APP, and control mice were monitored in the open field test to evaluate locomotor activity and anxiety-related behavior. Each animal’s movements in both the horizontal and vertical planes were recorded and quantified. Open field data can be used to evaluate a mouse’s anxiety-related response to being in a novel, well lit, and unsheltered area by calculating the ratio between the amount of time spent in the center of the open field and the total distance traveled during the 30-min observation period. In a brightly lit open area, mice will tend to stay near the walls of the open field rather than enter the center region; more so for highly anxious rodents. Genotype had no significant effect on locomotor activity at any of the ages tested (5 or 9 months of age). One-way ANOVA revealed no significant effect of genotype on total distance traveled, number of vertical rearings, time spent in the center of the grid, or in the center:distance ratio during the 30-min evaluation time (Table I). We conclude from these observations that PS-1, APP, or PS-1/APP animals are not abnormally active in the open field test, nor do they exhibit anomalous anxiety-related behavior.

Rotarod — The motor abilities of PS-1, APP, PS-1/APP, and control mice were probed using the rotarod test. The amount of time an animal can remain on an accelerating rotating wheel is an index of the animal’s motor coordination. In addition, it is expected that the animals gain proficiency in the task with increasing trial number, and this is taken as an indication of motor learning. As anticipated, each animal group remained on the rotarod for longer periods of time with increasing trial number over the course of two training days: 5 months, F(7, 312) = 7.87, p < 0.0001; 9 months, F(7, 296) = 25.08, p < 0.0001 (Fig. 2). Two-way ANOVA with repeated measures did not detect an interaction between genotype and trial number, demonstrating that all groups of mice learned the task at similar rates. Although the 9-month-old APP and PS-1/APP animals appeared to plateau in their performance at a lower total time spent on the rotarod, this effect was not statistically significant. Overall, these data indicate that PS-1, APP, and PS-1/APP mice are unimpaired in motor coordination and motor learning as compared with control animals.

Shock Threshold—After all other behavioral tests were complete a subset of 9-month-old animals was evaluated for their ability to perceive shock stimuli. The shock threshold to flinch (Fig. 3a), to jump (Fig. 3b), and to vocalize (Fig. 3c) was quantified for each animal group. One-way ANOVA and Tukey post hoc analysis did not detect any significant differences between any of the genotypes except between the PS-1 transgenic and PS-1/APP transgenic animals in their threshold to vocalize (F(3, 22) = 4.11, p < 0.05; Fig. 3). The implications of this difference are unclear. On the whole, transgenic animals do not appear to be impaired in their ability to detect mild foot-shock.

Associative Learning—Although no differences in baseline motor and sensory behaviors were detected between each genotypic group of mice, we sought to determine whether there might be defects in higher order cognitive function. A well defined cognitive deficit is both a hallmark of human AD and an important component of valid animal models of AD. Thus far, learning and memory impairments described for various
transgenic animal models of AD have been detected with behavioral tasks that require multiple days of training and testing (7, 14, 15, 35). We investigated the use of the fear conditioning associative learning paradigm in our mouse models of AD to potentially develop a rapid cognition assay for application in these systems.

### FIG. 2

All genotypic groups learn the rotarod task. Control, PS-1, APP, and PS-1/APP transgenic animals were placed on the rotarod apparatus for four trials over two consecutive days. With trial number and days of training, each animal group increased the amount of time they remained on the rotarod apparatus.

### TABLE I

Summary of open field test results
For each genotypic group of 5- and 9-month-old animals, total distance traveled (cm × 100), number of vertical rearings, amount of time spent in the center of the observation grid, and center: distance ratio are reported. Values below the mean value represent S.E.

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<tr>
<td>5 Months</td>
<td></td>
<td></td>
<td></td>
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<tr>
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<td>150.16</td>
<td>87.96</td>
<td>0.010</td>
</tr>
<tr>
<td>PS-1/APP</td>
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<td>309.53</td>
<td>389.33</td>
<td>0.057</td>
</tr>
<tr>
<td>9 Months</td>
<td></td>
<td></td>
<td></td>
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<tr>
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<td>540.00</td>
<td>176.41</td>
<td>0.026</td>
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<tr>
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<td>27.10</td>
<td>0.004</td>
</tr>
<tr>
<td>PS-1/APP</td>
<td>114.11</td>
<td>276.13</td>
<td>211.95</td>
<td>0.026</td>
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Fig. 2. All genotypic groups learn the rotarod task. Control, PS-1, APP, and PS-1/APP transgenic animals were placed on the rotarod apparatus for four trials over two consecutive days. With trial number and days of training, each animal group increased the amount of time they remained on the rotarod apparatus.
Fear conditioning is associative and comes in two forms: one that is hippocampus-dependent (contextual fear learning) and one that is hippocampus-independent (cued fear learning) (16–20). We chose this learning paradigm because we can probe cognitive function with a single training day followed in 24 h by tests for contextual and cued fear learning, each requiring just minutes to perform. Most relevant, contextual fear learning is dependent upon a brain area that has been implicated as a locus for cognitive decline in AD, the hippocampus.

PS-1, APP, PS-1/APP, and control mice were subjected to a standard fear conditioning paradigm in which the animals learn to associate neutral stimuli with an aversive one. The mice were placed in a novel context (fear conditioning box) and exposed to two pairings of a white noise cue and mild foot shock. Fear learning was assessed 24 h later by measuring freezing behavior in response to representation of the context or of the auditory cue within a completely different context.

At 5 months of age, there were no apparent differences in the freezing behavior of the different mouse genotypes during the two-pairing training phase of fear conditioning (Fig. 4a). In the contextual test for fear learning, the APP and PS-1/APP animals exhibited decreased freezing behavior compared with both the control and PS-1 groups (Figs. 4b, 5a). Two-way ANOVA with repeated measures did not detect a significant interaction between genotype and time spent in the context, demonstrating that time spent in the context did not differentially influence a genotypic group of mice. One-way ANOVA and Tukey post hoc analysis detected a significant difference in freezing behavior at the 1–4 min time epochs compared with controls and at the 1-, 2-, 3-, and 5-min epochs compared with the PS-1 group (Fig. 4b; min 1: \( F(3,60) = 5.60; p = 0.001 \); min 2: \( F = 7.68; p = 0.001 \); min 3: \( F = 8.51; p = 0.001 \); min 4: \( F = 4.26; p < 0.05 \) all groups). Analysis of total freezing behavior indicates that APP and PS-1/APP animals freeze significantly less than control and PS-1 animals (see Fig. 9a; Tukey’s multiple comparison test: \( F(5,16) = 27.97; p < 0.001 \) all groups). We conclude that APP and PS-1/APP animals have a deficit in contextual fear learning as measured by percent freezing during 1-min bins as well as measured by percent freezing during the entire observation period.

One-way ANOVA and Tukey post hoc analysis determined that all animals displayed similar and significant freezing in the cued test for associative learning, indicating that the impairment in contextual fear learning exhibited by the APP and PS-1/APP animal groups is not due to an inability to freeze or to an inability to detect the aversive foot shock stimulus (Fig. 4c; \( p < 0.001 \) all groups; also see “Shock Threshold” above). Therefore, 5-month-old APP and PS-1/APP mice appear to have a selective hippocampus-dependent impairment in associative learning following two pairings of conditioned and unconditioned stimuli for fear conditioning.

We then determined whether a more vigorous training paradigm could rescue the contextual fear learning impairment in the APP and PS-1/APP animals. One week later, the four groups of animals were subjected to a five-pairing fear conditioning paradigm (Fig. 5a). Statistical analysis of the freezing exhibited during the first 2 min of the five-pairing training protocol indicates that control and PS-1 animals’ freezing during this time period is significantly greater than during the first 3 min of two-pairing training 1 week prior when the mice were naïve (one-way ANOVA and Tukey post hoc analysis \( p < 0.01 \)). This result is indicative of the animals’ memory of the context.

Twenty-four hours following training, the animals were tested for contextual and cued fear learning. Two-way ANOVA with repeated measures did not detect a significant interaction between genotype and time spent in the context, demonstrating that time spent in the context did not differentially influence a genotypic group of mice. Again, the control and PS-1 mice performed comparably on the contextual fear learning test (Figs. 5b, 6b). Although the PS-1/APP mice were still impaired, the APP mice exhibited contextual fear learning indistinguishable from control and PS-1 mice. Analysis of total freezing behavior signifies that control, PS-1, and APP animals freeze significantly more than PS-1/APP transgenic animals.
Fig. 4. 5-month-old APP and PS-1/APP transgenic mice are impaired in contextual fear learning. Two pairings of CS–US for fear conditioning (a) is followed 24 h later by testing for contextual (b) and cued (c) fear learning. # indicates statistically significant difference from control; * indicates statistically significant difference from PS-1 transgenic animal group; see “Results” for p values.
5-month-old APP transgenic mice contextual fear learning impairment is rescued by a five-pairing training protocol. Five pairings of CS-US (a) lead to contextual fear learning (b) in APP transgenic animals that is indistinguishable from control and PS-1 transgenic animals. PS-1/APP transgenic mice are still impaired in the contextual test for fear learning. All genotypic groups learn to freeze upon presentation of the CS (cue) in the cued test for fear learning (c). * indicates that APP transgenic animals froze statistically significant difference from PS-1/APP transgenic animals; see “Results” for p values.
(see Fig. 8b; one-way ANOVA and Tukey’s multiple comparison test: \( F(3,16) = 7.04; \) all \( p < 0.05 \)). Statistical analysis of the 1-min epoch data (unpaired \( t \) test with Welch’s correction for significantly different variance) determined that APP mice performed contextual fear learning significantly better than PS-1/APP mice (Fig. 5b; min 3: APP versus PS-1/APP \( p < 0.03, df = 9 \); min 5: APP versus PS-1/APP \( p < 0.04, df = 16 \)). As before, all animal groups exhibited significant cued fear learning indicating no general impairment in amygdala function or the type of sensory processing necessary for fear learning (Fig. 5c; one-way ANOVA \( p < 0.001, \) all groups). Thus, this impairment in contextual fear learning is again suggestive of a hippocampus-specific behavioral defect.

A separate group of animals 9 months of age were subjected to an identical fear conditioning paradigm to test for an age-dependent decline in the contextual fear learning exhibited by the APP and PS-1/APP animals. Two-way ANOVA with repeated measures did not detect a significant interaction between genotype and time spent in the context, demonstrating that time spent in the context did not differentially influence a genotypic group of mice. Once again, one-way ANOVA detected no significant differences in the levels of freezing displayed by any of the animal groups during training (Fig. 6a). Control and PS-1 animal groups did not perform significantly different from each other in the contextual test for fear learning (Figs. 6b, 8c). APP and PS-1/APP mice were significantly impaired in the contextual fear learning test compared with control and PS-1 animals (Fig. 8c; Tukey’s multiple comparison test: \( F(3,16) = 37.12, \) control versus APP, control versus PS-1/APP, PS-1 versus APP, PS-1 versus PS-1/APP, all \( p < 0.001 \)). When evaluating 1-min epoch data, APP and PS-1/APP animals froze in the contextual test significantly less than control animals in epochs 1–3 (Fig. 6b; one-way ANOVA; min 1: \( F(3,37) = 4.73, p < 0.05, \) min 2: \( F = 5.15, p < 0.05, \) min 3: \( F = 4.38, p < 0.05, \) all animal groups exhibited significant cued fear learning (Fig. 6c; one-way ANOVA; \( p < 0.001, \) all groups).

When animals were tested for contextual fear learning 24 h after five pairings of CS-US, two-way ANOVA with repeated measures did not detect a significant interaction between genotype and time spent in the context, demonstrating that time spent in the context did not differentially influence a genotypic group of mice. Control and PS-1 transgenic animal groups once again did not perform significantly different from each other in the context test for fear learning. When assessing total freezing levels, APP and PS-1/APP transgenic animals displayed abnormal freezing behavior as compared with control and PS-1 transgenic animals (see Fig. 8d; Tukey’s multiple comparison test: \( F(3,16) = 20.21, \) control versus APP, control versus PS-1/APP, PS-1 versus APP, PS-1 versus PS-1/APP, all \( p < 0.05, \) all \( p < 0.05, \) both APP and PS-1/APP animals’ freezing behavior was significantly different from control animals at the second 1-min epoch (Fig. 7b; one-way ANOVA; \( F(3,37) = 10.36, p < 0.05, \) and APP animals’ freezing behavior was significantly different from control animals at every epoch (Fig. 7b; one-way ANOVA; min 1: \( F = 6.09, p < 0.01, \) min 3: \( F = 7.98, p < 0.001, \) min 4: \( F = 3.48, p < 0.05, \) min 5: \( F = 5.71, p < 0.05, \) in other words, at 9 months of age, the more robust five pairings of CS-US failed to rescue the contextual fear-learning impairment exhibited by APP animals following two pairings of CS-US. To test for an age-dependent decline in APP animals’ performance from 5 to 9 months of age, statistical analysis of total freezing following five pairings of CS-US was performed. Two-way ANOVA detected a significant interaction of age and genotype for performance in the contextual test at 5 and 9 months of age following five-pairing training (\( p < 0.0001, \) Tukey post hoc analysis indicates that APP animals’ performance in the contextual test significantly declined from 5 to 9 months of age following training with the five-pairing protocol (one-way ANOVA; \( F(7,32) = 12.20, p < 0.001, \) none of the other animal groups showed significant changes in performance between 5 and 9 months of age. Therefore, APP transgenic animals’ performance in contextual fear learning declines in an age-dependent manner.

As a control, we tested 2-month-old APP (\( n = 10, \) PS-1/APP (\( n = 10, \) and control (\( n = 15, \) all animals for contextual fear learning. The three genotypic sets of animals, 2 months of age, were subjected to two pairs of CS-US and tested for contextual fear learning 24 h later. None of the animal groups performed significantly different from any other animal group in the contextual test for fear learning following the two-pairing training protocol (one-way ANOVA with Tukey post hoc analysis, results not shown). Furthermore, all three groups demonstrated significant contextual fear learning; each animals’ freezing performance in the contextual test was significantly different from the pretraining baseline (all groups \( p < 0.005, \) Student’s \( t \) test). These results indicate that 2-month-old animals acquire contextual fear conditioning and suggest that our findings in the older animals are not due to overexpression of the transgene or position effects.

\[ \alpha 7 \text{nAChR Quantification—} \] We have previously observed an age-dependent up-regulation of \( \alpha 7 \text{nAChR} \) protein in the hippocampi of APP animals (6). Following behavioral assessment of each animal, their hippocampi were removed and subjected to quantitative immunoblot to measure \( \alpha 7 \text{nAChR} \) protein levels in area CA1 and DG. Consistent with our previous findings, the DG of 5-month-old APP animals had significantly elevated \( \alpha 7 \text{nAChR} \) protein (Fig. 9b; \( p < 0.003, df = 15, \) Tukey test). Furthermore, all three groups demonstrated significant contextual fear learning; each animals’ freezing performance in the contextual test was significantly different from the pretraining baseline (all groups \( p < 0.005, \) Student’s \( t \) test). These results indicate that 2-month-old animals acquire contextual fear conditioning and suggest that our findings in the older animals are not due to overexpression of the transgene or position effects.

\[ \alpha 7 \text{nAChR null mice, which included contextual fear conditioning, failed to detect any behavioral abnormalities } \] (21). By 9 months of age, a significant reduction in \( \alpha 7 \text{nAChR} \) protein was no longer detected in the DG of PS-1 animals, possibly indicating a trend toward increased \( \alpha 7 \text{nAChR} \) protein at older ages (Fig. 9d).

**DISCUSSION**

In this work, we have taken a genetic approach to study the effects of different \( \beta \) loads by crossing mice transgenic for PS-1 A246E FAD mutation with animals transgenic for the APP K670N/M671L FAD mutation. We tested the hypothesis that the combination of FAD mutations in PS-1/APP animals would lead to an acceleration of amyloid deposition by performing an immunohistochemical analysis of these animals at various ages. We have shown that the co-expression of mutant.
**Fig. 6.** 9-month-old APP and PS-1/APP transgenic animals are impaired in contextual fear learning. Two pairings of CS–US for fear conditioning (**a**) is followed 24 h later by testing for contextual (**b**) and cued (**c**) fear learning. Cued fear learning was comparable for all genotypic groups. * indicates a statistically significant difference from both control and PS-1 transgenic animal groups; see "Results" for $p$ values.
FIG. 7. 9-month-old APP transgenic mice exhibit an age-dependent decline in contextual fear learning. Five pairings of CS-US (a) lead to contextual (b) fear learning in control and PS-1 transgenic mice. APP and PS-1/APP transgenic animals exhibit less freezing in the contextual test of fear learning. All genotypic groups learn to freeze upon presentation of the CS (cue) in the cued test for fear learning (c). * indicates a statistically significant difference from both control and PS-1 transgenic animal groups; see "Results" for p values.
human APP and mutant human PS-1, but not the wild-type PS-1, facilitates deposition of amyloid by several months as compared with APP mice. The plaque deposition pattern in the PS-1/APP animals is characteristic of the plaques detected in the APP animals (7, 12, 13). Largely, our data agree well with similar studies reported for mice expressing different combinations of APP and PS-1 transgenes in that plaque deposition is accelerated and the pattern of deposition is similar to that found in the APP singly transgenic animals from which they are derived (22, 23).

It is now well established that impairment in the encoding of new episodic memories is typical of the earliest stages of AD (24–26). Converging lines of evidence have linked the early loss of episodic memory in AD to medial temporal pathology including the hippocampus (27–29). In the work described here, we have identified a hippocampus-dependent associative learning impairment that manifests itself relatively early in the animals’ lifetime, precedes detectable (plaques > 5 μm) plaque deposition in the hippocampus and coincides with increased α7 nAChR protein. These findings and the recent reports by Chishti et al. (15), Koistinaho et al. (35), and Westerman et al. (36) describe the earliest yet behavioral and biochemical phenotypes for an AD mouse model. In addition, the robust impairment in contextual fear conditioning detected in these transgenic mice provides a simple and rapid behavioral screen to evaluate potential therapeutic compounds in rodent models of AD.

No significant differences were detected between PS-1, APP, or PS-1/APP and control animals when assessed regarding general health, shock threshold, locomotor activity, motor coordination, and motor learning. Neither did we observe any deficits in cued fear conditioning; however, there were significant findings when the animals were evaluated for contextual fear learning. An impairment in contextual but not in cued fear learning demonstrates that these animals are capable of freezing, and indicates that they do not suffer general cortical or amygdala damage or alterations in sensory processing required for fear learning. These findings suggest that the impairment in contextual fear learning is localized to the hippocampus (16–20). Furthermore, we did not detect impairment in any genotypic set of 2-month-old animals in the contextual test 24 h following fear conditioning. Thus demonstrating that the cognitive deficits detected at later ages in the APP and PS-1/APP transgenic animals is not a consequence of transgene overexpression or position effects.

APP transgenic animals exhibit a contextual fear learning deficit 24 h after two pairings of CS-US. At this age, APP transgenic animals express significantly higher levels of α7 nAChR protein in the DG of hippocampus. These findings are consistent with our model that chronic elevated Aβ leads to up-regulation of α7 nAChR protein in hippocampus by interacting with these receptors in hippocampus. This contextual fear learning impairment is rescued by performing a more robust training paradigm of five pairings of CS-US. At 9-months of age, the contextual fear learning deficit in APP animals is no longer rescued by five pairings of CS-US, indic-
ative of an age-dependent decline in hippocampus function. At this age, the level of α7 nAChR protein had not significantly changed from the level measured at 5 months of age, indicating that the contextual fear learning impairment that is no longer rescued by five pairings of CS-US cannot be attributed to accumulating α7 nAChR protein. How α7 nAChR accumulation, Aβ production, and metabolism, accumulation, and deposition into plaques result in hippocampus dysfunction and age-related hippocampus-dependent behavioral impairments remains unclear. Possibly, changes in the level of α7 nAChR protein occur at a different rate than the functional consequences of chronic exposure to Aβ (30–33). Alternatively, the increase in α7 nAChR protein could occur independently of hippocampus dysfunction and impaired associative learning. Future studies examining the role of α7 nAChR function in hippocampal synaptic plasticity in these animals at different ages may begin to elucidate a mechanism.

Animals that express both PS-1 and APP transgenes have further elevation of Aβ, which apparently accelerates plaque pathology by several months; plaque deposition evident at 6 months of age in PS-1/APP mice is not detected to comparable level in APP animals until 9–10 months of age. PS-1/APP animals are impaired in contextual fear learning and have elevated α7 nAChR protein in both CA1 and DG of the hippocampus at 5 months of age. At this age, five pairs of CS-US do not rescue the contextual fear learning impairment exhibited by these mice. The different age-of-onset for the contextual fear learning deficit in APP versus PS-1/APP mice suggests that the phenotype is relevant to the overexpression of Aβ rather than a feature contributed by either strain of mice.

In previous studies, we found that Aβ42 couples via α7 nAChRs to the MAPK cascade, a critical element in hippocampal synaptic plasticity and learning (8–10, 34). We also showed that in vivo elevation of Aβ, such as that exhibited by the APP and PS-1/APP animals used in this study, leads to the upregulation of α7 nAChR protein in an age-dependent manner (6). α7 nAChR up-regulation occurs concomitantly with the dysregulation of ERK2 MAPK in DG of 4-month-old APP animals. ERK MAPK activity is known to be required for contextual fear learning; therefore, a model we have previously proposed that is consistent with our current findings is that hippocampus-dependent learning and memory impairments in early AD arise in part because of increases in Aβ burden and

**FIG. 9.** α7 nAChR protein level is elevated in the hippocampus of APP and PS-1/APP transgenic animals. Quantitative immunoblot for α7 nAChR protein was performed on CA1 and DG homogenates from the three groups of transgenic animals and compared with control animals. a, CA1, 5-month-old animals; b, DG 5-month-old animals; c, CA1 9-month-old animals; d, DG 9-month-old animals. * indicates a statistically significant difference from normalized control animals value; see “Results” for p values.
chronic activation of the ERK MAPK cascade in hippocampus through α7 nAChRs (6, 8, 34).

The work described here identifies a cognitive deficit in two animal models of AD that localizes to impaired hippocampal function. The extent of contextual fear learning deficits appears to correlate with Aβ production, as it is more pronounced in the PS-1/APP animals. In AD, the hippocampus is a locus for the earliest stages of impaired memory formation, and our findings that both APP and PS-1/APP animals exhibit impaired associative learning prior to frank plaque deposition may be relevant to early human AD. In addition, we may have identified a biochemical marker that is indicative of hippocampal dysfunction: α7 nAChR up-regulation. These findings of early onset behavioral and biochemical markers may prove useful in screening new therapies for AD.

REFERENCES

Accelerated Plaque Accumulation, Associative Learning Deficits, and Up-regulation of α7 Nicotinic Receptor Protein in Transgenic Mice Co-expressing Mutant Human Presenilin 1 and Amyloid Precursor Proteins

Kelly T. Dineley, Xuefeng Xia, Duy Bui, J. David Sweatt and Hui Zheng


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