Experimental Neurology **170,** 326–344 (2001)
doi:10.1006/exnr.2001.7715, available online at http://www.idealibrary.com on IDEAL®

Behavioral Phenotyping of GFAP-ApoE3 and -ApoE4 Transgenic Mice: ApoE4 Mice Show Profound Working Memory Impairments in the Absence of Alzheimer's-like Neuropathology

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Received December 4, 2000; accepted April 16, 2001

For the purpose of studying the potential neurobehavioral effects of different human apolipoprotein E (apoE) isoforms produced within the brain, transgenic (TG) mice were generated in which human apoE3 or apoE4 isoforms were under control of an astrocyte-specific, glial fibrillary acidic protein promoter and these TG mice were bred back to apoE knockout (KO) mice. Behavioral phenotypes of apoE3 and apoE4 TG mice were derived by conducting a longitudinal study in which apoE3 and apoE4 TG mice were compared with apoE KO and wild-type (WT) mice (all male) on several behavioral measures. Analysis of locomotor activity, "open-field" behaviors, acoustic startle/prepulse inhibition, and elevated plus maze data suggested that the apoE TG/KO groups were more "emotionally reactive" than WT mice, with apoE4 mice typically being the most reactive. The absence of performance differences among groups on the rotating holeboard and water navigation tasks suggested intact reference memory processing in apoE TG/KO mice. However, apoE4 mice were profoundly impaired on a working memory-based protocol in the radial arm maze (11–14 months). Nonassociative factors (sensorimotor capacities or emotionality differences) did not appear to confound interpretation of the learning/memory results. Western blot analysis revealed no alterations in the level of synaptic, neuronal, or glial markers in neocortex or hippocampus and histologic analysis revealed no evidence of Aβ deposition or neuritic plaques in the apoE KO/TG mice. Our findings suggest that apoE4 expression in the brain may have selective deleterious effects on memory function in the absence of typical Alzheimer's-like neuropathology.

Key Words: **Alzheimer's disease; apolipoprotein E; knockout mice; spatial learning and memory; reference memory; emotionality; sensorimotor,**

INTRODUCTION

Identifying the ε4 allele of apolipoprotein E (apoE) as a major risk factor for Alzheimer's disease (AD) (9, 17, 27, 31) has generated substantial interest in the role of apoE in age- and AD-related pathophysiology and cognitive decline. The development of transgenic (TG) mouse models of apoE expression offers the opportunity to learn more about the neurobehavioral effects of different human apoE isoforms produced within the brain by studying them under controlled conditions. Since CNS synthesis of apoE is carried out predominantly by glia in mammals (4, 18, 19, 33), we developed a TG mouse model of human apoE expression in which isoforms of apoE were elaborated in a similar fashion (33). Specifically, TG mice were developed in which human apoE3 or apoE4 isoforms were expressed in astrocytes under the control of a glial fibrillary acidic protein (GFAP) promoter. These TG mice were bred back onto a mouse apoE knockout (KO) background to examine the effects of human apoE in the absence of endogenous murine apoE. The goal of the present study was to begin deriving the behavioral phenotypes of these apoE TG mice, particularly in terms of their learning and memory capabilities.

Most of the work concerned with evaluating the role of apoE in learning and memory has involved studying the effects apoE deficiency on water maze performance and the results of these studies have often been contradictory and/or difficult to interpret. As a result, it is not clear whether apoE KO mice have reference and/or working memory deficits. Moreover, in a well-controlled study by Anderson and colleagues (1) it was concluded on the basis of several water navigation indices that the apoE KO mice were not impaired rel-

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0014-4886/01 $35.00

ative to WT controls. Spatial learning/memory capabilities of apoE KO mice have also been evaluated concurrently with those of apoE TG mice by Raber and colleagues (21, 22), who used a different TG mouse model of apoE expression from the one used in the present study. These investigators used a neuron-specific enolase (NSE) promoter to express human apoE3 or apoE4 at similar levels in neurons of TG mice that lacked endogenous apoE. They reported that NSE-apoE4 female (but not male) mice exhibited impaired performance on two different types of water navigation tasks when tested at 6 or 18 months of age.

In the present study, apoE3 and apoE4 TG mice were compared with apoE KO and wild-type (WT) mice (all male) on a variety of behavioral tasks including three learning and memory tests as well as other measures designed to evaluate sensorimotor capabilities and alterations in emotionality. This approach not only provides a full characterization of the behavioral phenotypes, but also aids in determining whether nonassociative factors likely influenced performance on the learning and memory tests. Comparing some of our results to those of Raber and colleagues (21, 22) will also help to establish whether glial or neuronal synthesis of these isoforms of apoE has a differential impact on behavior.

METHODS

Generation of apoE TG/KO Mice and Animal Husbandry

Human apoE TG mice were generated in which human apoE3 or E4 was expressed in astrocytes under the control of a GFAP promoter [see Sun *et al.* (33) for information pertaining to construct preparation]. For this experiment GFAP-apoE3 and GFAP-apoE4 mice hemizygous for the transgene (+/−) were bred to apoE KO mice on a C57BL/6J (B6) background. The pups (all F4 generation) were apoE3 +/−, apoE4 +/−, or apoE −/− and had been bred back onto a B6 background for four generations and expressed no mouse apoE. Expression levels of human apoE3 and apoE4 in the TG lines utilized are indistinguishable from each other and are very similar to that seen in the brains of mice wild-type for mouse apoE (12, 33). The presence of the apoE transgene was confirmed by PCR and immunocytochemistry on tissue sections from each mouse as described in Sun *et al.* (33). Since the apoE TG mice were bred to apoE KO mice, the apoE KO littermate mice served as controls for evaluating the effects of each of the transgenes. However, we also included a group of C57BL6 (B6) mice that were WT for mouse apoE and they were compared to the other three groups on the same tasks. It should be noted that the WT mice were on a similar though not identical genetic background relative to the other groups of mice. However, we felt that it was reasonable to include the WT mice in order to estimate the effects of the TG/KO manipulations in general. Animal care guidelines were in strict accordance with the rules and regulations set forth in the NIH Guide for Humane Care and Use of Laboratory Animals and all experimental protocols were approved by the Division of Comparative Medicine at the Washington University School of Medicine.

General Experimental Design

The four groups of mice were evaluated on several behavioral tests in a longitudinal manner throughout early adulthood and into middle age. The ages at which the mice were evaluated on the behavioral tests are listed below.

Behavioral test	Age
Rotating holeboard	5 and 14–17 months
Locomotor activity	6 and 10–13 months
Sensorimotor battery	6 months
Acoustic startle/PPI	6 months
Elevated plus maze	8–11 months
Morris water navigation	9–12 months
Radial arm maze	11–14 months

The mice were born at different times and were tested on the first holeboard, the locomotor activity, the sensorimotor, and the acoustic startle/PPI tests when they became 5 or 6 months of age as specified above. For the elevated plus maze, water navigation, and radial arm maze tests all the animals were tested together at the same time and thus were of different ages (as specified above) although all were mature adults.

Spatial Learning and Memory Tests

Rotating holeboard. This task provides a test of reference (trial-independent) memory and has been shown to be a sensitive methodology for evaluating the behavioral pharmacologic and neurotoxicologic effects of NMDA receptor antagonists on spatial learning and memory in mice (6, 7, 34). The apparatus consists of a square floor that contains a hole in each of the four corners and is enclosed by Plexiglas sides. Each hole contains a "Froot Loop" (Kellogg's), which is made inaccessible by being placed under a screen at the bottom of a hole. The screen allows the odor of the food to emanate from the hole, but does not allow access to it. When an individual hole is baited, a piece of Froot Loop is placed on top of the screen, making the food accessible. The apparatus rests on a turntable so that it may be easily rotated. Mice were handled, habituated to the apparatus, food-restricted, and trained to poke their heads into the exposed holes in order to retrieve the food reward. A trial consisted of placing a mouse in the start tube and then removing the tube so that it was

free to explore the apparatus and poke its head into the holes until it retrieved a food reward. Habituation sessions lasted until a mouse reached a criterion of eight correct trials out of nine consecutive trials. A correct trial was defined as one in which the mouse's first response was a nose poke into the baited hole. In order to prevent mice from using odor or other proximal cues to locate the correct hole, the apparatus was washed with a scented detergent and rotated 90° on a pseudorandom basis between each trial. Thus for a given trial (except the first), the baited hole was different from the one used on the previous trial although it was always located in the same relative spatial position. Essentially the same protocol used in the last phase of habituation was used during acquisition training except a different baited hole location was used. A criterion of eight correct trials out of nine consecutive trials was used to define learning. In addition, a retention test was conducted 24 h after the acquisition session using the same protocol to evaluate the extent to which the mice retained what they may have learned on the previous day. Trials to criterion was the dependent variable used to evaluate acquisition and retention performance. Mice were tested in early adulthood (5 months) and during later middle age (14–17 months). [See Brosnan-Watters and Wozniak (5) for a more detailed description of the rotating holeboard procedure.]

Morris water navigation task. Reference memory was also evaluated by testing the mice on the water navigation task at 10–13 months of age. The mice were trained in a round pool (100-cm inner diameter) of opaque water to learn the location of a platform in order to escape out of the water. The pool was located in the center of a room containing many distinct distal spatial cues. All trials were videotaped with an overhead camera, and the swim paths of the mice were recorded by a computerized tracking system (Polytrack, San Diego Instruments, San Diego, California) that was used to calculate the escape latency (latency to find the platform) and distance traveled (path length) to reach the platform for each trial. The mice were first trained in the "place" condition to learn the location of a platform that was submerged 1.5 cm beneath the surface of the water and was located 15 cm from the wall of the pool. For each mouse the location of the escape platform was randomly assigned to one of the four pool quadrants (N, S, E, or W; compass points), where it remained for all of the place trials. Release points (NE, SE, SW, and NW) were pseudorandomly assigned, with each point being used only once within each block of 4 trials and so that the same release point was never used on 2 consecutive trials. In the place condition, the mice received two blocks of 4 consecutive trials (60-s maximum for a trial) per day for 2 consecutive days (2-h rest between blocks) and for one block of 4 trials on the third day for a total of 20 trials. In order to evaluate retention capabilities, the mice were tested on two probe trials during place training. During the 60-s probe trial, the escape platform was removed from the pool and the mouse was released into the maze at a point that was diagonally opposite from the previous location of the platform. Time spent searching in the target quadrant where the platform had been located, number of crossings over the former platform location (platform crossings), and the percentage of total distance traveled in the target quadrant were recorded. One probe trial was conducted after the place trials were completed on day 2 and another one was given after the place trials were completed on day 3. After completing the place trials, the mice were tested in the "cued" condition where they were trained to navigate to a "visible" platform. In the cued condition, the platform was actually submerged beneath the surface of the water but its location was made apparent by a rod that was screwed into the base of the platform and which protruded 20 cm above the water surface and on top of which was placed a red tennis ball. In the cued condition, the mice received two blocks of 4 consecutive trials during which the location of the platform was moved to a different quadrant for each trial (each quadrant was used once for each block of trials). The cued condition was used to evaluate the possibility that nonassociative factors (i.e., sensorimotor disturbances or differences in motivation) might have affected acquisition performance during place training.

Radial arm maze. At 11–14 months of age, working (trial-dependent) memory capacities were evaluated using a win-shift spatial discrimination protocol in a standard eight-arm radial maze. Except for the use of a habituation criterion (see below), the procedure was very similar to previously published methods (34). The maze consisted of an octagonal central platform enclosed by a *Plexiglas* frame that contained eight experimenter-controlled doors that blocked access to the eight arms. The mice were habituated to handling and the experimental procedures and were shaped to traverse the arms and retrieve and consume a food reward (a "Fruity Pebble," Post) placed in a cup at the end of each arm. Neophobia (avoidance of a familiar food in a novel environment) was assessed by recording the time it took a mouse to first begin eating the Fruity Pebbles spread about the maze during the first 2 days of shaping. We have found that different strains of mutant mice may vary widely in the time it takes them to habituate to our procedures before they will freely traverse the arms of the maze and retrieve and consume the food reward. In order to control for differences in the times needed for habituation, we adopted a criterion that a mouse had to reach before formal spatial learning testing could be initiated. In the last phase of habituation, one Fruity Pebble was placed in each arm and a mouse qualified for acquisition training when it

ate all eight Fruity Pebbles within 5 min on 2 out of 3 consecutive days. Acquisition involved baiting each arm with a Fruity Pebble and training a mouse to remember the arms that it had been reinforced in, so that it would not revisit those arms (commit a retracing error). Acquisition was defined by an *a priori* criterion of at least eight correct responses out of the first nine responses for 4 consecutive days. Days and errors to criterion were the dependent variables used to assess acquisition performance.

Tests of Sensorimotor Capabilities and Emotionality

Sensorimotor battery. The general sensorimotor capabilities of the mice were evaluated using the four tests described below, which have previously been found to be sensitive for detecting acute drug-induced sensorimotor disturbances in mice (6, 7) and for evaluating the long-term sequelae following neurotoxic doses of drugs (34; see these articles for greater details concerning the procedures of these tests). A subset of the tests has been used to determine the onset of chronic disturbances in certain transgenic mice (8). The sensorimotor battery consists of four tests that reflect measures of balance, strength, coordination, and initiation of movement. Briefly, these tests involve timing how long a mouse can remain on a very narrow elevated Plexiglas ledge (*Ledge test*), on a small elevated circular platform with rounded edges (*Platform test*), or on an elevated wire mesh grid (*Inclined screen test*). In general, the ledge and platform tests are more sensitive tests for detecting sensorimotor disturbances than the inclined screen test. In the *Walking initiation test*, a mouse is placed in the middle of a square outlined on a smooth black surface of a large table top and is timed for how long it takes it to leave the square. Although initiation of movement is measured by this test, we have found it to be sensitive to drug-induced alterations in emotionality in rats and mice (6, 34, 35).

One-hour activity. General activity levels and "open-field" exploratory behaviors were evaluated during a 1-h test. When the mice were tested at 6 months of age, only general locomotor activity was assessed using previously published procedures (6, 7, 34). Briefly, this involved summing the number of beam breaks that occurred in three pairs of photoelectric cells that were placed across the width of each of two transparent (47.6 × 25.4 × 20.6 cm high) polystyrene cages, which divided each cage into four equal quadrants along its length. When the mice were tested at 10–13 months of age, a computerized, high-resolution, photobeam system (Hamilton-Kinder, LLC, Poway, California) was used to quantify open-field behaviors as well as variables related to general locomotor activity using the same enclosures that were used for the testing conducted at 6 months [see Schaefer *et al.* (28) for a more detailed account of the procedures]. Variables that were analyzed included measures reflecting open-field exploratory behaviors such as the number of entries, the time spent, and the distance traveled in the center area as well as the distance traveled in the periphery. Measures of general activity that were analyzed included the total number of ambulations (whole body movements), distance traveled, and total number of fine movements. The computerized high-resolution photobeam motor activity system was obtained after the mice had been tested on the 1-h activity test at 6 months of age and the decision was made to use the new, more sophisticated system when the mice were tested at 10–13 months. The new system was used because it provided much more information on various aspects of motor activity, included quantification of open-field behaviors, and involved using the same enclosures that had been used with the low-resolution photobeam system. Thus, since the new system provided more sensitive measures for quantifying movement, it was expected that it would detect any differences among the groups in terms of locomotor activity if they still existed at this older age and likely provide additional information on aspects of emotionality.

Acoustic startle/PPI. The acoustic startle response (ASR) is a contraction of the skeletal musculature in response to a brief, intense auditory stimulus and it is elicited in experimental situations by a tone or white noise burst. Prepulse inhibition of startle (PPI) is a form of startle plasticity that refers to a decrease in the ASR amplitude that occurs when the startle stimulus (e.g., a 120-dB white noise burst) is preceded 30–500 ms earlier by the presentation of a weak stimulus such as an 80- to 90-dB(A) noise burst. PPI is a measure of sensorimotor gating that has been found to be deficient in certain neuropsychiatric conditions such as schizophrenia. It is often used to study the effects of antipsychotic and psychotomimetic drugs as well as to characterize the behavioral phenotype of inbred WT and genetically mutant mice.

Testing was carried out in two startle chambers (SR-LAB, San Diego Instruments), each consisting of a nonrestrictive Plexiglas cylinder 5 cm in diameter mounted on a platform with a piezoelectric accelerometer unit attached below the cylinder, both of which were designed expressly for evaluating mice. The accelerometer detected all animal movements, which were then digitized and stored by a computer and interface assembly (San Diego Instruments). Beginning at stimulus onset, 65 1-ms readings were recorded and the startle amplitude was defined as the average of these 65 readings. The cylinder and platform were housed in a ventilated, well-lit, sound-attenuated cabinet. The cabinet also contained a high-frequency speaker that was mounted 33 cm above the platform and produced all acoustic stimuli. A vibrating stan-

dardization unit (San Diego Instruments) that emulates an animal's response was used to equilibrate the sensitivities of the two platforms (within 5%). The average readings in the two chambers was 1100 using the standardization unit. Sound pressure levels for the background noise level and for all acoustic stimuli were calibrated daily with a digital sound level meter (Radio Shack). The protocol used in the present study was similar to that used by Dulawa and Geyer (10) and involved five different types of trials: a 40-ms broadband 120-dB burst [STARTLE (pulse alone) trial]; three different PPI trials (prepulse+pulse) trials in which 20-ms-long stimuli that were 4, 12, or 20 dB above the 65-dB background level preceded the 120-dB pulse by 100 ms (onset to onset). Thus, three kinds of PPI trials were presented: PPI4, PPI12, and PPI20. The last trial type, NS (no stimulus), contained only background noise and no other acoustic stimuli. The five trial types were pseudorandomly ordered within a block of five trials in order to control for changes in responsivity over the test session. Trials were separated by an average of 15 s. Ten trials of each trial type were presented. In addition, each session began with a 5-min acclimation period during which only background noise was presented. This was followed by 5 consecutive STARTLE trials before presenting the blocks of the 5 different trial types as described above. The session ended with 5 consecutive STARTLE trials. Two data transforms were used to control for differences in startle in order to compare the amount of PPI induced across groups. The %PPI transform was similar to that used by Dulawa and Geyer (10) in that it involved subtracting the amplitude of the response resulting from a PPI (prepulse+pulse) trial from the mean amplitude of the middle 10 STARTLE (pulse alone) trials, dividing that quantity by the mean amplitude of the middle 10 STARTLE trials, and then multiplying that number by 100. The block%PPI transform involved subtracting the amplitude of the response resulting from a PPI trial from the amplitude of a STARTLE trial within a 5-trial block and then dividing that quantity by the amplitude of the STARTLE trial (within the block) and then multiplying that amount by 100.

Elevated plus maze. The elevated plus maze is an often-used test for studying anxiety-like behaviors in rodents. The apparatus is a four-arm "maze" configured in the shape of a "cross" or "plus sign" that is elevated above the floor. It contains one set of opposing arms that are enclosed with "walls" that surround the perimeter of the arms (closed arms) and another set of opposing arms that are not enclosed (open arms). "Anxiety levels" are inferred from the analysis of certain spontaneous behaviors with particular reference to avoidance of the open arms. Mice were tested in a maze consisting of two open arms [35 $\times$ 6.1 (outer width) $\times$ 0.3 cm] and two closed arms (35 $\times$ 6.1 $\times$ 15 cm) that extended from a central platform (5.5 $\times$ 5.5 cm) and that were constructed of black *Plexiglas*. A computerized, high-resolution photobeam system (Hamilton-Kinder, LLC) was used to quantify several variables including time spent, distance traveled, time spent at rest, and numbers of entries made into the open and closed arms and the center area. These data were used to calculate total arm entries, percentage open arm entries [e.g., (open arm entries/total arm entries) $\times$ 100], and percentage closed arm entries and percentage open arm time [e.g., (open arm time (min)/300 min) $\times$ 100], which refers to the percentage of the total session time spent in the open arms, and percentage closed arm time. Numbers of fine movements and *x*- and *y*-axis ambulations were also recorded throughout the maze. The numbers of *x*- and *y*-axis ambulations predominantly reflect locomotor activity in the closed and open arms, respectively, although a small percentage of *x*-ambulations are measured in the open arms and the same is true for *y*-ambulations in the closed arms. Certain behaviors related to risk assessment (11, 24) were quantified from a videotaped record of the test sessions including stretch attend postures (SAPs) in the open and closed arms, rearing, open arm head dips over the side, closed arm head dips around the exiting corners of the arms nearest the central area, and the time taken to leave the center area to first enter an arm. Mice were tested during 5-min sessions conducted over 3 consecutive days in a darkened room dimly illuminated by a single "NightLight SPOT" incandescent (60 W) bulb. Test sessions commenced by placing a mouse in an opaque plastic tube located in the middle of the central area and then removing the tube, allowing the mouse to explore the maze. [See Schaefer *et al.* (28) for a more detailed description of the elevated plus maze methods and definitions of certain behaviors.]

Histology and Western blot analysis. For tissue analysis, mice were anesthetized with pentobarbital (150 mg/kg, ip) and perfused transcardially with 0.1 M phosphate-buffered saline (PBS; pH 7.4) at 4°C. Brains were divided into the left and right hemispheres. The right hemisphere was immersion fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C overnight. After fixation, the brain was cryoprotected in 30% sucrose in PBS at 4°C and frozen in powdered dry ice. Brain regions from the left hemisphere were dissected and frozen in powdered dry ice prior to analysis.

For histological analysis, tissue sections were cut in the coronal plane at 40 μm on a freezing sliding microtome from the genu of the corpus callosum through the caudal extent of the hippocampus. Every sixth section was stained with cresyl violet. For Western blot analysis, levels of apoE, synaptophysin, GFAP, neuron-specific enolase, and microtubule-associated pro-

tein-2 (MAP-2) in brain tissue were determined by semiquantitative Western blotting as described in Holtzman *et al.* (12).

Statistical Analyses

In general, ANOVA models were used to analyze the data from each behavioral test. Typically, the ANOVA models contained one between-subjects variable, groups (apoE KO, apoE3, apoE4, and WT), and usually at least one within-subjects variable, such as blocks of trials or acquisition versus retention. When ANOVAs with repeated measures were conducted, the Huynh–Feldt adjustment of α levels was used for all within-subjects effects containing more than two levels in order to protect against violations of the sphericity/compound symmetry, which are assumptions underlying this ANOVA model. Planned (pairwise) comparisons were conducted following significant main effects or interactions and P values for comparisons exceeding Bonferoni corrected levels ($0.05/6 = 0.0083$) are signified in the text by asterisks.

RESULTS

Spatial Learning and Memory Tests

Rotating holeboard. The data from the two holeboard tests are displayed in Figs. 1A and 1B. The acquisition and retention data from the testing conducted at 5 months of age show that the groups performed similarly on both measures. In contrast, the acquisition data from testing done at 14–17 months show that the WT and apoE4 mice did not perform as well as the apoE KO and apoE3 mice, although the retention data from that time point did not show any substantial differences among the groups. An ANOVA on these data indicated a nonsignificant effect of Group, a significant effect of Age at testing, $F(1,23) = 14.64$, $P = 0.001$, and a significant effect of Acquisition versus Retention (Acq/Ret), $F(1,23) = 35.82$, $P = 0.0005$. The Age by Group and Acq/Ret by Group interactions were both nonsignificant. However, the Age by Acq/Ret and the Age by Acq/Ret by Group interactions were both significant [$F(1,23) = 7.28$, $P = 0.13$, and $F(3,23) = 5.73$, $P = 0.004$, respectively]. Subsequent analyses indicated that the groups of mice did not differ in terms of trials to criterion for either acquisition or retention when tested at 5 months of age or with regard to retention when tested at 14–17 months of age. However, the groups did differ significantly with regard to acquisition performance when tested at 14–17 months of age, $F(3,25) = 40.00$, $P = 0.019$. Subsequent pairwise comparisons showed that the WT group required significantly more trials to reach the acquisition criterion than either the apoE KO ($P = 0.013$) or the apoE3 mice ($P = 0.004$*). The acquisition performance of the apoE4 mice was not significantly different from that of the WT mice. Although the performance difference between the apoE4 and the apoE3 groups was substantial at this time point, it only "approached signficance" ($P = 0.072$). To further investigate the effect of age on holeboard performance, paired t tests were conducted within each group on the trials to criterion data for acquisition from the two tests. The WT ($P = 0.008$), apoE4 ($P = 0.035$), and KO ($P = 0.046$) groups all showed significant increases in trials to criterion scores when tested at 14–17 months compared to the scores obtained at 5 months of age. In contrast, the trials to criterion scores for acquisition were not different at the two ages of testing for the apoE3 mice. Note, however, the small sample size ($n = 5$) of the apoE3 mice at the 14- to 17-month time point.

Morris water navigation test. An unanticipated finding of testing the mice on the Morris water navigation task (10–13 months of age) was that several mice were unable to complete the last two trials within a block of four trials due to them being removed from the pool because of trouble remaining afloat while swimming, particularly during place training. This finding was surprising since our pilot work with younger C57BL/6 mice suggested that our protocol was a reasonable one, and we wanted to administer several trials per day to promote good retention performance on the probe trials, which is sometimes difficult to demonstrate with mice of advanced ages or with certain strains. Almost all the mice completed the first two trials within each block (see analysis described below) and the number of mice unable to complete all trials was similar across genotypic groups. In future studies with middle age or older apoE TG/KO mice, fewer trials per day will be used and spread out over a greater number of training days (e.g., 14 days), and other performance measures such as average proximity to the former platform location during probe trials may be implemented to increase test sensitivity (15, 16).

The water navigation data were analyzed in several ways and most of the evidence supported the conclusion that the groups of mice did not differ on the various performance measures. However, one method of data analysis suggested that the groups differed in terms of escape latency during the place trials. This method involved giving animals that did not complete a trial a score that was the mean derived from those in their group that did complete the trial. This was done so that the performance of a mouse on that (uncompleted) trial did not add to or detract from the performance of the mice in that group that did complete the trial, yet it allowed an ANOVA with repeated measures to be conducted without deleting animals from the analysis because of uncompleted trials, which would have seriously diluted statistical power. An ANOVA

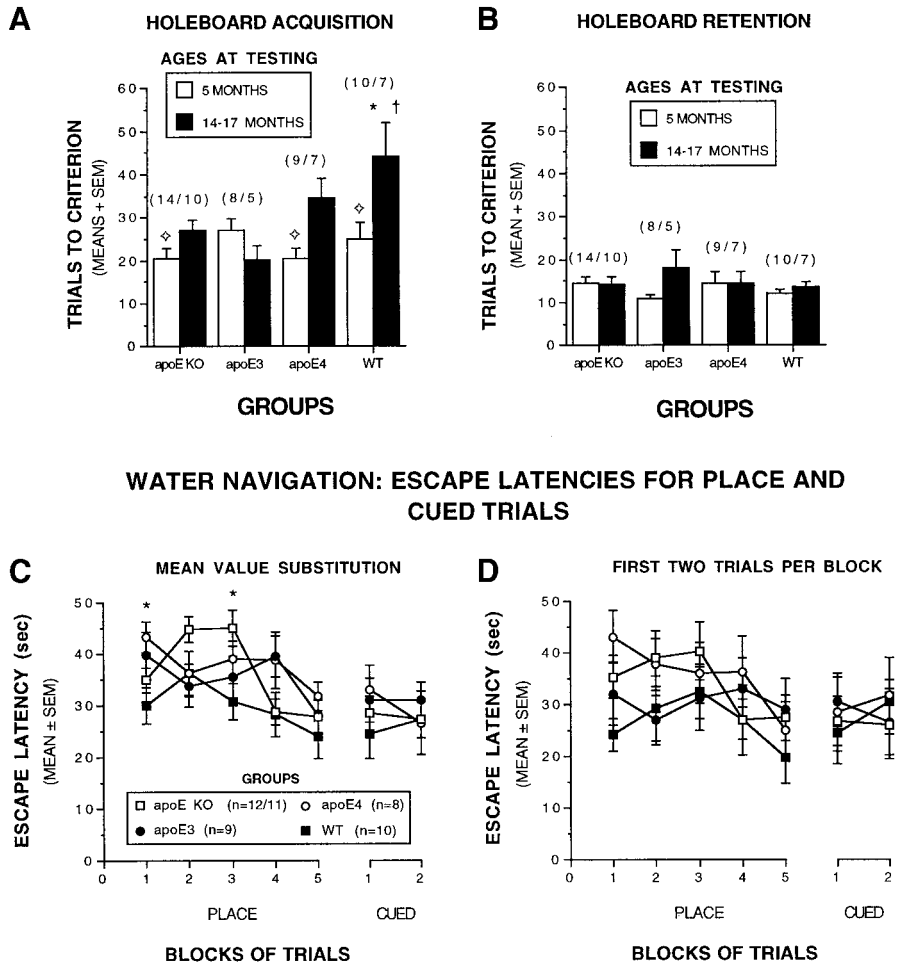

FIG. 1. Performance of apoE transgenic/knockout (TG/KO) mice and wild-type (WT) mice on two reference memory based spatial learning/memory tasks, the rotating holeboard (A and B) and the Morris water navigation task (C). The mice were tested on the rotating holeboard at 5 and 14–17 months of age. Groups did not differ in terms of acquisition (A) or retention (B) performance when tested at 5 months of age or on retention performance when evaluated at 14–17 months of age (sample sizes are in parentheses). However, acquisition performances were significantly different among the groups when the mice were tested at 14–17 months of age, with the WT group exhibiting the poorest performance, requiring significantly more trials to criterion than the apoE KO ($P = 0.013$) (*) and the apoE3 ($P = 0.004$) (†) mice. The data in A also show an age-related decrement in acquisition performance. Specifically, paired t tests conducted within each group indicated that the apoE KO, apoE4, and WT groups required significantly (✧) fewer trials to reach acquisition criterion on the 5-month test compared to the number of trials required for the 14- to 17-month test, whereas the performance of the apoE3 mice did not differ significantly between tests, although the sample size for the apoE3 group was particularly small ($n = 5$) at the 14 to 17-month test. See text for greater details concerning the results of the statistical analyses. (C) Performance of the mice during place and cued trials when tested on the Morris water navigation task at 10–13 months of age. Several mice did not complete all of the trials since they were removed from the pool because they appeared to have trouble remaining afloat. Group mean values were assigned to trials that were not completed (see text). An analysis of these data showed that the groups were found to differ significantly (*) on the place trials in terms of escape latency during the first and third blocks (Ps = 0.042 and 0.05, respectively). The apoE4 mice had significantly longer escape latencies than the WT or the apoE KO mice on the first block of trials, while the apoE KO mice had significantly longer latencies than the WT mice on the third block of trials during place training. However, analyses of the path length data (not shown; see text) did not support the latency data in that no differences were found among the groups in terms of path length during the place trials. The groups also did not differ on several probe trial performance variables (see text). In addition, the groups performed similarly in terms of both escape latency (C) and path length (not shown) during the cued trials, suggesting that nonassociative factors did not affect performance. (D) These data represent the first two trials from each block of four trials during which almost all of the mice completed all of the trials. When these data were analyzed, no differences were found between the groups during place or cued trials. In summary, the bulk of the evidence suggested that the genotypic groups did not differ in terms of place or cued learning performance.

conducted on the escape latency data (Fig. 1C) yielded a nonsignificant main effect of Group, a significant effect of Blocks of Trials [$F(4,140) = 5.88$, $P < 0.0005$], and a significant Group × Blocks of Trials interaction [$F(12,140) = 2.00$, $P = 0.028$], indicating that the groups differed in their average latencies over the five blocks. Subsequent one-way ANOVAs showed that the groups differed significantly during the first and third

blocks (Fig. 1C) [$F(3,35) = 3.04$, $P = 0.042$; and $F(3,35) = 2.87$, $P = 0.05$, respectively]. Subsequent pairwise comparisons conducted within Block 1 revealed that the apoE4 mice had significantly longer escape latencies than the WT mice ($P = 0.011$) and the apoE KO mice ($P = 0.040$). In addition, the apoE KO mice were found to have significantly longer escape latencies than the WT mice ($P = 0.007$*) during the third block of trials. However, the escape path length data (not shown) did not corroborate the latency data in that the ANOVA indicated a nonsignificant effect of Group and a nonsignificant Group by Blocks of Trials interaction as well. The effect of Blocks of Trials was marginally nonsignificant ($P = 0.059$). In addition, no significant differences were found on any of the "probe" trial measures (latency to reach the target quadrant, latency to reach the former platform location, percentage of time spent searching the platform quadrant, and number of swim passes over the former platform location; -data not shown). In general, the mice showed inconsistent retention performance during the probe trials. Analysis of the cued trials indicated that no group differences were apparent for either escape latency (Fig. 1C) or path length, suggesting that nonassociative factors were not selectively affecting performance as a function of group. Swimming speeds (data not shown) were also analyzed in an effort to determine whether the groups differed in swimming abilities. An ANOVA of these data yielded a marginally nonsignificant main effect of Group ($P = 0.064$), a significant effect of Blocks of Trials [$F(4,140) = 12.95$, $P < 0.0005$], and a marginally nonsignificant Group by Blocks of Trials interaction ($P = 0.057$). The lack of significant differences in swimming speeds suggests that sensorimotor disturbances or other nonassociative factors were not differentially affecting group performances.

Another method of data analysis involved analyzing the data from only the first two trials within each block during which almost all of the animals completed every trial (Fig. 1D). Specifically, one mouse did not complete a trial in Block 2, three mice did not complete a trial in Block 4, and two mice did not complete a trial in Block 5. These results appear similar to those presented in Fig. 1C, although an ANOVA of these escape latency data yielded a nonsignificant effect of Group and a nonsignificant Group by Blocks interaction, while the effect of Blocks of Trials was significant [$F(4,124) = 3.29$, $P < 0.013$]. In addition, the groups did not differ significantly on any block although differences approached significance during Block 1. An ANOVA of the path length data revealed similar findings. Other analyses involving the assignment of a maximum score of 60 s for uncompleted trials also yielded nonsignificant findings. In summary, the bulk of evidence suggests that genotypic groups did not differ in terms of their performance on the water navigation task, although the physical demands of the test protocol may have blunted the sensitivity of this test to detect subtle differences.

Radial arm maze. The mice were tested on the working (trial dependent) memory protocol used with the radial arm maze at 11–14 months of age. Results from the radial maze testing suggested that the groups of mice differed in terms of both emotionality and spatial learning/memory capabilities (Fig. 2). For example, the groups differed in terms of the amount of neophobia (reluctance to consume familiar food in a novel environment) exhibited during habituation as measured by the time taken to first begin eating in the maze (Fig. 2A). Figure 2A shows that the WT and apoE4 mice tended to consume the food more quickly than the apoE3 and apoE KO mice. An overall ANOVA of these data yielded a significant main effect of Group [$F(3,32) = 3.48$, $P = 0.027$], a significant effect of Days (of habituation) [$F(8,256) = 25.77$, $P < 0.0005$], and a significant Group by Days interaction [$F(24,256) = 2.19$, $P = 0.006$]. Subsequent one-way ANOVAs conducted within each test day indicated that the groups differed on Days 1 [$F(3,32) = 7.52$, $P = 0.001$] and 2 [$F(3,32) = P < 0.0005$] but not on Day 3 or on subsequent days. On Day 1, the WT mice ate significantly sooner than either the apoE KO ($P = 0.007$*) or the apoE3 ($P = <0.0005$*) mice but not sooner than the apoE4 mice. On Day 2, the WT mice ate significantly sooner than the apoE KO ($P = 0.007$*), apoE3 ($P < 0.0005$*), or apoE4 ($P = 0.004$*) mice. Another indication of differences in emotionality was suggested by the data pertaining to the number of days required to reach the habituation criterion (Fig. 2B) before formal spatial learning testing could be initiated. Specifically, groups differed significantly on these habituation scores [$F(3,32) = 4.16$, $P = 0.014$], with the WT mice requiring significantly less time for habituation than the apoE KO ($P = 0.007$*), apoE3 ($P = 0.033$), or apoE4 ($P = 0.003$*) mice. No differences were noted among the apoE TG/KO groups on the habituation scores.

The most important findings from the radial maze testing involved the acquisition data. Figures 2C and 2D show that the acquisition performance of the apoE4 group was substantially inferior to that of the other three groups. This was the case for both days and errors to reach the acquisition criterion demonstrating learning (Fig. 2C) and with regard to the cumulative percentage of mice reaching acquisition criterion as a function of blocks of Test Days (Fig. 2D). An ANOVA on the days and errors to criterion data showed that the groups did differ significantly [$F(3,31) = 6.75$, $P = 0.001$; $F(3,31) = 5.60$, $P = 0.003$, respectively] and the apoE4 mice were the most impaired, requiring significantly more trials and errors to criterion than the WT (Ps < 0.0005*), the apoE KO (Ps = 0.002* and 0.003*, respectively), or the apoE3 mice (Ps = 0.012 and 0.025, respectively). No other differences were found among

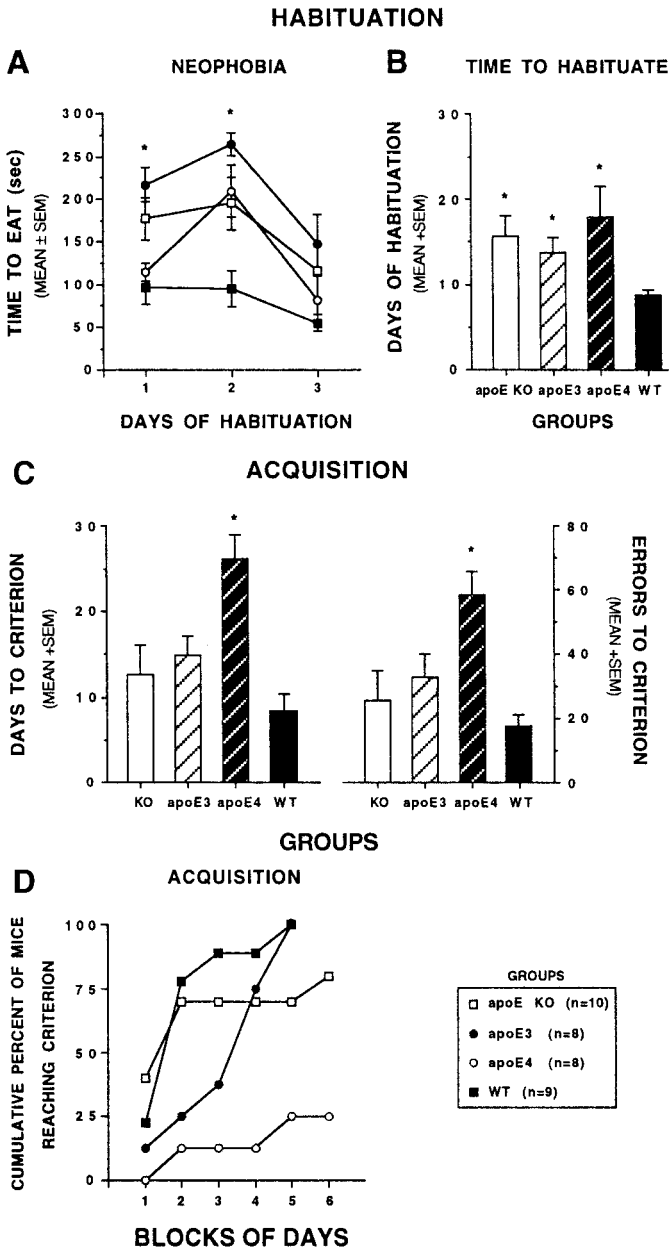

FIG. 2. Performance of the mice on the win-shift spatial discrimination (working memory) protocol in the radial arm maze when tested at 11–14 months of age. (A) The groups differed in terms of the amount of neophobia (reluctance to eat a familiar food in a novel environment) exhibited during early phases of habituation. The groups differed significantly (*) with regard to the times at which they began eating in the maze on Days 1 and 2 ($P = 0.001$ and $P < 0.0005$, respectively) although no significant group effects were found on Day 3 or on any other subsequent day. On Day 1, the WT mice ate significantly sooner than the apoE KO ($P = 0.007$) or apoE3 ($P < 0.0005$) mice while on Day 2 they ate significantly sooner than the apoE KO ($P = 0.007$), apoE3 ($P < 0.005$), or apoE4 ($P = 0.004$) mice. (Note that data from only the first three days of habituation are shown since significant main effects of Group were found only during Days 1 and 2). (B) The groups also differed significantly on the number of days required to reach the habituation criterion ($P = 0.014$) with the apoE KO ($P = 0.007$), apoE3 ($P = 0.033$), and apoE4 ($P = 0.004$) groups each requiring significantly (*) more days to reach criterion than the WT mice. The apoE TG/KO mice required similar amounts of habituation. (C) Most importantly, the groups differed significantly in days and errors to criterion during acquisition training. This effect was mostly due to the profound impairment of the apoE4 mice, which required significantly (*) more days and errors to reach criterion than the WT (Ps < 0.0005), the apoE KO (Ps $= 0.002$ and 0.003, respectively), and the apoE3 (Ps $= 0.012$ and 0.025, respectively) mice. (D) This graph shows the cumulative percentage of mice reaching the acquisition criterion over time, which further illustrates the extensive impairment of the apoE4 mice.

the groups with regard to the days to criterion scores. Acquisition training was terminated when mice could not reach criterion within 30 days and they were assigned scores that represented the earliest time they could have reached criterion (e.g., 30 days). Comparing across groups, the number of mice that never reached criterion further highlights the acquisition impairment in the apoE4 mice. Specifically, 6/8 apoE4 mice never learned the task (reached criterion) compared to 2/10 apoE KO, 0/8 apoE3, and 0/9 WT mice. In summary, although the apoE TG/KO mice showed signs of altered emotionality, only the apoE4 mice showed a profound acquisition impairment on the working memory protocol in the radial arm maze.

Possible Effects of Age Differences on Learning and Memory

All of the mice were evaluated on the first rotating holeboard test when they were the same age, i.e., when they became 6 months of age. The remaining learning/memory tests were conducted on all of the mice during the same period of time. Thus, at the time of each test, the groups being compared to each other were composed of mice that were slightly different in age. Table 1 shows the mean (±SEM) and range of ages for each group as well as the results of correlational analyses (Pearson r) that were used to assess the relationship between age and performance on each of the tests. For the water navigation task, the correlation coefficients reflect the association between age and escape latency and path length scores averaged across the first two trials in each block of trials (i.e., data shown in Fig. 1D). As Table 1 shows, age was not significantly related to learning/memory performance on any of the tests. For the water navigation data we also calculated correlation coefficients for age versus the average performance for each block of trials and for the data where group means were assigned to individual mice that did not complete a trial (i.e., data shown in Fig. 1C). None of these calculations yielded significant correlation coefficients. Note that the apoE4 group tended to be slightly younger than the other groups (means and ranges), thus indicating that differences in ages could not be used to account for the radial maze deficits.

TABLE 1

Relationship between Age and Performance on Spatial Learning and Memory Tests

Group	Ages and correlation coefficients for learning and memory tests [means (±SEM) and ranges]		
	Water navigation	Radial arm maze	Holeboard (second)
apoE KO	11.1 ± 0.33 (9.7–12.4)	12.0 ± 0.39	16.0 ± 0.35
apoE3	11.4 ± 0.09 (10.8–11.6)	12.4 ± 0.33	16.2 ± 0.11
apoE4	10.0 ± 0.23 (9.0–10.9)	11.1 ± 0.32	15.2 ± 0.40
WT	10.9 ± 0.03 (10.5–11.4)	11.9 ± 0.00	16.4 ± 0.28
r	PL L: 0.05 (ns) PL P: 0.24 (ns) CU L: 0.13 (ns) CU P: 0.26 (ns)	Days: −0.27 (ns) Errors: −0.24 (ns)	Acq: −0.16 (ns) Ret: 0.07 (ns)

Note. PL L, escape latency averaged across place trial blocks; PL P, escape path length averaged across place trial blocks; CU L, escape latency averaged across cued trial blocks; CU P, escape path length averaged across cued trial blocks; Days, days to criterion; Errors, errors to criterion; Acq, acquisition; Ret, retention.

Tests of Sensorimotor Capabilities and Altered Emotionality

Sensorimotor battery. One-way ANOVAs conducted on the data from each of the four tests yielded negative results except for the walking initiation test [$F(3,38) = 3.80$, $P = 0.018$; see Figure 3]. Pairwise comparisons conducted subsequent to the significant ANOVA indicated that it took the WT group a significantly longer time to leave the square than was the case for the apoE KO group ($P = 0.006$*) or the apoE3 group ($P = 0.005$*), although there were no differences among the apoE TG/KO groups (Fig. 3C). In summary, the negative findings on the ledge, platform, and inclined screen suggest that the groups did not differ in terms of balance, strength, or coordination while the significant findings on the walking initiation test suggest that the WT mice were more reluctant to move in a wide open area than the apoE3 or apoE KO mice.

One-hour activity. Data from the 1-h activity tests are shown in Fig. 4. The results of a one-way ANOVA on the general locomotor activity data from testing the mice using a low-resolution photobeam system at 6 months of age (Fig. 4A) revealed that the groups of mice differed significantly [$F(3,38) = 4.48$, $P = 0.009$] in terms of activity counts (photocell beam breaks). Subsequent pairwise comparisons indicated that the apoE KO group had significantly higher activity counts than the WT ($P = 0.004$*), apoE3 ($P = 0.022$), and apoE4 ($P = 0.006$*) groups. Differences among WT, apoE3, and apoE4 groups were not significant. When the mice were tested using a high-resolution photobeam system when they were 10–13 months of age, the groups were not found to differ in terms of total ambulations (whole body movements; Fig. 4B). However, the groups did differ significantly on numbers of fine movements [$F(3,33) = 3.39$, $P = 0.029$], which are defined as any motor activities exhibited within a small area such that they do not qualify as ambulations (Fig. 4C). Significantly greater numbers of fine movements were observed in the WT mice relative to the apoE KO ($P = 0.011$) and apoE4 mice ($P = 0.008$*) while compared to the apoE3 mice the differences were marginally nonsignficant ($P = 0.063$). However, no differences were found among the apoE TG/KO groups. The groups of mice also differed in terms of the time spent in the center of the field (Fig. 4D), $F(3,33) = 3.09$, $P = 0.04$. Subsequent pairwise comparisons showed that the apoE3 and apoE4 mice spent significantly less time in the center area than the WT mice ($P = 0.048$ and 0.011, respectively). Also, the apoE4 mice spent significantly less time in the center than the apoE KO mice ($P =$

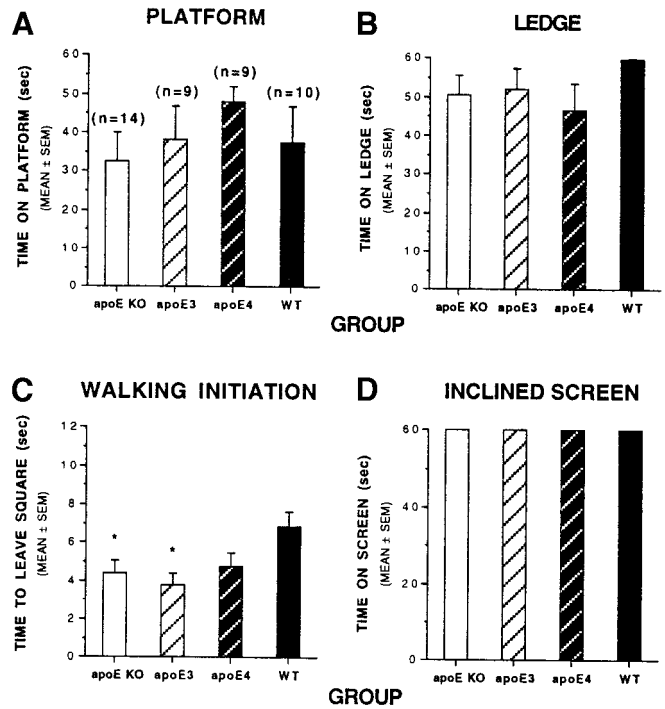

FIG. 3. Results from the sensorimotor battery derived from testing the mice when they were 6 months old. No significant performance differences were found among the groups on the platform (A), ledge (B), or inclined screen (D) tests, suggesting that the groups of mice did not differ in terms of balance, strength, or coordination. However, significant differences were found among the groups on the walking initiation test (C), with the apoE KO and apoE3 mice each taking significantly (*) less time to leave the square than the WT mice (*P*s = 0.006 and 0.005, respectively), while no differences were found among the apoE TG/KO groups. Although this test measures initiation of movement, results from drug studies and other data from the present study suggest that these differences may reflect altered emotionality.

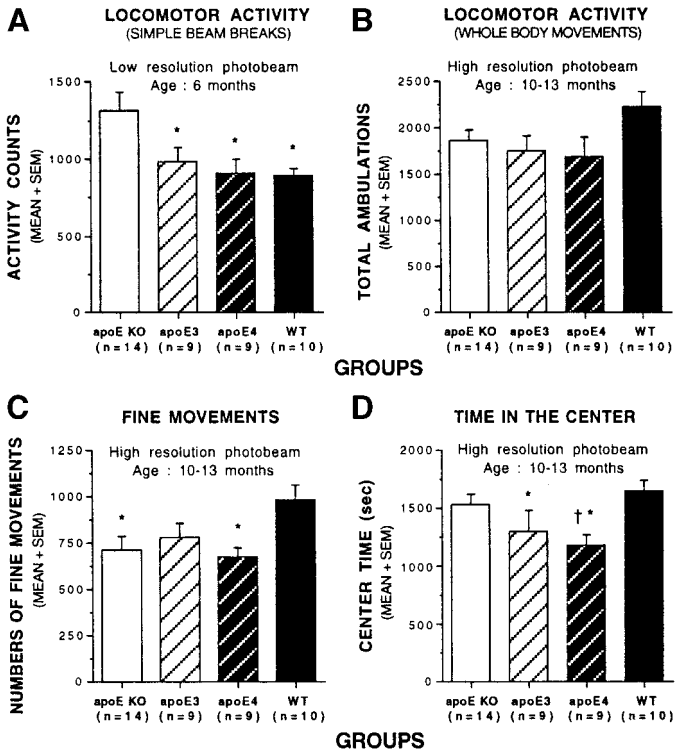

FIG. 4. Measures of locomotor activity and open-field behavior during a 1-h test conducted when the mice were 6 and 10–13 months of age. (A) At 6 months of age the mice were tested using a low-resolution photobeam system and the results indicated that the groups differed in terms of total activity (beam breaks), with the apoE KO mice exhibiting significantly (*) higher levels than the WT ($P = 0.004$), apoE3 ($P = 0.022$), and apoE4 ($P = 0.006$) groups. (B) In contrast, no differences were found in total ambulations (whole body movements) when a computerized, high-resolution photobeam system was used to evaluate activity and open-field behaviors when the mice were 10–13 months of age. (C) However, the mice were found to differ in terms of the numbers of fine movements (small movements without ambulation) exhibited, with the apoE KO or the apoE4 mice displaying significantly (*) fewer movements than the wild-type mice (Ps = 0.011 and 0.008, respectively). No differences were found among the apoE TG/KO groups. (D) The groups differed in terms of certain open-field behaviors, suggesting alterations in emotionality. Specifically, the groups differed in the time spent in the center of the "field," with the apoE3 and apoE4 mice spending significantly (*) less time in the center than the WT mice (Ps = 0.048 and 0.011, respectively). The apoE4 mice also spent significantly (†) less time in the center than the apoE KO mice ($P = 0.045$).

0.045) Thus, the apoE TG mice, particularly the apoE4 mice, spent the most time outside the center area.

Acoustic startle/PPI. Data from the acoustic startle/PPI testing carried out when the mice were 6 months old are shown in Fig. 5. With regard to the acoustic startle data, Fig. 5A shows that the apoE4 mice consistently displayed the highest startle amplitudes across the four blocks of trials while the WT mice consistently responded with the lowest amplitude. The apoE KO and apoE3 mice showed startle amplitudes that were consistently in between those of the apoE4 and WT mice. An ANOVA revealed that the groups differed significantly in terms of the amplitude of their startle response [$F(3,38) = 5.13$, $P = 0.004$] and this was the case for each of four blocks (five trials each) of startle trials. The startle amplitude of the apoE4 mice was significantly higher than that of the WT group for each of the four blocks of trials (Ps = 0.000*–0.006*)

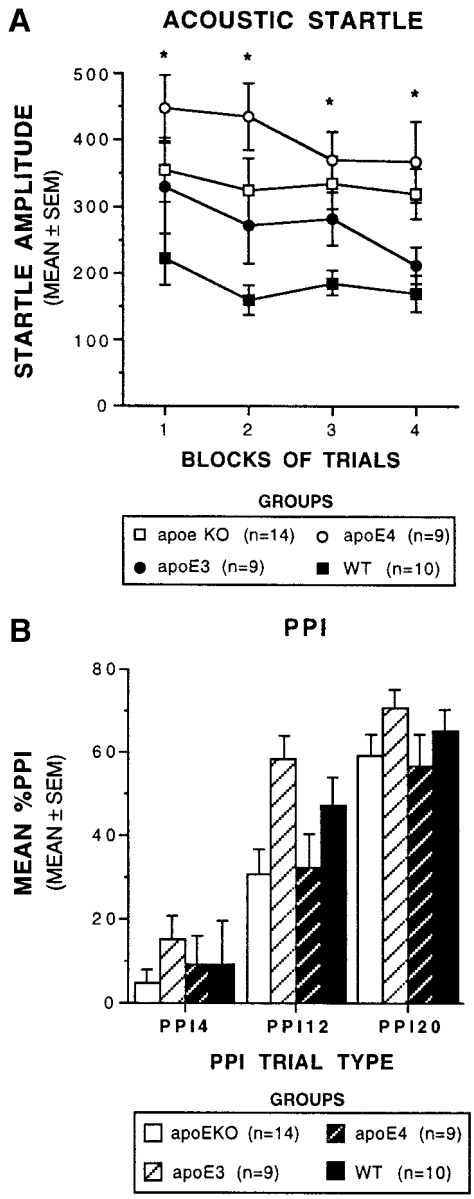

FIG. 5. Data pertaining to the acoustic startle response and prepulse inhibition of the startle response (PPI) resulting from testing the mice when they were 6 months of age. (A) The groups differed significantly in terms of startle amplitude [$F(3,38) = 5.13$, $P = 0.004$], with the apolipoprotein E4 mice showing significantly higher startle amplitudes than the wild-type mice for each of the four blocks of trials (Ps = 0.0009–0.006) and significantly higher amplitudes than those of the apolipoprotein E3 mice on blocks 2 ($P = 0.029$) and 4 ($P = 0.015$). The apolipoprotein E knockout mice had significantly higher amplitudes than the wild-type mice for blocks 2–4 (Ps = 0.004–0.013). (B) The groups did not differ in terms of %PPI or block%PPI (not shown).

TABLE 2

Significant Main Effects of Group and Group by Test Days Interactions for Elevated Plus Maze Performance Variables.

Variable	Main effect of Group	Group by Test Days interaction
% of time in open arms		$F(6,76) = 3.79$, $P = 0.006$
Time at rest in open arms	$F(3,38) = 3.63$, $P = 0.021$	$F(6,76) = 4.20$, $P = 0.007$
% of time in open arms at rest	$F(3,38) = 3.38$, $P = 0.028$	
Total arm entries	$F(3,38) = 2.96$, $P = 0.044$	
x-ambulations (closed arm activity)	$F(3,38) = 3.12$, $P = 0.037$	
y-amublations (open arm activity)		$F(6,76) = 2.19$, $P = 0.053$
Fine movements	$F(3,38) = 3.80$, $P = 0.018$	
Stretch attend postures in the closed arms	$F(3,34) = 3.55$, $P = 0.024$	
Corner dips in the closed arms		$F(6,68) = 3.43$, $P = 0.005$

Note. A significant main effect of Test Days was also found for each of the above variables.

and higher than that of the apoE3 mice on blocks 2 ($P = 0.029$) and 4 ($P = 0.015$). In addition, the apoE KO mice also showed significantly higher startle amplitudes than the WT group for blocks 2–4 (Ps = 0.004*–0.013). In contrast to the startle amplitude data, ANOVAs conducted on the PPI data (Fig. 5B) did not yield similar between-groups effects. Data generated from both types of PPI transforms (%PPI and block%PPI) were subjected to ANOVAs, and each revealed a significant effect of PPI trial type [$F(2,76) = 150.79$, $P < 0.0005$; and $F(2,76) = 64.86$, $P < 0.0005$, respectively], although the main effect of Group and the Group by Trial Type interaction were nonsignificant using both types of transforms.

Elevated plus maze testing. When the mice were tested on the elevated plus maze at 8–11 months of age, the groups exhibited different patterns of behavior on several variables (see Table 2 for the specifics concerning important variables for which significant main effects and interactions were found following "overall" ANOVAs). One of the most important findings concerned the percentage of the total session time that the mice spent in the open arms (Fig. 6A). Following a significant Group by Test Day interaction (Table 2), one-way ANOVAs were conducted for each test day and the groups were found to differ significantly only for Test Day 1 [$F(3,38) = 3.66$, $P = 0.021$]. Subsequent pairwise comparisons showed that the apoE4 mice spent a significantly greater percentage of time in the open arms on Day 1 than the WT ($P = 0.005$*) and apoE KO ($P = 0.006$*) mice, while the difference between the apoE3 and the apoE4 groups was marginally nonsignificant ($P = 0.075$). Additional analyses clarified the nature of the apoE4 mice spending more time in the open arms. Specifically, the groups differed significantly in terms of the percentage of time spent at rest in the open arms (Table 2; Fig. 6B) and subsequent analyses showed that they differed only on Day 1 [$F(3,38) = 4.16$, $P = 0.012$]. Additional pairwise comparisons showed that the apoE4 mice spent a significantly greater percentage of time "at rest" in the open arms than the WT ($P = 0.005$*), apoE KO ($P = 0.003$*), or apoE3 ($P = 0.019$) mice on Day 1. The relative immobility of the apoE4 mice in the open arms was further supported by evidence from the *y*-axis ambulation data, which predominantly reflect activity in the open arms (Fig. 5C). These data show that apoE4 mice exhibited the least amount of open arm activity on Day 1. Although the Group by Test Day interaction for the *y*-axis ambulations was marginally nonsignificant ($P = 0.053$), we decided to evaluate group differences on Day 1 since differences in open-arm activity were likely to have been severely restricted as a result of the mice spending almost no time in the open arms by Day 3. An ANOVA of the Day 1 *y*-axis ambulation data indicated that the groups differed significantly, $F(3,38) = 2.95$, $P = 0.045$, and subsequent pairwise comparisons showed that the apoE4 mice were significantly less active in the open arms (had fewer *y*-axis ambulations) than the WT ($P = 0.016$) and apoE3 ($P = 0.012$) mice. Note that all groups of mice showed extensive decreases across test days in the percentage of time spent in the open arms and in the locomotor activity exhibited in the open arms such that by the third day, the mice spent almost no time in the open arms and virtually no activity was recorded. Similar results were found with regard to entries made into the open arms (data not shown). This almost complete avoidance of the open arms that occurred as a function of repeated plus maze testing is a well-documented finding that is thought to reflect heightened anxiety that results from conditioning or sensitization of the fear-inducing properties of the open arms (e.g., 25, 26).

Analysis of other plus maze variables suggested that the apoE4 mice were hypoactive in general in the plus maze. For example, the groups differed significantly concerning *x*-axis ambulations (Table 2, Fig 6D), which predominantly reflect activity in the closed arms. Groups differed significantly on Days 1 [$F(3,38) = 4.10$, $P = 0.013$] and 3 [$F(3,38) = 4.36$, $P = 0.010$], with the apoE4 mice exhibiting significantly fewer *x*-axis ambulations than the WT, apoE KO, and apoE3 groups on

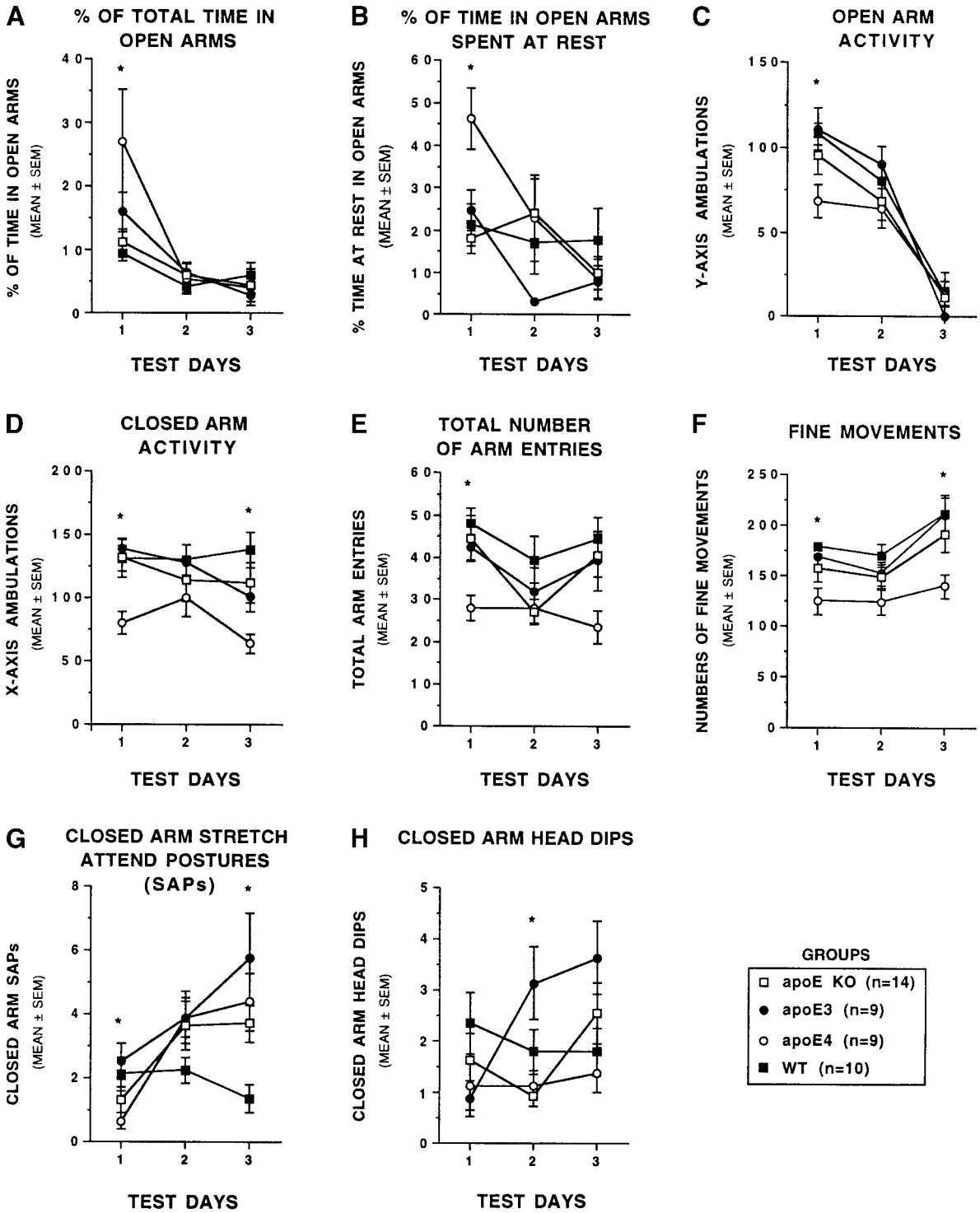

FIG. 6. Elevated plus maze data from selected variables resulting from testing the mice over 3 consecutive days when they were 8–11 months of age. (A) Upon initial exposure to the plus maze (Test Day 1), the apoE4 mice spent a significantly greater percentage of the total session time in the open arms than the WT ($P = 0.005$) and apoE KO ($P = 0.006$) mice, thus suggesting that the apoE4 mice were "less anxious" when first tested. However, this is not likely to be the case since the apoE4 mice spent a greater percentage of the open arm time "at rest" than the WT ($P = 0.002$), apoE KO ($P < 0.0005$), or apoE3 mice ($P = 0.008$) on Test Day 1 (B) and since they were significantly less active in the open arms (made fewer *y*-axis ambulations) on Day 1 (C) than the WT ($P = 0.016$) and apoE3 mice ($P = 0.012$). The high degree of immobility exhibited by the apoE4 mice in the open arms and the lack of group differences in open arm entries strongly argue against

Day 1 (*P*s = 0.010, 0.005*, and 0.004*, respectively) and significantly fewer than the WT and apoE KO mice on Day 3 (*P*s = 0.001* and 0.018, respectively). Also, note how the *x*-axis ambulation data are in striking contrast to the *y*-axis ambulation data in that the groups in general did not show the precipitous decline in activity over the 3 test days as occurred with the *y*-axis ambulations. This suggests that heightened anxiety with reference to the closed arms did not occur as a result of repeated testing in the plus maze. Data from the total arm entries (Fig. 6E) were similar to those of the *x*-axis ambulations in that there was a significant main effect of Group (Table 2) and that the groups differed significantly on Day 1, $F(3,38) = 3.61$, $P = 0.022$. This effect was mostly due to the small number of total arm entries of the apoE4 mice who had significantly fewer entries than the WT, apoE KO, and apoE3 groups (*P*s = 0.004*, 0.010, and 0.040, respectively). A significant main effect of Group was also found for fine movements (Table 2, Fig. 6F), with significant differences occurring between groups on Days 1 [$F(3,38) = 3.72$, $P = 0.019$] and 3 [$F(3,38) = 3.34$, $P = 0.029$]. Again, the apoE4 mice were the least active in terms of small nonambulatory movements that likely reflect either preening or stereotypical behaviors. For example, they exhibited significantly fewer fine movements compared to the WT mice on Days 1 ($P = 0.003$*) and 3 ($P = 0.008$*), compared to the apoE KO mice on Days 1 ($P = 0.046$) and 3 ($P = 0.036$), and compared to the apoE3 mice on Day 1 ($P = 0.015$).

Behavioral variables likely related to risk assessment, which were quantified by videotape analyses, are shown in Figs. 6G and 6H. In contrast to the open arm variables, the numbers of SAPs in the closed arms actually increased over the three test sesions in the apoE TG/KO groups while the number of "protected" (closed arm) SAPs in WT mice slightly decreased over time (Fig. 6G). An ANOVA of these data yielded a significant main effect of Group and a significant Group by Test Days interaction (Table 2) and subsequent analyses showed that the groups differed in terms of protected SAPs on Days 1 [$F(3,34) = 3.03$, $P = 0.042$] and 3 [$F(3,34) = 4.51$, $P = 0.009$]. Initially, the apoE4 mice emitted significantly fewer protected SAPs than the WT ($P = 0.034$) and apoE3 mice ($P = 0.011$) on Day 1 but the apoE 4 mice increased their numbers of protected SAPs over the 3-day test session along with the other apoE TG/KO mice. By Day 3 the numbers of SAPs in the WT group were significantly lower than those observed in the apoE KO, apoE3, and apoE4 groups (*P*s = 0.04, 0.001*, and 0.019, respectively). With regard to the "protected" head dips emitted around the corners of the closed arms (Fig. 6H), the significant Group by Test Day interaction (Table 2) showed that groups emitted the corner dips in differing amounts across the test sessions. Subsequent analyses showed that the groups differed significantly on Day 2, with the apoE3 mice showing significantly greater numbers of corner dips than the WT ($P = 0.035$), apoE KO ($P < 0.0005$*), and apoE4 mice ($P = 0.003$*).

In summary, the relative immobility of the apoE4 mice in the open arms and the lack of group differences with regard to entries made into the open arms on Day 1 (data not shown) suggest that it is not appropriate to interpret the increased time spent in the open arms by the apoE4 mice on Day 1 as being indicative of abnormally low anxiety levels on the part of the apoE4 mice. The interaction of certain cognitve and emotionality factors may provide a reasonable explanation for the initial behavioral responses of the apoE mice to being tested in the plus maze (see Discussion). However, the apoE4 mice responded like the other groups in terms of increasing their avoidance of the open arms as a function of repeated testing. In contrast, the apoE3 mice tended to be the most reactive in terms of protected risk assessment behaviors.

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interpreting the increased open arm time exhibited by the apoE4 mice as being indicative of "lowered anxiety." The possible role of certain cognitive factors in accounting for the increased open arm time observed in the apoE4 mice on Day 1 is discussed in the text. The relative immobility of the apoE4 mice was also apparent in other behaviors such as in their hypoactivity in the closed arms compared to that of the other groups and in terms of other general movement measures in the maze such as total arm entries and fine movements as shown in D through F. Note the extensive decreases in the percentage of time spent in the open arms (A) and in the locomotor activity exhibited in the open arms (C) across test days in all the groups of mice such that by the third day the mice spent almost no time in the open arms and virtually no activity was recorded. These findings reflect "phobic avoidance" of the open arms, which is a well-documented effect in rodents occurring as a result of exposing animals to multiple plus maze test sessions. Certain cognitive factors that might account for the increased time spent in the open arms by the apoE4 mice are discussed in the text. Note also that the apoE4 mice were as capable of becoming conditioned (or sensitized) to these effects as were the other groups of mice. In contrast to the open arm activity data shown in C, activity in the closed arms (*x*-axis ambulations; D) did not significantly decrease over the test sessions, indicating that there was no phobic avoidance of the closed arms. Videotape analyses of two risk assessment-related behaviors are depicted in G and H. (G) On Day 1 the apoE4 mice exhibited significantly lower numbers of "protected" (closed arm) SAPs relative to the apoE3 ($P = 0.011$) and WT ($P = 0.034$) mice. Interestingly, the numbers of protected SAPs increased over the three test sessions in the apoE TG/KO groups while they stayed the same or slightly decreased in the WT mice such that by Day 3, the WT group emitted significantly fewer protected SAPs than each of the other three groups (*P*s = 0.001–0.04). (H) With regard to the "protected" head dips emitted around the corners of the closed arms, the apoE3 mice showed increased numbers over the test sessions and demonstrated significantly greater numbers of dips compared to each of the other groups (*P*s = 0.0005–0.035) on Day 2. The asterisk indicates a significant effect of Group for a given test day. See text and Table 2 for a detailed description of the statistical results.

Neuronal and Glial Markers

At the completion of behavioral testing, the mice were sacrificed in order to subject the right hemisphere to histological analyses while the left hemisphere was subdissected and assessed for neuronal and glial markers by Western blot analyses. As previously shown (12, 33), we found that levels of apoE protein were indistinguishable between apoE3 and apoE4 TG mice and were ≈70% of WT mouse apoE levels. We performed semiquantitative Western blotting and compared the levels of synaptophysin, neuron-specific enolase, and GFAP between WT, apoE KO, apoE3, and apoE4 mice (Fig. 7). There were no significant differences in the levels of any of these proteins in cortex or hippocampus between any of the groups of mice (Fig. 7C). Similar results were obtained with MAP-2 (data not shown). We also examined the brains from each group after staining with cresyl violet. There were no structural abnormalities noted in any brain region nor was there any qualitative evidence of cell loss in the hippocampus or neocortex in any group. We also looked for evidence of amyloid-β (Aβ) deposition through the use of immunostaining and for neuritic degeneration using the de Olmos silver technique as previously described (13, 34). These histologic techniques yielded no evidence of Aβ deposition or neuritic plaques.

DISCUSSION

The present work is the first to report on the behavioral phenotyping of GFAP-apoE3 and GFAP-apoE4 TG mice in which the two isoforms of human apoE are expressed in glia, the predominant cell type in the CNS that synthesizes apoE in mammals (4, 18, 19, 30). Our results suggest that the apoE TG/KO and WT groups of mice differed in terms of emotionality profiles and that the acquisition performance of the apoE4 mice was severely impaired on the radial arm maze, a working (trial-dependent) memory-based spatial learning task. In contrast, no convincing evidence was found for deficits in the acquisition of reference (trial-independent) memory-based tasks (holeboard or Morris water navigation) in the apoE TG/KO mice. Although the apoE TG/KO and WT groups differed on several indices of emotionality (e.g., open-field behaviors, acoustic startle response, plus maze variables), these differences did not appear to have an impact on acquisition or retention performance. Our behavioral findings and the absence of any AD-like pathology in the apoE TG/KO mice suggest that apoE and apoE genotype may play a role in age-and/or AD-related cognitive impairments in humans, which may be distinct from any of their effects on Aβ structure and deposition.

The most robust effect of apoE genotype on behavior was the finding that apoE4 mice were profoundly impaired in terms of their acquisition performance (days and errors to criterion) on the working memory protocol used with the radial arm maze. Moreover, 6/8 apoE4 mice were unable to reach the acquisition criterion of demonstrating learning within 30 days compared to 2/27 of all of the other groups of mice combined. The working memory deficits exhibited by the apoE4 mice suggest that they have difficulties in executing correct responses that are dependent on accurately recalling recent events. Additional studies are necessary to further explore the nature of this radial maze acquisition deficit in the apoE4 mice, particularly to rule out the possibility that somehow differences in olfaction capabilities (pertaining to scent marking) could be responsible for the performance deficits and to determine at what age the impairment becomes manifest.

Although the radial maze data suggested severe working memory deficits in the apoE4 mice, there was little evidence that reference memory capabilities were compromised in any of the apoE TG/KO groups as indexed by the first rotating holeboard test and the water navigation task. These findings are consistent with those of Raber *et al.* (22), who reported that both 6- and 18-month old NSE-apoE4 male mice did not differ from NSE-apoE3 or apoE KO male mice in terms of place learning performance in the water maze. In contrast to these data on male mice, the authors found that NSE-apoE4 female mice were not impaired during place learning acquisition at 6 months of age, although they demonstrated retention deficits on a probe trial. However, 18-month old NSE-apoE4 female mice were impaired during both place learning acquisition and probe trials. The results from our holeboard testing when the mice were 14–17 months of age also suggested age-related deficits in reference memory processing in some of our mice. For example, the WT mice were impaired during acquisition compared to the apoE KO and apoE3 mice and all of the groups, except for the apoE3 mice, exhibited age-related (within-groups) decrements in acquisition performance relative to levels observed on the earlier holeboard test. Although these data suggest that the apoE3 genotype may have somehow protected against age-associated impairments in reference memory processing, the lack of differences in acquisition performance among the apoE TG/KO mice and the small sample sizes seriously limit this interpretation. In another study, Raber *et al.* (20) reported that 6-month-old NSE-apoE4 (but not NSE-apoE3) female mice were impaired, relative to WT mice, on a different type of water navigation task that resembled a series of reversal tests possibly invoking elements of both reference and working memory. In contrast to the female NSE-apoE TG data, 6- or 18-month-old male NSE apoE TG/KO mice were not impaired on this task. Clearly, much information pertaining to the similarity of behavioral phenotypes resulting from the two apoE TG/KO models would be provided by

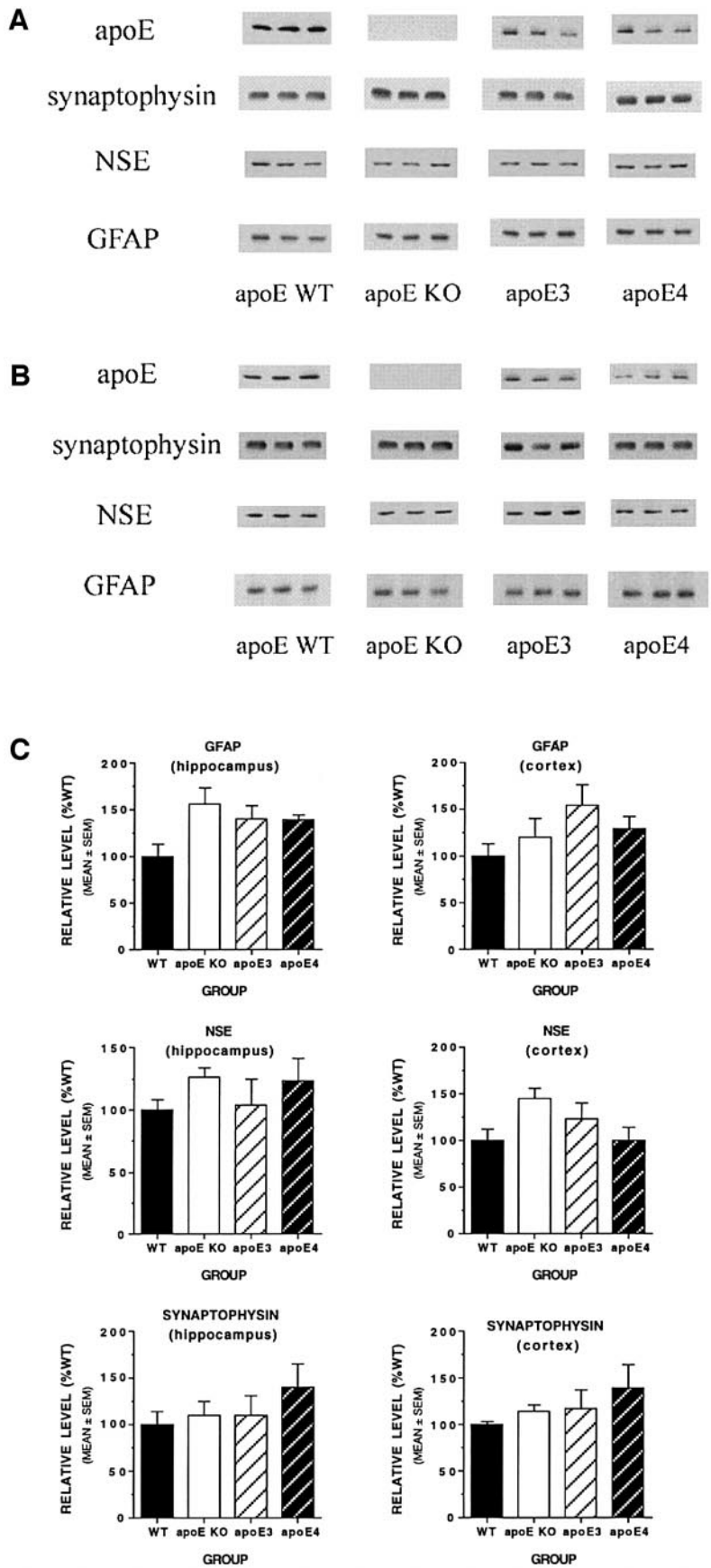

FIG. 7. Western blot analysis of glial and neuronal markers in WT and apoE TG/KO mice. Fifty micrograms of detergent-soluble protein from individual 14- to 15-month-old mice of each genotype (apoE KO, apoE3, apoE4, WT) from either cortex (A) or hippocampus (B) was loaded per lane and analyzed by Western blot for apoE, synaptophysin, NSE, or GFAP. Representative examples of results from three individual mice per genotype are shown in A and B. Each sample was run in triplicate from five mice of each genotype and the level of each protein was determined relative to WT mice by densitometry. Quantitation of these data is shown in C as means (±SEMs). There were no significant differences in the levels of GFAP, NSE, or synaptophysin among the four groups of mice in either the cortex or the hippocampus at 14–15 months of age.

evaluating females in our GFAP-apoE TG model and by testing NSE-apoE TG mice on "pure" working memory tasks.

The interpretation of learning and memory data is oftentimes problematic due to an inability to gauge the influence of nonassociative factors in contributing to altered performance. Thus, fully characterizing the behavioral phenotypes of TG/KO mice may be important, not only to determine the spheres of behavior affected by the genetic manipulation, but also to aid in interpreting the effects of genotype on learning and memory. In this regard, the lack of group differences on the platform, ledge, and inclined screen tests from the sensorimotor battery suggest that the groups did not differ in terms of balance, strength, or coordination. Differences on the walking initiation test were mostly due to the reluctance of the WT mice to leave the square since no differences were observed among the apoE TG/KO groups. Given the above data and evidence that the apoE TG/KO and WT mice differed in terms of emotionality profiles, it seems likely that the results of the walking initiation test reflect altered emotionality rather than differences in motoric capabilities. Furthermore, the finding that the groups did not differ in terms of performance on the cued trials portion of the water navigation task suggests that sensorimotor disturbances did not develop selectively as a function of genotype during middle age (10–13 months). The results of a recent study by Raber *et al.* (20) suggest that the motivational properties of food reward may be another nonassociative factor that could influence performance of the apoE TG/KO mice on appetitive learning/memory tests. Specifically, these authors reported that apoE KO mice showed significant increases in food intake at 12 and 18 months of age (but not at 6 months) compared to WT mice. We directly assessed this possibility in our radial arm maze protocol by measuring the amount of time taken to first begin eating in the maze during daily habituation sessions. Although there were initial differences among groups, they were no longer evident by the third day of habituation and thus they did not likely have an impact on acquisition.

Interpretation of the results from the activity, acoustic startle/PPI, and elevated plus maze tests is complicated although certain trends emerged suggesting that, in general, the apoE TG/KO groups were more emotionally reactive than the WT mice and that the apoE4 mice were often the most reactive of the apoE TG/KO groups. For example, the apoE4 mice spent the least amount of time in the center of the open field, consistently demonstrated the greatest acoustic startle response, and exhibited a unique behavioral response pattern upon initial exposure to the elevated plus maze. With regard to the locomotor activity data, we initially found using a low-resolution photobeam system that the apoE KO mice were more active than the other groups of mice when they were tested at 6 months of age. However, we found no differences among groups when the mice were retested at 10–13 months using a computerized, high-resolution photobeam system that offered quantification of open-field behaviors as well as various measures of locomotor activity. It is unlikely that the discrepant findings were due to the use of different measurement techniques, since identical enclosures were used for both tests and since the high-resolution photobeam system provides a more sensitive index of movement. It seems more likely that additional handling or some other aspect of the Morris maze testing that preceded the second 1-h activity test, or age, was responsible for the difference in results. Future studies should include longitudinal testing with the high-resolution system so that changes in locomotor activity can be compared with changes in open-field behaviors as a function of age.

Results from the elevated plus maze testing also point to the potential importance of alterations in emotionality in that they may interact with certain cognitive factors to produce complex behavioral outcomes. For example, based on percentage of time spent in the open arms on day 1, the apoE4 mice initially appeared to be less anxious than the WT or apoE KO mice. However, additional analyses that characterized the immobility of the apoE4 mice suggested that they may not have been less anxious upon initial exposure to the maze but rather less discriminating of the potential dangers associated with open arms. However, the high avoidance levels of the open arms exhibited by all of the groups of mice on Test Days 2 and 3 show that the apoE4 mice were as capable as the other groups of mice of becoming conditioned (or sensitized) to the anxiety-provoking aspects of the open arms as a result of repeated exposures to the test procedures, consistent with the results of previously published studies (e.g., 25, 26) on nontransgenic mice. Other plus maze results suggested that, within the relatively protected environs of the closed arms, the apoE3 mice were the most likely to engage in risk assessment behaviors such as SAPs or corner head dips and that the apoE3 mice showed a steady increase in these behaviors across the test sessions. Similar differences in emotional reactivity between apoE TG/KO and WT mice may have relevance in accounting for the extended time required for radial maze habituation in apoE TG/KO mice. However, the pattern of results concerning acquisition performance among the groups of mice suggests that spatial working memory was not similarly affected.

The working memory deficits exhibited by the apoE4 TG mice in the radial arm maze in the absence of discernible neurodegenerative changes suggest that apoE and apoE genotype may influence aspects of mammalian behavior and cognition, including certain forms of learning and memory that are independent of AD-related pathology. Although recent evidence demonstrates that apoE plays an important role in the

formation of neuritic and cerebrovascular plaques (2, 13, 14, 23, 31), results from the present study suggest that apoE may also influence the cognitive decline observed in AD and/or in advancing age through additional mechanisms. To help clarify this and other issues, it will be important for future research to reassess, using this and other models (3, 29, 32, 36), whether the apoE4 genotype leads to profound working memory deficits and/or changes in emotionality and to establish when these alterations in behavioral function become manifest.

ACKNOWLEDGMENTS

This work was supported in part by NIH Grants AG13956 (D.M.H.) and AG11355-06 (D.M.H., D.F.W., J.W.O.), Alzheimer's Association Grant RG3-96-26 (D.M.H.), and a Paul Beeson Physician Faculty Scholar Award from the American Federation for Aging Research (D.M.H.).

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