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REFERENCE MEMORY, ANXIETY AND ESTROUS CYCLICITY IN C57BL/6NIA MICE ARE AFFECTED BY AGE AND SEX

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Abstract—Age-related changes in learning and memory are common in rodents. However, direct comparisons of the effects of aging on learning and memory in both males and females are lacking. The present study examined whether memory deteriorates with increasing age in C57BL/6NIA mice, and whether age-related changes in learning and memory are similar in both sexes. Male and female mice (five, 17 and 25 months of age) were tested in a battery of behavioral tasks including the Morris water maze (spatial and non-spatial reference memory), simple odor discrimination (olfactory reference memory), plus maze (anxiety/exploration), locomotor activity, and basic reflexes. Five-month-old mice learned the water maze and odor discrimination tasks rapidly. Relative to five-month-old mice, 25-month-old mice exhibited impaired spatial and olfactory reference memory, but intact non-spatial reference memory. The spatial reference memory of 17-month-old mice was also impaired, but less so than 25-month mice. Seventeen-month-old mice exhibited intact non-spatial (visual and olfactory) reference memory. Five and 25-month-old mice had similar levels of plus maze exploration and locomotor activity, whereas 17-month-old mice were more active than both groups and were slightly less exploratory than five-month-old mice. Although sex differences were not observed in the five- and 25-month groups, 17-month-old females exhibited more impaired spatial reference memory and increased anxiety relative to 17-month-old males. Estrous cycling in females deteriorated significantly with increased age; all 25-month-old females had ceased cycling and 80% of 17-month-old females displayed either irregular or absent estrous cycling.

This study is the first to directly compare age-related mnemonic decline in male and female mice. The results suggest that: (i) aged mice exhibit significant deficits in spatial and olfactory reference memory relative to young mice, whereas middle-aged mice exhibit only a moderate spatial memory deficit and; (ii) spatial reference memory decline begins at an earlier age in females than in males, a finding that may be related to the cessation of estrous cycling. © 1999 IBRO. Published by Elsevier Science Ltd.

Key words: aging, sex differences, mice, spatial memory, estrous cycle, olfactory discrimination.

Age-related deterioration of learning and memory is characteristic of a variety of mammals, from humans to rodents. In elderly humans, several types of memory are affected, including spatial memory and memory for recent events, with declines beginning at approximately 60 years of age.[8,53] In rats, age-related deficits in spatial memory, particularly spatial reference and working memory, have been extensively documented.[10,13,16] Reference memory refers to memory for items that remain constant from trial to trial, whereas working memory refers to memory for items that vary between trials.[47] Different patterns of age-related alteration have been reported for spatial and non-spatial reference and working memory. Spatial reference memory, commonly tested in a spatial version of the Morris water maze, gradually deteriorates from youth (four months) to old age (24 months) in rats, with mild deficits appearing as early as 11 months of age.[16] In contrast, non-spatial reference memory, commonly tested in a cued version of the Morris water maze, does not exhibit age-related decline.[19] Impairments of spatial working memory occur later in life, appearing at approximately 24 months of age.[16]

Age-related mnemonic deterioration has also been reported in mice.[14,15,31,32,37] However, it can be difficult to reconcile the findings of age-related decline in different mouse strains because of robust strain differences in such cognitive tasks as the Morris water maze[57,58] and six-unit T-maze.[33] For example, some mouse strains do not learn the spatial Morris water maze task rapidly, whereas other strains learn the task very easily,[58] thus confounding the observation of age-related changes in performance. Given the increased use of genetically altered mice in models of age-associated diseases such as Alzheimer's disease, it has become imperative to determine the magnitude of age-related changes in mice, such as the C57BL/6 strain, that commonly provide genetic backgrounds for transgenic mouse lines.[1]

A pair of studies in female NMRI mice reported that memory for the spatial water maze task begins to deteriorate at approximately nine months of age, whereas memory for the cued task remains intact until about 22 months.[31,32] As is the case in rats,[17,39] these studies found that age-related cognitive changes were independent of motor changes, such that mice impaired in one domain (e.g., cognitive) were not necessarily impaired in the other (e.g., motor). In male C57BL/6 mice, spatial water maze deficits are evident at 22–26 months of age,[14,15,37] and have been observed as early as 10 months of age.[37] In contrast, 26-month-old male C57BL/6 mice are not impaired in the cued water maze task.[37] Male 27–28-month-old C57BL/6 mice are also not impaired in a radial arm maze task testing spatial working memory,[6] suggesting that, similarly to rats, spatial reference and working memory may deteriorate at different rates in aging mice.

Thus far, studies of age-related spatial memory decline in rodents have focused on either males or females. To date, no study in mice has directly compared the effects of aging on spatial learning and memory in both sexes. There are several reasons to suspect that age-related spatial memory decline may occur differently in males and females. First, the spatial perception and memory of males have been reported to be superior to those of females in adolescent and adult humans,[21,43,52] rats[36,51,61] and mice.[18] Second, sex differences in spatial ability persist well into old age in humans.[34,48,55]

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Abbreviation: SOD, simple odor discrimination.

Third, women are more at risk for Alzheimer's disease than men,[29] and of those who have the disease, women exhibit both more severe cognitive decline[7,25] and a faster rate of behavioral deterioration[35] than men. Together, the findings of sex differences in normal spatial cognition and age-associated cognitive decline in a variety of species raise the possibility that sex differences in the decline of spatial and other cognitive abilities may occur in normal aging mice.

The present study was designed to examine the following questions. (i) Do spatial and non-spatial reference memory deteriorate with increasing age in C57BL/6NIA mice? (ii) Are age-related changes in memory similar in both sexes? Two different behavioral paradigms were used to test reference memory. Spatial and non-spatial (visual) reference memory were examined using the Morris water maze, a task that requires rodents to use either extramaze cues (spatial) or intramaze cues (nonspatial/cued) to locate the position of a platform in the water maze. Based on previously published results from rats and mice, we hypothesized that C57BL/6NIA mice would exhibit age-related impairments in the spatial, but not cued, version of the task. To examine whether the preservation of non-spatial memory was a general phenomenon, or was limited to visually based tasks, the mice were also tested in a non-spatial reference memory task based on olfactory cues. In this task, termed a simple odor discrimination (SOD), the mice were trained to discriminate between two scents to obtain a food reward.[5] A principal components analysis was performed on measures from the spatial, cued and olfactory reference memory tasks to address the question of whether these operationally defined memory tasks measured qualitatively different aspects of memory. Because performance in learning and memory tasks may be influenced by motor and motivational differences, a battery of nonmnemonic tasks was also conducted. To examine differences in anxiety and exploration behavior that may confound the observation of age- and sex-related differences in memory, mice were tested on an elevated plus maze. Because aged C57BL/6 mice are impaired in some sensorimotor and activity measures,[9,15] general reflexes and general locomotor activities were measured. Finally, to examine the potential influence of age-related ovarian hormone decline on cognition in females, the regularity of estrous cycling in female mice was monitored at the conclusion of behavioral testing.

EXPERIMENTAL PROCEDURES

Subjects

Subjects were 36 male and 36 female C57BL/6NIA mice obtained from the National Institutes on Aging colony at Charles River Laboratories (Stoneridge, NY). Three ages of mice were tested, representing young, middle-aged and aged time-points. At the beginning of behavioral testing, the mice were the following ages: five months (10 male, 10 female), 17 months (10 male, 10 female), 25–26 months (16 males at 25 months, 10 females at 25 months, six females at 26 months; this group will hereafter be referred to as the 25-month group). An additional six 25-month-old mice (two male, four female) were tested in the odor sensitivity task. All mice were handled for five days prior to testing to habituate them to being picked up by the experimenter. A time line describing the sequence of behavioral testing and the duration of each task is provided in Table 1. Mice were housed up to six/cage in a room with a 12:12 h light/dark cycle (lights on at 06.00) and behavioral testing was performed during the light phase of the cycle. Food (Harlan Tecklad 2215 Rodent Diet) and water were provided *ad libitum*, except during SOD testing, when food was removed at approximately 21.00 h. Food was returned after the mice had completed their daily test session (approximately 15.00–16.00 h).

Table 1. Time line of behavioral testing

Task	No. of sessions	Test week*
Handling	5	1
Reflexes	1	1
Morris water maze		
Shaping	1	1
Spatial task	5	2
Spatial reversal task	3	3
Cued task	3	3
Elevated plus maze	1	4
Locomotor activity	1	4
Simple odor discrimination		
Shaping	2	5
Simple odor discrimination	3	5
Vaginal lavage (females only)	10	6,7

*Test weeks varied from five to seven days in length, depending on the task.

At the completion of testing in the odor task, mice were returned to an *ad libitum* diet. Body weights of the 17- and 25-month-old mice were monitored during food restriction to ensure that the mice did not drop below 80% of their free feeding weight. The weights of five-month-old mice were not monitored because young mice do not decrease to 80% of their free feeding weight using this food deprivation procedure.[5] All procedures conformed to the standards set forth in the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and were approved by the Animal Care and Use Committee of Wellesley College.

Reflexes

Two basic reflexes were tested. The righting reflex was tested by holding each mouse by its tail and attempting to flip it on its back. A score of 1 indicated the presence of a righting reflex and a score of 0 meant the mouse could be flipped over. The placing reflex was tested by holding each mouse by its tail and lowering it towards a table. If the mouse extended its forearms towards the table, a score of 1 was recorded. If not, a score of 0 was recorded.

Morris water maze

A white circular tank (103 cm in diameter) was filled with water (26°C) and was surrounded by a variety of extramaze cues. The tank was divided into four quadrants, and four start positions were located at the intersections of the quadrants. Data were recorded using an automated tracking system (HVS Image, Hampton, U.K.). Prior to water maze testing, all mice were habituated to the water using a four-trial shaping procedure in which a smaller ring (55 cm) was inserted inside of the larger 103 cm ring to decrease the total swimming area. The purpose of this procedure was to habituate the mice to the water and to teach them to escape from the water by climbing on to a platform. Each mouse was first placed on a visible yellow lucite platform (10 × 10 cm) for 10 s, and then placed at three progressively further distances from the platform where it was allowed 10 s to escape on to it. No data were collected during this procedure. For water maze testing, three different water maze tasks were conducted during two weeks as follows.

Spatial task. In the spatial task (a test of spatial reference memory), the mice were trained to find a submerged platform using extramaze cues. A transparent lucite platform (10 × 10 cm) was submerged 0.5 cm underneath the water in the north-west quadrant of the tank, where it remained for the duration of the five spatial test sessions. The sequence of the four start positions in the tank (north, west, south and east) varied each trial for each mouse. Six trials/mouse were conducted for five consecutive days (one session/day). During the first five trials (platform trials), the platform was available to the mouse for escape. Each mouse was given 120 s to locate the platform, after which time the experimenter placed the mouse on the platform and allowed it to remain there for 10–15 s. Each mouse was dried with a cloth towel upon removal from the platform and placed in its home cage for an intertrial interval of approximately 20 min. The sixth trial was a variable-interval probe trial[38] in which the platform was collapsed, remaining under the water and unavailable for escape for either 20,

30, or 40 s. After this interval, the platform was raised and available for escape.

Multiple measures of water maze performance were recorded. Swim time (s), path length (cm) and swim speed (cm/s) were recorded during trials 1–5 of each session. A "corridor" measure was also recorded during these trials to examine whether the mice swam in a straight path from the start location to the platform.[60] A corridor (14 cm wide) was drawn between the start location and the platform, and the mouse's location was sampled 10 times/s. The ratio of time spent in the corridor per total swim time was calculated as follows: (number of samples in the corridor/total number of samples) × (area of tank/area of the corridor). The total area of the corridor varied with the start location. During the probe trial (trial 6 of each session), the number of "platform crossings" (the number of times/10 s that the mice crossed the exact location of the platform) were recorded, as was "quadrant time" (the per cent time that each mouse spent in the quadrant containing the platform).

Spatial reversal task. The spatial reversal task tested how well the mice could learn a new platform position in the opposite quadrant of the tank. The reversal was conducted in the same manner as the spatial task except that the platform was moved to the south-east quadrant. Six trials/mouse were conducted for three consecutive days. Trials 1–5 were platform trials, and trial 6 was a variable-interval probe trial. The intertrial interval was approximately 20 min. The same measures of performance were recorded as in the spatial task.

Cued task. In the cued task, a test of non-spatial reference memory, the platform was made visible using several local cues. Thus, intramaze cues on the platform predicted platform location rather than extramaze cues. The platform was made visible by: (i) lowering the water level such that the platform was 0.25 cm above the surface of the water, (ii) covering the platform with yellow tape, and (iii) attaching the top of a plastic container (8 cm in diameter, 0.5 cm wide) to the platform oriented perpendicular to the surface of the platform and water. Six platform trials were conducted in which the platform was located in a different quadrant for each trial. Swim time, path length, the corridor measure, and swim speed were recorded. No probe trials were conducted. The intertrial interval was approximately 20 min.

Simple odor discrimination

In the SOD task, a food-deprived mouse learns to dig in scented sand to retrieve a chocolate reward. This test of non-spatial (olfactory) reference memory trained the mice to discriminate between two odors.[5] The SOD was conducted using standard mouse cages (18.5 cm wide, 29.5 cm long, 13 cm high) and small white cups (2.5 cm in diameter, 1 cm high) attached to the floor of the cage with Velcro. A small amount of bedding was placed at the far end of the cage and either one or two sand-filled caps were placed at the near end of the cage. Powdered odors were diluted in natural play sand and presented in the cups. Prior to SOD testing, all mice were trained to dig in the sand to retrieve a piece of chocolate during two shaping sessions. On the first day of shaping, one cup full of unscented sand was presented in the middle of the near end of the cage. One piece of chocolate ($\approx$15 mg) was placed in each of three locations in the cup: on top of the sand, partially buried and completely buried. Four trials of 15 min each were conducted. If the mouse did not retrieve the buried piece within 8 min, another piece of chocolate was placed on top of the sand. During the 1 min intertrial interval, the mouse was placed in a separate holding cage. For the second day of shaping, two cups were presented at the near end of the cage. One cup contained unscented sand and the other contained garlic-scented sand. The garlic cup was rewarded throughout the session. Six trials of 7 min each were conducted. For the first two trials, two chocolate pieces were placed in the cup, one on top and one buried. For the other four trials, only the buried piece was given. The trial ended when the mouse retrieved the buried chocolate in the garlic cup. If the mouse did not dig in this cup after 5 min, then it was rebaited with another piece on top. The intertrial interval was 1 min.

Simple odor discrimination testing. Two scents were used throughout SOD testing, cinnamon and curry. Each mouse was randomly assigned one scent to be consistently rewarded. Four trials of 5 min each were conducted for three consecutive days. For each trial, the mouse was presented with two cups, one with curry-scented sand and the other with cinnamon-scented sand. Chocolate was buried in one of the cups. A choice was defined as a dig in a cup. If, after 3 min, no choice was made or a choice was made but the chocolate was not retrieved, then a piece of chocolate was placed on top of the correct cup and an additional 2 min allowed to retrieve the buried chocolate. Time (s) to retrieve the buried chocolate (latency), number of errors (digs) in the unrewarded cup, and the cup of first choice (choice accuracy (%)) were recorded. The intertrial interval was about 1 min, during which the mouse was placed in a separate holding cage. The position of the correct cup (left or right) varied randomly for each trial such that spatial location was irrelevant. The day after completion of SOD, two probe trials were conducted to eliminate the possibility that the mice discriminated between cups by smelling the chocolate. The mice were again presented with the cinnamon- and curry-scented sand; however, neither cup contained chocolate. During each 5 min trial, choice accuracy and latency were recorded as in the SOD.

Odor sensitivity. This task was designed to ensure that any sex- or age-related differences in the SOD were not due to differences in olfactory ability. The procedure was similar to SOD testing, except that six trials were conducted per day. Mice were first trained to a criterion of five correct choices out of six at 100% strength of cinnamon or curry (100% being the strength used in SOD testing). The particular scent rewarded for each mouse was the same as in the SOD. After reaching criterion, each mouse was tested on a series of decreasing odor strengths (75%, 50%, 25%, 10%, 5%, 2.5%) for six sessions (six trials/session, one session/strength). Choice accuracy was recorded. Only a subset of mice was trained in odor sensitivity; some of these mice were tested at a later date and were not included in the original experiment. A total of nine young mice (four male, five female), 10 middle-aged mice (five male, five female) and six aged mice (two male, four female) was tested in this task.

Elevated plus maze

The plus maze was constructed of wood and consisted of a central platform (5 × 5 cm), two open arms (30 × 5 cm) and two closed arms (30 × 5 cm) with walls extending 15 cm high and no ceilings. The open arms were painted white and all other surfaces were painted black. The arms were arranged in a plus shape with the two open arms facing each other and the two closed arms facing each other. The maze was positioned 90 cm above the testing room floor on a stand anchored beneath the central platform. One 5 min trial was conducted for each mouse. Each mouse was placed in the central platform facing a random arm and was allowed to roam freely about the maze for the duration of the trial. Six measures were recorded: number of closed arm entries, number of open arm entries, time spent in closed arms, time spent in open arms, number of defecations in closed arms, and number of defecations in open arms. A mouse was scored as entering an arm when all four paws had crossed into the arm.

Locomotor activity

Locomotor activity was recorded in a chamber consisting of a clear plastic cage used for housing rats (25 cm wide, 47 cm long, 21 cm high) inserted into a rectangular arena. Along the length of the rectangle were placed three photobeams. An automated recording system (San Diego Instruments, San Diego, CA) recorded the number of photobeam breaks/15 min interval for 2 h, beginning approximately at 17.30 h.

Estrous cycle determination

Changes in vaginal cytology are closely correlated with circulating levels of the sex-steroid hormones estrogen and progesterone in the bloodstream. Thus, daily examination of vaginal cell types can be used to determine the regularity of hormone, or estrous, cycling. Estrous cycles in mice typically last four or five days and are divided into four distinct stages: proestrus, estrus, metestrus and diestrus.[56] These cycles become prolonged and eventually cease in aging females.[46] To determine whether female mice in this study were cycling regularly, stages of the estrous cycle were recorded using vaginal lavage, which was conducted each morning between 9.00 and 11.00 h for 12 days. A drop of distilled water was placed over the vaginal opening and vaginal cells were extracted with a transfer pipette. These vaginal smears were immediately placed on slides and the stage of the cycle was determined microscopically using the following guidelines:[56] Proestrus was indicated by predominantly nucleated epithelial cells, estrus by

Table 2. Summary of age-related impairments in spatial and nonspatial memory tasks relative to five- and 17-month groups

Type of memory	Task	Measure	Age	
			17 months	25 months
Spatial	Spatial	Swim time	–	XX
		Path length	X	X
		Corridor	–	XX
		Swim speed	X	X
		Platform crossings	X	XX
		Quadrant time	–	–
Spatial	Spatial reversal	All measures	–	–
Non-spatial (visual)	Cued	All measures	–	–
Non-spatial (olfactory)	SOD	Choice accuracy	–	X
		Errors	–	X
		Latency	–	–
		Probe choice accuracy	–	–

X represents an impairment relative to the five-month-old group ($P < 0.05$); XX represents an impairment relative to the five- and 17-month-old groups ($P < 0.05$); – represents no difference from the five-month-old group.

predominantly non-nucleated cornified cells, metestrus by a mixture of nucleated epithelial cells, cornified cells and leukocytes, and diestrus by predominantly leukocytes. Slides were later stained with a Hematoxylin–Eosin stain, which stains cell bodies and nuclei, respectively, to confirm categorization of each smear.

Data analysis

Water maze, SOD and activity measures were averaged within a group for each session (water maze and SOD) or 15-min interval (activity), and analysed using a two-way repeated-measures ANOVA with sex and age as independent variables (SuperANOVA, Abacus Concepts, Berkeley, CA). Fisher's protected least significant difference (to compare the three age groups) or least squared means (to compare among the six groups) post hocs were performed on significant age effects and sex × age interactions, respectively. Two-way ANOVAs without repeated measures were performed on the SOD probe (trials 1–2 were averaged together) and plus maze measures. Because differential patterns of age-related decline in the two sexes may be obscured in the two-way ANOVAs, separate one-way ANOVAs were performed on each sex for measures revealing a significant main effect of age. These ANOVAs were termed "single-sex" ANOVAs.

To differentiate among the operationally defined spatial and nonspatial reference memory tasks, measures from the spatial and cued water maze tasks and the simple odor discrimination task were entered into a principal components analysis. Although the multivariate data reduction technique of principal components analysis has been used in studies of aged humans[42] and rats[16] to differentiate between measures of different memory systems, this type of analysis has not previously been utilized in studies of aged mice. Because performance in most spatial task measures improved rapidly in early test sessions and reached asymptotic levels by session 4, mean values representing either initial (sessions 1–3) or asymptotic (sessions 4–5) performance were computed for spatial measures. Because asymptotic performance levels were not reached in the cued water maze and SOD tasks, values for all three sessions were averaged. The platform crossings and quadrant time measures were not included in this analysis because they were not recorded in the cued task. Also, measures from the spatial reversal task were not included because this task measures the same type of memory as the spatial task, and is therefore redundant. In the principal components analysis, the initial factor pattern was rotated using a Varimax rotation algorithm. Factors with Eigen values >1 were retained in the analysis.

RESULTS

Subjects

Three 25-month-old mice (two female, one male) died of natural causes before the start of behavioral testing. One 17-month-old male was killed prior to testing owing to the presence of excessive skin lesions. The general health of the remaining animals varied with their age. All young animals were healthy. All middle-aged and aged animals were in general good health; they were able to walk, swim, and complete all tasks. None of the mice lost more than 20% of their free-feeding body weight during food restriction for the SOD task. No evidence of tumors or other gross pathological abnormalities was present in any of the animals. Alopecia was evident in all 25-month-old females and to a lesser extent in 17-month-old females. However, skin lesions were not observed in any mouse tested. All mice showed normal righting and placing reflexes. Five 25-month-old females and one 17-month-old female had a cataract on one eye; these mice were tested in all tasks, but excluded from the water maze data analysis. Three five-month-old mice (one female and two males) were also excluded from the water maze analyses because of their tendency to float during the majority of trials. One five-month-old male was excluded from the analyses of platform crossings and quadrant time in the spatial task because of missing data for session 3. Two five-month-old females were excluded from the SOD analyses because they did not dig within the allotted time.

Water maze

Spatial task. Table 2 presents a summary of age-related impairments in the memory tasks. The five-month group learned the task extremely rapidly, with most learning occurring during the first two sessions. Although the majority of learning in all three age groups occurred early in testing (sessions 1–3), all mice exhibited similar performance (i.e. swim times, path lengths and time in the corridor) during trial 1 of session 1 (data not shown), suggesting that the three age groups began training at a similar level of performance. Five of the six spatial measures were affected by age (swim time: $F_{2,53} = 6.56$, $P < 0.01$; path length: $F_{2,53} = 7.96$, $P < 0.01$; corridor: $F_{2,53} = 8.35$, $P < 0.01$; swim speed: $F_{2,53} = 7.87$, $P < 0.01$; platform crossings: $F_{2,52} = 10.16$, $P < 0.01$). The 25-month group was significantly impaired relative to the five-month group (Ps < 0.01) in five measures: swim time (Fig. 1A, B), path-length (Fig. 1C, D), time in corridor (Fig. 2A, B), swim speed (Fig. 2C, D) and platform crossings (Fig. 3A, B). Twenty-five-month-old mice were also significantly impaired relative to 17-month-old mice (Ps < 0.05) in the swim time (Fig. 1A, B), corridor (Fig. 2A, B) and platform

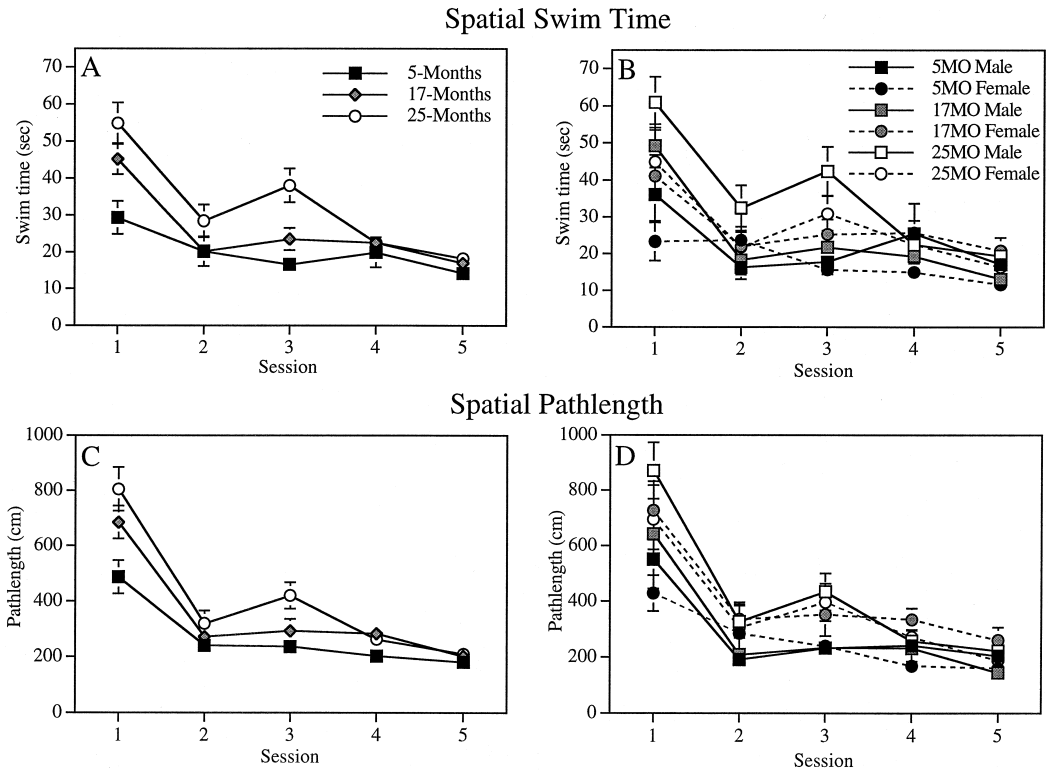

Fig. 1. Performance in the spatial water maze task as assessed by swim time (A, B) and path length (C, D). Twenty-five-month mice were impaired relative to five-month mice in both measures, whereas 17-month mice were impaired relative to five-month mice in path length only. Although sex differences were not observed at five and 25 months of age in either measure, 17-month females performed similarly to 25-month females in both measures, whereas 17-month males performed more like five-month males. Each symbol represents the group mean performance (±S.E.M.) for each session. MO, month. Sample sizes for A and C were as follows: 5 months ($n=17$), 17 months ($n=18$), 25 months ($n=24$). Sample sizes for B and D were: 5-month males ($n=8$), 25-month males ($n=15$), and all other groups ($n=9$).

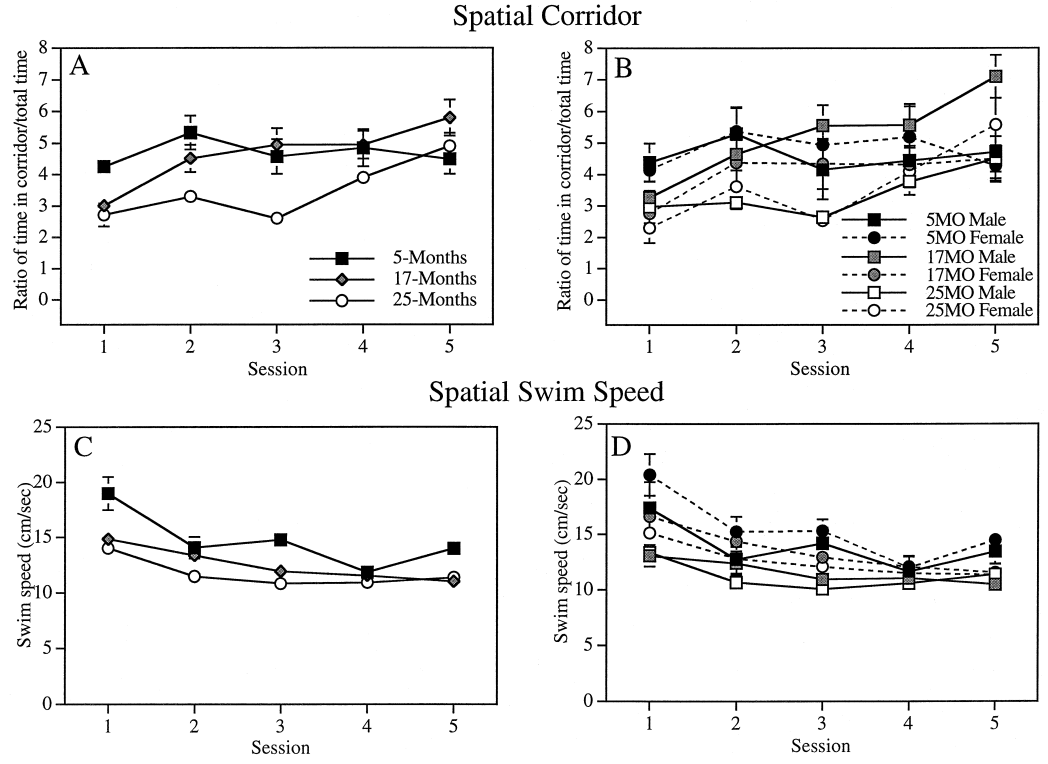

Fig. 2. Performance in the spatial water maze task as assessed by the corridor measure (A, B) and swim speed (C, D). Twenty-five-month mice were impaired relative to five-month mice in both measures, whereas 17-month mice were impaired relative to five-month mice in swim speed only. Although sex differences were not observed at five and 25 months of age in either measure, 17-month males spent a higher proportion of time in the corridor than 17-month females. Each symbol represents the group mean performance (±S.E.M.) for each session. MO, month. Sample sizes are the same as in Fig. 1.

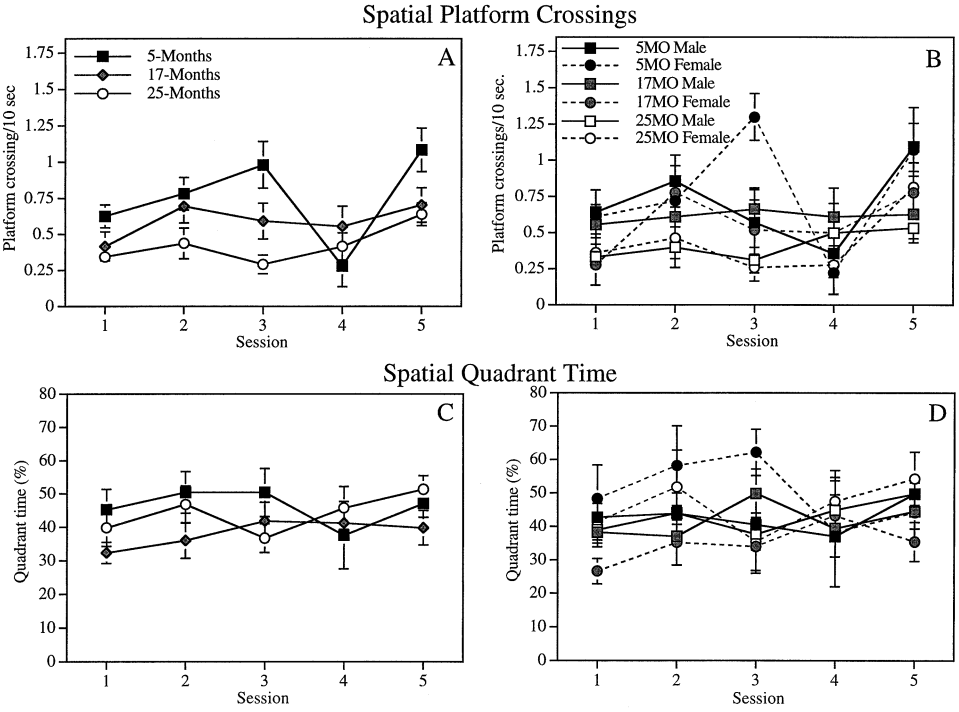

Fig. 3. Performance in the probe trials of the spatial water maze task as assessed by platform crossings (A, B) and quadrant time (C, D). Overall, 25-month mice had significantly fewer crossings than both five- and 17-month mice. Although the 17-month group exhibited significantly fewer crossings than five-month mice, only 17-month females were impaired relative to their same-sex five-month group. Quadrant time was not affected by age or sex. Each symbol represents the group mean performance (±S.E.M.) for each session. MO, month. Sample sizes for A and C were as follows: 5 months ($n = 16$), 17 months ($n = 18$), 25 months ($n = 24$). Sample sizes for B and D were: five-month males ($n = 7$), 25-month males ($n = 15$), and all other groups ($n = 9$).

crossings measures (Fig. 3A, B). The 17-month group was significantly impaired relative to the five-month group (Ps < 0.05) in three measures: path length (Fig. 1C, D), swim speed (Fig. 2C, D) and platform crossings (Fig. 3A, B). No differences between the five- and 17-month groups were observed in the swim time, corridor or quadrant time measures. The quadrant time measure was not affected by age or sex (Fig. 3C, D), and performance in this measure did not significantly improve over testing. All groups improved over training in all other measures, as suggested by significant session effects in the remaining measures ($Fs_{4,212} = 8.57$–51.5, Ps < 0.05; $F_{4,208} = 6.5$, $P < 0.01$ for platform crossings). However, significant session × age interactions in swim time ($F_{8,212} = 2.54$, $P < 0.05$), time in corridor ($F_{8,212} = 2.8$, $P < 0.01$) and swim speed ($F_{8,212} = 3.25$, $P < 0.01$) suggested a differential rate of improvement among the three age groups. The session × age interaction was also significant for platform crossings ($F_{8,208} = 2.04$, $P < 0.05$); however, this interaction was probably caused by the poor performance of five-month females during session 4.

The main effect of sex was significant in the swim speed measure only ($F_{1,53} = 7.4$, $P < 0.01$; Fig. 2D); females swam more quickly than males. A significant sex × age interaction in the path-length measure (Fig. 1D) indicated that, although males and females in the five- and 25-month groups performed similarly, 17-month-old females had significantly longer path lengths than 17-month-old males ($P < 0.05$). No other interactions involving sex were significant in any measure.

Spatial reversal. Age-group means for the spatial reversal are presented in Table 3. None of the reversal measures was

Table 3. Age-group means for the spatial reversal and cued water maze tasks

		Age		
Task	Measure	Five months	17 months	25 months
Spatial reversal	Swim time	30.55 ± 3.34	28.61 ± 2.76	32.5 ± 2.85
	Path length	428.4 ± 46.66	409.58 ± 41.68	445.67 ± 40.98
	Corridor	3.92 ± 0.38	3.12 ± 0.26	2.79 ± 0.19
	Swim speed	14.36 ± 0.64	13.17 ± 0.35	13.13 ± 0.31
	Platform crossings	0.58 ± 0.08	0.49 ± 0.07	0.42 ± 0.04
	Quadrant time	41.19 ± 3.63	37.31 ± 2.55	39.13 ± 2.53
Cued	Swim time	15.55 ± 2.44	10.91 ± 0.65	10.36 ± 0.64
	Path length	204.97 ± 36.39	151.78 ± 8.45	142.56 ± 7.26
	Corridor	4.97 ± 0.44	4.13 ± 0.4	4.41 ± 0.28
	Swim speed	14.95 ± 0.72	14.28 ± 0.53	14.33 ± 0.39

Values represent the mean ± S.E.M. Units are as follows: swim time = s, path-length = cm, swim speed = cm/s, quadrant time = %.

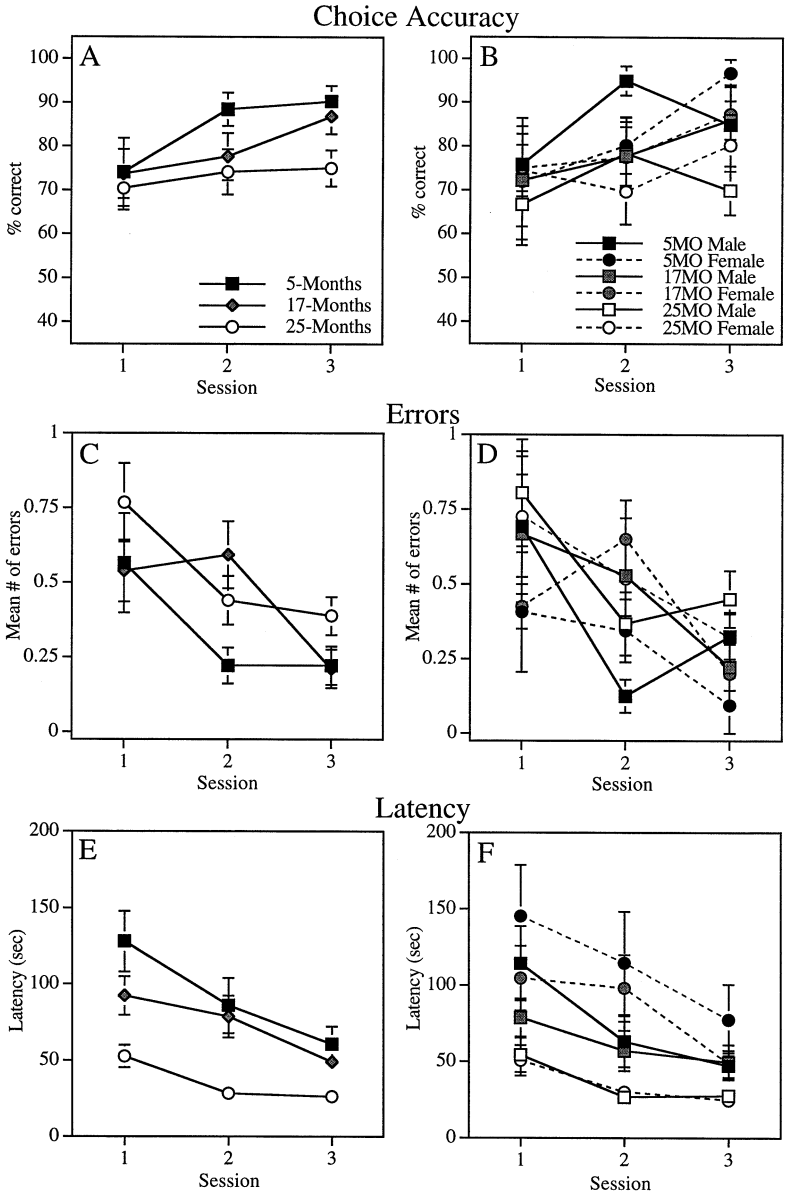

Fig. 4. SOD performance as assessed by choice accuracy (A, B), errors (C, D) and latency (E, F). Twenty-five-month mice had lower choice accuracies and more errors, but performed the task more rapidly, than five-month mice. Relative to their same-sex five-month group, only 25-month males had lower choice accuracy, but only 25-month females committed more errors. Both 25-month groups exhibited similar digging latencies. Neither male nor female 17-month mice were impaired in any measure. Each symbol represents the mean group performance (±S.E.M.) for each session. Sample sizes for A, C and E were as follows: 5 months ($n = 18$), 17 months ($n = 19$), 25 months ($n = 29$). Sample sizes for B, D and F were as follows: five-month male ($n = 10$), five-month female ($n = 8$), 17-month male ($n = 9$), 17-month female ($n = 10$), 25-month male ($n = 15$), and 25-month female ($n = 14$).

affected by age or sex, and no sex × age interactions were significant. All groups improved throughout testing, as suggested by significant session effects in all measures but swim speed ($Fs_{2,106} = 13.52–75.37$, $Ps < 0.01$). A significant session × sex interaction in swim speed suggested that, whereas females maintained consistent swim speeds during the three sessions, males decreased swim speeds from session to session. However, *t*-tests performed on each session revealed that swim speeds were not significantly different between the sexes within each session ($Ps > 0.05$). A significant session × sex × age interaction in the platform crossings measure suggested that all groups increased their number of crossings in each session, except for five-month-old males who maintained a high number of crossings in all three sessions. No other interactions were significant.

Cued task. Age-group means for the cued task are presented in Table 3. No sex, age or sex × age effects were significant in any measure. All groups improved similarly in swim time, corridor and swim speed, as suggested by significant session effects ($Fs_{2,106} = 4.66–33.55$, $Ps < 0.05$) in the absence of any significant interactions. The session effect was also significant in the path-length measure ($F_{2,106} = 19.99$, $P < 0.01$), but the significant session × age interaction ($F_{4,106} = 2.66$, $P < 0.05$) suggested differential improvement among the three age groups. In this measure, the five-month group had slightly higher path lengths during sessions 1 and 2 than the 17- and 25-month groups, possibly indicating a strong memory for the hidden platform location or a reluctance to switch from a spatial strategy to a cued strategy.

Table 4. Sex differences in the spatial water maze, simple odor discrimination, plus maze and activity tasks

		Male			Female		
Task	Measure	Five months	17 months	25 months	Five months	17 months	25 months
Spatial	Swim time	22.6 ± 2.7	24.4 ± 2.6	35.5 ± 2.9*†	17.9 ± 1.9	27.0 ± 2.1*	27.3 ± 2.7*
	Path length	284.3 ± 33.2	292.3 ± 30.9§	422.7 ± 39*†	256.7 ± 23.8	402.1 ± 38.9*§	371.6 ± 41.4*
	Corridor	4.6 ± 0.3	5.2 ± 0.3§	3.4 ± 0.2*†	4.8 ± 0.3	4.1 ± 0.3§	3.6 ± 0.3*
	Swim speed	13.9 ± 0.7	11.6 ± 0.4*	11.2 ± 0.3*	15.5 ± 0.7	13.5 ± 0.5	12.6 ± 0.4*
	Platform crossings	0.7 ± 0.01	0.6 ± 0.1	0.4 ± 0.1*†	0.8 ± 0.1	0.6 ± 0.1*	0.4 ± 0.1*
	Quadrant time	42.7 ± 4.0	41.8 ± 3.4	43.0 ± 2.6	50.2 ± 4.8	34.9 ± 2.8*‡	46 ± 3.8
SOD	Choice accuracy	84.3 ± 3.8	78.7 ± 4.8	71.7 ± 4.0*	82.9 ± 5.6	80.0 ± 3.7	74.8 ± 3.8
	Errors	0.4 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.3 ± 0.1	0.4 ± 0.1	0.5 ± 0.9*
	Latency	74.8 ± 11.2	61.7 ± 7.2	36.2 ± 5.1*†	112.3 ± 17.9	83.7 ± 11.2	35.1 ± 4.0*†
SOD probe	Choice accuracy	90.0 ± 6.67	83.3 ± 8.3	73.3 ± 8.3	77.8 ± 12.1	90.0 ± 6.7	67.9 ± 6.7
	Latency	74.7 ± 15.4	41.5 ± 13.6*	21.3 ± 4.8*	116.9 ± 31.1	43.3 ± 10.9*	30.7 ± 2.8*
Plus maze	Time in closed arms	220.2 ± 7.1	223.2 ± 6.3§	229.1 ± 6.7	203.2 ± 8.3	244.6 ± 7.8*‡§	212.4 ± 5.1
	Time in open arms	2.5 ± 1.0	7.1 ± 3.8	6.8 ± 3.6	5.8 ± 2.3	1.3 ± 0.9*	4.6 ± 1.1
	Closed-arm entries	12.7 ± 1.3	13.9 ± 1.5§	11.5 ± 1.0	12.2 ± 0.8	10.2 ± 1.0‡§	13.5 ± 1.1
	Open-arm entries	0.5 ± 0.2	1.3 ± 0.7	0.7 ± 0.4	1.4 ± 0.6	0.2 ± 0.1*	1.1 ± 0.2
	Defecations in closed arms	0.9 ± 0.5	0.1 ± 0.1	0.3 ± 0.2	0.3 ± 0.3	0.7 ± 0.3	0.4 ± 0.1
Activity	Photobeam breaks	135.1 ± 9.1	203.2 ± 8.1*‡	116.5 ± 7.6	156.5 ± 8.5	197.5 ± 9.2	159.1 ± 6.7

Values represent the mean ± S.E.M. Units are as follows: times and latencies = s, path length = cm, swim speed = cm/s, quadrant time and choice accuracy = %.
*Represents a significant difference from the same-sex five-month group ($P < 0.05$).
†Represents a significant difference from the same-sex 17-month group ($P < 0.05$).
‡Represents a significant difference from the same-sex 25-month group ($P < 0.05$).
§Represents a significant difference between 17-month males and females ($P < 0.05$).

Simple odor discrimination

Choice accuracy ($F_{2,60} = 3.8$, $P < 0.05$), errors ($F_{2,60} = 3.23$, $P < 0.05$) and latency ($F_{2,60} = 13.71$, $P < 0.01$) were significantly affected by age (Fig. 4, Table 2). The 25-month group was significantly impaired relative to the five-month group in the choice accuracy (Fig. 4A, B) and error measures (Fig. 4C, D; Ps < 0.05), but performed the task significantly more quickly than both the five- and 17-month groups (Fig. 4E, F; Ps < 0.01). The choice accuracy and errors of 17-month group were intermediate between those of five- and 25-month groups, although they were not significantly different from either (Fig. 4A, C). The five- and 17-month groups did not differ in terms of latency (Fig. 4E, F). A sex difference was significant only in the latency measure (Fig. 4F), in which females were faster than males ($F_{1,60} = 4.07$, $P < 0.05$). The performance of all groups improved similarly throughout testing, as suggested by significant session effects in all three measures (Fs$_{2,120} = 3.56$–23.28, Ps < 0.05) in the absence of any other significant interactions.

Table 4 presents the group means of both sexes for the SOD probe trials. In the probe trials, choice accuracy was not significantly affected by age or sex, and the sex × age interaction was not significant. Consistent with the SOD task, probe trial latency was significantly affected by age ($F_{2,60} = 13.39$, $P < 0.01$), such that 25-month-old mice dug more quickly than five-month-old mice ($P < 0.01$). Seventeen-month-old mice also performed faster in the probe trials than five-month-old mice ($P < 0.01$). Latency was not affected by sex, as suggested by a non-significant sex effect and sex × age interaction.

Performance in the odor sensitivity task was not affected by sex ($F_{1,19} = 0.92$, $P > 0.05$) or Age ($F_{2,19} = 0.61$, $P > 0.05$). All age groups had similar choice accuracies across all seven odor strengths tested (Fig. 5). The main effect of odor strength was significant ($F_{6,114} = 3.0$, $P < 0.01$), probably because of poor performance of the five-month group at the 50% strength. The only significant interaction was the strength × sex × age effect ($F_{12,114} = 2.18$, $P < 0.05$), which suggested that five-month-old females had higher choice accuracies than five-month-old males at the 2.5% strength.

Elevated plus maze

Group means for plus maze measures are presented in Table 4. No mouse ever defecated in the open arms of the maze, so this measure is not included the table. Time spent in the open arms and number of defecations in the closed arms were not affected by sex or age. The age effect was also not significant for the number of closed and open arm entries, but was significant in the time in closed arms measure ($F_{2,62} = 4.54$, $P < 0.05$), in which the 17-month group spent more time in the closed arms than the five-month group ($P < 0.01$). The significant sex × age interactions in the number of closed and open entries and the time in closed arms (Fs$_{2,62} = 3.21$–4.66, Ps < 0.05) suggested that 17-month females were more anxious than the other females and 17-month-old males. Seventeen-month-old females had fewer closed-arm entries than 17-month-old males and 25-month-old females (Ps < 0.05), fewer open-arm entries and less time in the open arms than five-month-old females ($P < 0.05$), and spent more time in the closed arms than 17-month-old males, 25-month-old females and both five-month groups (Ps < 0.05).

Locomotor activity

Group means for locomotor activity are presented in Table 4. Activity was significantly affected by Age ($F_{2,62} = 9.77$, $P < 0.01$; Fig. 6A). All groups decreased activity over the 2 h observation period (Fig. 6A and B), as suggested by a

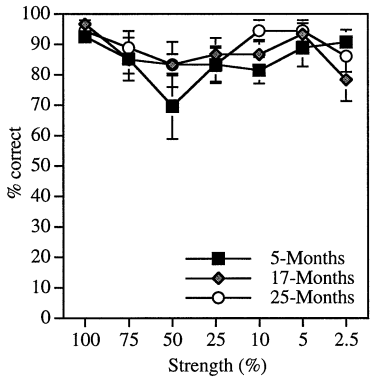

Fig. 5. Odor sensitivity performance as assessed by choice accuracy. No significant differences were observed among the age groups at any odor strength, suggesting similar olfactory abilities among all mice. See Experimental Procedures for sample sizes.

significant interval effect ($F_{7,434} = 58.97$, $P < 0.01$). However, the rate of habituation to the activity chamber was not similar among the age groups, as suggested by a significant interval × age interaction ($F_{14,434} = 3.47$, $P < 0.01$). Although the activities of the five- and 25-month groups were similar, the 17-month group was more active than these groups in all but the first 15 min interval ($Ps < 0.01$; Fig. 6A). Locomotor activity was not affected by sex (Fig. 6B), as suggested by a non-significant main effect sex and non-significant sex-related interactions.

Regularity of estrous cycling

Cycling activity was divided into three categories: (i) regular cycling, which denoted a four to five day estrous cycle in which the estrus phase was observed at least twice during the sampling period, (ii) irregular cycling, which denoted a prolonged (>five days) cycle in which the estrus phase was observed only once, and (iii) no cycling, which denoted the absence of the estrus or metestrus phases throughout sampling. The number of mice in each category for each age group is presented in Table 5. Nine five-month-old females cycled regularly and one did not cycle. By 17 months, regular estrous cyclicity was more infrequent. Only two 17-month-old females cycled regularly, whereas six did not cycle at all. Two 17-month-old females exhibited irregular cycling. By 25 months, estrous cycling had ceased completely, as indicated by the lack of estrous cycling in all 14 25-month-old females.

Sex differences in the pattern of age-related behavioral changes

To more closely examine patterns of age-related change within each sex, separate ANOVAs were performed on each sex for behavioral tasks with significant age effects. Group means and significant post hoc comparisons are presented in Table 4. Similar patterns of age-related change were found in a few measures: SOD latency, SOD probe choice accuracy, SOD probe latency, and number of defecations in the closed arms of the plus maze (see above for specific age-related differences). However, different patterns of age-related decline in females and males were revealed in all four behavioral paradigms.

In the spatial water maze, both male and female 25-month

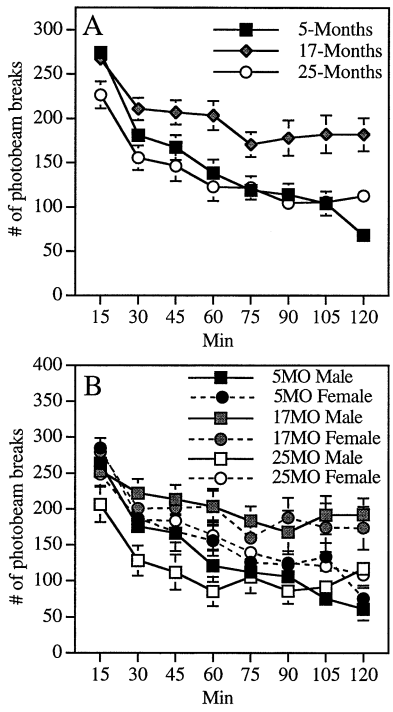

Fig. 6. Locomotor activity recorded during eight 15 min intervals (A, B). Seventeen-month mice were more active than five- and 25-month mice. Each symbol represents the mean (±S.E.M.) of each group's activity. Sample sizes for A were as follows: 5 months ($n = 20$), 17 months ($n = 19$), 25 months ($n = 29$). Sample sizes for B were as follows: five-month male ($n = 10$), five-month female ($n = 10$), 17-month male ($n = 9$), 17-month female ($n = 10$), 25-month male ($n = 15$), and 25-month female ($n = 14$).

Table 5. Estrous cyclicity in female mice

	Number of mice/category		
Age	Regular cycling	Irregular cycling	No cycling
Five months	9	0	1
17 months	2	2	6
25 months	0	0	14

groups were significantly impaired relative to their respective five-month-old groups in all measures except quadrant time, suggesting a similar magnitude of impaired spatial memory in the two sexes at very advanced ages. However, sex differences in the extent of spatial memory impairment were observed in the 17-month group in all five cognitive measures. In the swim time, path length and platform crossings measures, 17-month-old females performed more like 25-month-old females; they were significantly worse than five-month-old females, but were not significantly different from 25-month-old females (Table 4). In contrast, 17-month-old males performed more like five-month-old males; they were significantly better than 25-month-old males and were not different from five-month-old males. Furthermore, 17-month-old males had significantly shorter path lengths and spent more time in the corridor than 17-month-old females (Table 4). Seventeen-month-old females also spent significantly less time than other females in the correct quadrant during the probe trial, whereas 17-month-old males did not differ from other males. This differential pattern of spatial

Table 6. Components loadings for spatial, cued, and simple odor discrimination tasks

Task	Measure	Sessions	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5
Spatial	Swim time	1–3	− 0.50*	0.11	− 0.06	0.81*	− 0.01
	Path length	1–3	− 0.14	0.08	− 0.02	0.93*	0.04
	Corridor	1–3	− 0.10	− 0.33	0.05	− 0.71*	0.15
	Swim speed	1–3	0.87*	− 0.02	0.14	− 0.22	0.09
	Swim time	4–5	− 0.49	0.79*	0.21	0.07	− 0.06
	Path length	4–5	− 0.10	0.90*	0.12	0.12	− 0.06
	Corridor	4–5	0.03	− 0.73*	− 0.13	− 0.25	− 0.01
	Swim speed	4–5	0.89*	− 0.09	− 0.14	− 0.04	0.09
Cued	Swim time	1–3	− 0.49	0.25	0.72*	− 0.11	− 0.13
	Path length	1–3	− 0.35	0.22	0.71*	− 0.13	− 0.14
	Corridor	1–3	− 0.01	− 0.29	− 0.66*	− 0.20	0.22
	Swim speed	1–3	0.71*	− 0.19	− 0.14	− 0.08	0.06
SOD	Choice accuracy	1–3	− 0.003	− 0.09	− 0.04	0.02	0.93*
	Errors	1–3	− 0.22	0.002	0.10	0.06	− 0.89*
	Latency	1–3	0.24	− 0.08	0.80*	− 0.11	0.17
Eigen values			4.83	2.45	1.83	1.57	1.05
% variance accounted for			32.2	16.4	12.2	10.5	7.0

*Loadings higher than 0.5.

memory decline suggests a more preserved spatial memory ability in 17-month-old males than in 17-month-old females.

Five- and 25-month mice of both sexes performed similarly in all plus maze measures. However, sex differences in four plus maze measures were evident in the 17-month group (Table 4). Seventeen-month-old females spent significantly more time in the closed arms, less time in the open arms, and had fewer open-arm entries than five-month-old females. Seventeen-month-old females also spent more time in the closed arms and had fewer open arm entries than 25-month-old females and 17-month-old males. In contrast, 17-month-old males did not differ from five- and 25-month-old males in any measure. This pattern of results suggests that 17-month-old females were more anxious than both other females and all males.

Differences in the pattern of age-related change in the SOD task were limited to the 25-month group. Although a significant effect of age was observed in the two-sex ANOVA for SOD choice accuracy, the single-sex ANOVAs revealed that only 25-month-old males had significantly lower choice accuracies than their respective five-month group (Table 4). However, this difference was reversed for SOD errors, such that only 25-month-old females were impaired. Both 25-month groups were significantly faster than their respective five-month groups in performing both the regular SOD task and the SOD probe. No sex differences were observed between 17-month-old males and females.

Sex differences in locomotor activity were limited to the 17-month group (Table 4). Although the activity of both 17-month groups was higher than that of the other age groups, only 17-month males exhibited significantly higher activity than their respective five- and 25-month groups.

Principal components analysis

Five significant factors were retained in the principal components analysis. The component loadings of the variables on to the rotated factors are presented in Table 6. In this analysis, variables that measure qualitatively similar aspects of memory or motor performance will load on to the same factor. Swim speeds in the spatial and cued tasks loaded highly on to factor 1. Swim times from both water maze tasks loaded less strongly on this factor, confirming a role of swim speed in time to find the platform. Measures of initial and asymptotic performance in the spatial task loaded highly on to separate factors (factors 4 and 2, respectively). The other three cued task measures loaded highly on to factor 3, as did SOD latency. SOD choice accuracy and errors loaded highly on to factor 5. The finding that measures from the spatial, cued and SOD tasks loaded primarily on to different components suggests that each task may measure a qualitatively different aspect of memory function.

DISCUSSION

Aged mice

Aged C57BL/6NIA mice were significantly impaired relative to young mice in all measures of the spatial water maze and SOD tasks, indicating both spatial and olfactory reference memory deficits in this age group. The finding of an age-related spatial memory deficit in this study is consistent with previous findings in C57BL/6 mice[15,37] and other strains of mice,[32] as well as with previous studies in rats.[13,16,20] Although the aged mice acquire the spatial task more slowly than young mice, aged mice improved with testing to the level of young mice. Furthermore, the fact that the aged mice were not impaired on the spatial reversal task indicates that they were capable of learning to find the platform using spatial cues, and that they could quickly learn a new platform location, suggesting intact ability to utilize learned information.

The magnitude of the spatial memory impairment observed in these aged mice does not appear to be as robust as has been previously seen in rats. A previous study that tested the spatial reference memory of male Fischer-344 rats using the same behavioral protocol[16] found that 24-month-old rats were severely impaired relative to four-month-old rats, and their performance never improved to the level of young rats. Furthermore, the deficit of aged rats was particularly striking in the probe trial measures quadrant time and platform crossings. In the present study, 25-month-old mice were impaired in the platform trial measures during early test sessions (particularly sessions 1 and 3), but performed as well as young rats by session 4. Similarly, the probe trial measures

were not as sensitive to age-related changes in C57BL/6 mice as in Fischer-344 rats.[16] The reason for these species differences in the magnitude of the effects of aging on spatial memory is currently unclear. It is possible that differences in perseverance between the two species may contribute to the reduced sensitivity of the probe trials to aging. Performance in the variable-interval probe trials is more affected by differences in perseverance than the platform trials because the animals are required to swim to the platform location and wait until the platform reappears.[38] Young rats easily learn to stay in the correct quadrant by the third probe trial.[16] However, the young C57BL/6 mice in this study appeared to be less persistent, as suggested by their low number of platform crossings during the fourth probe trial and their lack of improvement in the quadrant time measure. The difference in the magnitude of the spatial memory impairment observed in rats and mice may have important implications for the development of C57BL/6-based transgenic mouse models of age-related neurodegeneration.

The present study is the first to describe an age-related olfactory reference memory deficit in C57BL/6 mice. The 25-month group exhibited lower choice accuracies and more errors than both the five- and 17-month groups, although the aged group performed the task significantly more quickly than the five-month group. This pattern of results suggests that five-month-old mice practised a more advantageous strategy of slowly and carefully selecting the correct cup in which to dig, whereas 25-month-old mice dug quickly at either cup regardless of accuracy. A similar result has been found in rats with lesions of the fimbria–fornix, the axonal pathway connecting the basal forebrain and hippocampus; lesioned rats exhibited impaired acquisition of a simultaneous two-choice odor discrimination but faster response latencies relative to controls.[12] Thus, age-related basal forebrain or hippocampal degeneration may contribute to the performance of the aged mice in this study. Degeneration of these brain areas is also associated with age-related spatial memory decline, suggesting that the spatial water maze and simple odor discrimination tasks involve similar hippocampally dependent memory processes. If so, then we might expect behavioral measures from these tasks to be correlated. The fact that they were not suggests fundamental differences between these tasks. Although the hippocampus may be involved in forming both visual and olfactory-based memories, differences in sensory processing (perhaps in the visual or olfactory cortices) prior to hippocampal processing may contribute to this discrepancy. Performance of this odor discrimination in mice also involves basal forebrain projections to the cortex.[5] Thus, age-related degeneration of cortical, rather than hippocampal, basal forebrain projections may underlie the olfactory reference memory deficit in aged mice.

It is unlikely that the spatial and olfactory reference memory deficits observed in the aged mice are due to age-related differences in motor ability or anxiety. First, although swim speeds in the spatial task were significantly slower in aged mice than in young mice, this probably reflects a different strategy for learning the spatial layout of the room and platform location rather than a true motor deficit. For instance, aged rats are less likely to use a spatial place learning strategy in the water maze than young rats, preferring to use more simple egocentric response or cued-navigation strategies if possible.[20] Second, no age-related differences were observed in swim speed or any mnemonic measure in the spatial reversal and cued tasks, suggesting that once the 25-month-old mice had learned to navigate in the pool, they swam to the platform as quickly as the other age groups. Third, the fact that aged mice performed the SOD task more quickly than young mice demonstrates that aged mice can sufficiently walk and dig in the sand. Further, the absence of significant differences in SOD probe trials and odor sensitivity testing excludes the possibility that the aged mice cannot make accurate odor discriminations due to impaired olfactory abilities. Fourth, the absence of alterations in the aged group in locomotor activity or impairments in the placing and righting reflexes indicates similar levels of general motor functioning between five- and 25-month-old mice. Although deficits have been previously observed in 22–23-month-old C57BL/6 mice in an initiation of walking task and a rod-walking task,[9,26] other sensorimotor tasks, including placing, have revealed no impairment.[9,32] Fifth, age-related differences in anxiety also do not contribute to the memory deficits, as no differences were observed between the five- and 25-month groups in any plus maze measure. This finding conflicts with the plus maze deficit previously observed in 22-month-old female NMRI mice;[32] strain differences may account for this inconsistency. The fact that young and aged mice in this study did not have significantly different motor abilities or anxiety levels suggests that the observed age-related deficits in spatial and olfactory memory were primarily attributable to impairments of learning and memory.

Consistent with previous findings in aged C57BL/6 mice,[37] aged mice in this study were not impaired on the cued water maze task, suggesting that non-spatial reference memory does not deteriorate with increasing age. However, aged mice did exhibit impaired acquisition of the SOD task, suggesting that olfactory reference memory is affected by increasing age. The fact that age-related deficits were observed in one non-spatial task (the SOD task) but not the other (the cued water maze task) suggests that not all types of non-spatial reference memory are impaired with increased age. One possible explanation for the discrepancy between these two non-spatial tasks involves the different stimuli used to motivate performance (aversive in the cued task vs appetitive in SOD). Alternatively, differences in task demand may contribute to the inconsistency between the non-spatial tasks. The cued task involves a simple stimulus–response association; visual cues on the platform mark its location such that the platform can be easily located from a distance. In contrast, two stimuli (the rewarded and unrewarded scent-cups) are presented simultaneously in the odor discrimination task and, because the spatial location of the rewarded scent varies, the mouse must interact with both cups to determine which contains the reward. Thus, the mouse must simultaneously process two odor stimuli, as well as the response-reinforcement contingencies of each, creating an increased level of complexity not present in the cued task. Finally, similar odor discrimination tasks are hippocampally dependent,[11] whereas the cued task is non-hippocampally dependent,[44] suggesting that aging may have a more detrimental effect on the hippocampus than on the neural substrate underlying cued task performance. Further studies will be necessary to distinguish among these alternatives.

A cessation of estrous cycling was observed in 25-month-old female mice, which is consistent with previous findings of acyclicity in 20-month-old C57BL/6J females.[45] Although

estrogen is thought to play a role in cognition, and some studies suggest that loss of regular ovarian hormone cycling during menopause deleteriously affects cognitive functioning in women,[2] the loss of regular estrous cycling in our 25-month-old females did not seem to affect their water maze performance relative to their male counterparts; 25-month-old males and females were not differentially impaired relative to their respective young groups in any spatial water maze measure. However, loss of estrous cycling may have affected performance of the SOD task. In this task, only aged males were significantly impaired relative to their respective young group in SOD choice accuracy, whereas only aged females were significantly impaired in the SOD error measure. Although both choice accuracy and errors measure how well the mice have learned the discrimination, the error measure also reflects perseverance after an initial incorrect choice has been made.[5] Thus, these sex differences could be interpreted as a deficit of choice accuracy in aged males but an increased amount of perseverance in aged females. Age-related decreases in serum testosterone in aged males may also contribute to this behavioral sex difference. Serum testosterone levels are decreased in 25-month-old male C57BL/6NIA mice as a group, although approximately 25% of aged males have normal testosterone levels, suggesting that this decrease is highly variable.[49] Testosterone replacement in older men modestly improves spatial cognition,[27] but in aged rats does not improve spatial memory and actually impairs spatial memory in middle-aged rats.[23] Whether age-related declines in sex-steroid hormone levels contribute to sex differences in performance of the SOD task remains to be investigated.

Middle-aged mice

Middle-aged mice also exhibited a significant spatial reference memory deficit in the water maze. However, the fact that the 17-month groups performed intermediately between the five- and 25-month groups in almost all measures suggests a less severe deficit than that of 25-month-old mice. This result is consistent with previous findings of less severe impairments in 17-month-old male rats,[16] but not with a study of NMRI mice which reported deficits of a similar magnitude in 17- and 22-month-old females.[32] Because the spatial reference memory of 17-month-old C57BL/6 mice has been not previously tested in the water maze, it is difficult to know whether this discrepancy between NMRI and C57BL/6 mice is the result of strain differences or, as is suggested by this study, sex differences within the 17-month age group. Nevertheless, the performance of the middle-aged mice on the spatial task improved throughout testing and no deficit was present in the spatial reversal or cued tasks, suggesting that middle-aged mice were capable of learning to find both the hidden and visible platforms in the pool.

Middle-aged mice exhibited slower swim speeds than young mice in the spatial task, raising the possibility that a motor impairment in this group confounded the observation of cognitive deficits. However, this is not likely given that their swim speeds were similar to those of young mice in the spatial reversal and cued tasks. In addition, middle-aged mice were more active in the general locomotor activity apparatus than both young and aged mice, suggesting that their ambulatory ability was not impaired. This increased activity probably reflects an impaired ability to habituate to the activity chamber, suggesting a heightened level of anxiety. The fact that the middle-aged mice spent more time in the closed arms of the plus maze than the young group also suggests that this group was more anxious than the young or aged groups. In particular, middle-aged female mice seemed less likely to repeatedly explore the maze, as suggested by a lower number of open and closed arm entries and more time in the closed arms than middle-aged male mice. Seventeen-month-old female NMRI mice also exhibit significantly higher levels of anxiety than young females in the plus maze,[32] suggesting that this finding of increased anxiety in females may be generalizable to other strains of mice.

Although middle-aged mice performed intermediately between the young and aged groups in the SOD, they were not significantly impaired relative to the young group in any SOD measure, suggesting that their ability to acquire an olfactory reference memory task remained intact. The finding that the 17-month group was unimpaired in non-spatial reference memory tasks using both appetitive and aversive motivations may suggest a more global preservation of this type of memory in middle-age. One possible interpretation of these results, in light of those from the 25-month group, is that spatial and non-spatial reference memory decline at different rates, with spatial reference memory declining gradually from an earlier point in the life-span. The apparent differential rate of decline of spatial and non-spatial reference memory may reflect differential decay of separate neural systems that underlie these types of memory. A longitudinal study, rather than the cross-sectional study employed here, could better address these issues.

The differential patterns of age-related behavioral change evidenced in the single-sex ANOVAs suggest that spatial memory may deteriorate earlier in females than in males. In the spatial task, 25-month-old mice of both sexes were similarly impaired relative to five-month-old mice, suggesting a similar magnitude of spatial memory decline at advanced ages. However, the different patterns of impairment of 17-month males and females suggest that spatial memory decline may begin at a younger age in females. Seventeen-month-old females performed more like 25-month-old females in several spatial measures; they were impaired relative to five-month-old females and were not different from 25-month-old females. This pattern of results is consistent with the finding of similar deficits in middle-aged and aged female NMRI mice.[32] In contrast, 17-month-old males performed more like five-month-old males than 25-month-old males in these measures; they performed significantly better than 25-month-old males and were not different from five-month-old males. Seventeen-month-old males also spent significantly more time (better performance) than 25-month-old males in the corridor between the start location and the platform, whereas this time was similar for 17- and 25-month-old females. Interestingly, 17-month-old males, but not 17-month-old females, had significantly slower swim speeds in the spatial task relative to their respective five-month group, suggesting that the 17-month-old males may have searched the pool slower and, therefore, more carefully than 17-month-old females, which may aid males in finding the platform. The higher anxiety levels observed in 17-month-old females may contribute to these observed sex differences; 17-month-old females, but not 17-month-old males, spent more of their time in the plus maze in the closed arms than either five- or 25-month-olds,

indicating an increased level of anxiety. This finding is consistent with the increased anxiety observed in 17-month-old NMRI female mice relative to young female mice.[32] Together with the data from the aged mice, this pattern of results suggests that the mnemonic ability of mice of both sexes declines to similar levels in old age, but that this decline may begin at an earlier age in females.

The behavioral sex differences observed in the 17-month-old mice may be related to the loss of estrous cycling in 17-month-old females. Regular estrous cycling was observed in only 20% of the 17-month-old females, which is consistent with previous findings in 16–20-month-old C57BL/6NIA females[50] but slightly different from results in C57BL/6J (the same strain supplied by The Jackson Labs, Bar Harbor, ME) females in which estrous cyclicity ceased at 13–16 months.[45] Although, in this study, the loss of estrous cyclicity did not appear to affect the performance of the 25-month-old females compared with 25-month-old males, it is possible that the hormonal changes occurring during middle age accelerated age-related degeneration in females. Low levels of estrogen and progesterone in rats, either after ovariectomy or during certain phases of the estrous cycle, are associated with significant decreases in the density of dendritic spines on hippocampal CA1 neurons[24,62] and reduced long-term potentiation,[59] suggesting that decreased levels of ovarian hormones can profoundly alter hippocampal morphology and physiology. Perhaps the decreasing levels of estrogen and progesterone during the early stages of estrous cycle cessation alter hippocampal connectivity, thereby triggering age-related neurobiological changes that interfere with learning and memory. Recent studies of aging female rats that experienced long-term ovarian hormone deprivation have reported significant reductions of hippocampal dentate granule cell spine density[40] and basal forebrain choline acetyltransferase mRNA,[22] suggesting that the loss of ovarian hormones may have adverse effects on hippocampal and basal forebrain neurons beyond those of aging. Alternatively, given that sex differences were observed in the 17-month group in several behavioral domains (memory, motor, emotional), the loss of estrous cyclicity may affect general mood or motivation, which in turn may affect performance on a variety of tasks. It is common for middle-aged women to experience a variety of uncomfortable physiological and emotional side-effects of menopause,[54] and perhaps 17-month-old female mice also experience a period of discomfort and anxiety at the onset of estrous cycle loss, which then disappears as they get older and more accustomed to the loss of ovarian hormones. These possibilities will need to be investigated further to determine whether the loss of circulating ovarian hormones contributes to the earlier age of onset of age-related spatial memory decline in females.

Overall sex differences

Of all of the behaviors tested in this study, only two measures revealed a main effect of sex in the two-way ANOVAs: swim speed in the spatial task and SOD latency. Both of these measures assess response time. Females were faster than males in both measures, suggesting that males have slower motor responses than females. Although this result is consistent with previous findings of increased activity in female rats relative to male rats,[3,28] sex differences in activity levels have not been reported in several strains of mice.[4,30,41] In the present study, the fact that swim speeds in the spatial reversal and cued tasks, as well as general locomotor activities, were not significantly different between the sexes suggests that motor differences in mice are mild and perhaps task specific. The absence of sex differences in the five-month group is consistent with previous studies of young C57BL/6 mice in which no sex differences were observed in water maze[4] and T-maze[33] tasks. However, sex differences in memory have been observed in other mouse strains,[41] suggesting that the observation of sex differences in memory may be dependent on experimental factors such as task, procedure, age and strain.

Dissociations among measures of learning and memory

The finding that measures from the spatial, cued and olfactory tasks were associated with different factors in the principal components analysis suggests that these tasks may measure different aspects of reference memory. Furthermore, measures of initial and asymptotic spatial task performance were strongly associated with different factors, suggesting that spatial learning may be qualitatively different from spatial memory. The finding that measures of swim speed from both spatial and cued tasks were highly correlated with each other, but associated with a different factor than the more mnemonic measures (path length and corridor) suggests that the motor performance of aging mice in these tasks may be independent of mnemonic performance, a result consistent with previous work in aged mice[31,32] and rats.[17,39] It is unclear why SOD latency was associated with the same factor as the mnemonic measures of the cued task, rather than swim speed. This relationship could reflect a common aspect of SOD latency and the cued task that is not readily apparent. It is tempting to speculate that the different factors and different tasks are associated with different neural systems; however, further studies will be necessary to shed light on this possibility.

CONCLUSIONS

In light of the increased use of mice in transgenic models of age-related neurodegenerative and cognitive decline, it is of paramount importance to understand how behavior changes normally with age in mouse strains, such as C57BL/6, which provide genetic background for transgenic lines.[1] The results of this study demonstrate that aged male and female C57BL/6 mice are impaired on tasks designed to measure spatial and olfactory reference memory, and that these deficits are not attributable to alterations in sensorimotor abilities or anxiety. The finding that the performance of middle-aged females in the spatial water maze task was more like that of aged females, whereas that of middle-aged males was more similar to young males, suggests that spatial memory decline may begin at a younger age in females than in males. Given that only three ages were tested in this study, these results reflect merely a snapshot of age-related mnemonic decline in male and female C57BL/6 mice. Although these results suggest a sex difference in the rate of age-related mnemonic decline, clearly more work is needed to determine: (i) the age at which the spatial learning and memory abilities of female C57BL/6 mice begin to decline relative to males; and (ii) whether the apparently premature age-related

mnemonic decline in females is related to loss of circulating ovarian hormones. These sex differences highlight the importance of directly comparing males and females in studies of aging and cognition, as well as suggesting caution when generalizing results from one sex to the other.

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