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Spatial Reference Memory and Neocortical Neurochemistry Vary With the Estrous Cycle in C57BL/6 Mice

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Estrous cycle-related variations of spatial reference memory and neurochemistry in intact female mice were examined. Spatial reference memory was tested in cycling females, ovariectomized (OVX) females, and males by using a 1-day water maze protocol. Choline acetyltransferase (ChAT) and glutamic acid decarboxylase (GAD) activities were measured in the hippocampus and neocortex. Estrus females exhibited worse spatial acquisition and 30-min retention than did proestrus and metestrus females, higher neocortical ChAT activity than proestrus females, and higher neocortical GAD activity than OVX females and males. Neocortical, rather than hippocampal, neurochemistry was more sensitive to hormonal modulation, suggesting that hormonal mediation of neocortical function may play a critical role in regulating spatial reference memory in female mice.

Brain regions critical for memory, such as the hippocampus, basal forebrain, and neocortex, are influenced by cyclic fluctuations of the ovarian hormones estrogen and progesterone. Hormone levels vary during the rodent estrous cycle, rising gradually during metestrus and diestrus, peaking during proestrus, and plummeting during estrus (Carter, 1992). In the rat hippocampus, the density of dendritic spines (Woolley & McEwen 1992), neuronal proliferation (Tanapat, Hastings, Reeves, & Gould, 1999), and long-term potentiation (LTP) (Warren, Humphreys, Juraska, & Greenough, 1995) are enhanced during proestrus relative to estrus, suggesting that rapid changes in hormone levels may alter hippocampal excitability. Estrogen-induced decreases in hippocampal glutamic acid decarboxylase (GAD) synthesis in vitro are associated with reduced inhibitory activity and increased synaptogenesis (Murphy, Cole, Greenberger, & Segal, 1998), implicating gamma-aminobutyric acid (GABA)-ergic neurons in hormonal modulation of hippocampal function. Cyclic fluctuations of GABAergic markers in rat neocortex (Al-Dahan, Jalilian Tehrani, & Thalmann, 1994) and cholinergic markers, including choline acetyltransferase (ChAT), in basal forebrain and hippocampus (Gibbs, 1996), suggest that modulation of the cholinergic and GABAergic systems by ovarian hormones may influence hippocampal or neocortical excitability or both and, in turn, memory.

If increased ovarian hormone levels augment hippocampal or neocortical function, then memory should be enhanced when hormone levels are elevated (i.e., during proestrus). Numerous studies in humans have reported no effect of the menstrual cycle on memory (for a review, see Epting & Overman, 1998), whereas others have demonstrated cyclic, albeit conflicting, fluctuations in visuo-spatial memory (Hampson, 1990; Phillips & Sherman, 1992). Similarly, some studies in rat report no cyclic variations of spatial memory (Berry, McMahan, & Gallagher, 1997; Stackman, Blasberg, Langan, & Clark, 1997), whereas others describe impaired spatial memory during proestrus (Frye, 1995; Warren & Juraska, 1997) or estrus (Healy, Braham, & Braithwaite, 1999) relative to other stages of the cycle. The magnitude of mnemonic fluctuation, when reported, has been modest, suggesting that hormonal mediation of memory is subtle and likely task dependent.

Given the increased development of transgenic mouse models of Alzheimer's disease (Neve & Robakis, 1998) and the potential use of estrogen as a treatment for this disease (Henderson, 1997), it is imperative to establish how ovarian hormones affect memory and neurotransmitter function in mice. Estradiol given to ovariectomized (OVX) C57BL/6 mice has either improved (Rissanen, Puoliväli, van Groen, & Riekkinen, 1999) or impaired (Fugger, Cunningham, Rissman, & Foster, 1998) performance in the spatial version of the Morris water maze, depending on the dose and duration of treatment. Progesterone treatment in OVX CD-1 mice impairs acquisition of footshock avoidance, further suggesting that ovarian hormones influence memory in mice. However, recent preliminary data collected in C57BL/6 mice suggest that, unlike in female rats, estrogen given to OVX female mice does not increase hippocampal CA1 spine density (Li, Magariños, Alves, & McEwen, 1999). Because cyclic fluctuations of hippocampal dendritic spines and memory have yet to be examined in mice, it is unclear whether hormonal regulation of these processes occurs in naturally cycling mice. To date, only two mouse studies have examined mnemonic fluctuations in naturally cycling females. Using footshock avoidance paradigms, these studies reported that CD-1 mice in estrus displayed impaired learning and retention relative to mice in other stages of the cycle (Farr et al., 1995; Gray, 1977). These

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data indicate that at least one type of memory is influenced by hormonal fluctuations during the natural estrous cycle in mice.

The present study was designed to examine whether the estrous cycle modulates spatial reference memory in the Morris water maze, a task commonly used to assess learning and memory in transgenic and knockout mouse studies. Activities of the enzymes ChAT and GAD, which synthesize acetylcholine and GABA, respectively, were measured to determine whether hormone-related fluctuations in these transmitter systems play a role in cyclic variations in memory. Intact females were tested during proestrus, estrus, or metestrus to examine the effect of naturally occurring hormone fluctuations on memory and neurochemistry. Intact females were also compared with OVX females and males to assess whether the absence of ovarian hormone cycling influences spatial memory and neurochemistry. Spatial learning and memory were tested in a 1-day water maze protocol with retention probe trials at 30 min and 24 hr. ChAT and GAD activities were then measured in the hippocampus and frontoparietal cortex. Our results suggest that spatial learning and memory are impaired and that neocortical ChAT activity is increased during estrus relative to proestrus in mice.

Methods

Subjects

Subjects were 11 male and 45 female C57BL/6J mice obtained from Jackson Laboratories (Bar Harbor, ME) at 6 weeks of age. C57BL/6J mice were selected for use in this study because this mouse strain commonly provides a genetic background for transgenic mouse lines (Banbury Conference on Genetic Background in Mice, 1997). Mice were housed up to 5 per cage in a room with a 12-hr light–dark cycle (lights on at 6 a.m.), and behavioral testing was performed during the light phase of the cycle. Food (Harlan Teklad 2215 Rodent diet) and water were provided ad libitum. Mice were allowed to mature in our colony until 8 weeks of age, at which point they were handled daily for 14 days. Beginning at 10 weeks of age, all females were lavaged for 2 weeks to establish the presence of regular 4–5-day cycling. At 12 weeks, 11 females were chosen randomly to be OVX. The remaining females were lavaged daily and were assigned randomly to be tested in one of three estrous cycle stages: proestrus, estrus, or metestrus. Females in diestrus were not examined. Females were tested in their assigned stage for the spatial acquisition and 30-min retention tasks, and were also killed in this stage at a later date. For the shaping, 24-hr retention, and cued tasks, females were tested irrespective of estrous stage, which allowed for examination of hormone-related effects on spatial memory consolidation.

Behavioral testing of males and cycling females was conducted from 14 to 23 weeks of age. Cycling females were lavaged 5–6 days/week until they were killed. OVX females were tested behaviorally at 22 weeks of age (10 weeks postsurgery) and were killed at 23 weeks (11 weeks postsurgery). The body weights of OVX females, cycling females, and a subset of males were recorded at 22 weeks. Behavioral tests were conducted in the order listed in this article and occurred each day immediately after all cycling females were lavaged. All procedures conformed to the standards set forth in the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (1986) and were approved by the Institutional Animal Care and Use Committee of Wellesley College.

Estrous Cycle Determination

In mice, cyclic changes in vaginal cytology are closely correlated with hormone-related changes in ovarian structure (Allen, 1922). Thus, daily examination of vaginal cell types is an excellent noninvasive method of determining the hormonal state of a female. Estrous cycles in mice typically last 4 to 6 days and are generally divided into four stages: proestrus, estrus, metestrus, and diestrus (Allen, 1922; Snell, 1941). Stages of the estrous cycle were determined by vaginal lavage. A drop of distilled water was placed over the vaginal opening, and vaginal cells were extracted with a transfer pipette. These vaginal smears were placed immediately on slides, and estrous stage was determined microscopically using the following guidelines (Allen, 1922; Snell, 1941): Proestrus was indicated by predominantly nucleated epithelial cells; estrus by predominantly cornified epithelial cells; metestrus by a mixture of nucleated epithelial cells, cornified cells, and leukocytes; and diestrus by predominantly leukocytes. Slides were later stained with hematoxylin and eosin to confirm categorization of each smear. Vaginal lavage was conducted each morning between 9 and 11 a.m. to allow sufficient time for examination of vaginal smears and behavioral testing. On the day of proestrus, estrogen and progesterone rise until they peak in the late afternoon. Thus, our proestrus females were behaviorally tested just prior to peak estrogen and progesterone levels.

Ovariectomy

Each mouse was anesthetized (0.2 cc/10 g of Avertin, ip), and two incisions were made on the dorsal surface of the body, each one just above the lateral extent of the upper pelvic bone. After the ovary, oviduct, and uterine horn were isolated, the ovary and oviduct were clamped off and removed, and the uterine horn was placed back into the abdominal cavity. This procedure was repeated for the other ovary. Mice were given 1 week to recovery from surgery before being lavaged for 1 week to confirm loss of estrous cycling.

Morris Water Maze

The water maze apparatus and pretraining shaping procedure have been described in detail elsewhere (Berger-Sweeney, Arnold, Gabeau, & Mills, 1995; Frick, Burlingame, Arters, & Berger-Sweeney, 1999). No data were collected during the shaping procedure. The spatial water maze task is generally administered over the course of several days (e.g., Frick et al., 1999; Morris, 1984), which limits its potential to measure spatial learning and memory within a single stage of the estrous cycle. To address this issue, we developed a spatial task that mice can learn within 2 hr. Thirty minutes after the last spatial acquisition trial, retention of the spatial task was tested during the same estrous stage. Twenty-four-hour retention and performance of a cued water maze task were tested the following day, irrespective of estrous stage. Although cycling females were no longer in their assigned stage during 24-hr retention, this trial allowed us to examine whether consolidation of spatial memory was affected by the estrous cycle. Previous studies in rats have revealed that estradiol, given immediately after spatial water maze training, can significantly enhance memory (Packard, Kohlmaier, & Alexander, 1996; Packard & Teather, 1997). Thus, a lingering effect of elevated estradiol during proestrus may affect retention at the 24-hr time point. To differentiate between cycling females tested on Days 1 and 2, the designation "former" was applied to each group tested on Day 2 (e.g., former proestrus group). This designation also applied to cycling females tested in the cued task. The water maze tasks were conducted as follows.

Spatial task acquisition (Day 1). In the spatial task, a test of spatial reference memory (Olton, Becker, & Handelmann, 1979), the mice learned to find a submerged platform using extramaze cues. The tank (103 cm diameter) was divided into four quadrants, with four start positions located near the edge of the tank at the junctions between the quadrants. A transparent Lucite platform (10 × 10 cm) was submerged 0.5 cm in the water in the northwest quadrant of the tank, where it remained for all trials. Each mouse participated in twelve trials, organized into three blocks of four trials (1 trial/start position within a block), with each block separated

by 30 min. For each trial, the mouse was given a maximum time of 60 s to locate the platform, on which it remained for 10–15 s. If the platform was not located within 60 s, the mouse was placed on it by the experimenter. The next trial started immediately after removal from the platform. After the completion of the fourth trial of the block, the mouse was removed from the platform and placed in its home cage for 30 min. Swim time (s), path length (cm), and swim speed (cm/s) were recorded (HVS Image, Hampton, England).

Thirty-minute retention trial (Day 1). Thirty minutes after completion of the spatial task, one probe trial was conducted. During this trial, the platform was collapsed, remaining underneath the water and unavailable for escape for 30 s. After 30 s, the platform was raised and available for escape for 30 additional seconds. The following measures were recorded during the first 30 s of the probe trial: quadrant time (percentage of time spent in the training quadrant), proximity (average distance to the platform in cm; distances sampled 10 times/s), and platform crossings (the number of times the mice crossed the exact location of the platform).

Twenty-four-hour retention trial (Day 2). One probe trial was conducted 24 hr after spatial testing using the same procedure as the 30-min retention trial.

Cued task (Day 2). In the cued task, the mice learned to swim to a visible platform. This nonspatial task was designed to examine nonmnemonic aspects of water maze performance, such as swimming ability, motivation, and visual ability. The visible platform was raised just above the water level, covered with yellow tape, and had a small yogurt cup cover (8 cm o.d., 0.5 cm wide) attached perpendicularly to it (Frick et al., 1999). This task was conducted approximately 5 min after completion of the 24-hr retention trial. Four cued trials were conducted, with start locations randomized and the platform located in a different quadrant during each trial. Swim time, pathlength, and swim speed were recorded. The intertrial interval was approximately 5 min.

Enzyme Activity Assays

Cycling females were killed in their assigned estrous stage. Each mouse was sedated briefly with CO_2 (Berger-Sweeney, Berger, Sharma, & Paul, 1994) and decapitated. The brain was removed immediately, and frontoparietal cortex and hippocampus were dissected bilaterally on ice. Tissue samples were weighed and stored at −70°C until assay, at which point they were resuspended in 50 mM TrisCl (pH 7.4) and 0.02% Triton X-100 at a concentration of 50 mg/ml. Samples were sonicated with a probe sonicator (Branson Ultrasonics Corporation, Danbury, CT) and centrifuged for 10 min at 10,000 *g*. The supernatant was diluted 1:5 and designated as the crude extract. This crude extract was used for all assays. The protein content of the samples was measured using a Bio-Rad (Bio-Rad Laboratories, Hercules, CA) protein assay (Bradford, 1976). Enzyme activities were expressed as nanomoles of product · hr · mg protein. All chemicals were obtained from Sigma Chemical Company (St. Louis, MO) unless otherwise noted.

Choline acetyltransferase. Activity of the enzyme ChAT, which synthesizes acetylcholine, was measured by the formation of [^{14}C]Acetylcholine from [acetyl-1-^{14}C]-acetyl-coenzymeA (55.7 mCi/mmol; New England Nuclear, Boston, MA) and choline (Fonnum, 1975). A detailed description of the assay procedure is provided elsewhere (Berger-Sweeney et al., 1994).

Glutamic acid decarboxylase. Activity of the enzyme GAD, which synthesizes GABA, was measured from L-[1-^{14}C]-glutamic acid (40–60 mCi/mmol; New England Nuclear) with a [^{14}C]CO_2 trapping technique (O'Connor, Nock, & McEwen, 1988). Reactions contained 50 μl of crude extract and 0.5 M KH_2PO_4, 5 mM ethylenediaminetetraacetic acid, 1 mM 2-aminoethylisothiouronium bromide, 10 mM glutamate, 1 mM pyridoxal phosphate and L-[1-^{14}C]-glutamic acid in a total volume of 100 μl. Samples were incubated for 1 hr at 37 °C in test tubes containing #32 glass fiber filters (Schleicher and Schuell, Kenne, NH) coated with 0.5 M Solvable (Packard Instruments, Meriden, CT). Each filter was suspended at the top of the tube, just underneath a rubber stopper that sealed the tube. The reaction was terminated by the injection of 15% trichloroacetic acid through the stopper. The tubes were incubated at room temperature for another 90 min to ensure complete release and absorption of [^{14}C]CO_2 into the filter paper. The filter papers were then removed from the tubes and placed in scintillation vials for measurement of the [^{14}C]CO_2 product with a scintillation counter.

Data Analysis

Two sets of data analyses were performed to address the following questions: (a) Do memory and neocortical or hippocampal neurochemistry vary during the estrous cycle? and (b) Does the absence of ovarian hormone cycling affect spatial memory and either neocortical or hippocampal neurochemistry? To address the first question, analyses were performed including only the three cycling female groups (proestrus, estrus, and metestrus). To address the second question, all three cycling groups plus OVX females and males were included.

Spatial acquisition and cued task measures were averaged within a group for each block of four trials (spatial task) or single trial (cued task) and analyzed using a one-way repeated-measures analysis of variance (ANOVA; SuperANOVA, Abacus Concepts, Berkeley, CA) with "Estrous-Stage" or "Group" as the independent variables for analyses 1 and 2, respectively. The main effect of Estrous-Stage had three levels (proestrus, estrus, and metestrus), whereas the main effect of Group had five (proestrus, estrus, metestrus, OVX, and male). To examine whether the groups differed in their performance of the spatial task by the end of testing, one-way ANOVAs were performed on Block 3 for the swim time and path length measures. One-way ANOVAs without repeated measures were performed on the 30-min and 24-hr retention trial measures (each retention trial was analyzed separately), ChAT activity, and GAD activity (each brain region was analyzed separately). To examine the effect of OVX on body weight, a one-way ANOVA on body weight included cycling females, OVX females, and males. Cycling females were not weighed in their assigned stage and thus were combined for the body weight analysis. For all measures, Fisher's Protected Least Significant Difference post hoc tests were performed on significant main effects.

Results

Subjects

All OVX females recovered from surgery and exhibited no cycling activity. Sample sizes for the water maze analyses were as follows: proestrus (n = 8), estrus (n = 11), metestrus (n = 12), OVX (n = 11), and male (n = 11). The brains of only two proestrus mice and ten estrus mice tested in the water maze were included in the enzyme assays. An additional three females killed during proestrus were included in the enzyme assays. Also, three metestrus ChAT samples were excluded from the ChAT analyses as statistical outliers. Therefore, sample sizes for the ChAT assays were as follows: proestrus (n = 5), estrus (n = 10), metestrus (n = 9), OVX (n = 11), and male (n = 11). Sample sizes for the GAD assays were the same as for ChAT, except for the metestrus group (n = 12).

Effects of the Estrous Cycle

Spatial task acquisition was impaired in estrus during the last block of training trials. ANOVAs performed on the last block of training trials revealed a significant main effect of estrous cycle stage in both swim time, $F(2, 28) = 4.8$, $p < .05$, and path length,

$F(2, 28) = 4.6$, $p < .05$. In this third block, estrus females exhibited slower swim times (see Figure 1A) and longer path lengths (Figure 1C) than both proestrus and metestrus females (post hocs: $ps < .05$), suggesting better learning of the platform location by proestrus and metestrus females. When examined over the course of all three spatial test blocks, performance was not significantly affected by estrous cycle stage. The nonsignificant main effects of estrous stage suggested similar swim times and path lengths among the groups during Blocks 1 and 2. In general, swim times and path lengths decreased during testing, main effects of test block: $Fs(2, 56) = 7.6$ and 9.0, respectively, $ps < .01$.

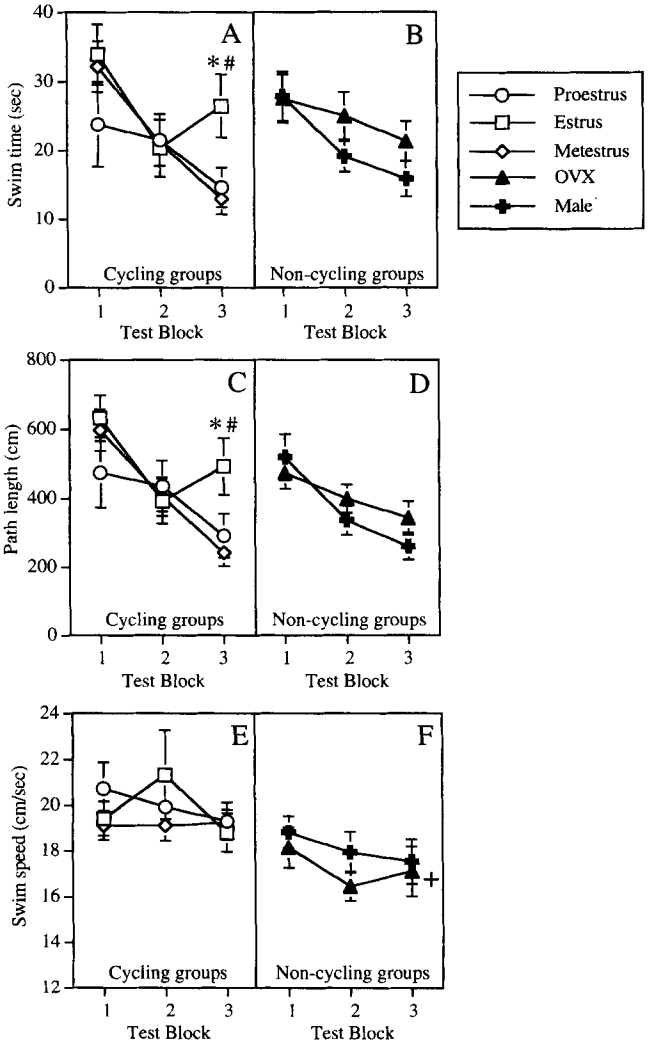

Figure 1. Spatial task acquisition as illustrated by swim time (A, B), path length (C, D), and swim speed (E, F). Each symbol represents the mean ± standard error of the mean of each group for a block of four trials. For swim time and path length, lower values indicate better performance. Estrus females exhibited longer swim times and path lengths than did proestrus or metestrus females and males during Block 3. Swim speed was not affected by estrous cycle stage or sex, but was decreased by ovariectomy (OVX). $*p < .05$, relative to proestrus and metestrus during Block 3. $\#p < .05$, relative to males during Block 3. $+ p < .05$, relative to proestrus and estrus throughout testing.

Estrous-Stage × Test-Block interactions were not significant for either measure. Swim speeds (Figure 1E) did not vary significantly during the estrous cycle or throughout training, as suggested by nonsignificant main effects and Estrous-Stage × Test-Block interaction.

Thirty-minute spatial retention was significantly impaired during estrus. Thirty-minute retention of the former platform location varied significantly during the cycle, as suggested by significant main effects in both quadrant time and proximity to the platform, $Fs(2, 28) = 3.4$ and 4.2, respectively, $ps < .05$. Estrus females spent significantly less time in the target quadrant (Figure 2A) than did proestrus females (post hoc: $p < .05$) and swam significantly farther from the platform (Figure 2C) than did both proestrus and metestrus females (post hocs: $ps < .05$). The proestrus and metestrus groups exhibited similar quadrant times and proximities. In contrast to the quadrant time and proximity measures, the number of platform crossings during the retention trial (Figure 2E) did not fluctuate significantly during the estrous cycle.

Neither 24-hr retention nor the cued task differed among the groups. Twenty-four-hour retention of the platform location was unaffected by prior testing in assigned estrous stages (see Table 1), as suggested by nonsigificant main effects of estrous stage in all three measures. Similarly, cued task performance did not differ among the three cycling groups, as suggested by nonsignificant main effects of estrous stage in swim time, path length, and swim speed (see Table 1). Performance improved somewhat during testing; the main effect of trial was significant for path length, $F(3, 84) = 3.6$, $p < .05$, but not for swim time, $F(3, 84) = 2.6$, $p = .056$, or swim speed. No Estrous Stage × Trial interactions were significant in any measure.

Neocortical ChAT activity was significantly elevated during estrus relative to proestrus, whereas GAD activity did not vary during the estrous cycle in either the neocortex or hippocampus. Table 2 presents mean ChAT and GAD activities for each group in the neocortex and hippocampus. Neocortical ChAT activity varied significantly during the estrous cycle, $F(2, 21) = 3.5$, $p < .05$; estrus females had significantly higher neocortical ChAT activity than did proestrus females, $p < .05$. The neocortical ChAT activity of estrus females also tended to be elevated relative to metestrus females, although this difference was not significant ($p = .057$). Hippocampal ChAT activity did not fluctuate significantly during the estrous cycle. Similarly, GAD activity in the neocortex and hippocampus was not significantly influenced by the estrous cycle.

Effects of Ovarian Hormone Removal and Sex

OVX females gained a significant amount of weight postsurgery. Body weight significantly differed among the cycling, OVX, and male groups, $F(2, 39) = 92.4$, $p < .01$. The mean body weight (g) of OVX females (28.6 ± 0.6) was intermediate between that of males (31.3 ± 1.0) and cycling females (22.8 ± 0.2). Cycling females weighed significantly less than did both OVX females and males, whereas OVX females weighed significantly more than did cycling females and less than males ($ps < .05$).

Males exhibited better performance during spatial Block 3 than estrus females did, whereas OVX females differed from cycling females only in swim speed. ANOVAs conducted on Block 3 revealed a significant effect of Group in both swim time [Figure 1,

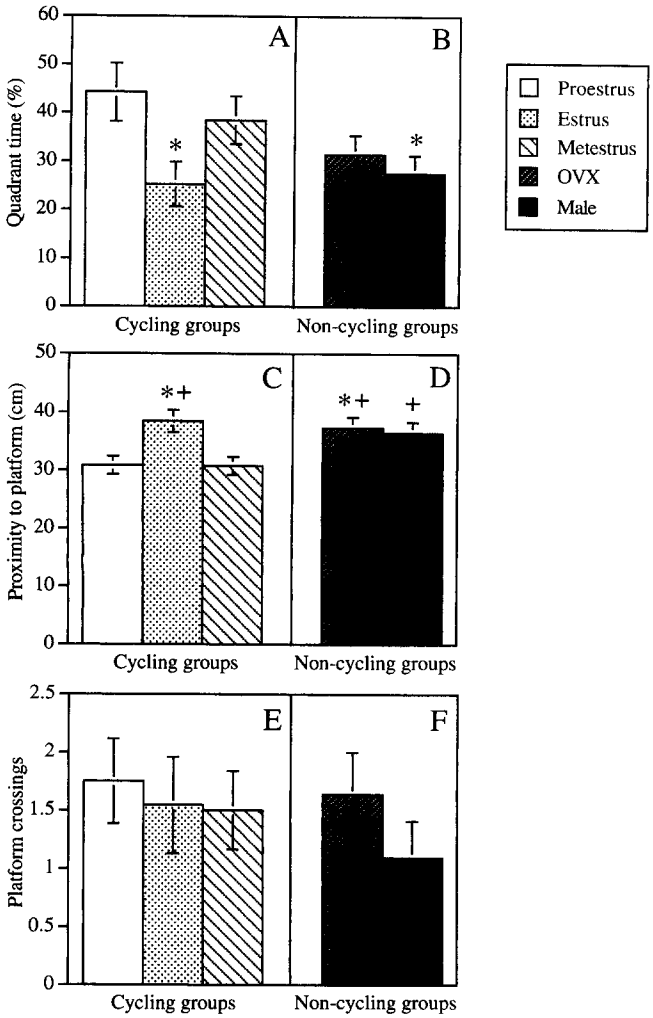

Figure 2. Performance in the 30-min retention trial as illustrated by quadrant time (A, B), proximity to the platform (C, D), and platform crossings (E, F). Each bar represents the mean ± standard error of the mean of each group for the single probe trial. For quadrant time and crossings, higher values indicate better performance, whereas lower proximity values indicate better performance. Estrus females exhibited lower quadrant times than proestrus females showed and farther proximities than both proestrus and metestrus females exhibited. Males exhibited lower quadrant times and farther proximities than did proestrus and metestrus females. Ovariectomized (OVX) females displayed farther proximities than did proestrus and metestrus females. Platform crossings did not differ among the groups. * $p < .05$, relative to the proestrus group. + $p < .05$, relative to the metestrus group.

A and B, $F(2, 96) = 3.4, p < .05$] and path length [Figure 1, C and D, $F(2, 96) = 3.4, p < .05$]. In both measures, these effects were due in part to the poor performance of estrus females relative to males ($p < .05$). When examined over the course of all three spatial test blocks, swim time and path length did not differ significantly among the groups, as suggested by nonsignificant main effects and interactions. Swim time, $F(2, 96) = 14.4, p < .01$, and path length, $F(2, 96) = 18.4, p < .01$, decreased in all groups during the three blocks.

Swim speeds (Figure 1, E and F) differed significantly among the groups, $F(4, 48) = 2.6, p < .05$; OVX females swam slower than did proestrus and estrus females, $ps < .05$. Swim speeds in all groups remained constant throughout testing, as illustrated by a nonsignificant test block effect and Condition × Block interaction.

Thirty-minute retention was impaired in OVX females and males relative to cycling females; however, 24-hr retention was similar among all five groups. The main effect of group was significant for the quadrant time [Figure 2, A and B, $F(4, 48) = 2.6, p < .05$] and proximity measures [Figure 2, C and D, $F(4, 48) = 3.2, p < .05$]. OVX females exhibited farther proximities than did the proestrus and metestrus groups (post hocs: $ps < .05$). Males also displayed farther proximities than the metestrus group (post hoc: $p < .05$) and, in addition, spent less time in the correct quadrant than did the proestrus group (post hoc: $p < .05$). Platform crossings did not differ among the groups due to the large variability in this measure. The five groups did not differ in any measure of 24-hr retention (Table 1).

Time to find the visible platform was increased and swim speeds decreased by ovariectomy. Differences among the groups are indicated in Table 1. OVX females displayed significantly longer swim times, $F(4, 48) = 3.9, p < .01$, than all cycling females displayed (post hocs: $ps < .05$), whereas both OVX females and males exhibited slower swim speeds, $F(4, 48) = 7.4, p < .01$, than did all cycling females (post hocs: $ps < .05$). The fact that path length did not differ among the groups suggests that the adverse effect of OVX on swim time may be the result of the slower swim speeds of OVX females rather than an effect on cognition per se. Swim times and path lengths decreased throughout testing, $Fs(3, 144) = 8.1$ and 8.3, respectively, $ps < .05$, whereas swim speeds remained constant. Group × Trial interactions were not significant for any measure.

Neocortical GAD activity was significantly reduced in OVX females and males relative to cycling females, whereas ChAT activity was not altered in either the neocortex or hippocampus. Table 2 presents mean ChAT and GAD activities for each group in the neocortex and hippocampus. ChAT activity was not significantly different among the five groups in the neocortex and hippocampus. In contrast, neocortical GAD activity was significantly decreased, $F(4, 44) = 3.1, p < .05$, in OVX females and males relative to estrus females (post hocs: $ps < .05$). Male neocortical GAD activity was also significantly decreased relative to metestrus females ($p < .05$). In the hippocampus, GAD activity did not differ significantly among the five groups, despite a tendency toward elevated GAD activity in estrus females (post hocs: $ps > .05$ vs. all other groups).

Discussion

The present study is the first to document estrous-cycle-related variations in spatial learning and memory in mice. These findings indicate that the estrous cycle, as measured by vaginal lavage, influences spatial learning and memory in intact female mice. Relative to proestrus (high ovarian hormone levels) and metestrus (intermediate hormone levels) females, estrus (low hormone levels) females displayed an impaired ability to learn the hidden platform location and remember it after 30 min. Estrous-related differences in acquisition of the spatial task were limited to the last block of training, suggesting a subtle effect of ovarian hormones

Table 1
Spatial Retention (24 hr) and Cued Water Maze Means

Task and measure	Cycling			Noncycling	
	Former proestrus	Former estrus	Former metestrus	OVX	Male
24-hr spatial probe trial					
Quadrant time (%)	27.9 ± 5.1	19.7 ± 4.1	26.1 ± 4.0	32.8 ± 5.6	20.3 ± 4.0
Proximity (cm)	35.4 ± 2.3	40.0 ± 2.8	36.4 ± 2.2	36.7 ± 2.4	38.7 ± 2.4
No. platform crossings	2.0 ± 0.7	1.1 ± 0.3	1.3 ± 0.3	0.9 ± 0.3	1.3 ± 0.2
Cued task					
Swim time (s)	16.2 ± 1.9	16.4 ± 2.0	18.9 ± 2.0	28.9 ± 2.2*	22.2 ± 2.2
Path length (cm)	265.4 ± 31.4	265.0 ± 32.5	292.8 ± 32.9	336.3 ± 34.0	258.8 ± 24.7
Speed (cm/s)	17.3 ± 0.7	17.0 ± 0.5	16.0 ± 0.6	11.7 ± 0.6*	12.6 ± 0.6*

Note. Values represent $M \pm SEM$. OVX = ovariectomized.
* $p < .05$, relative to all cycling groups.

on spatial learning. The impairment evinced by estrus females during Block 3 of the spatial task likely contributed to the 30-min retention deficit, as memory for the platform location may have been compromised by impaired learning. Estrus females also exhibited significantly higher neocortical ChAT activity than did proestrus females and higher neocortical GAD activity than OVX females and males. Surprisingly, neocortical enzyme levels were more sensitive than were hippocampal enzyme levels to differing fluctuations across the cycle.

Estrous Cycle Variations

The present findings of impaired spatial water maze acquisition and retention during estrus are consistent with previous reports from mice tested in nonspatial footshock avoidance paradigms. In these prior studies, CD-1 mice in estrus exhibited both impaired learning and retention of footshock avoidance tasks relative to females in other stages of the cycle (Farr et al., 1995; Gray, 1977). These findings, together with those of the current report, suggest that learning and memory in naturally cycling mice are compromised when estrogen levels are low and indicate that cyclic fluctuations of ovarian hormones may alter several types of learning and memory in mice. The specific roles of either estrogen or progesterone in modulating learning and memory in mice remains unclear. In the current study, we have used intact mice with normal levels of estrogen and progesterone. Therefore, we cannot tease apart the relative contributions of each hormone to spatial memory modulation. However, the effects of estrogen and progesterone replacement in OVX females suggest a considerable effect of both hormones on memory. For example, estrogen treatment given to ovariectomized female mice affects both spatial and nonspatial memory; it facilitates spontaneous alternation (Miller et al., 1999) and either improves or impairs spatial water maze performance (Fugger et al., 1998; Rissanen et al., 1999) depending on the dose and duration of treatment. Furthermore, progesterone replacement impairs acquisition of footshock avoidance (Farr et al., 1995). Future behavioral testing of hormone-receptor knockout mice will undoubtedly help elucidate the extent to which estrogen and progesterone influence memory.

The apparent spatial learning impairment observed in estrus females was due primarily to their poor performance during Block 3. The cause of this deterioration in performance compared to Block 2 is unclear. Although the Morris water maze is commonly used to assess spatial memory in mice (e.g., Fordyce & Wehner, 1993; Fugger et al., 1998; Rissanen et al., 1999; Silva, Paylor, Wehner, & Tonegawa, 1992), recent studies suggest that mice may rely less on spatial strategies to find the platform than do rats, for whom the task was originally designed (Frick, Stillner, & Berger-Sweeney, 2000; Whishaw, 1995; Whishaw & Tomie,

Table 2
Enzyme Activities in the Frontoparietal Cortex and Hippocampus

Enzyme and brain region	Cycling			Noncycling	
	Proestrus	Estrus	Metestrus	OVX	Male
ChAT					
Neocortex	59.4 ± 2.2*	67.4 ± 1.9	61.7 ± 2.2	63.9 ± 2.7	73.1 ± 5.9
Hippocampus	65.4 ± 2.8	76.2 ± 2.6	73.1 ± 3.9	74.4 ± 3.1	74.3 ± 4.0
GAD					
Neocortex	234.6 ± 11.0	252.4 ± 13.3	232.0 ± 9.9	220.3 ± 8.4*	202.9 ± 9.5*†
Hippocampus	191.1 ± 12.7	211.0 ± 12.5	202.1 ± 8.3	201.8 ± 11.9	174.9 ± 8.8

Note. Values are given in nanomoles of product/hour/milligrams of protein ($M \pm SEM$). OVX = ovariectomized; ChAT = choline acetyltransferase; GAD = glutamic acid decarboxylase.
* $p < .05$, relative to the estrus group. † $p < .05$, relative to the metestrus group.

1996). Mice often appear to use nonspatial strategies such as swimming a fixed distance from the edge of the tank, random spiraling, stereotyped motor response strategies, or predatory avoidance strategies (Frick et al., 2000; Lipp & Wolfer, 1998). The use of these strategies can confound interpretation of this "spatial" memory task for mice. However, it has not been demonstrated convincingly that mice ignore spatial cues entirely and rely exclusively on nonspatial strategies. Moreover, some mice may rely on nonspatial strategies more than others do. Perhaps, search strategies are influenced by the estrous cycle, such that mice are more reliant on spatial strategies during proestrus or metestrus and nonspatial strategies during estrus. This idea is supported by data from the probe trial, in which estrus females exhibited less of a spatial bias than proestrus or metestrus females did. In aging rodents, for example, the lack of a spatial bias (i.e., spending less time in the correct quadrant and swimming farther from the platform location) during probe trials is indicative of a reduction in spatial strategy utilization (Frick et al., 1999; Gallagher & Pelley-mounter, 1988). Thus, estrus mice displaying reduced quadrant time and farther proximities during the probe trial raises the possibility of differing search strategies among cycling females.

Although 30-min retention varied across the estrous cycle, 24-hr spatial retention was not influenced by prior training in the assigned estrus stage. These findings suggest that cyclic fluctuations of 30-min retention were transient and did not affect 24-hr spatial memory consolidation. However, the potential state-dependency of spatial memory and that most cycling females were in a different hormonal state on Day 2 of testing cannot be excluded as explanations for the lack of effect in this task. Similarly, performance in the cued task did not differ among cycling females. Because this task measures similar nonmnemonic aspects of water maze performance as the spatial task, it is unlikely that differences in swimming ability, motivation, and visual acuity contribute to cyclic variations of spatial learning and memory.

Hippocampal ChAT activity was not altered during the estrous cycle. In contrast, neocortical ChAT activity varied cyclically; ChAT levels were elevated during estrus relative to proestrus. Recent data illustrating that the neocortex expresses mRNAs for ERα, ERβ, and progesterone receptors (Butler, Kalló, Sjöberg, & Coen, 1999; Kato, Hirata, Nozawa, & Yamada-Mouri, 1994; Shughrue, Lane, & Merchenthaler, 1997; Shughrue, Scrimo, Lane, Askew, & Merchenthaler, 1997) suggest a mechanism by which ovarian hormones may influence the neocortical function. A previous study of Albino-Swiss mice did not find altered cerebral cortical ChAT activity during the estrous cycle (Ladinsky, Consolo, Bianchi, Peri, & Garattini, 1976), although an examination of the means reveals a trend, in that ChAT activity was the highest during estrus, less during metestrus, and the least during proestrus. It is important to note that this previous study examined the entire cerebral cortex. It is therefore likely that regional variations in cortical cholinergic innervation (Mesulam, Hersh, Mash, & Geula, 1992), as well as estrogen and progesterone receptor distribution (Kato et al., 1994; Shughrue et al., 1997), were obscured in the Ladinsky et al. (1976) study. Limiting our dissection to the frontoparietal cortex may have enabled us to observe more pronounced estrous-cycle variations than is possible in a sample of the entire neocortex.

The association between elevated neocortical ChAT activity and impaired spatial retention in estrus mice raises two interesting points. First, the response of cholinergic neurons to elevated estrogen levels may be delayed for 1–2 days. This hypothesis is supported by the present finding of elevated ChAT activity during estrus (1 day after peak estrogen) and previous reports that estrogen replacement in OVX rats increases basal forebrain ChAT mRNA 24–53 hr after treatment (Gibbs, 1996). Second, this association is contrary to the notion that enhanced cholinergic function improves memory (Bartus, Dean, Pontecorvo, & Flicker, 1985). However, similar relationships between elevated ChAT activity and impaired spatial reference memory have been reported in aging mice (Frick, Burlingame, & Berger-Sweeney, 1998) and rats (Baxter et al., 1999; Frick, Gorman, & Markowska, 1996). Furthermore, reducing hippocampal ChAT activity can improve memory in middle-aged rats (Frick et al., 1996). These data suggest that ovarian hormones may modulate ChAT activity such that transmitter levels optimal for spatial memory are achieved during proestrus rather than estrus.

Hippocampal and neocortical GAD activity did not vary significantly during the estrous cycle. The lack of cyclic variation in hippocampal GAD activity is surprising, given recent findings in vitro that estrogen-induced downregulation of hippocampal GAD immunoreactivity promotes CA1 dendritic spine formation (Murphy et al., 1998). However, it is unknown whether these in vitro effects of exogenous estrogen resemble those of estrogen in vivo. In the present study, proestrus females tended to have lower hippocampal GAD activity that estrus females had, which lends some support to the idea that estrogen-induced GAD reductions may promote hippocampal spine formation (Murphy et al., 1998). More discrete dissections of hippocampal and neocortical subregions may help elucidate this effect.

Effects of Ovariectomy

Consistent with previous findings in mice, ovariectomy did not substantially affect spatial reference memory in the water maze (Wilson, Puoliväli, Heikkinen, & Riekkinen, 1999). Ovariectomy in this experiment did adequately remove circulating ovarian hormones, as suggested by the significant postsurgical weight gain in OVX females. The finding is consistent with previous reports of increased body weight in OVX rats (Bimonte & Denenberg, 1999; Geary is Asarian, 1999; O'Neal, Means, Poole, & Hamm, 1996). Although estrogen treatment can improve spatial memory in OVX mice (Rissanen et al., 1999), it is unclear why removal of the ovaries (the main female source of estrogen and progesterone) does not disrupt spatial memory. The behavioral disparity between OVX and estrus females may reflect a fundamental difference between the temporary low hormone levels of estrus and the permanent hormone reductions of ovariectomy. Perhaps chronic hormone reductions allow the OVX brain more time to compensate for prolonged hormone loss. Alternatively, the task used here may not be sufficiently sensitive to reveal adverse effects of hormone acyclicity on memory. This possibility may be supported by findings in OVX rats of a spatial memory deficit in a more challenging water maze task (O'Neal et al., 1996).

Of the neurochemical variables examined in this study, only neocortical GAD activity was affected by ovariectomy. The lack of effect of ovariectomy on neocortical ChAT activity in mice agrees with a previous report in rats, but is inconsistent with decreased hippocampal ChAT activity reported in the same animals (Singh,

Meyer, Millard, & Simpkins, 1994). Discrepant tissue dissections or species differences may account for these inconsistencies. Alternatively, the long duration of ovariectomy in the present study may have permitted compensation after initial disruptions of cholinergic function. In contrast to ChAT activity, neocortical GAD activity was reduced in OVX females relative to estrus females. In mice, GABAergic transmission is reportedly reduced in male mice relative to intact females (Pericic, Manev, & Geber, 1986), raising the possibility that decreased neocortical GAD activity in our OVX females reflects a masculinization of neocortical GABA transmission. The potential functional correlates of reduced neocortical GAD activity in OVX females remain to be explored.

Sex Differences

Consistent with previous studies of young C57BL/6 mice (Frick et al., 1999; Lindzey & Winston, 1962), sex differences in spatial memory were not observed in this study. Males were impaired relative to one cycling female group in one (30-min proximity) of ten measures of spatial and nonspatial memory, which did not represent a considerable effect of sex on memory. Hippocampal and neocortical ChAT activity were also similar between the sexes, which is consistent with previous reports in the basal forebrain and hippocampal CA1 region (Luine, Renner, Heady, & Jones, 1986). In contrast, neocortical GAD activity was reduced in males relative to estrus females. As previously mentioned, male mice are more sensitive to the convulsive effects of the GABAergic antagonist picrotoxin than are females (Pericic et al., 1986), suggesting reduced GABAergic transmission in males. Although our results are consistent with this finding, the sex difference in GAD activity does not appear to influence spatial memory.

Conclusions

The present study provides the first evidence that spatial learning and memory in mice are sensitive to hormone fluctuations during the estrous cycle. Our finding of impaired spatial learning and retention in estrus mice is consistent with previous findings from mice tested in nonspatial tasks. The finding that neocortical neurochemistry in mice is more sensitive to ovarian hormone levels than is hippocampal neurochemistry highlights the need for further investigation of hormonal modulation of neocortical function. The vast majority of data on the effects of hormone cycling on the brain and behavior has been collected in rats. Due to the increasing use of transgenic mice to model dementing disorders such as Alzheimer's disease, it is crucial that the effects of ovarian hormones on cognition be studied further in mice.

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