

ESTROGEN REPLACEMENT IMPROVES SPATIAL REFERENCE MEMORY AND INCREASES HIPPOCAMPAL SYNAPTOPHYSIN IN AGED FEMALE MICE

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Abstract—Estrogen deficiency during menopause is often associated with memory dysfunction. However, inconsistencies regarding the ability of estrogen to improve memory in menopausal women highlight the need to evaluate, in a controlled animal model, the potential for estrogen to alleviate age-related mnemonic decline. The current study tested whether estrogen could ameliorate spatial reference memory decline in aged female mice. At the conclusion of testing, levels of the presynaptic protein synaptophysin, and activities of the synthetic enzymes for acetylcholine and GABA, were measured in the hippocampus and neocortex. Aged (27–28-month-old) female C57BL/6 mice were given daily subcutaneous injections of 1 µg or 5 µg of β-estradiol-3-benzoate dissolved in sesame oil. Control mice received daily injections of sesame oil or no injections. Estradiol treatment began 5 days prior to behavioral testing and continued throughout testing. Spatial and non-spatial memory were assessed in the Morris water maze. The 5 µg dose of estradiol significantly improved spatial learning and memory in aged females. The performance of 5 µg females improved significantly more rapidly than that of control females; estradiol-treated females performed at asymptotic levels by session 2. Furthermore, 5 µg females exhibited a more robust spatial bias than controls during probe trials. In contrast, 1 µg of estradiol did not improve spatial task performance. Neither dose affected performance of the non-spatial task. In the hippocampus, synaptophysin was increased in 5 µg females relative to controls. Estrogen did not affect enzyme activities in either brain region.

This study is the first to examine the effects of estrogen replacement on spatial reference memory and synaptophysin expression in aged post-estropausal female rodents. The results suggest that: (1) estrogen can profoundly improve spatial reference memory in aged females, and (2) this improvement may be related to increased hippocampal synaptic plasticity, but not modulation of the synthetic enzymes for acetylcholine and GABA.

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Key words: aging, estradiol, Morris water maze, hippocampus, neocortex, ELISA.

The profound reductions of estrogen associated with menopause have been linked to mnemonic decline in both normal aging and dementia. Recent evidence suggests that estrogen replacement therapy in non-demented menopausal women can improve both verbal (Sherwin, 1999) and spatial memory (Duff and Hampson, 2000). However, others have found little or no cognitive benefit of estrogen treatment in women (see Yaffe et al., 1998 and Hogervorst et al., 2000 for reviews). These discrepancies may be due, in part, to subject pools that vary widely in terms of the total duration of estrogen treatment prior to testing and the delay between onset of menopause and initiation of estrogen replacement. The methodological shortcomings of these clinical studies highlight the need to evaluate the potential of estrogen

to attenuate or prevent age-related memory decline in established animal models.

Considerable evidence in rodents suggests that estrogen profoundly influences brain regions that are critical for learning and memory (e.g. the hippocampus, neocortex, and basal forebrain) and deteriorate in normal aging and dementia. In female rodents, elevated levels of estrogen are associated with a variety of alterations in the hippocampus, including reduced long-term depression (Vouimba et al., 2000), increased CA1 dendritic spine density (Woolley and McEwen, 1992; Woolley and McEwen, 1993) and synaptic proteins (Stone et al., 1998), and enhanced long-term potentiation (Warren et al., 1995; Foy et al., 1999) and neurogenesis (Tanapat et al., 1999). Increased spine density may result from the activation of *N*-methyl-D-aspartate (NMDA) receptors on pyramidal neurons (Woolley and McEwen, 1994; Woolley et al., 1997), made possible in part by estrogen-induced inhibition of GABA synthesis in the inhibitory interneurons that regulate pyramidal neuron function. The latter notion is supported by evidence from cultured hippocampal neurons that within 24 h, estradiol decreases the expression of the GABAergic synthetic enzyme glutamic acid decarboxylase (GAD), reduces GABA synthesis, and suppresses inhibitory elec-

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Abbreviations: ANOVA, analysis of variance; ChAT, choline acetyltransferase; CO₂, carbon dioxide; EB, β-estradiol-3-benzoate; ELISA, enzyme-linked immunosorbent assay; GAD, glutamic acid decarboxylase; MBS, mouse brain standard.

trical activity (Murphy et al., 1998a,b), although GAD expression increases relative to controls after 2 days of estradiol exposure (Weiland, 1992; Murphy et al., 1998a). In the basal forebrain, estrogen enhances the function of hippocampal- and cortically projecting cholinergic neurons (e.g. Gibbs and Aggarwal, 1998a; Wu et al., 1999; Gibbs, 2000a), which are involved in attention and cortical information processing, as well as some aspects of learning and memory (e.g. Bartus et al., 1985; Baxter and Chiba, 1999; Berger-Sweeney et al., 2000). Furthermore, we recently demonstrated that activity of the enzyme choline acetyltransferase (ChAT), which synthesizes acetylcholine, fluctuates in the neocortex of female mice during the estrous cycle (Frick and Berger-Sweeney, 2001). Although associations between estrogen-induced alterations in the brain and behavior are scant, some evidence suggests that estrogen given to young ovariectomized rodents can improve both spatial and non-spatial learning and memory (Farr et al., 1995; O'Neal et al., 1996; Packard and Teather, 1997; Gibbs et al., 1998b; Bimonte and Denenberg, 1999; Gibbs, 1999; Rissanen et al., 1999; Sandstrom and Williams, 2001) (although see Fugger et al., 1998; Chesler and Juraska, 2000).

As in humans (e.g. Evans et al., 1984; Sharps and Gollin, 1987; Moffat et al., 2001), aging in rodents is typically accompanied by impairments in spatial memory. Spatial reference memory, commonly tested in a hidden-platform version of the Morris water maze, deteriorates with age in both males and females (Gage et al., 1984; Gallagher and Pelleymounter, 1988; Lamberty and Gower, 1990; Lamberty and Gower, 1991; Fischer et al., 1992; Fordyce and Wehner, 1993; Frick et al., 1995), although recent work suggests that the onset of this decline occurs earlier in females than in males (Markowska, 1999; Frick et al., 2000). For example, spatial reference memory deterioration in female mice begins at approximately 17 months, whereas this memory in males remains preserved until more advanced ages (Frick et al., 2000). Furthermore, the onset of this premature decline in females coincides with the cessation of ovarian hormone (estrogen and progesterone) cycling (Frick et al., 2000). Similar to humans, the loss of cycling in middle-aged mice is accompanied by drastic reductions in serum levels of estrogen and progesterone (Nelson et al., 1982; Nelson et al., 1995). This deficiency may substantially impact spatial memory, as the hippocampus and neocortex come to rely on estrogen as a trophic factor in adulthood (see Brinton, 2001 for review). The age-related loss of estrogen may render these neurons more vulnerable to deterioration and exacerbate emerging spatial memory deficits.

If the age-related loss of estrogen contributes to spatial memory decline in females, then estrogen replacement may restore spatial memory functioning. The hippocampus of aged females and males remains responsive to estrogen (Miranda et al., 1999; Vouimba et al., 2000), and thus estrogen may be a potent mnemonic enhancer for aged subjects. In previous studies examining spatial working memory, estrogen improved the performance of both aged male (Luine and Rodriguez, 1994) and female

rats (Gibbs, 2000b). Non-spatial working memory is also improved by estrogen in aged female mice (Miller et al., 1999; Vaucher et al., 2002). However, no study has yet examined whether estrogen can ameliorate spatial reference memory decline in aged females. Thus, the present study was designed to address this issue. Female mice, 27–28 months of age, were tested in spatial and non-spatial versions of the Morris water maze. To examine whether mnemonic improvements are related to estrogen-induced alterations in cholinergic and GABAergic function, activities of ChAT and GAD were examined in the hippocampus and neocortex after completion of behavioral testing. To examine relationships between estrogen-induced mnemonic improvements and synaptic plasticity, levels of the protein synaptophysin were also measured in these two brain regions. Synaptophysin is a 38-kDa calcium-binding glycoprotein found in the membranes of neurotransmitter-containing presynaptic vesicles (Jahn et al., 1985; Wiedenmann and Franke, 1985). In non-demented humans, synaptophysin decreases with age in the hippocampus and various cortical regions including temporal, prefrontal, and parietal (Masliah et al., 1993; Eastwood et al., 1994; Liu et al., 1996). In patients with Alzheimer's disease, synaptophysin immunoreactivity is significantly reduced in frontal cortex and hippocampus, and is correlated with impaired cognitive abilities (Terry et al., 1991; Zhan et al., 1993; Liu et al., 1996; Sze et al., 1997). In rodents, some studies have found similar age-related reductions in cortical and hippocampal synaptophysin (Saito et al., 1994; Chen et al., 1995), whereas other studies report no age-related changes in this protein (Calhoun et al., 1998; Nicolle et al., 1999). Nevertheless, findings from several rodent studies indicate that estrogen can significantly increase hippocampal synaptophysin expression (Murphy and Segal, 1996; Stone et al., 1998; Pozzo-Miller et al., 1999), and thus, modulation of synaptophysin may play a role in estrogen-induced alterations in memory. The present study is the first to examine this potential relationship.

EXPERIMENTAL PROCEDURES

Subjects

Subjects were 28 aged female C57BL/6 mice obtained at age 26–27 months from Hilltop Lab Animals (Scottsdale, PA, USA). Upon arrival at Yale, the mice were quarantined at our medical school animal facility for 3 weeks. They were then moved to an animal facility adjacent to the laboratory in the Department of Psychology, and handled for 5 days prior to testing to habituate them to being picked up by the experimenter. Thus, at the beginning of behavioral testing, the mice were approximately 27–28 months of age. Mice were housed up to five per shoebox cage in a room with a 12-h light/dark cycle (lights on at 06:00) and behavioral testing was performed during the light phase of the cycle. Food (LabDiet 5P00 ProLab RMH 3000) and water were provided *ad libitum*. 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 Institutional Animal Care and Use Committee of Yale University. All efforts were made to minimize the number of animals used.

Estrogen administration

Because female mice of this advanced age are reproductively quiescent (Nelson et al., 1992; Nelson et al., 1995) and it was felt that an ovariectomy surgery would endanger the health of the animals, all mice in this experiment remained gonadally intact. β -Estradiol-3-benzoate (EB; Sigma, St. Louis, MO, USA) dissolved in sesame oil was injected subcutaneously and administered daily in two doses, 1 μ g/0.05 ml ($n=8$) or 5 μ g/0.05 ml ($n=8$). This form of estradiol was used because it is metabolized slowly and results in consistent serum levels of estradiol with chronic administration. EB-treated mice were compared to control mice receiving either no injection ($n=6$) or daily subcutaneous sesame oil vehicle ($n=7$). EB and oil treatment began 5 days prior to behavioral testing and continued throughout testing until the mice were killed.

Morris water maze

A white circular tank (97 cm in diameter) was filled with water ($24 \pm 2^\circ\text{C}$) 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 HVS 2020 automated tracking system (HVS Image, Hampton, UK). 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 97-cm ring to decrease the total swimming area. This procedure habituated the mice to the water and taught them to escape from the water by climbing onto a platform. Each mouse was first placed on a visible red lucite platform (10×10 cm) for 10 s, and then placed at three progressively further distances from the platform where it was allowed 30 s to escape onto the platform. No data were collected during this procedure. For water maze testing, two water maze tasks were conducted as follows.

Spatial task. In this task, 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 northeast 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 5 consecutive days (1 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 (Markowska et al., 1993) 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), *swim distance* (cm), and *swim speed* (cm/s) were recorded during trials 1–5 of each session. During the probe trial (trial 6 of each session), *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 percent time each mouse spent in the quadrant containing the platform) and *proximity* (average distance in cm to the platform; distances sampled 10 times/s).

Cued task. In this non-spatial reference memory task, the platform was made visible using several local cues. In addition, the platform was located in a different quadrant for each trial. Thus, intramaze cues on the platform predicted platform location rather than extramaze cues. The platform was made visible by: (1) lowering the water level such that the platform was 0.25 cm above the surface of the water, (2) covering the platform with red tape, and (3) attaching the circular 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. For each of 3 days, six platform trials were conducted. *Swim time*, *swim distance*, and *swim speed* were recorded. No probe trials were conducted. The intertrial interval was approximately 20 min.

Tissue collection

Each mouse was sedated briefly with CO_2 and decapitated (Berger-Sweeney et al., 1994). The brain was removed immediately, and frontoparietal cortex and hippocampus were dissected bilaterally on ice. The uterus of each mouse in the oil control group and both EB groups was dissected and weighed (g). Brain tissue samples were weighed and stored at -70°C until the day of assay. Samples were resuspended in 0.02% Triton X-100 in 0.1 mM Tris, pH 7.4, sonicated with a probe sonicator, and centrifuged for 10 min at $10000 \times g$. The supernatant was diluted 1:5 with the same reagent and the resulting homogenate was used for all assays.

Enzyme activity assays

Activity of the enzyme ChAT, which synthesizes acetylcholine, was measured by the formation of [^{14}C]acetylcholine from [^{14}C]acetyl-coenzymeA (55.7 mCi/mmol; New England Nuclear, Boston, MA, USA) and choline (Fonnum, 1975). Activity of the enzyme GAD, which synthesizes GABA, was measured from L-[^{14}C]glutamic acid (40–60 mCi/mmol; New England Nuclear, Boston, MA, USA) using a [^{14}C]CO $_2$ trapping technique (O'Connor et al., 1988). Detailed descriptions of the assay procedures are provided elsewhere (Berger-Sweeney et al., 1994; Frick and Berger-Sweeney, 2001; Frick et al., 2002). The protein content of the samples was measured using a Bradford protein assay (Bradford, 1976). Enzyme activities were expressed as nmol of product/h/mg protein.

Synaptophysin assay

Synaptophysin was measured using an enzyme-linked immunosorbent assay (ELISA) adapted from Schlaf et al. (1996) for use in mice by N. Stearns and J. Berger-Sweeney (Berger-Sweeney et al., 2001). Because purified synaptophysin was not available for use as a standard, synaptophysin levels in the samples are expressed as 'equivalents' relative to synaptophysin immunoreactivity from whole mouse brain homogenate [termed mouse brain standard (MBS)]. An antibody sandwich ELISA assay using two different anti-synaptophysin antibodies (monoclonal anti-synaptophysin Clone SY 38 and polyclonal rabbit anti-synaptophysin; Dako, Carpinteria, CA, USA) was used to determine the relative amounts of synaptophysin in the samples. Samples were identical to those used for the enzyme assays (except diluted to 1:32000 from the original homogenate) and were similarly assayed in triplicate. Optical density was measured at a wavelength of 405 nm using a Labsystems Multiskan Plus microplate reader. The average absorbance of three wells containing no MBS was subtracted from each reading.

To calculate the relative amount of synaptophysin in the samples, the absorbance of each of four MBS concentrations was plotted versus the log of the total protein concentration. The equation of the straight line that resulted and the absorbance of each sample was used to determine the concentration of MBS which would have the absorbance exhibited by the sample. This apparent MBS concentration of the sample was divided by the total protein concentration of the sample (obtained from the protein assay described in the previous section) to yield the relative amount of synaptophysin in the sample versus the amount of synaptophysin in the MBS homogenate (termed 'MBS synaptophysin equivalent').

Data analysis

Because it was anticipated that treatment effects on memory in mice of this age would be subtle and the power of our anal-

yses too low to detect this subtle change, separate analyses were performed comparing each EB dose to the controls. Water maze measures were averaged within a group for each session and analyzed using a one-way repeated-measures analysis of variance (ANOVA) with Treatment as the independent variable (SuperANOVA, Abacus Concepts; Berkeley, CA, USA). One-way ANOVAs without repeated measures were performed on uterine weights, neurochemical measures, and synaptophysin measures. To compensate for the multiple ANOVAs, an alpha level of 0.025 was used to reject the null hypothesis.

RESULTS

Subjects

At the beginning of the experiment, all mice appeared in good health. Alopecia was evident in most mice. Two animals died of natural causes during the experiment: one 1 μg mouse died during spatial water maze testing and is excluded from all data analyses, and one 5 μg mouse died after the completion of spatial testing and is included in that analysis (but is excluded from analysis of the cued task). One 5 μg mouse was killed during the week of spatial testing due to very poor health and one oil control mouse was eliminated from behavioral testing after the first day of the spatial task due to difficulty swimming. Both of these mice were excluded from all behavioral data analysis, however, the oil control mouse continued to be injected and her uterine weight and brain were included in those analyses. Five mice completed testing, but were eliminated from the behavioral data analysis due to cataracts on one or both eyes: one no-injection control, three oil controls, and one EB 5 μg (all but the no-injection control were included in the neurochemical assays). Uteri of the oil control and EB 5 μg mice were dissected and these weights included in the data analysis. One mouse from each group was perfused for anatomical analysis of their brains (data not shown).

Because there was no discernible difference between the behavioral performance of the no-injection controls and oil-injected controls, these groups were combined into one control group. Thus, the numbers of animals included in the spatial data analysis were as follows: control ($n=8$), EB 1 μg ($n=7$), EB 5 μg ($n=6$). Numbers are identical for the cued analysis except for the EB 5 μg group, where $n=5$. For the enzyme and synaptophysin assays, the three oil control mice with cataracts were included and thus the sample sizes were as follows: control ($n=11$), EB 1 μg ($n=6$), EB 5 μg ($n=5$). For uterine weights, sample sizes were as follows: oil control ($n=6$), EB 1 μg ($n=6$), EB 5 μg ($n=5$).

Uterine weights

Uterine weights differed significantly among the groups ($F(2,14)=12.02$, $P<0.001$). Uterine weights were as follows: oil controls = 0.06 ± 0.007 g, EB 1 μg = 0.23 ± 0.04 g, and EB 5 μg = 0.21 ± 0.03 g. The uterine weights of oil controls were significantly lower than those of both EB groups ($P_s < 0.0001$), whereas the EB groups did not differ from each other.

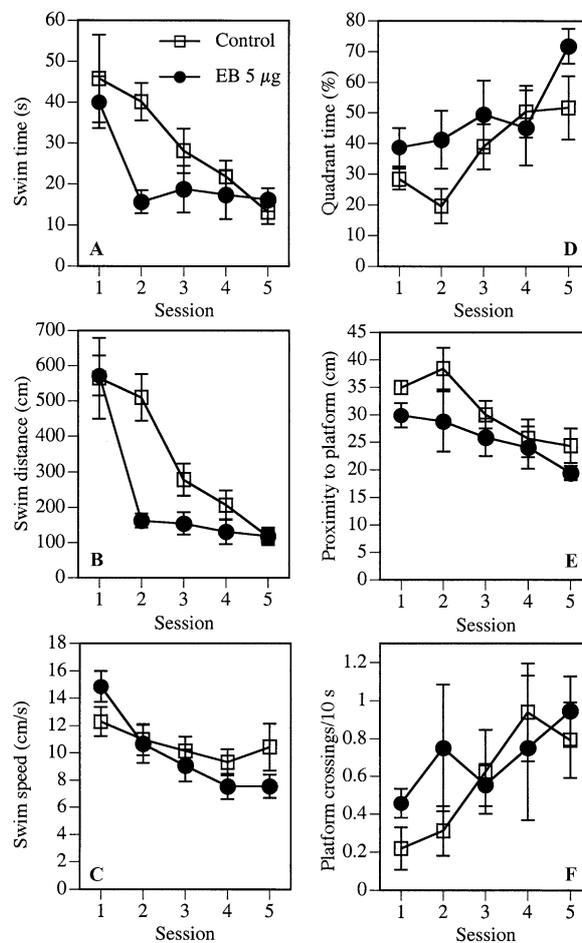


Fig. 1. Five μg of EB significantly improved spatial reference memory. Swim time (A), swim distance (B), and swim speed (C) were recorded during the platform trials of the spatial water maze task (trials 1–5 of each session), and quadrant time (D), proximity (E), and platform crossings (F) were recorded during the daily probe trials (trial 6). Each point represents the mean \pm S.E.M. of each group for trials 1–5 (A–C) or trial 6 (D–F). EB 5 μg females exhibited significantly faster swim times and shorter swim distances than controls by session 2. A significant interaction suggested that EB 5 μg females swam slightly slower than controls. No significant main effects or interactions were observed during the course of training in the probe trials.

Spatial water maze

Control vs. EB 5 μg . The main effect of treatment was not significant in any measure of the spatial task, although the main effect of session was significant for all measures ($F(4,48) = 3.5\text{--}27.5$, $P_s < 0.02$). Significant session \times treatment interactions in several spatial task measures indicated differential spatial memory improvement between the two groups. Specifically, interactions were observed in swim time ($F(4,48) = 3.17$, $P < 0.03$), swim distance ($F(4,48) = 4.47$, $P < 0.01$), and swim speed ($F(4,48) = 5.05$, $P < 0.01$). As illustrated in Fig. 1A, B, the swim times and swim distances of EB 5 μg mice improved significantly more rapidly than those of controls, such that EB 5 μg mice reached asymptotic levels of performance by session 2. Although the interaction in swim speed indicates that EB 5 μg mice swam more slowly than controls (Fig. 1C), t -tests performed for

each session revealed no difference between the groups ($P_s > 0.1$). Given that slower swim speeds would actually mitigate an improvement in swim time in the EB 5 μg group, the swim time effect reflects enhanced spatial learning rather than a motoric effect.

Interactions were not significant in the probe trial measures (quadrant time, proximity, and platform crossings) over the course of testing, suggesting no effect of EB on these measures (Fig. 1D–F). However, because visible inspection of the quadrant time data suggested a difference between the groups, we analyzed more closely the time spent in each quadrant during each session. This type of analysis allowed us to determine whether the groups differed in the development of a spatial bias for the quadrant containing the platform. The percent time spent in each of the four quadrants was calculated and compared within each group using one-way ANOVAs with ‘Quadrant’ as the between-subjects variable. Differences between percent time spent in various quadrants were assessed using Fisher’s Protected LSD post-hocs. A spatial bias was demonstrated if a group spent signifi-

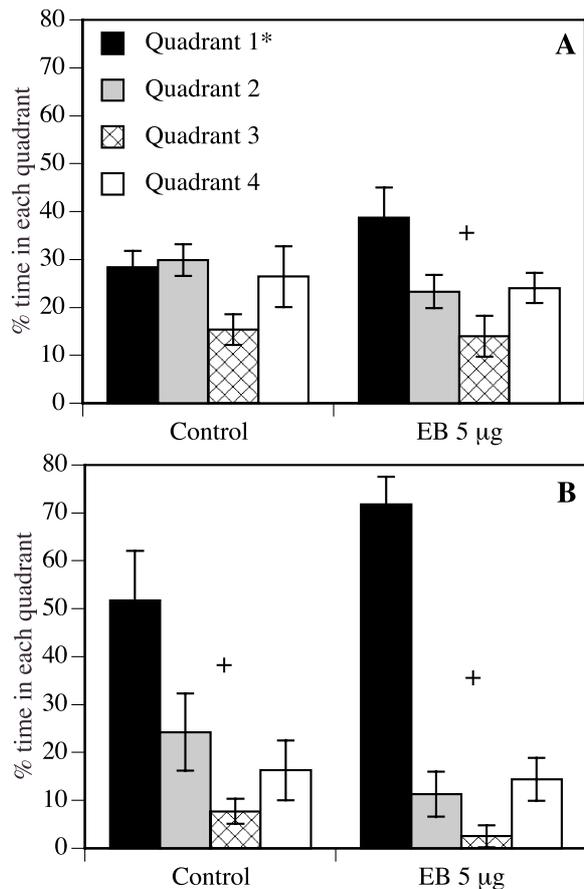


Fig. 2. EB 5 μg females displayed a more robust spatial bias during the probe trials for the quadrant containing the platform than controls. During session 1 (A), only EB 5 μg females exhibited a spatial bias. By session 5 (B), both groups had developed spatial biases, although that of the EB 5 μg females was of a greater magnitude. Each bar represents the mean (\pm S.E.M.) time spent by a group in an individual quadrant. Quadrant 1* contained the platform (inset at right = maze with four quadrants). ‘+’ indicates a significant main effect of Quadrant in the one-way ANOVA.

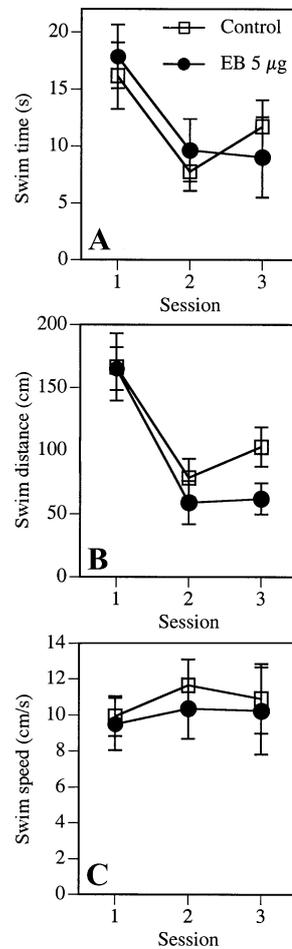


Fig. 3. EB 5 μg did not affect non-spatial memory, as assessed by the cued water maze task (A–C). Each point represents the mean \pm S.E.M. of each group for trials 1–6. No treatment-related differences were found.

cantly more time in the correct quadrant compared to the other quadrants. Data from sessions 1 and 5 are presented, as the most interesting differences were observed in these sessions. As illustrated in Fig. 2A, EB 5 μg females, but not control females, exhibited a modest but significant spatial bias during session 1 for the quadrant containing the platform ($F(3,20) = 5.3$, $P < 0.01$). By the end of the first day of testing, EB 5 μg females spent 39% of their time in the correct quadrant, which was significantly higher than the percent time spent in quadrants 2–4 ($P_s < 0.05$). This bias for quadrant 1 indicates a focused search for the platform, even after only five previous platform trials. In contrast, controls searched all four quadrants about equally, suggesting a more random search for the platform. By session 5, both controls and EB 5 μg females exhibited a spatial bias (Fig. 2B), as indicated by the one-way ANOVAs ($F(3,28) = 6.7$, $P = 0.0015$ for controls; $F(3,20) = 50.1$, $P < 0.0001$ for EB 5 μg) and post-hoc comparisons between quadrant 1 and quadrants 2–4 ($P_s < 0.01$). However, the magnitude of the bias in the EB 5 μg group was more robust than that of the control group, as illustrated by the large difference in F values and the

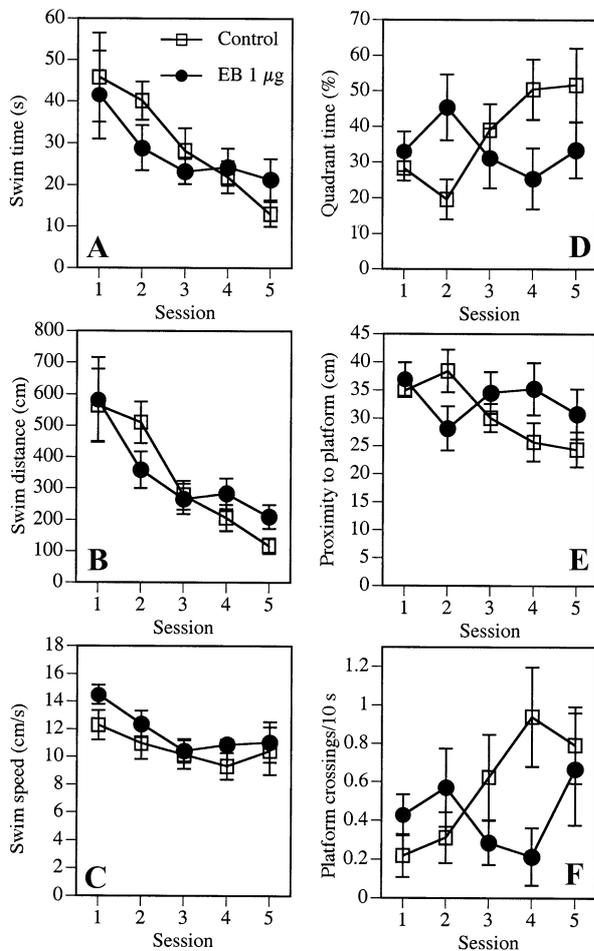


Fig. 4. EB 1 μg did not affect the platform trial measures in the spatial water maze task (A–C), but impaired performance in the probe trial (D–F). Each point represents the mean \pm S.E.M. of each group for trials 1–5 (A–C) or trial 6 (D–F).

20% more time spent in the correct quadrant by EB 5 μg females relative to controls.

The main effect of treatment was not significant for any measure from the cued task. Significant session effects in swim time ($F(2,22)=17.3$, $P < 0.0001$) and swim distance ($F(2,22)=21.6$, $P < 0.0001$) in the absence of significant session \times treatment interactions in these measures (Fig. 3A, B) indicate that both groups learned the cued task similarly. Neither the main effects nor the interactions were significant for the swim speed measure (Fig. 3C), suggesting similar motor abilities in the two groups.

Control vs. EB 1 μg . The main effect of treatment was not significant for any measure from the spatial task. Significant session effects in swim time, swim distance, and swim speed ($F(4,52)=8.7$, 14.0, and 7.9, respectively, $P_s < 0.0001$) in the absence of significant session \times treatment interactions in these measures (Fig. 4A–C), indicate that both groups learned the spatial task similarly. However, significant session \times treatment interactions in quadrant time, proximity, and platform crossings ($F(4,52)=4.5$, 5.4, and 3.3, respectively, $P_s < 0.018$) in the absence of significant main effects of

both treatment and session, suggest that one of the groups did not improve during testing. As illustrated in Fig. 4D–F, the performance of control females improved during training, whereas that of EB 1 μg females did not. These data suggest that 1 μg of EB may actually impair performance in the spatial task. To examine further performance during the probe trials, the percent time spent in each of the four quadrants was calculated and one-way ANOVAs with post-hocs were performed as described for EB 5 μg females. Similar to controls, EB 1 μg mice did not exhibit a spatial bias during session 1 (Fig. 5A). However, control females did display a spatial bias by session 5 (see previous section). In contrast, a spatial bias did not develop in EB 1 μg females throughout testing (Fig. 5B); they spent the same amount of time in the correct quadrant during the first and last sessions (33%).

Treatment was not significant for any measure from the cued task. Significant session effects in swim time, swim distance, and swim speed ($F(2,26)=12.7$, 11.7, and 4.9, respectively, $P_s < 0.02$) in the absence of significant session \times treatment interactions in these measures (Fig. 6A, B), indicate that both groups performed the cued task similarly.

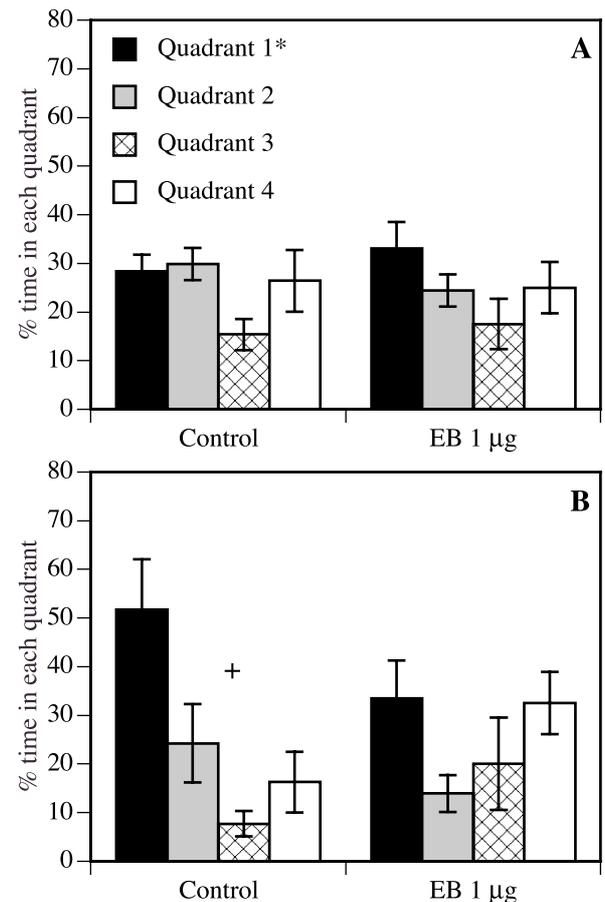


Fig. 5. EB 1 μg females did not exhibit a spatial bias in the probe trials of either session 1 (A) or session 5 (B). Each bar represents the mean (\pm S.E.M.) time spent by a group in an individual quadrant. Quadrant 1* contained the platform (inset at right = maze with four quadrants). '+' indicates a significant effect of Quadrant in the one-way ANOVA.

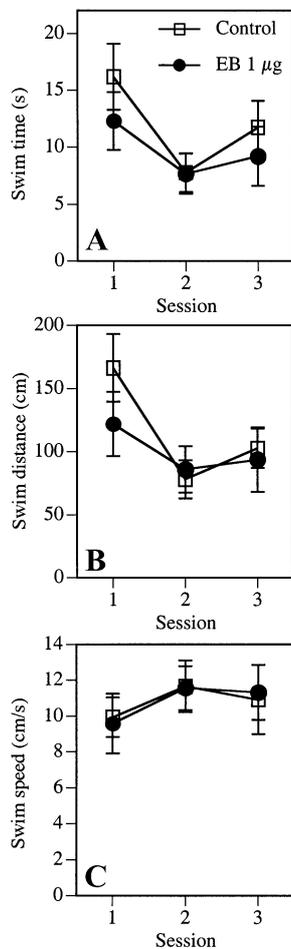


Fig. 6. EB 1 µg did not affect non-spatial memory in the cued task (A–C). Each point represents the mean ± S.E.M. of each group for trials 1–6.

Enzyme activities. Neither dose of estrogen affected ChAT or GAD activities in either brain region (all $F_s < 1.0$ and $P_s > 0.05$). See Table 1 for mean values.

Synaptophysin. Females receiving 5 µg of EB exhibited more synaptophysin immunoreactivity than controls in the hippocampus ($F(1,14) = 5.3$, $P = 0.037$), but not neocortex ($F(1,14) = 0.05$, $P > 0.05$). In contrast, EB 1 µg females displayed more synaptophysin immunoreactivity than controls in the neocortex ($F(1,15) = 4.54$,

$P = 0.05$), but not in hippocampus ($F(1,15) = 0.06$, $P > 0.05$). See Table 1 for mean values.

DISCUSSION

The present study is the first to demonstrate that estrogen can improve spatial reference memory in aged female mice. Mice receiving daily injections of 5 µg of EB, but not those receiving 1 µg of EB, displayed accelerated acquisition of the task compared to controls. By the second session of training, EB 5 µg females reached asymptotic levels of swim time and swim distance in the platform trials. In addition, EB 5 µg females displayed a spatial bias in the probe trial as early as session 1 and developed a more robust spatial bias than controls by the last session. In contrast, controls and EB 1 µg females learned the platform location gradually during training. Unlike the other groups, EB 1 µg females did not show evidence of improvement in the spatial probe trial, suggesting that this dose may impair spatial memory. No effect of estradiol was observed in the non-spatial version of the task, indicating that the effects observed in the estradiol-treated females were specific to spatial memory and not due to non-mnemonic aspects of performance such as increased motivation, swimming ability, or visual acuity. Interestingly, ChAT and GAD activities in the hippocampus and neocortex were not altered by estradiol, suggesting that the mechanism of estradiol-induced spatial memory enhancement in aged females does not involve regulation of acetylcholine and GABA synthesis. In contrast, synaptophysin immunoreactivity was altered by estradiol such that increased hippocampal synaptophysin was associated with improved spatial memory, whereas increased neocortical synaptophysin was associated with mild spatial memory impairment. To our knowledge, the present study is the first to examine the effects of estrogen on synaptophysin in behaviorally characterized rodents.

The significant interactions observed in the spatial platform trial measures and the early development of a spatial bias strongly suggest that 5 µg of EB accelerated acquisition of the spatial water maze task. This finding of improved spatial reference memory in aged female mice is consistent with previous reports in aged rats tested in spatial working memory tasks. In one such report, estradiol improved the radial arm maze performance of 25-

Table 1. Neurobiological data for each treatment group

Assay	Brain region	Control	1 µg	5 µg
ChAT ^a	neocortex	51.9 ± 2.7	55.3 ± 4.9	51.1 ± 3.1
	hippocampus	58.3 ± 2.6	55.2 ± 2.8	55.8 ± 4.4
GAD ^a	neocortex	114.3 ± 5.8	124.4 ± 9.2	120.9 ± 11.9
	hippocampus	90.0 ± 4.2	97.8 ± 5.6	93.4 ± 8.6
Synaptophysin ^b	neocortex	1.01 ± 0.06	1.2 ± 0.04*	1.04 ± 0.07
	hippocampus	0.93 ± 0.06	0.95 ± 0.06	1.18 ± 0.09*

* $P \leq 0.05$ relative to controls.

^aValues represent the mean nmol product/h/mg protein ± S.E.M.

^bValues represent mean ± S.E.M. 'MBS synaptophysin equivalents' expressed as sample immunoreactivity relative to that of an equal amount of MBS.

month-old male rats when a 2- or 3-h delay was interspersed between the 4th and 5th arm choices (Luine and Rodriguez, 1994). However, these rats had already undergone extensive training on the radial arm maze prior to estradiol treatment, and thus the effect of estrogen on acquisition of the task could not be observed. In 25-month-old female rats ovariectomized at 12 months of age, estradiol did improve the acquisition of a discrete-trial delayed matching-to-position task in the T-maze (Gibbs, 2000b). In this study, estradiol administered over the course of 9–12 months via silastic capsules or weekly injections of 10 μg estradiol plus progesterone reduced both the days to reach criterion and number of errors. Our data are also consistent with previous studies in aged C57BL/6 mice in which estradiol improved acquisition of two different non-spatial working memory tasks (Miller et al., 1999; Vaucher et al., 2002). Thus, the present study adds to our understanding of estrogen as a mnemonic enhancer in aging by demonstrating that estrogen can improve more than one type of spatial memory in aged rodents.

Interestingly, the spatial probe trial measures suggest that the 1 μg dose of EB impaired memory. None of the previous studies of estrogen replacement in aged rats or mice reported memory impairments (Luine and Rodriguez, 1994; Miller et al., 1999; Gibbs, 2000b; Vaucher et al., 2002), however, none tested more than one dose of estrogen. In young rats, various doses of estradiol reportedly improve spatial working memory (O'Neal et al., 1996; Daniel et al., 1997; Bimonte and Deneberg, 1999; Fader et al., 1999). However, in spatial reference memory tasks, different estrogen doses and administration methods given to rats have led to findings of both estrogen-induced memory improvement and impairment (e.g. Packard and Teather, 1997; Chesler and Juraska, 2000). Similar discrepant findings have also been reported in young female mice; performance in the spatial water maze has been found to be impaired by daily subcutaneous injections of 20 μg of estradiol dissolved in sesame oil (Fugger et al., 1998) and improved by minipellets releasing 0.18 mg of estradiol (Rissanen et al., 1999). Because ovariectomy has minimal effects on spatial reference memory in mice (Frick and Berger-Sweeney, 2001), all but an ideal dose of estradiol is likely to disrupt the perfectly functional system of young mice. Estrogen treatment in older subjects will presumably have a wider therapeutic window given the widespread hippocampal and neocortical dysfunction evident in the aged brain (e.g. Geinisman et al., 1986; Walker et al., 1988; Peters et al., 1994; Barnes, 1998; Frick et al., 2002). Nevertheless, it may be possible, even in aged subjects, that different doses of estrogen are optimal for different kinds of memory. Thus, evaluating multiple doses in a wide variety of tasks will be critical to determining the utility of estrogen as a treatment for age-related memory decline.

It is also important to note that the doses of estrogen administered in this study were likely supra-physiological doses, based on the large size of the uteri. Due to the difficulty in collecting sufficient serum from mice of this age, we could not accurately measure serum estradiol in

our mice. We routinely use uterine weight as a bioassay for the physiological effects of our treatment. Given that the 1 μg dose did not improve memory, but produced equivalent effects on uterine weight, it is possible that lower doses in the physiological range would not have enhanced memory. Rather, the data suggest that high pharmacological doses are necessary to improve spatial reference memory in aged females.

Neither dose of estradiol affected ChAT or GAD activities in hippocampus or neocortex. This finding is surprising given that estrogen modulates hippocampal and/or cortical ChAT activity in young intact mice (Frick and Berger-Sweeney, 2001) and ovariectomized rats (Luine, 1985; Lapchak et al., 1990; Singh et al., 1994; Wu et al., 1999), decreases GAD expression in cultured hippocampal neurons (Murphy et al., 1998a,b), and that ChAT and GAD activities in both brain regions are significantly higher in estropausal middle-aged female mice relative to middle-aged males (Frick et al., 2002). However, with the exception of hippocampal ChAT activity, we find that sex differences in 25-month-old mice are absent (Frick et al., 2002), suggesting that these enzymes may no longer be sensitive to estrogen at advanced ages. Although this explanation seems somewhat unlikely given that other aspects of neuronal functioning, such as long-term depression (Vouimba et al., 2000), dentate granule dendritic spine density (Miranda et al., 1999), and synaptophysin expression (current study) remain responsive to estrogen in aged rats and mice, we cannot presently exclude this possibility. Interestingly, alterations in muscarinic receptor binding in neocortex, hippocampus, and basal forebrain are unrelated to estrogen-induced improvements of non-spatial working memory in aged female mice (Vaucher et al., 2002). This finding supports the notion that estrogenic modulation of cholinergic function in these regions may not play a role in memory improvement. However, other aspects of cholinergic and GABAergic functioning (e.g. transmitter release, reuptake, enzyme activities in basal forebrain cell bodies) will need to be assessed to definitively establish that estrogenic modulation of these transmitter systems is not involved in the observed mnemonic enhancement.

In contrast to ChAT and GAD activities, synaptophysin levels were increased by both doses of estradiol. Interestingly, each dose was effective in different brain regions. Relative to controls, the 5 μg dose increased synaptophysin levels in the hippocampus only, whereas the 1 μg dose elevated synaptophysin in the neocortex only. Very few studies have examined estrogen modulation of synaptic proteins such as synaptophysin, and these have focused on the hippocampus exclusively. Our data are somewhat inconsistent with one previous study in which an approximate dose of 2 μg of estradiol failed to alter synaptophysin protein the whole hippocampus of adult ovariectomized rats (Brake et al., 2001). However, the fact that, in the present study, the 1 μg dose had a similar null effect on synaptophysin suggests that dose may play a key role in this effect. In studies examining hippocampal subregions, estradiol elevated synaptophysin immunoreactivity in CA1 (Brake et

al., 2001) and ameliorated a lesion-induced reduction of synaptophysin immunoreactivity in the dentate gyrus (Stone et al., 1998), which is consistent with the present effect of 5 μ g of EB on hippocampal synaptophysin. Similarly, estradiol increased synaptophysin immunoreactivity in hippocampal neurons cultured from neonatal rats (Murphy and Segal, 1996; Pozzo-Miller et al., 1999). However, in none of these studies were the animals behaviorally tested during estrogen administration, and thus, no relationship between estrogen-induced alterations in synaptophysin and memory has been previously established. The present data indicate that increased synaptophysin in the hippocampus is associated with improved spatial reference memory, whereas increased synaptophysin in neocortex is related to unaltered or impaired spatial reference memory. It is important to note that elevated synaptophysin as measured by ELISA may reflect three potential changes in synaptic plasticity: (1) increased number of presynaptic boutons, (2) increased numbers of vesicles in presynaptic boutons, or (3) increased amount of synaptophysin in presynaptic vesicle membranes. Increases in synaptophysin immunoreactivity have most often been interpreted as an increase in presynaptic terminals (e.g. Murphy and Segal, 1996; Stone et al., 1998; Pozzo-Miller et al., 1999), suggesting that estrogen in the present study enhances synaptogenesis. Although this conclusion cannot be established definitively with an ELISA, the present data demonstrate that estrogen can modulate synaptic plasticity in aged females and suggest a relationship between this regulation and spatial memory.

Estradiol-induced regulation of spatial memory and synaptophysin is likely mediated by two nuclear receptors (Warner et al., 1999), estrogen receptors alpha (ER α) and beta (ER β), although a membrane-bound estrogen receptor has also been hypothesized based on reported rapid, non-genomic effects of estrogen (McEwen and Alves, 1999; Toran-Allerand, 2000). ER α and ER β are both expressed throughout the neocortex and hippocampus (Shughrue et al., 1997a; Shughrue et al., 1997b; Shughrue and Merchenthaler, 2000), and thus either receptor could contribute to the mnemonic alterations observed in the present study. Given that estradiol binds readily to both receptors, it is impossible to determine which estrogen receptor may have mediated the spatial reference memory improvements described in this study. It will undoubtedly be of interest to pursue this question in future work.

The present findings may have significant implications for estrogen replacement therapy in menopausal women. Some previous work has suggested that estrogen replacement can improve verbal (Phillips and Sherwin, 1992; Maki and Resnick, 2000; Verghese et al., 2000; Maki

et al., 2001), spatial (DUFF AND HAMPSON, 2000), AND FIGURAL MEMORY (Resnick et al., 1998; Maki and Resnick, 2000), increase cerebral blood flow in the hippocampus and related temporal lobe structures (Maki and Resnick, 2000), and reduce the risk of developing Alzheimer's disease (Henderson et al., 1994; Tang et al., 1996; Kawas et al., 1997; Yaffe et al., 1998). However, other studies do not reveal a cognitive benefit of estrogen replacement (e.g. Barrett-Connor and Kritz-Silverstein, 1993; Polo-Kantola et al., 1998; Hogervorst et al., 1999) and a recent study of estrogen replacement in women with mild to moderate Alzheimer's disease reported no beneficial effect of estrogen on indices of global cognitive functioning or memory (Mulnard et al., 2000). One recent meta-analysis suggested that estrogen is most beneficial to women who experience menopausal symptoms at the time of assessment, rather than women who are post-menopausal (Yaffe et al., 1998). Although this conclusion is appropriate given the data, it must be noted that the studies reviewed differed in many ways, including the types and duration of estrogen replacement given, patient selection criteria, and the neuropsychological test batteries administered. Our results suggest that an appropriate dose of estrogen can effectively improve memory, even in aged subjects who are well past the point of initial hormone loss. By administering estrogen to rodents in a variety of mnemonic and cognitive tasks, we might gain a better understanding of the conditions under which estrogen can be an effective treatment for the prevention or reduction of cognitive decline in elderly women.

In summary, our finding that 5 μ g of estradiol can improve spatial reference memory and increase hippocampal synaptophysin immunoreactivity in aged female mice provides important groundwork for future study of estrogen replacement in aged rodents. Further research is needed to ascertain, among other things, detailed dose-response curves and the types of memory that can be improved. The answers provided by this research in rodents will hopefully serve to increase the utility of hormone replacement therapy for cognitive impairments in menopausal women.

Acknowledgements—The authors would like to thank Dr. Greg Rose of Memory Pharmaceuticals (Montvale, NJ, USA) for the generous donation of the mice used in this study, Tovia Martirosian, Anthony Taglienti, Julie Rosenthal, and Sue Vanderhill for assistance with behavioral testing, Nancy Stearns and Dr. Joanne Berger-Sweeney for the development of the synaptophysin ELISA protocol, and Dr. Mark Baxter for critical comments on the manuscript. Funding was provided by Yale University and an American Federation for Aging Research/Pfizer Research Grant in Hormones and Aging to KMF.

REFERENCES

- Barnes, C.A., 1998. Memory changes during normal aging: Neurobiological correlates. In: Martinez, J.L.J., Kesner, R.P. (Eds.), *Neurobiology of Learning and Memory*. Academic Press, New York, pp. 247–287.
- Barrett-Connor, E., Kritz-Silverstein, D., 1993. Estrogen replacement therapy and cognitive function in older women. *JAMA* 269, 2637–2641.
- Bartus, R.T., Dean, R.L., Pontecorvo, M.J., Flicker, C., 1985. The cholinergic hypothesis: A historical overview, current perspective, and future directions. *Ann. NY Acad. Sci.* 444, 332–358.

- Baxter, M.G., Chiba, A.A., 1999. Cognitive functions of the basal forebrain. *Curr. Opin. Neurobiol.* 9, 178–183.
- Berger-Sweeney, J., Berger, U.V., Sharma, M., Paul, C.A., 1994. Effects of carbon dioxide-induced anesthesia on cholinergic parameters in rat brain. *Lab. Anim. Sci.* 44, 369–371.
- Berger-Sweeney, J., Stearns, N.A., Frick, K.M., Beard, B., Baxter, M.G., 2000. Cholinergic basal forebrain is critical for social transmission of food preferences. *Hippocampus* 10, 729–738.
- Berger-Sweeney, J.E., Stearns, N.A., Pan, J.Y., Frick, K.M., 2001. Effects of environmental enrichment on GAD activity and synaptophysin in mice. *Soc. Neurosci. Abstr.* 27, Program 86.14.
- Bimonte, H.A., Denenberg, V.H., 1999. Estradiol facilitates performance as working memory load increases. *Psychoneuroendocrinology* 24, 161–173.
- Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248–254.
- Brake, W.G., Alves, S.E., Dunlop, J.C., Lee, S.J., Bulloch, K., Allen, P.B., Greengard, P., McEwen, B.S., 2001. Novel target sites for estrogen action in the dorsal hippocampus: An examination of synaptic proteins. *Endocrinology* 142, 1284–1289.
- Brinton, R.D., 2001. Cellular and molecular mechanisms of estrogen regulation of memory function and neuroprotection against Alzheimer's disease: Recent insights and remaining challenges. *Learn. Mem.* 8, 121–133.
- Calhoun, M.E., Kurth, D., Phinney, A.L., Long, J.M., Hengemihle, J., Mouton, P.R., Ingram, D.K., Jucker, M., 1998. Hippocampal neuron and synaptophysin-positive bouton number in aging C57BL/6 mice. *Neurobiol. Aging* 19, 599–606.
- Chen, K.S., Masliah, E., Mallory, M., Gage, F.H., 1995. Synaptic loss in cognitively impaired aged rats is ameliorated by chronic human nerve growth factor infusion. *Neuroscience* 68, 19–27.
- Chesler, E.J., Juraska, J.M., 2000. Acute administration of estrogen and progesterone impairs the acquisition of the spatial Morris water maze in ovariectomized rats. *Horm. Behav.* 38, 234–242.
- Daniel, J.M., Fader, A.J., Spencer, A.L., Dohanich, G.P., 1997. Estrogen enhances performance of female rats during acquisition of a radial arm maze. *Horm. Behav.* 32, 217–225.
- Duff, S.J., Hampson, E., 2000. A beneficial effect of estrogen on working memory in postmenopausal women taking hormone replacement therapy. *Horm. Behav.* 38, 262–276.
- Eastwood, S.L., Burnet, P.W.J., McDonald, B., Clinton, J., Harrison, P.J., 1994. Synaptophysin gene expression in human brain: a quantitative in situ hybridization and immunocytochemical study. *Neuroscience* 59, 881–892.
- Evans, G.W., Brennan, P.L., Skorpanich, M.A., Held, D., 1984. Cognitive mapping and elderly adults: Verbal and location memory for urban landmarks. *J. Gerontol.* 39, 452–457.
- Fader, A.J., Johnson, P.E.M., Dohanich, G.P., 1999. Estrogen improves working but not reference memory and prevents amnesic effects of scopolamine on a radial-arm maze. *Pharmacol. Biochem. Behav.* 62, 711–717.
- Farr, S.A., Flood, J.F., Scherrer, J.F., Kaiser, F.E., Taylor, G.T., Morley, J.E., 1995. Effect of ovarian steroids on footshock avoidance learning and retention in female mice. *Physiol. Behav.* 58, 715–723.
- Fischer, W., Chen, K.S., Gage, F.H., Björklund, A., 1992. Progressive decline in spatial learning and integrity of forebrain cholinergic neurons in rats during aging. *Neurobiol. Aging* 13, 9–23.
- Fonnum, F., 1975. A rapid radiochemical method for the determination of choline acetyltransferase. *J. Neurochem.* 24, 407–409.
- Fordyce, D.E., Wehner, J.M., 1993. Effects of aging on spatial learning and hippocampal protein kinase C in mice. *Neurobiol. Aging* 14, 309–317.
- Foy, M.R., Xu, J., Xie, X., Brinton, R.D., Thompson, R.F., Berger, T.W., 1999. 17 β -estradiol enhances NMDA receptor-mediated EPSPs and long-term potentiation. *J. Neurophysiol.* 81, 925–929.
- Frick, K.M., Baxter, M.G., Markowska, A.L., Olton, D.S., Price, D.L., 1995. Age-related spatial reference and working memory deficits assessed in the water maze. *Neurobiol. Aging* 16, 149–160.
- Frick, K.M., Berger-Sweeney, J., 2001. Spatial reference memory and neocortical neurochemistry vary with the estrous cycle in C57BL/6 mice. *Behav. Neurosci.* 115, 229–237.
- Frick, K.M., Burlingame, L.A., Arters, J.A., Berger-Sweeney, J., 2000. Reference memory, anxiety, and estrous cyclicity in C57BL/6NIA mice are affected by age and sex. *Neuroscience* 95, 293–307.
- Frick, K.M., Burlingame, L.A., Delaney, S.S., Berger-Sweeney, J., 2002. Sex differences in neurochemical markers that correlate with behavior in aging mice. *Neurobiol. Aging* 23, 145–158.
- Fugger, H.N., Cunningham, S.G., Rissman, E.F., Foster, T.C., 1998. Sex differences in the activational effect of ER α on spatial learning. *Horm. Behav.* 34, 163–170.
- Gage, F.H., Dunnette, S.B., Björklund, A., 1984. Spatial learning and motor deficits in aged rats. *Neurobiol. Aging* 5, 43–48.
- Gallagher, M., Pellemounter, M.A., 1988. Spatial learning deficits in old rats: a model for memory decline in the aged. *Neurobiol. Aging* 9, 549–556.
- Geinisman, Y., De Toledo-Morrell, L., Morrell, F., 1986. Loss of perforated synapses in the dentate gyrus: Morphological substrate of memory deficit in aged rats. *Proc. Natl. Acad. Sci. USA* 83, 3027–3031.
- Gibbs, R.B., 1999. Estrogen replacement enhances acquisition of a spatial memory task and reduces deficits associated with hippocampal muscarinic receptor inhibition. *Horm. Behav.* 36, 222–233.
- Gibbs, R.B., 2000a. Effects of gonadal hormone replacement on measures of basal forebrain cholinergic function. *Neuroscience* 101, 931–938.
- Gibbs, R.B., 2000b. Long-term treatment with estrogen and progesterone enhances acquisition of a spatial memory task by ovariectomized aged rats. *Neurobiol. Aging* 21, 107–116.
- Gibbs, R.B., Aggarwal, P., 1998a. Estrogen and basal forebrain cholinergic neurons: implications for brain aging and Alzheimer's disease-related cognitive decline. *Horm. Behav.* 34, 98–111.
- Gibbs, R.B., Burke, A.M., Johnson, D.A., 1998b. Estrogen replacement attenuates effects of scopolamine and lorazepam on memory acquisition and retention. *Horm. Behav.* 34, 112–125.
- Henderson, V.W., Paganini-Hill, A., Emanuel, C.K., Dunn, M.E., Buckwalter, J.G., 1994. Estrogen replacement therapy in older women: Comparisons between Alzheimer's disease cases and nondemented control subjects. *Arch. Neurol.* 51, 896–900.
- Hogervorst, E., Boshuisen, M., Riedel, W.J., Willekes, C., Jolles, J., 1999. The effect of hormone replacement therapy on cognitive function in elderly women. *Psychoneuroendocrinology* 24, 43–68.
- Hogervorst, E., Williams, J., Budge, M., Riedel, W., Jolles, J., 2000. The nature of the effect of female gonadal hormone replacement therapy on cognitive function in post-menopausal women: A meta-analysis. *Neuroscience* 101, 485–512.
- Jahn, R., Schiebler, W., Ouimet, C., Greengard, P., 1985. A 38,000-dalton membrane protein (p38) present in synaptic vesicles. *Proc. Natl. Acad. Sci. USA* 82, 4137–4141.
- Kawas, C., Resnick, S., Morrison, A., Brookmeyer, R., Corrada, M., Zonderman, A., Bacal, C., Lingle, D.L., Metter, E., 1997. A prospective study of estrogen replacement therapy and the risk of developing Alzheimer's disease: The Baltimore Longitudinal Study of Aging. *Neurology* 48, 1517–1521.

- Lamberty, Y., Gower, A.J., 1990. Age-related changes in spontaneous behavior and learning in NMRI mice from maturity to middle age. *Physiol. Behav.* 47, 1137–1144.
- Lamberty, Y., Gower, A.J., 1991. Age-related changes in spontaneous behavior and learning in NMRI mice from middle to old age. *Physiol. Behav.* 51, 81–88.
- Lapchak, P.A., Araujo, D.M., Quirion, R., Beaudet, A., 1990. Chronic estradiol treatment alters central cholinergic function in the female rat: Effect on choline acetyltransferase activity, acetylcholine content, and nicotinic autoreceptor function. *Brain Res.* 525, 249–255.
- Liu, X., Erikson, C., Brun, A., 1996. Cortical synaptic changes and gliosis in normal aging, Alzheimer's disease and frontal lobe degeneration. *Dementia* 7, 128–134.
- Luine, V., Rodriguez, M., 1994. Effects of estradiol on radial arm maze performance of young and aged rats. *Behav. Neural Biol.* 62, 230–236.
- Luine, V.N., 1985. Estradiol increases choline acetyltransferase activity in specific basal forebrain nuclei and projection areas of female rats. *Exp. Neurol.* 89, 484–490.
- Maki, P.M., Resnick, S.M., 2000. Longitudinal effects of estrogen replacement therapy on PET cerebral blood flow and cognition. *Neurobiol. Aging* 21, 373–383.
- Maki, P.M., Zonderman, A.B., Resnick, S.M., 2001. Enhanced verbal memory in nondemented elderly women receiving hormone-replacement therapy. *Am. J. Psychiatry* 158, 227–233.
- Markowska, A.L., 1999. Sex dimorphisms in the rate of age-related decline in spatial memory: Relevance to alterations in the estrous cycle. *J. Neurosci.* 19, 8122–8133.
- Markowska, A.L., Long, J.M., Johnson, C.T., Olton, D.S., 1993. Variable-interval probe test as a tool for repeated measurements of spatial memory in the water maze. *Behav. Neurosci.* 107, 627–632.
- Masliah, E., Mallory, M., Hansen, L., DeTeresa, R., Terry, R.D., 1993. Quantitative synaptic alterations in the human neocortex during normal aging. *Neurology* 43, 192–197.
- McEwen, B.S., Alves, S.E., 1999. Estrogen actions in the central nervous system. *Endocr. Rev.* 20, 279–307.
- Miller, M.M., Hyder, S.M., Assayag, R., Panarella, S.R., Tousignant, P., Franklin, K.B.J., 1999. Estrogen modulates spontaneous alternation and the cholinergic phenotype in the basal forebrain. *Neuroscience* 91, 1143–1153.
- Miranda, P., Williams, C.L., Einstein, G., 1999. Granule cells in aging rats are sexually dimorphic in their response to estradiol. *J. Neurosci.* 19, 3316–3325.
- Moffat, S.D., Zonderman, A.B., Resnick, S.M., 2001. Age differences in spatial memory in a virtual environment navigation task. *Neurobiol. Aging* 22, 787–796.
- Mulnard, R.A., Cotman, C.W., Kawas, C., van Dyck, C.H., Sano, M., Doody, R., Koss, E., Pfeiffer, E., Jin, S., Gamst, A., Grundman, M., Thomas, R., Thal, L.J., 2000. Estrogen replacement therapy for treatment of mild to moderate Alzheimer disease: A randomized controlled trial. *JAMA* 283, 1007–1015.
- Murphy, D.D., Cole, N.B., Greenberger, V., Segal, M., 1998a. Estradiol increases dendritic spine density by reducing GABA neurotransmission in hippocampal neurons. *J. Neurosci.* 18, 2550–2559.
- Murphy, D.D., Cole, N.B., Segal, M., 1998b. Brain-derived neurotrophic factor mediates estradiol-induced dendritic spine formation in hippocampal neurons. *Proc. Natl. Acad. Sci. USA* 95, 11412–11417.
- Murphy, D.D., Segal, M., 1996. Regulation of dendritic spine density in cultured rat hippocampal neurons by steroid hormones. *J. Neurosci.* 16, 4059–4068.
- Nelson, J.F., Felicio, L.S., Osterburg, H.H., Finch, C.E., 1992. Differential contributions of ovarian and extraovarian factors to age-related reduction in plasma estradiol and progesterone during the estrous cycle of C57BL/6J mice. *Endocrinology* 130, 805–810.
- Nelson, J.F., Felicio, L.S., Randall, P.K., Sims, C., Finch, C.E., 1982. A longitudinal study of estrous cyclicity in aging C57BL/6J mice: I. Cycle frequency, length and vaginal cytology. *Biol. Reprod.* 27, 327–339.
- Nelson, J.F., Karelus, K., Bergman, M.D., Felicio, L.S., 1995. Neuroendocrine involvement in aging: Evidence from studies of reproductive aging and caloric restriction. *Neurobiol. Aging* 16, 837–843.
- Nicolle, M.M., Gallagher, M., McKinney, M., 1999. No loss of synaptic proteins in the hippocampus of aged, behaviorally impaired rats. *Neurobiol. Aging* 20, 343–348.
- O'Connor, L.H., Nock, B., McEwen, B.S., 1988. Regional specificity of gamma-aminobutyric acid receptor regulation by estradiol. *Neuroendocrinology* 47, 473–481.
- O'Neal, M.F., Means, L.W., Poole, M.C., Hamm, R.J., 1996. Estrogen affects performance of ovariectomized rats in a two-choice water-escape working memory task. *Psychoneuroendocrinology* 21, 51–65.
- Packard, M.G., Teather, L.A., 1997. Posttraining estradiol injections enhance memory in ovariectomized rats: cholinergic blockade and synergism. *Neurobiol. Learn. Mem.* 68, 172–188.
- Peters, A., Leahu, D., Moss, M.B., McNally, K.J., 1994. The effects of aging on area 46 of the frontal cortex of the rhesus monkey. *Cereb. Cortex* 6, 621–635.
- Phillips, S.M., Sherwin, B.B., 1992. Effects of estrogen on memory function in surgically menopausal women. *Psychoneuroendocrinology* 17, 485–495.
- Polo-Kantola, P., Portin, R., Polo, O., Helenius, H., Irjala, K., Erkkola, R., 1998. The effect of short-term estrogen replacement therapy on cognition: A randomized double-blind, cross-over trial in postmenopausal women. *Obstet. Gynecol.* 91, 459–466.
- Pozzo-Miller, L.D., Inoue, T., Murphy, D.D., 1999. Estradiol increases spine density and NMDA-dependent Ca²⁺ transients in spines of CA1 pyramidal neurons from hippocampal slices. *J. Neurophysiol.* 81, 1404–1411.
- Resnick, S.M., Maki, P.M., Golski, S., Kraut, M.A., Zonderman, A.B., 1998. Effects of estrogen replacement therapy on PET cerebral blood flow and neuropsychological performance. *Horm. Behav.* 34, 171–182.
- Rissanen, A., Puoliväli, J., van Groen, T., Riekkinen, P.J., 1999. In mice tonic estrogen replacement therapy improves non-spatial and spatial memory in a water maze task. *NeuroReport* 10, 1369–1372.
- Saito, S., Kobayashi, S., Ohashi, Y., Igarashi, M., Komiya, Y., Ando, S., 1994. Decreased synaptic density in aged brains and its prevention by rearing under enriched environment as revealed by synaptophysin contents. *J. Neurosci. Res.* 39, 57–62.
- Sandstrom, N.J., Williams, C.L., 2001. Memory retention is modulated by acute estradiol and progesterone replacement. *Behav. Neurosci.* 115, 384–393.
- Schlaf, G., Salje, C., Poethke, R., Felgenhauer, K., Mäder, M., 1996. A novel enzyme-linked immunosorbent assay for determination of synaptophysin as compared with other quantification procedures. *J. Neuroimmunol.* 67, 59–65.
- Sharps, M.J., Gollin, E.S., 1987. Memory for object locations in young and elderly adults. *J. Gerontol.* 42, 336–341.
- Sherwin, B.B., 1999. Can estrogen keep you smart? Evidence from clinical studies. *J. Psychiatry Neurosci.* 24, 315–321.
- Shughrue, P., Scrimo, P., Lane, M., Askew, R., Merchenthaler, I., 1997a. The distribution of estrogen receptor- β mRNA in forebrain regions of the estrogen receptor- α knockout mouse. *Endocrinology* 138, 5649–5652.

- Shughrue, P.J., Lane, M.V., Merchenthaler, I., 1997b. Comparative distribution of estrogen receptor- α and - β mRNA in the rat central nervous system. *J. Comp. Neurol.* 388, 507–525.
- Shughrue, P.J., Merchenthaler, I., 2000. Evidence for novel estrogen binding sites in the rat hippocampus. *Neuroscience* 99, 605–612.
- Singh, M., Meyer, E.M., Millard, W.J., Simpkins, J.W., 1994. Ovarian steroid deprivation results in a reversible learning impairment and compromised cholinergic function in female Sprague-Dawley rats. *Brain Res.* 644, 305–312.
- Stone, D.J., Rozovsky, I., Morgan, T.E., Anderson, C.P., Finch, C.E., 1998. Increased synaptic sprouting in response to estrogen via an apolipoprotein E-dependent mechanism: implications for Alzheimer's disease. *J. Neurosci.* 18, 3180–3185.
- Sze, C.-I., Troncoso, J.C., Kawas, C., Mouton, P., Price, D.L., Martin, L.J., 1997. Loss of the presynaptic vesicle protein synaptophysin in hippocampus correlates with cognitive decline in Alzheimer disease. *J. Neuropath. Exp. Neurol.* 56, 933–944.
- Tanapat, P., Hastings, N.B., Reeves, A.J., Gould, E., 1999. Estrogen stimulates a transient increase in the number of new neurons in the dentate gyrus of the adult female rat. *J. Neurosci.* 19, 5792–5801.
- Tang, M.-X., Jacobs, D., Stern, Y., Marder, K., Schofield, P., Gurland, B., Andrews, H., Mayeux, R., 1996. Effect of oestrogen during menopause on risk and age at onset of Alzheimer's disease. *Lancet* 348, 429–432.
- Terry, R.D., Masliah, E., Salmon, D.P., Butters, N., DeTeresa, R., Hill, R., Hansen, L.A., Katzman, R., 1991. Physical basis of cognitive alterations in Alzheimer's disease: Synapse loss is the major correlate of cognitive impairment. *Ann. Neurol.* 30, 572–580.
- Toran-Allerand, C.D., 2000. Novel sites and mechanisms of oestrogen action in the brain. *Novartis Found. Symp.* 230, 56–69.
- Vaucher, E., Reymond, I., Najaffe, R., Kar, S., Quirion, R., Miller, M.M., Franklin, K.B.J., 2002. Estrogen effects on object memory and cholinergic receptors in young and old female mice. *Neurobiol. Aging* 23, 87–95.
- Vergheze, J., Kuslansky, G., Katz, M.J., Sliwinski, M., Crystal, H.A., Buschke, H., Lipton, R.B., 2000. Cognitive performance in surgically menopausal women on estrogen. *Neurology* 55, 872–874.
- Vouimba, R.-M., Foy, M.R., Foy, J.G., Thompson, R.F., 2000. 17 β -estradiol suppresses expression of long-term depression in aged rats. *Brain Res. Bull.* 53, 783–787.
- Walker, L.C., Kitt, C.A., Struble, R.G., Wagster, M.V., Price, D.L., Cork, L.C., 1988. The neural basis of memory decline in aged monkeys. *Neurobiol. Aging* 9, 657–666.
- Warner, M., Nilsson, S., Gustafsson, J.-A., 1999. The estrogen receptor family. *Curr. Opin. Obstet. Gynecol.* 11, 249–254.
- Warren, S.G., Humphreys, A.G., Juraska, J.M., Greenough, W.T., 1995. LTP varies across the estrous cycle: Enhanced synaptic plasticity in proestrus rats. *Brain Res.* 703, 26–30.
- Weiland, N.G., 1992. Glutamic acid decarboxylase messenger ribonucleic acid is regulated by estradiol and progesterone in the hippocampus. *Endocrinology* 131, 2697–2702.
- Wiedenmann, B., Franke, W.W., 1985. Identification and localization of synaptophysin, an integral membrane glycoprotein of Mr 38,000 characteristic of presynaptic vesicles. *Cell* 41, 1017–1028.
- Woolley, C.S., McEwen, B.S., 1992. Estradiol mediates fluctuation in hippocampal synapse density during the estrous cycle in the adult rat. *J. Neurosci.* 12, 2549–2554.
- Woolley, C.S., McEwen, B.S., 1993. Roles of estradiol and progesterone in regulation of hippocampal dendritic spine density during the estrous cycle in the rat. *J. Comp. Neurol.* 336, 293–306.
- Woolley, C.S., McEwen, B.S., 1994. Estradiol regulates hippocampal dendritic spine density via an *N*-methyl-D-aspartate receptor-dependent mechanism. *J. Neurosci.* 14, 7680–7687.
- Woolley, C.S., Weiland, N.G., McEwen, B.S., Schwartzkroin, P.A., 1997. Estradiol increases the sensitivity of hippocampal CA1 pyramidal cells to NMDA receptor-mediated synaptic input: Correlation with dendritic spine density. *J. Neurosci.* 17, 1848–1859.
- Wu, X., Glinn, M.A., Ostrowski, N.L., Su, Y., Ni, B., Cole, H.W., Bryant, H.U., Paul, S.M., 1999. Raloxifene and estradiol benzoate both fully restore hippocampal choline acetyltransferase activity in ovariectomized rats. *Brain Res.* 847, 98–104.
- Yaffe, K., Sawaya, G., Lieberburg, I., Grady, D., 1998. Estrogen therapy in postmenopausal women: Effects on cognitive function and dementia. *JAMA* 279, 688–695.
- Zhan, S.-S., Beyreuther, K., Schmitt, H.P., 1993. Quantitative assessment of the synaptophysin immuno-reactivity of the cortical neuropil in various neurodegenerative disorders with dementia. *Dementia* 4, 66–74.

(Accepted 14 June 2002)