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Low Doses of 17α-Estradiol and 17β-Estradiol Facilitate, Whereas Higher Doses of Estrone and 17α- and 17β-Estradiol Impair, Contextual Fear Conditioning in Adult Female Rats

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Estrogens are known to exert significant structural and functional effects in the hippocampus of adult rodents. In particular, 17β-estradiol can improve, impair, or have no effect on hippocampus-dependent learning and memory depending on dose and time of administration. The effects of other forms of estrogen, such as estrone and 17α-estradiol, on hippocampus-dependent learning have not been as thoroughly investigated. Therefore, the purpose of this study was to investigate the effects of 17β-estradiol, estrone, and 17α-estradiol at three different doses on two different tasks: hippocampus-dependent contextual fear conditioning and hippocampus-independent cued fear conditioning. Adult ovariectomized female rats were injected with one of the estrogens at one of the three doses 30 mins before conditioning to assess the rapid effects of these estrogens on acquisition. Twenty-four hours later memory for the context was examined and 1 h later memory for the cue (tone) was assessed. Levels of synaptophysin were examined in the dorsal hippocampus of rats to identify a potential synaptic correlate of hormonal effects on contextual fear conditioning. Low 17β-estradiol and 17α-estradiol enhanced, whereas high 17β-estradiol and 17α-estradiol impaired, contextual fear conditioning. Only the middle dose of estrone severely impaired contextual fear conditioning. Estrogens did not alter performance in the hippocampus-independent cued task. Synaptophysin expression was increased by estrone (at a middle and high dose) and 17β-estradiol (at a middle dose) in the CA3 region of the hippocampus and was not correlated with cognition. The results of this study indicate that estradiol can positively or negatively influence hippocampus-dependent learning and memory, whereas estrone impairs hippocampus-dependent learning and memory in a dose-dependent manner. These results have important therapeutic implications, as estrone, a main component of a widely used hormone replacement therapy, was shown to have either a negative effect or no effect on learning and memory. It may be possible to use 17α-estradiol and lower doses of estrogens as potential alternatives in hormone replacement therapies.

INTRODUCTION

Gonadal hormones influence hippocampus-dependent learning and memory (for review see Daniel, 2006; Frick, 2009). In particular estradiol has equivocal effects on cognition, with some studies showing improvements (eg, Luine and Rodriguez, 1994; Daniel *et al*, 1997; Bimonte and Denenberg, 1999), whereas other studies either show no effect (eg, Berry *et al*, 1997; Luine *et al*, 1998; Fader *et al*, 1999) or impairments with estradiol (eg, Frye, 1995; Warren and Juraska, 1997; Wilson *et al*, 1999). These differential

effects are likely based on dose and the different types of cognitive tasks used in which performance depends on the integrity of different brain regions. Both endogenous and exogenous estrogen levels influence hippocampus-dependent learning and memory. For example, female rats in proestrus, when endogenous plasma levels of estrogen are highest, show impaired performance on different hippocampus-dependent tasks compared with female rats in estrus, when endogenous plasma levels of estrogen are lowest (Frye, 1995; Warren and Juraska, 1997; Markus and Zecevic, 1997). Intriguingly however, rats during proestrus are more likely to use a spatial strategy when solving certain tasks (Korol *et al*, 2004). In addition, exogenous administration of estradiol to ovariectomized female rats shows a dose-dependent relationship between estradiol and memory. Low but physiological levels of exogenous estradiol enhance performance (Fader *et al*, 1999; Luine *et al*, 1998; Holmes *et al*, 2002), whereas administration of high

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548

exogenous pharmacological or physiological levels of estradiol impair performance (Galea et al, 2001; Holmes et al, 2002) on a hippocampus-dependent spatial working/reference memory version of the radial arm maze task. Therefore, it is necessary to investigate dose-dependent effects of estradiol on different types of learning and memory tasks that are subserved by different memory systems.

Fear conditioning relies on the integrity of the amygdala and contextual fear conditioning relies on the integrity of the hippocampus and amygdala. Estradiol influences hippocampus-dependent contextual fear conditioning, but not hippocampus-independent cued fear conditioning (Markus and Zecevic, 1997; Gupta et al, 2001), indicating that estradiol may mediate hippocampus-dependent conditioning, but has less impact on amygdala-dependent fear conditioning. Physiologically high levels of estradiol influence contextual fear conditioning negatively, as female rats in proestrus or treatment with high estradiol reduces the amount of freezing after conditioning compared with females in estrus (Markus and Zecevic, 1997; Gupta et al, 2001; Altemus et al, 1998), whereas ovariectomy increases freezing behavior in response to a context associated with shock (Gupta et al, 2001). Interestingly, a similar relationship between estrogen and contextual fear conditioning is seen in humans. Milad et al (2006) found that women had greater context memory in the early follicular phase (low physiological estrogen levels) of the menstrual cycle compared with the midfollicular phase (high physiological estrogen levels) and men. Collectively, these studies indicate a function for estradiol in contextual fear conditioning, although to date, it is not known whether a dose-dependent effect of estradiol exists for contextual fear conditioning as it does for other hippocampus-dependent tasks or whether contextual fear conditioning is influenced by other types of estrogens.

17β-estradiol is the most potent form of estrogen and is the most prominent estrogen in young pre-menopausal women, whereas estrone is a weaker estrogen that is the most prominent estrogen in postmenopausal women (Rannevik et al, 1995). Interestingly, estrone is the main component of Premarin, the most popular hormone replacement therapy given to menopausal women. A recent meta-analysis found that Premarin does not have as many cognitive-enhancing properties as other hormone replacement therapies that use different types of estrogen, such as 17β-estradiol (Ryan et al, 2008). Furthermore, Premarin can alter estrogen receptor (ER) subtypes independently of the effects of 17β-estradiol. Jin et al (2005) found that treatment with Premarin led to decreased levels of ERα, but did not affect ERβ levels, whereas treatment with 17β-estradiol increased ERβ, but did not affect ERα levels in the hippocampus and cortex of adult female rats. Given that ERs in the brain show tissue and region-specific patterns of expression (Shughrue et al, 1997), and the diverse behaviors they regulate (Kudwa et al, 2006; Rhodes and Frye, 2006), it is plausible that different forms of estrogen could have profound and distinct effects on neural plasticity and/or behavior. Despite the fact that estrone is centrally active (Barha et al, 2009) and is the main component of the HRT Premarin, to our knowledge only one study has directly examined the effects of estrone on learning (Farr et al, 2000). Thus, in this study we sought to determine whether

there were differential effects of the estrogens, estradiol, and estrone, on contextual fear conditioning.

In addition to estrone, there are naturally occurring optical isomers of estradiol: 17α-estradiol and 17β-estradiol. Although 17β-estradiol binds to ERα and ERβ with an approximately 40-fold higher affinity, 17α-estradiol (Perez et al, 2005) may be the preferred ligand of ER-X, a plasma membrane-associated ER (Toran-Allerand et al, 2002). In addition, 17α-estradiol has been shown to affect the structure and function of the hippocampus by rapidly increasing spine density in the CA1 region of the hippocampus (Maclusky et al, 2005), enhancing place memory (Rhodes and Frye, 2006; Luine et al, 2003), and increasing cell proliferation in the dentate gyrus of adult female rats (Barha et al, 2009). Therefore, it is possible that 17α-estradiol could have effects on hippocampus-dependent contextual fear conditioning.

Estradiol's effects on behavior may be linked to its ability to alter the number and properties of synapses in the hippocampus. Specifically, 17β-estradiol increases the number of spines and synapses in the CA1 field of the hippocampus (Woolley et al, 1990; Woolley and McEwen, 1992), and synaptophysin, a presynaptic protein that is a critical component of synaptic vesicle exocytosis, in the hippocampus (Murphy and Segal, 1996; Stone et al, 1998; Pozzo-Miller et al, 1999; Frick et al, 2002). Importantly, the increase in synaptophysin expression in the whole hippocampus is associated with enhanced spatial reference memory (Frick et al, 2002). Interestingly, contextual fear conditioning is reliant on an intact, functioning dorsal hippocampus as seen from lesion and immediate early gene studies (Barrientos et al, 2002; for review see Kubik et al, 2007). Therefore, it is of interest to see whether different forms of estrogen affect synaptophysin expression in the dorsal hippocampus after training in the contextual fear conditioning task.

This study aimed to determine the effects of 17β-estradiol, estrone, and 17α-estradiol at different doses on acquisition of hippocampus-dependent contextual fear conditioning and hippocampus-independent cued fear conditioning. Adult ovariectomized female rats were injected with either a low, middle, or high dose of one of the three hormones or vehicle, and later tested for contextual and cued fear conditioning. Levels of synaptophysin, a synaptic vesicle-associated integral membrane protein and a general marker of presynaptic nerve endings, were also assessed in the dorsal hippocampus of rats to identify a potential synaptic correlate of hormonal effects on contextual fear conditioning. We hypothesized that low doses of estradiol would facilitate, and high and middle doses of all three hormones would impair contextual fear conditioning, but have no significant effect on cued fear conditioning. Furthermore, we expected higher levels of synaptophysin expression in rats given estrogen treatment compared with controls.

MATERIALS AND METHODS

Animals

Eighty-one adult female Sprague–Dawley rats, weighing 200–250 g, obtained from the University of British Columbia Animal Care Centre (Vancouver, BC, Canada), were used in

the study. Rats were initially housed in pairs in opaque polyurethane bins (48 × 27 × 20 cm) with aspen chip bedding and were given Purina rat chow and tap water *ad libitum*. Rats were maintained under a 12:12 h light/dark cycle (lights on 07.30 h). Beginning the day after arrival, rats were handled every other day for 5 min. All experiments were conducted in accordance with the ethical guidelines set by the Canada Council for Animal Care and were approved by the University of British Columbia Animal Care Committee. All efforts were made to reduce the number and the suffering of animals.

Surgery

Approximately 1 week after arrival, all females were bilaterally ovariectomized using aseptic procedures. Rats were placed in a chamber and anesthetized with isoflurane, which was delivered at an induction flow rate of 5% (the flow rate of O$_2$ was approximately 1.5%). Rats were then maintained on a flow rate of 2.5–3% to sustain a stable respiratory rate and were given an injection of lactated ringer's solution (100 ml/kg, s.c.) and an injection of a nonsteroidal anti-inflammatory analgesic (Anafen, MERIAL Canada Inc., Baie d'Urfé, Quebec, Canada; 5 ml/kg). After surgery, rats were placed singly into a clean, sterile opaque polyurethane bin and kept warm until recovery from anesthetic was complete. A topical antibacterial ointment was externally applied to the incision (Flamazine, Smith & Nephew, St Laurent, Quebec, Canada). Rats were weighed daily to monitor recovery from surgery. The rats were given 7 days to recover before any experimental manipulations were initiated.

Apparatus

Conditioning and testing occurred in two identical observation chambers (30.5 × 24 × 21 cm; Med Associates, St Albans, VT), consisting of aluminum (side walls) and Plexiglass (ceiling, hinged front door, and rear wall). The chambers were enclosed within sound-attenuating boxes located in a brightly lit and quiet room. A video camera was positioned above each chamber to record subject's behavior for video scoring. The floor of each chamber consisted of 19 stainless steel rods spaced 1.5 cm apart that were connected to a shock generator and scrambler for the delivery of an unconditioned stimulus footshock. Each chamber was illuminated by a single 100 mA houselight positioned in the top center of one wall. In the left corner of the same wall, a speaker connected to a programmable audio generator (ANL-926, Med Associates) was located. On the wall opposite to the houselight, a speaker and two 100 mA stimulus lights (2.5 cm diameter) were located 7 cm above the floor. Ventilation fans in each box supplied background noise (70 dB, A scale).

Each chamber was positioned on a load-cell platform that recorded chamber displacement in response to a subject's motor activity (Med Associates). The output from each load cell was set to a gain (vernier knob 8) that was optimized for detecting freezing behavior, and the output was digitized and acquired online using Threshold Activity software (Med Associates). The load-cell activity was digitized at 5 Hz, resulting in one observation per rat every 200 ms. Freezing

is a defensive fear response that consists of an immediate suppression of behavior that is accompanied by immobility, shallow breathing, increased heart rate, and pilo-erection (Barrientos *et al*, 2002). Freezing was quantified by calculating the number of observations for each rat that had a value less than the freezing threshold, allowing for exclusion of behaviors such as grooming, sniffing, and head turning. The same freezing threshold was used for each animal throughout the duration of the experiment. Importantly, an observation was only considered to be freezing if it was a part of at least five continuous observations that fell below the threshold; therefore, freezing was only scored if the animal was immobile for at least 1 s (Maren, 1998). The threshold output of freezing was verified by comparing with results from video scoring of an animal's behavior. The amount of time spent actively exploring the chamber, self-grooming, and rearing was quantified by calculating the number of load-cell values above appropriate thresholds for each measure. These thresholds were determined by comparing load-cell output with an observer's ratings of these behaviors.

To assess the effects of different forms of estrogen on contextual fear conditioning as well as on cued fear conditioning, animals were exposed to both a conditioning context (Context A, for context-associated fear) and a novel environment (Context B, for cue-associated fear). Context A consisted of a standard operant chamber as described above with an illuminated houselight, bare aluminum and Plexiglass walls, and a 'strawberry' car air freshener used as an odor cue. Context B was a standard operant chamber that was illuminated by the two stimulus lights, had stripped and dotted inserts covering the Plexiglass walls, the stainless steal rods on the floor were covered by a smooth, white plastic insert, and a 'vanilla' air freshener provided an odor cue. Each animal was exposed to both Context A and Context B as described below.

Drug Treatment

Approximately 1 week after ovariectomy, rats were randomly assigned to 1 of 10 treatment groups ($n = 7$–9 per group) and received a single s.c. injection of either vehicle (Control, 0.10 ml sesame oil), 17β-estradiol, estrone, or 17α-estradiol. The estrogens were given at one of three doses: low (0.30 μg/0.10 ml sesame oil), middle (1 μg/0.10 ml sesame oil), or high (10 μg/0.10 ml sesame oil). These doses were chosen based on earlier studies investigating spatial learning and hippocampal neurogenesis (Tanapat *et al*, 1999; Ormerod *et al*, 2003; Holmes *et al*, 2002; Tanapat *et al*, 2005; Barha *et al*, 2009). A high dose of 10 μg 17β-estradiol was chosen because it results in circulating levels of estradiol observed on the morning of proestrus (Viau and Meaney, 1991) and has been shown to enhance cell proliferation 30 min (Barha *et al*, 2009), 2 h (Tanapat *et al*, 1999; Tanapat *et al*, 2005), and 4 h (Ormerod *et al*, 2003; Barha *et al*, 2009) after injection. However, this same dose produces supraphysiological levels of estradiol shortly after administration (Woolley and McEwen, 1993). We chose a middle dose of 1 μg 17β-estradiol as earlier studies have shown that this dose of 17β-estradiol slightly increases cell proliferation (Tanapat *et al*, 2005) and impairs working memory on the spatial working/reference memory version

of the radial arm maze (Holmes *et al*, 2002). On the other hand, a 0.3 μg dose of 17β-estradiol facilitates working memory (Holmes *et al*, 2002), increases cell proliferation 30 min after injection (Barha *et al*, 2009), and results in circulating levels of estradiol found during diestrus (Viau and Meaney, 1991).

The doses chosen for 17β-estradiol were also used for 17α-estradiol and estrone allowing for direct comparison of effects between estrogens. It is well established that the low and middle doses of 17β-estradiol are physiological levels as reviewed above, but that the high dose is pharmacological 30 min after administration (Woolley and McEwen, 1993, personal observation). We found that using radioimmunoassay, estrone levels range from 27.57 to 108.96 pg/ml across the estrous cycle. We have also established that the low, middle, and high doses of estrone correspond roughly to 85.59, 108.82, and 516.32 pg/ml 30 min after administration. Thus, this indicates that the low and medium doses of estrone produce physiological levels, whereas the high does is pharmacological. It is not at the moment possible to determine the levels of 17α-estradiol by a commercially available radioimmunoassay kit. However, using a different technique Toran-Allerand *et al* (2005) has shown that 17α-estradiol is undetectable in serum, but is found in the brain of adult mice. We chose the same doses for 17α-estradiol and estrone to directly compare all three doses with all three estrogens. Furthermore, we have used these same doses of all three estrogens in an earlier paper examining the effects of these estrogens on cell proliferation in the dentate gyrus of adult female rats (Barha *et al*, 2009). All rats received a single s.c. injection of 0.10 ml of either hormone or vehicle. All injections were given between 8:30 and 9:00 a.m. and hormones were given 30 min before conditioning to assess the effects of hormone administration on memory acquisition 24 h later. Earlier studies have shown that these same estrogens can influence hippocampus structure and function within 30 min of exposure (Barha *et al*, 2009; Maclusky *et al*, 2005; Luine *et al*, 2003). Effort was made to ensure that groups did not differ in post-operative body weights because ovariectomy is known to lead to increased body weight.

Estrogens (Sigma-Aldrich Chemicals, Oakville, ON, Canada) were dissolved in sesame oil (Sigma-Aldrich Chemicals) over low heat to a concentration of 0.3, 1, or 10 μg of hormone per 0.10 ml oil. Estrogen solutions were stored in opaque containers at room temperature.

Behavioral Procedure

This experiment was conducted over 2 days, conditioning day and testing day. On day 1, the conditioning day, rats were injected with hormone or vehicle by one experimenter. Thirty minutes later, rats were transported to the testing room in their home cages on a four-wheeled cart by a different experimenter. Rats were then placed in the Context A operant chamber. Three minutes after placement into the chamber, rats were given three presentations of the tone CS (4 kHz, 80 dB, 30 s) each co-terminating with a footshock (2 s, 1.0 mA) with 60 s intershock intervals. Sixty seconds after the third and final tone/shock pairing (after 3 min of exposure to Context A),

rats were immediately returned to their home cage and colony room. To assess whether there were group differences in pain sensitivity, we recorded freezing behavior during the minute after each shock presentation. To assess contextual fear conditioning, 24 h after conditioning, rats were transported back to the testing room in their home cages on a four-wheeled cart by the same experimenter as the day before. The same procedure was followed as the conditioning day to activate the representation of the context. Rats were placed back into Context A for 8 min and freezing behavior was assessed. After the 8 mins, rats were immediately returned to the colony room in their home cages. To assess cued fear conditioning, rats were transported approximately 1 h later (Winocur *et al*, 2006) to the testing room in a clean, unused, transparent cage (48 × 27 × 20 cm) by a different experimenter along a completely novel route without a cart. Rats were placed into the novel Context B and 3 min later were presented with three tones (4 kHz, 80 dB, 30 s) 60 s apart. A timeline of the experiment is presented in Table 1. Time spent freezing to the tone (cue) was measured during each tone presentation. In addition, time spent freezing for 3 min before the first tone presentation was also recorded as an index of whether the groups differed in how much they recognized the new Context B as the old Context A.

Immunohistochemistry

A random subset of rats (*n* = 4) from each group were perfused 4 h after exposure to Context B (cued fear conditioning) to assess levels of synaptophysin in the hippocampus. Briefly, rats were deeply anesthetized with a lethal dose of sodium pentobarbital and then perfused with 4% paraformaldehyde. After extraction, brains were stored at 4°C in 4% paraformaldehyde for 24 h before being transferred to 30% sucrose for a minimum of 72 h. Brains were sliced into 30 μm sections through the entire extent of the hippocampus in a bath of Tris-buffered saline (TBS) (pH 7.4) using a vibratome (Leica VT1000S; Leica Microsystems, Inc., Richmond Hill, ON, Canada). The sections were stored at −20°C in phosphate-buffered saline (PBS) antifreeze sterile culture plates. Sections were washed 3 × for 10 min each with TBS and then stored in sterile culture plates filled with TBS for the 24 h before immunohistochemistry processing.

All histological procedures were based on modification of earlier work (Burton *et al*, 2007). Between all steps free-floating sections were rinsed 3 × for 10 min each in PBS (0.1 M sodium phosphate buffer in 0.9% saline; pH 7.4) unless stated otherwise. Sections were blocked with 5% normal horse serum (NHS; Vector Laboratories, Burlington, ON, Canada) and 3% Triton-X (Boehringer Mannheim, Laval, QC, Canada) for 15 min and then incubated for 2 h in mouse monoclonal antibody against synaptophysin (1 : 200; Sigma-Aldrich Chemicals) at room temperature. Sections were then incubated in mouse secondary antisera (1 : 200; Vector Laboratories) for 2 h at room temperature. Sections were then incubated in avidin-biotin horseradish peroxidase complex (ABC Elite Kit; 1 : 50; Vector Laboratories) for 90 min. Sections were reacted in 0.01% diaminobenzidine (DAB; Sigma-Aldrich Chemicals) with 0.0003%

Table 1 Experimental Procedure

Conditioning				Testing				
				Contextual fear conditioning		Cued fear conditioning		
Hormone injection	→30 min later	Context A: 3-min exploration, three pairings of 30-s tone and 2-s 1.0 mA shock	→24 h later	Context A: 8-min in conditioning chamber (no tone or shock)	→1 h later	Context B: 3-min in novel chamber, three presentations of 30-s tone (no shock)	→4 h later	Perfusion and extraction of brains for synaptophysin analysis

H_2O_2 for approximately 5 min. The sections were mounted on super frost slides (Fisher Scientific, Edmonton, AB, Canada), dried overnight, dehydrated, and then cover-slipped with Permount (Fisher Scientific).

Quantitative Image Analysis

Quantitative densiometric assessment of synaptophysin protein was carried out at 40 × magnification by using SimplePCI image analysis system Ver.5 (Compix, Cranberry Township PA). The light intensity used was adjusted so that the range of gray level intensities for each slide was 0–255 (black to white). Gray levels were measured on the photomicrographs by placing open circles with diameters of 100 μm along different regions of the hippocampus (see Figure 1a). We followed procedures of Gao et al (2006). Specifically, optical density (OD) of synaptophysin was assessed in each of the hilus and inner molecular layer of the dentate gyrus (DGhilus and DGin), the stratum radiatum of the CA3 and CA1 (CA3ra and CA1ra), and the stratum oriens of the CA3 and CA1 (CA3or and CA1or). The background gray level for each section was obtained from the mean of eight circles placed within the corpus callosum to control for variations in staining and illumination. The mean gray levels for each hippocampal region of interest (ROI) were obtained by averaging the gray levels of all circles within that region. Then the mean OD was obtained by subtracting each mean gray level from 255 (the maximum white intensity). Then the mean OD for each ROI was obtained by subtracting the mean reference OD from the corpus callosum from the mean OD for each ROI on each section (Gao et al, 2006).

Data Analyses

To assess the effect of hormone treatment on freezing and any differences between groups in pain sensitivity to the shock on conditioning day, the total amount of time in seconds spent freezing after each shock presentation was calculated and analyzed using a one-way ANOVA, with group (Control, 17β-estradiol-low, 17β-estradiol-middle, 17β-estradiol-high, estrone-low, estrone-middle, estrone-high, 17α-estradiol-low, 17α-estradiol-middle, 17α-estradiol-high) as the between-subjects factor. Total amount of time in seconds spent freezing in Context A (contextual fear conditioning) and Context B (cued fear conditioning) on testing day were each analyzed using a one-way ANOVA, with group as the between-subjects factor. Total amount of
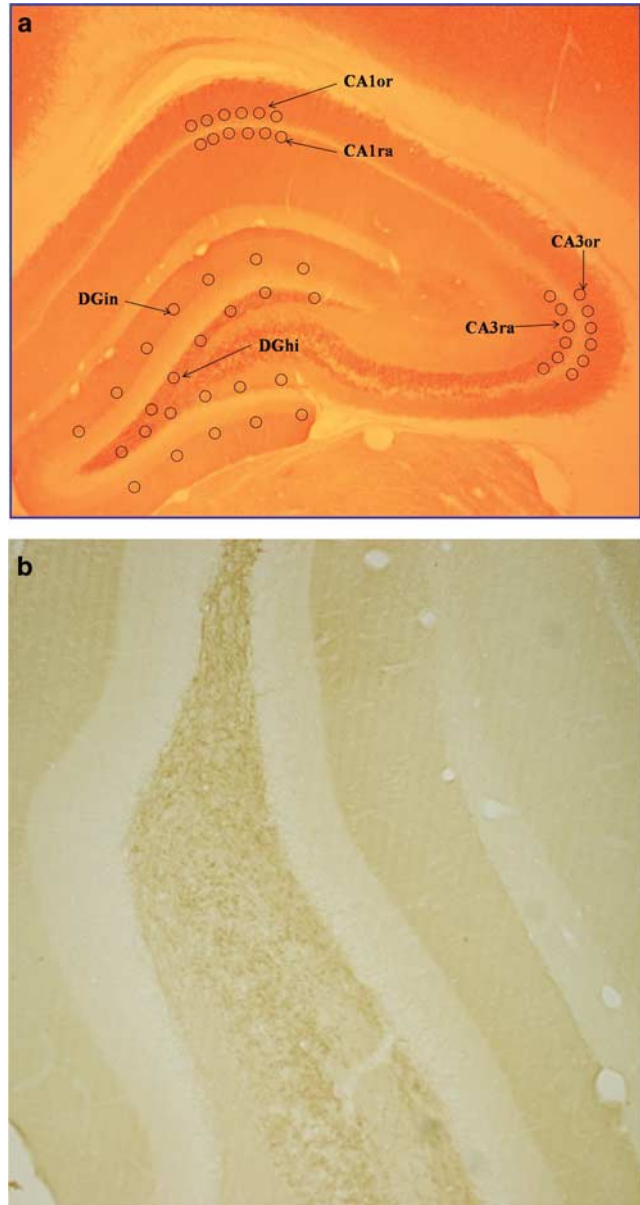

Figure 1 (a) Representative photomicrograph of a dorsal hippocampus section showing the placement of open circles used for densiometric analysis. 12 circles each with diameter of 100 μm were placed along each of the DGin and DGhi, and 6 circles were placed along each of the CA3ra, CA3or, CA1ra, and CA1or. All hippocampal sections were viewed at 40 × magnification. (b) Photomicrograph of dentate gyrus under 400 × magnification contrasting the signal integrity of the synaptophysin expression in the hilus to what is seen in the granule cell layer.

Table 2 Total (±SEM) Amount of Time in Seconds Spent in Locomotion, Grooming, and Rearing on the Conditioning Day in Ovariectomized Female Rats

Group	Locomotion (s)	Grooming (s)	Rearing (s)
Control	152.04 ± 12.08	41.51 ± 6.53	157.76 ± 6.32
17β-Estradiol			
Low	163.54 ± 17.46	54.57 ± 9.26	150.54 ± 19.54
Medium	151.97 ± 14.54	37.49 ± 6.08	192.63 ± 10.98
High	181.63 ± 14.94	44.40 ± 5.81	115.06 ± 20.97
Estrone			
Low	170.00 ± 11.85	40.17 ± 6.75	144.89 ± 32.66
Medium	162.74 ± 7.23	39.17 ± 11.65	200.46 ± 13.84
High	171.31 ± 12.93	62.20 ± 7.90	80.14 ± 10.06*
17α-Estradiol			
Low	161.11 ± 14.51	47.23 ± 6.80	118.51 ± 27.66
Medium	159.13 ± 12.00	41.03 ± 2.56	181.58 ± 10.58
High	185.78 ± 12.72	50.78 ± 4.38	104.55 ± 15.64

Groups did not significantly differ in locomotion or grooming. Rats administered high dose of estrone spent less time rearing than control rats. *$p < 0.05$ vs control.

Table 3 Total (±SEM) Percentage of Freezing During the Three Postshock Minutes on the Conditioning Day in Ovariectomized Female Rats

Group	Total percentage
Control	36.81 ± 7.41
17β-Estradiol	
Low	29.53 ± 5.54
Medium	25.11 ± 7.99
High	36.90 ± 8.52
Estrone	
Low	31.64 ± 10.42
Medium	15.97 ± 5.34
High	53.53 ± 4.49
17α-Estradiol	
Low	39.94 ± 9.40
Medium	24.89 ± 5.50
High	39.42 ± 6.47

Groups exhibit immediate postshock freezing and did not significantly differ in postshock freezing during conditioning.

time spent freezing before (to assess recognition of the different context) and during the three tone presentations in Context B (cued fear conditioning) on testing day were each analyzed using a one-way ANOVA, with group as the between-subjects factor. Owing to equipment malfunction, data from one control animal was not included in the analysis of cued fear conditioning. Differences in OD of synaptophysin expression were analyzed using repeated-measures ANOVA, with group as the between-subjects factor and region (DGhilus, DGin, CA3ra, CA3or, CA1ra, and CA1or) as the within-subjects factor. Spearman's rank correlations were conducted between OD of synaptophysin expression in each region and total amount of time in seconds spent freezing during contextual fear conditioning testing in Context A. Data were further analyzed using the Newman–Keuls *post hoc* test. All statistical procedures were set at $\alpha = 0.05$ unless otherwise stated.

RESULTS

Treatment with Estrogens Did Not Influence Sensorimotor Activity of Rats During Conditioning

The amount of time spent in locomotion, grooming, and rearing during conditioning 30 min after hormone administration are shown in Table 2. Groups did not differ in the amount of time spent in motion [$F(9,64) = 0.77$; $p < 0.70$] or in the amount of time spent grooming [$F(9,64) = 1.22$; $p < 0.30$]. Groups did differ in the amount of time spent rearing [$F(9,64) = 4.85$; $p < 0.0001$]. *Post hoc* tests indicate that only the high dose of estrone decreased the time spent rearing compared to control ($p < 0.05$).

All Groups Exhibit Immediate Postshock Freezing and Estrone Tended to Alter the Amount of Postshock Freezing During Conditioning

The percentage of total time spent freezing during the three postshock minutes during conditioning for all groups is shown in Table 3. The results indicate that all groups showed immediate freezing after presentation of the shock and that groups tended to differ in the amount of time spent freezing after presentation of the shock [$F(9,71) = 1.86$, $p = 0.07$]. A closer look at Table 3 suggests that a lower percentage of freezing after the shocks is seen in the group given a middle dose of estrone compared with controls (15.97% vs 36.81%) and a higher percentage of freezing after the shocks is seen in the group give a high dose of estrone compared with controls (53.53% vs 36.81%). Therefore, because groups tended to differ in initial response to shock, we used this factor as a covariate in all subsequent analyses.

Low Physiological Doses of 17β-Estradiol and 17α-Estradiol Increase, Whereas High Pharmacological Doses Decrease, Contextual Fear Conditioning and the Middle Dose of All Three Estrogens Dramatically Decreases Contextual Fear Conditioning

The percentage of time spent freezing during the contextual fear conditioning test for all groups is shown in Figure 2. Groups tended to differ in the amount of time spent freezing after presentation of the shock on the conditioning day; therefore, an analysis of covariance was conducted on the percentage of total time spent freezing across the eight minutes of contextual fear conditioning with total time spent freezing during the conditioning day as a covariate.
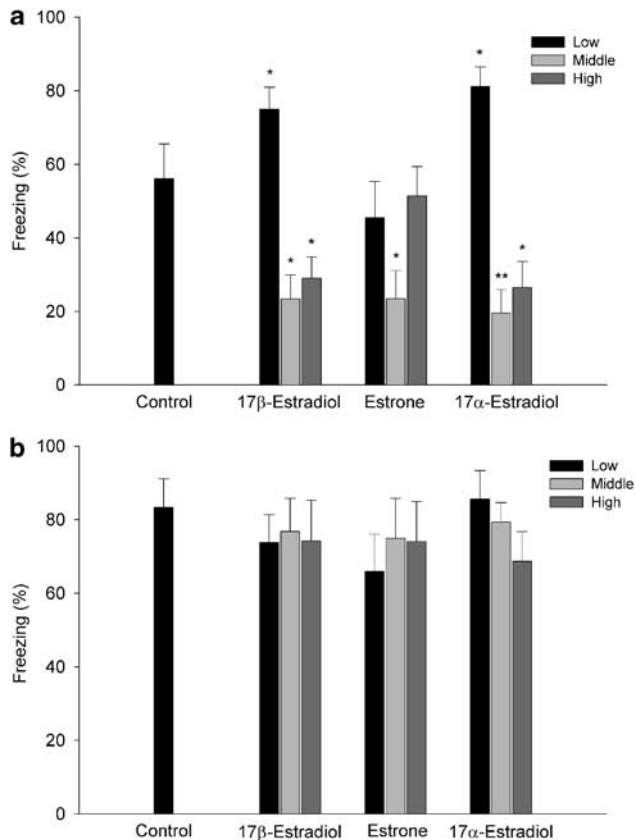
Figure 2 (a) Total (+ SEM) percentage of freezing during the 8-min contextual fear-conditioning test (Context A) in ovariectomized female rats when tested 24 h after conditioning. Rats given a low dose (0.3 μg) of 17β-estradiol and 17α-estradiol had higher levels of freezing compared with controls (both p-values <0.05). Rats given a middle dose (1.0 μg) of 17β-estradiol, estrone, and 17α-estradiol and a high dose (10 μg) of 17β-estradiol and 17α-estradiol had lower levels of freezing compared with controls (all p-values <0.05). (b) Total (+ SEM) percentage of freezing during the presentation of the three tones during the cued fear conditioning test (Context B) in ovariectomized female rats when tested 1 h after contextual fear conditioning test. Groups did not differ in freezing to the tone during the cued fear-conditioning test compared with controls. *p<0.05 vs control; **p<0.01 vs control.

The analysis yielded a significant main effect of the covariate [F(1,70) = 9.28, p < 0.01], and a main effect of Group [F(9,70) = 8.61, p < 0.0001]. Post hoc tests revealed that high doses of 17β-estradiol and 17α-estradiol decreased the percentage of time spent freezing (p < 0.04 and p < 0.03, respectively), and middle doses of 17β-estradiol, estrone, and 17α-estradiol decreased the percentage of time spent freezing compared with control (all p-values <0.03). In contrast, the low dose of 17α-estradiol increased the percentage of time spent freezing compared with control (p < 0.04). On the basis of the earlier studies (Holmes et al, 2002), we expected to find an enhancement in contextual fear conditioning with the low dose of 17β-estradiol; therefore, an a priori comparison was conducted and the low dose of 17β-estradiol was found to increase the percentage of time spent freezing compared with control (p < 0.05).

Different Doses of Different Estrogens Do Not Influence Cued Fear Conditioning

To determine whether groups differed on their recognition of Context B as being distinct from Context A, we measured freezing in the first 3 min of the cued fear conditioning test, before the tone presentation. Importantly, groups did not differ in the percentage of freezing at baseline, the first 3 min of the cued fear conditioning test [F(9,70) = 0.87, p = 0.56; see Table 4], indicating that all groups viewed Context B as being distinct from Context A. The percentage of time spent freezing during the presentation of the three tones during the cued fear conditioning test for all groups is shown in Figure 2. An analysis of covariance was conducted on the percentage of total time spent freezing during the presentation of tones during the cued fear conditioning test with time spent freezing on the conditioning day as a covariate. A main effect of the covariate was not found [F(1,69) = 3.50, p = 0.07] nor was a main effect of group found [F(9,69) = 0.57, p = 0.82], indicating that groups did not differ in the amount of time spent freezing during the presentation of the tones during the cued fear conditioning test in Context B.

Middle and High Dose of Estrone and the Middle Dose of 17β-Estradiol Increase Synaptophysin in Only the CA3 Region of the Hippocampus

Intense and consistent staining for synaptophysin throughout the CA1, CA3, and DG was observed in all groups with very low levels of staining observed in the pyramidal cell layers or the granule cell layer as expected (see Figure 1a and b). As section thickness can influence OD, we also determined section thickness across the groups. Section thickness did not differ between groups, with thickness ranging between 11.50 and 14.00 μm [F(9, 32) = 1.10, p < 0.40; data not shown]. Repeated-measures ANOVA conducted on OD of synaptophysin expression across treatment groups found a significant interaction between group and region [F(45,145) = 1.57, p < 0.03; see Figure 3]. Further analyses showed that in the CA3 striatum oriens region, the high dose of estrone and the middle dose of 17β-estradiol and estrone increased synaptophysin expression compared with control (all p-values < 0.02). The low dose of 17β-estradiol tended to increase synaptophysin expression in this same area (p < 0.08). Only the high and middle dose of estrone increased synaptophysin levels in the CA3 striatum radiatum region (both p-values < 0.002). Estrogens did not statistically influence synaptophysin expression in the dentate gyrus (all p-values > 0.77) or the CA1 (all p-values > 0.44) compared with control.

No significant correlations were found between synaptophysin expression in the DGhilus, DGin, CA3ra, CA3or, CA1ra, or CA1or regions and total amount of time in seconds spent freezing during the contextual fear conditioning test (all p-values > 0.13).

DISCUSSION

The results from this study show that different forms of estrogen dose dependently influence the acquisition of hippocampus-dependent contextual fear conditioning in

Table 4 Total (± SEM) Percentage of Freezing During the Three-Minute Baseline During the Cued Fear Conditioning Test (Context B) in Ovariectomized Female Rats

Group	Total percentage
Control	27.43 ± 7.05
17β-Estradiol	
Low	13.46 ± 4.83
Medium	26.52 ± 7.82
High	15.86 ± 6.20
Estrone	
Low	20.49 ± 10.81
Medium	20.86 ± 6.69
High	21.08 ± 5.76
17α-Estradiol	
Low	31.44 ± 9.96
Medium	25.89 ± 7.39
High	13.06 ± 6.16

Groups do not significantly differ in freezing durations during baseline indicating that Context B was viewed as a novel environment.

adult, ovariectomized female rats. Specifically, a low dose of 17β-estradiol and 17α-estradiol enhanced contextual fear conditioning, whereas middle and high doses of these estrogens impaired contextual fear conditioning. In contrast, estrone impaired contextual fear conditioning at a middle dose (resulting in high physiological levels), whereas a low and high dose of estrone did not significantly influence contextual fear conditioning. Although these estrogens affected hippocampus-dependent contextual fear conditioning, they did not affect hippocampus-independent cued fear conditioning, indicating that the effects of 17β-estradiol, estrone, and 17α-estradiol on these tasks are limited to those involving the hippocampus. Interestingly, 17β-estradiol (middle dose) and estrone (middle and high dose) increased synaptophysin levels in the CA3 region of the hippocampus and were not correlated with the behavioral effects, suggesting synaptophysin expression was disassociated from learning and memory performance in this task.

Different Forms of Estradiol Influence Hippocampus-Dependent Contextual Fear Conditioning in a Dose-Dependent Manner

Administration of a low 0.3 μg dose of 17β-estradiol and 17α-estradiol 30 min before training enhanced contextual fear conditioning performance 24 h after hormone injection, whereas administration of a high 10 μg dose 17β-estradiol and 17α-estradiol impaired contextual fear conditioning. These findings are consistent with and further extend earlier work showing that high physiological and pharmacological levels of exogenous or endogenous estradiol impair contextual fear conditioning after administration of 10 μg dose of estradiol benzoate, a conjugated form of

17β-estradiol (Gupta et al, 2001) and during proestrus (Markus and Zecevic, 1997). This study is the first demonstration that a low physiological dose of estradiol (17α and β) facilitates contextual fear conditioning. In addition, we found that a middle (1.0 μg) dose of 17β-estradiol, estrone, and 17α-estradiol impairs contextual fear conditioning compared with ovariectomized controls. This 1.0 μg dose has been shown to produce physiological levels of estradiol and estrone similar to levels seen during the range of proestrus (Holmes et al, 2002; Shaikh 1971; this study). To our knowledge, the effects of the low and middle doses of these estrogens and the high dose of estrone and 17α-estradiol on fear conditioning have not previously been investigated.

The pattern of effects seen in this study with the different doses of 17β-estradiol support findings using other hippocampus-dependent cognitive tasks (Galea et al, 2001; Holmes et al, 2002). Specifically, acute treatment with the low physiological dose of 17β-estradiol increases, whereas the middle physiological and high pharmacological doses disrupt working memory and reference memory performance, respectively, on the radial arm maze (Galea et al, 2001; Holmes et al, 2002). Furthermore, pharmacological levels of estradiol benzoate resulting from a 40 μg dose (but not 10 μg and 20 μg) enhance associative learning in trace eye-blink conditioning, in which performance depends on the hippocampus and cerebellum (Leuner et al, 2004). These same types of curvilinear relationships of hormonal effects are not unprecedented in the literature. For example, very low and very high physiological levels of adrenal steroids are associated with increased cell death in the hippocampus, whereas intermediate levels are associated with decreased cell death (Joels, 2007). In addition, dopamine activation of the D1 receptor in the prefrontal cortex mediates working memory performance with an inverted U-shape (Floresco and Magyar, 2006). Furthermore, physiologically low and pharmacologically high levels of 17β-estradiol and estrone increase hippocampal cell proliferation, whereas medium levels do not have an effect (Barha et al, 2009). Overall, low levels of 17β-estradiol enhance acquisition of different types of hippocampus-dependent cognitive tasks, and may have less consistent effects on recall (for review see Luine, 2008).

The effects of 17α-estradiol on learning and memory have not been afforded as much attention in the literature as the more potent optical isomer 17β-estradiol. One study reported that 17α-estradiol rapidly enhances acquisition of spatial memory in a similar manner as 17β-estradiol (Luine et al, 2003), similar to findings in this study. Interestingly, 17α-estradiol is a more potent inducer of CA1 pyramidal spine synapse density than is 17β-estradiol (MacLusky et al, 2005) and the greatest increase in spine density was seen 30 mins after hormone injection (MacLusky et al, 2005) along the same timeline used in this study. Although 17α-estradiol was once considered biologically and functionally inactive (Toran-Allerand et al, 2005), it rapidly influences hippocampus-dependent learning (this study; Luine et al, 2003) and upregulates hippocampal cell proliferation (Barha et al, 2009) and spine density (MacLusky et al, 2005) in a dose-dependent manner. Together, these findings indicate that 17α-estradiol is biologically active and may be a potent and viable alternative for hormone replacement therapy.
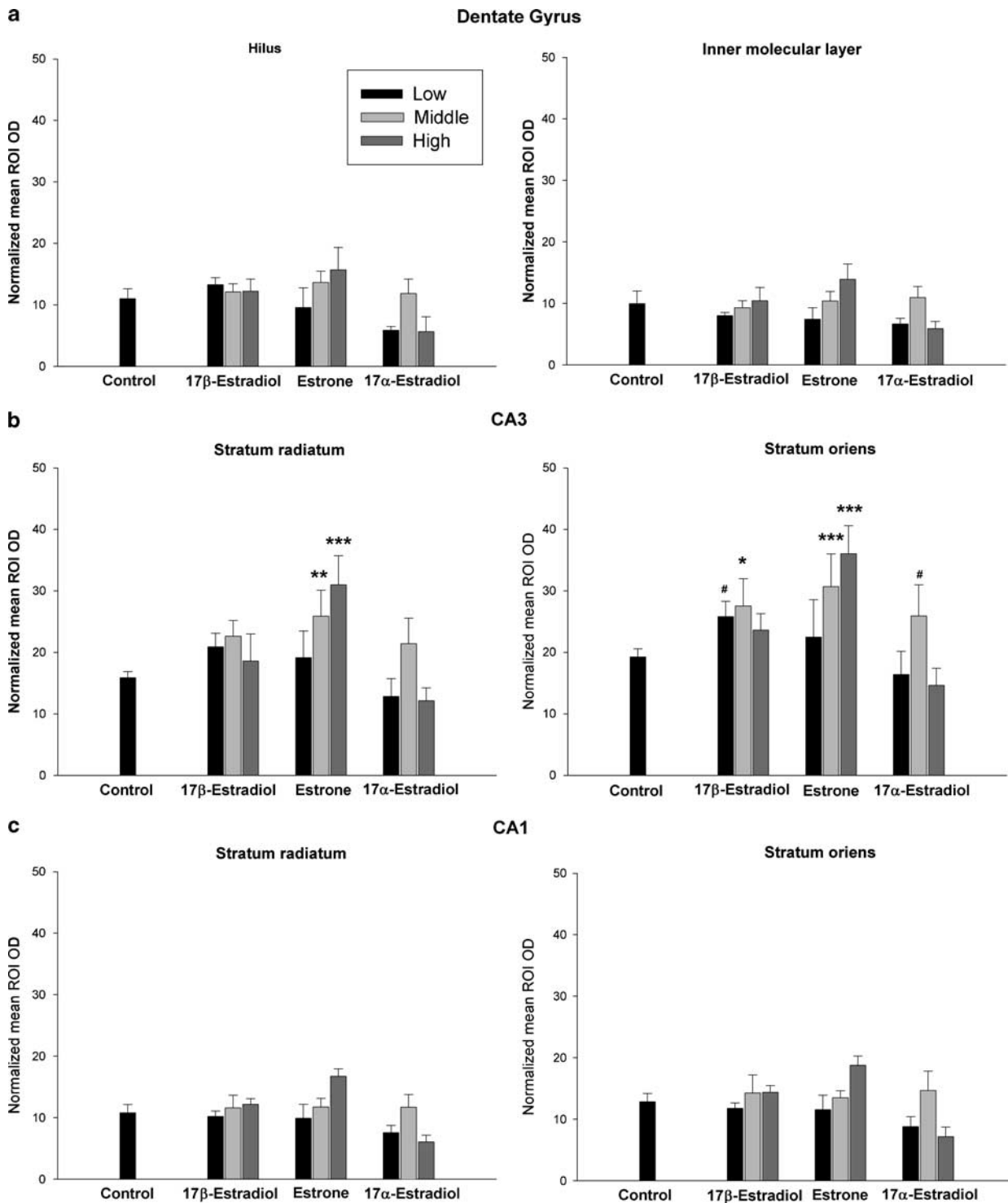
Figure 3 Average normalized OD for each ROI in ovariectomized female rats approximately 30 h after hormone treatment and 5 h after contextual fear conditioning test in the dentate gyrus (a), CA3 region (b), and the CA1 region (c). Rats given a high dose of estrone and a middle dose of 17β-estradiol and estrone had increased synaptophysin expression in the CA3 striatum oriens region ($p < 0.0001$, $p < 0.02$, and $p < 0.0001$, respectively). Rats given a low dose of 17β-estradiol and a middle dose of 17α-estradiol tended to have higher synaptophysin expression in the CA3 striatum oriens ($p = 0.08$ and $p = 0.11$, respectively). Rats given a high and a middle dose of estrone had higher synaptophysin expression in the CA3 striatum radiatum ($p < 0.0001$ and $p < 0.002$, respectively). *$p < 0.05$ vs control; **$p < 0.01$ vs control; ***$p < 0.0001$ vs control. #$p < 0.11$.

Estrone Impaired Contextual Fear Conditioning

Although different doses of 17α-estradiol and 17β-estradiol both facilitated and impaired contextual fear conditioning, estrone did not facilitate contextual fear conditioning at any dose and dramatically impaired contextual fear conditioning at the middle dose. This is in agreement with conclusions reached in a meta-analysis conducted by Ryan

et al (2008), indicating that hormone replacement therapies consisting primarily of estrone do not have a beneficial effect on cognition in post-menopausal women and may even enhance risk for dementia, whereas therapies using 17β-estradiol are more consistently associated with improved cognition. In this study, the low physiological and high pharmacological doses of estrone did not influence contextual fear conditioning. This may be partly related to the lower affinity that estrone has for the ERs, potentially rendering this estrogen less functionally active. Although estrone can be converted to 17β-estradiol through the enzyme 17β-hydroxysteriod dehydrogenases, we do not believe that this was the mechanism through which estrone influenced contextual fear conditioning in this study for three important reasons: (1) the oxidative pathway converting 17β-estradiol into estrone is favored over the reverse reaction (estrone into 17β-estradiol) in rodent tissue (Martel *et al*, 1992); (2) the effects of estrone on behavior in this study occur very rapidly (within 30 mins); and (3) the pattern of results is different for estrone and 17β-estradiol. The results from this study are not in complete agreement with those of Farr *et al* (2000), who found that direct infusion of estrone into the hippocampus enhanced footshock avoidance learning in a similar manner as 17β-estradiol in ovariectomized mice. However, this may be due to different modes of hormone administration, as well as due to the different type of task used. Furthermore, the results from this study are also not in complete agreement with a recent study that found that chronic cyclic (2 days on, 2 days off) treatment with Premarin enhanced spatial working memory and spatial reference memory in long-term ovariectomized middle-aged female rats (Acosta *et al*, 2009). However, many discrepancies between these two studies exist. For example, different strains of rat, different ages, and different post-ovariectomy times were used in these studies and all of these factors can profoundly influence the ability of estrogens to modulate neuroplasticity in the hippocampus (Miranda *et al*, 1999; Tanapat *et al*, 1999; Tanapat *et al*, 2005; Barha *et al*, 2009). Furthermore, although Premarin largely consists of estrone, it also contains other forms of estrogen including 17β-estradiol and 17α-estradiol. In this study we gave an acute injection of purified estrone thus making direct comparison between these studies problematic. Our results in combination with other studies indicate that therapies using certain doses of estrone may not have the same cognitive-enhancing benefits as other forms of estrogen. Our results further emphasize the need for research conducted in both animals and humans focusing on the effects of different doses of estrone and other estrogens on cognition across the lifespan.

Different Estrogens Did Not Influence Amygdala-Dependent Cued Fear Conditioning

Exposure to different forms of estrogens at different doses did not significantly affect hippocampus-independent cued fear conditioning, consistent with past literature examining behavior across the estrous cycle (Markus and Zecevic, 1997). Different Pavlovian fear conditioning paradigms depend on different brain structures and systems. In particular, cued fear conditioning with a tone does not require an intact hippocampus, whereas contextual fear conditioning does (Kim and Fanselow, 1992; Phillips and LeDoux, 1992). The amygdala is important for both contextual and cued-based fear learning (Phillips and LeDoux, 1992), with both the lateral and central nuclei of the amygdala implicated in cued fear conditioning (see Maren, 2008 for a review). Therefore, it is perhaps not surprising that exposure to estrogens, which influence the hippocampus also influence contextual fear conditioning, but not cued fear conditioning. However, in contrast with our results, Galea *et al* (2001) found that pharmacologically high levels of estradiol disrupted acquisition of the conditioned place-preference task, an associative learning task in which performance is reliant on the integrity of the basolateral amygdala. There are a number of differences between these two tasks that may account for these discrepant findings (Galea *et al*, 2001; this study), such as the different types of motivation underlying each task, aversive *vs* appetitive, and the fact that performance on each task relies to different extents on different nuclei of the amygdala. It may be that aversive learning is more natural and ethologically valid and, therefore, naturally occurring hormones, such as estrogen, would be expected to have a greater influence on tasks that involve this type of motivation. There is evidence that the different nuclei of the amygdala can respond differentially to estradiol (Osterlund *et al*, 1998; Schiess *et al*, 1988; Womble *et al*, 2002), which could contribute to discrepancies between studies. For example, different nuclei of the amygdala differentially contain ER subtypes (ERα and ERβ; Osterlund *et al*, 1998) and estradiol reduces excitatory postsynaptic potential (EPSP) in the basolateral amygdala, but increases EPSP occurrence in the medial nucleus of the amygdala (Schiess *et al*, 1988; Womble *et al*, 2002). Furthermore, infusion of estradiol into the medial amygdala has antianxiety and antidepressant effects in ovariectomized female rats (Frye and Walf, 2004). Therefore, it may be that estradiol has different effects on amygdala-based learning depending on the nuclei being recruited by the task and/or the different tasks being performed.

Estrone and Estradiol Increase Synaptophysin Expression in the CA3 Region of the Dorsal Hippocampus

Synaptophysin is a presynaptic protein that is a critical component of synaptic vesicle exocytosis and has been identified as a molecular correlate of learning and memory. In this study, increases in synaptophysin levels were only seen in the CA3 region of the hippocampus after administration of the middle and high doses of estrone and the middle dose of 17β-estradiol compared with controls. Our results are consistent with a study showing that 8 days of treatment with 17β-estradiol increased synaptophysin expression in the CA3 region of the hippocampus *in vitro* (Rune *et al*, 2002). In contrast, short-term (2 days) treatment with 10 μg estradiol benzoate led to increases in synaptophysin expression in only the CA1 region of the dorsal hippocampus in ovariectomized female rats (Brake *et al*, 2001). Estradiol benzoate is a conjugated form of 17β-estradiol and is metabolized at a much slower rate. Therefore, discrepancies in our results and those found by Brake *et al* (2001) could reflect a number of differences

between the studies (different estrogens, timing, etc.). Interestingly, long-term (4 weeks) treatment with 17β-estradiol increased the ovariectomy-induced reduction in synaptophysin expression in the CA1 region (no other region was examined) of the whole hippocampus in ovariectomized female rats (Sharma *et al*, 2007), indicating that longer-term treatment with estradiol may affect other regions of the hippocampus.

In this study estrogens increased synaptophysin expression only in the CA3 region of the hippocampus. The CA3 region of the dorsal hippocampus is preferentially associated with acquisition, whereas the CA1 region is associated with retrieval of contextual fear memory (Lee and Kesner, 2004). Furthermore, studies suggest that it is the NMDA receptors in the CA3 region, particularly in the dorsal hippocampus, that are involved in the rapid, one-trial encoding of complex associations between contextual stimuli (Cravens *et al*, 2006; Rajji *et al*, 2006).

The changes in synaptophysin levels seen in this study do not consistently reflect behavioral changes, as the middle dose of 17β-estradiol and estrone impaired contextual fear conditioning and the high dose of estrone did not affect contextual fear conditioning, but all three treatments increased synaptophysin levels in the CA3 region. Furthermore, we did not find any significant correlations between synaptophysin levels and amount of freezing. This is in contrast with one study, which found that increases in synaptophysin expression with chronic 17β-estradiol were associated with enhanced spatial reference memory in older mice (Frick *et al*, 2002). In this study synaptophysin expression was assessed 4 h after testing and 30 h after acute hormone treatment. Thus, it is possible that in this study a consistent association between contextual fear conditioning performance and synaptophysin expression in the hippocampus may be found if other time points were examined. Furthermore, it has been shown that training in a hippocampus-dependent task interferes with the ability of estradiol to increase spine density in the dorsal hippocampus (Frick *et al*, 2004). Therefore, our results could also be reflecting an interference of training on estrogen's ability to enhance synaptophysin.

CONCLUSIONS

This study shows that acquisition of hippocampus-dependent contextual fear conditioning is influenced by 17β-estradiol, estrone, and 17α-estradiol in a dose-dependent manner, with a low dose of 17β-estradiol and 17α-estradiol enhancing and a middle or high dose of either form of estradiol impairing contextual fear conditioning. Interestingly, impairments in contextual fear conditioning were seen with a middle dose of estrone. These results are particularly important in lieu of recent findings that estrone-based hormone replacement therapies are ineffective in preventing cognitive decline in post-menopausal women. Our results emphasize the need for further systematic research into the effects of different doses of estrone in both human and animal models of menopause. Furthermore, we found that estrone and 17β-estradiol, but not 17α-estradiol, upregulated synaptophysin expression in the CA3 region of the hippocampus, which was uncoupled

with the behavioral findings. Importantly, we have shown that a low physiological dose of both 17β-estradiol and 17α-estradiol can enhance hippocampus-dependent learning and memory. This could have therapeutic importance, as it may be possible to use lower doses of estrogens in hormone replacement therapies. Our results also suggest that 17α-estradiol is as potent as 17β-estradiol in influencing cognition. Therefore, future research is required to determine whether it may be possible to use 17α-estradiol, as well as lower doses, in hormone replacement therapies potentially circumventing some of the adverse effects associated with current therapies.

DISCLOSURE

The authors declare no conflict of interest.

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