Males show stronger contextual fear conditioning than females after context pre-exposure.

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Abstract

Estradiol affects the structure and function of the hippocampus. We have found that repeated estradiol affects neurogenesis and cell death in the hippocampus of adult female, but not male rats. In the present study we sought to determine whether using the same regimen of estradiol would influence hippocampus-dependent behaviour. Adult male and female rats were given estradiol or sesame oil for 15 days, and then tested using a contextual pre-exposure paradigm in which performance depends on the hippocampus. The time spent freezing displayed by rats was scored on subsequent days in (1) the training context, (2) a novel context in which rats had never been shocked, and (3) the training context a second time. Irrespective of treatment, males showed stronger memory for the context by exhibiting greater freezing in both the training context exposures and the novel context. Previous estradiol treatment, in either sex, did not affect the ability to learn and retain information about the training context. However, female rats treated with estradiol and exposed to a novel context after fear conditioning exhibited less freezing behaviour than controls. Taken together, our results demonstrate that gonadectomized male rats outperform females, regardless of previous treatment with estradiol, on a hippocampus-contextual fear conditioning test, and that previous estradiol treatment has a subtle effect on performance in female but not male rats.

Keywords: sex differences, estradiol, fear conditioning, context learning, novelty, hippocampus, gonadectomy, context fear conditioning, amygdala

Sex of subject and gonadal hormones affect performance on a variety of learning and memory tasks (for reviews, see [1, 2]). In general, males tend to outperform females on tasks that are considered hippocampus-dependent (for reviews, see [2, 3]) or amygdala-dependent [4-6]. Contextual fear conditioning is dependent on the integrity of the hippocampus and amygdala [7, 8] and performance is influenced by sex and estradiol levels. For example, intact male rats show more rapid conditioning and greater retention of contextual fear (i.e. greater time spent freezing) than intact females [9]. This sex difference is related to ovarian hormone levels, as females in proestrus spend less time freezing than females in estrus [10], and ovariectomized rats freeze more than estradiol-treated females, but to the same extent as intact males [11]. Estradiol modulates the inhibition of fear [12] and extinction of contextual fear in females [13, 14]. Estradiol also improves retention of passive avoidance in male rats [15], but inhibits passive avoidance in females [16]. Thus estradiol modulates performance on a variety of fear conditioning tasks, and can affect performance in both sexes.

The ability of estradiol to modulate fear conditioning may be related to estradiol-induced neuroplasticity in the hippocampus and/or amygdala. In the hippocampus of the adult rat, estradiol increases synaptogenesis in the hippocampus of females [17], but not males [18, 19]. Repeated estradiol treatment also decreases the survival of new neurons generated in the dentate gyrus of the female, but not male, hippocampus [20]. In the amygdala, there are sex differences in the size, morphology and responsiveness to sex steroids within various nuclei. For example, the volume of subnuclei of the medial amygdala is larger in males than females [21-23]. Dendritic spine density in the posteromedial cortical amygdala decreases in males, but not in females after gonadectomy [24]. Within the basolateral nucleus of the amygdala, which is involved in fear conditioning, the volume and total number of neurons are similar in males and

females [25]. However, males have higher spine density in principle neurons [26] and a lower number of GABA-immunoreactive neurons than females in this region [27]. Adult male and female rats have similar distributions of estrogen receptor alpha (ERα) in the hippocampus and amygdala [28-30], suggesting a similar capacity to respond to estradiol in both sexes. Estradiol is normally present in these regions in both sexes [31-33], in similar concentrations in both males and females [34]. However, estradiol given to gonadectomized rats has region- and sex-specific effects on tissue estradiol concentration [34]. In the amygdala, repeated administration of estradiol increases levels in both sexes to levels above those found in intact animals, whereas estradiol levels in the hippocampus are increased more in males than females [34]. Thus estradiol may play a role in mediating sex differences in cognitive tasks involving these regions.

Our group and others have demonstrated effects of chronic estradiol on the hippocampus and hippocampus-dependent behaviours (e.g. [35-43]). The effects of estradiol to modulate hippocampus-dependent behavior depend on dose [44], age [45], continuous or intermittent exposure to estradiol [46] and timing of exposure of estradiol in relation to learning [47-49]. For example, high estradiol can impair hippocampus dependent learning when estradiol is on board at the time of learning [50] or can facilitate learning if administered after training [47-49]. Intact rats are not often exposed to continuous high levels of estradiol, but as they age females typically enter persistent estrus and then persistent diestrus, both stages with continuous moderate serum levels of estradiol [51]. In addition, estrogens are frequently administered to women over relatively long periods of time (weeks to months at a time) in a variety of hormone replacement therapy regimens (e.g. [52-55]). It is therefore important to elucidate what effects such treatments might have on the brain and cognition. In the present study we examined whether 3 weeks of previous exposure to estradiol altered hippocampus-dependent learning and memory.

We sought to determine whether 1) there was a sex difference in contextual fear conditioning using a task that more heavily relies on the hippocampus and 2) previous repeated estradiol altered performance differentially in males versus females in context pre-exposure contextual fear conditioning. We used a contextual pre-exposure protocol which is a contextual fear conditioning task that cannot be performed if the hippocampus is compromised at any one or multiple stages of training and testing, unlike other contextual fear conditioning tasks [56]. To our knowledge this is the first use of this particular task to investigate sex differences in contextual fear. We administered repeated estradiol injections to gonadectomized rats of both sexes and tested them using a contextual pre-exposure fear conditioning paradigm [57, 58]. We included additional post-training test phases and a novel context phase and a cued-conditioning training phase, to determine whether any differences in freezing during the contextual conditioning/testing phases could be explained by effects on amygdala-based learning. In concordance with past experiments [9, 11, 20] we expected overall sex differences, favoring males, in contextual fear conditioning. we expect estradiol to have little or no effect on the freezing behavior of males [20].

Materials and methods

All experiments were conducted in accordance with the Canadian Council on Animal Care guidelines regarding appropriate treatment of animals and were approved by the University of British Columbia. Every effort was made to minimize the number of animals used per group and to minimize the suffering of animals used throughout all experimental procedures.

Experimental Subjects

Sixteen male and sixteen female adult (80-90 days old) Sprague-Dawley rats (Charles River Canada, Quebec, Canada) were kept on a 12h:12h light/dark cycle (lights on at 0700h), housed in same-sex pairs in opaque polyurethane bins (48 x 27 x 20 cm) with aspen chip bedding and Purina rat chow and tap water ad libitum.

Procedure

All subjects (males: n = 8 per group, females: n = 16 per group) were gonadectomized under isoflurane anaesthesia one week after their arrival in the colony. Briefly, all rats were anaesthetized with 1-chloro-2, 2,2-trifluoroethyl difluoromethyl ether (isoflurane) using an initial flow rate of 4% and a maintenance flow rate of 2% during surgery. All females were bilaterally ovariectomized through bilateral flank incisions, and all males were bilaterally castrated through an incision in the scrotal sac.

Eight days after surgery (Day 1), rats received s.c. injections of either estradiol benzoate (EB; 33 μg/kg, dissolved in 0.1 mL sesame oil) or sesame oil (vehicle, 0.1 mL) each day for 15 consecutive days. All animals were injected between 1000 h and 1230 h each day. This regimen has been previously shown to affect hippocampal neurogenesis in the female rat [20] and to increase estradiol levels in the serum and the brain to similar levels in male and female rats [34]. The remainder of the experimental timeline is described below, and shown in Figure 1.

Apparatus

All training and testing occurred in two identical operant conditioning training chambers (30.5 cm x 24.1cm x 21.0 cm) enclosed in sound-attenuating boxes (Med Associates Inc., St. Albans VT, USA). The chambers were constructed of aluminum (two side walls) and Plexiglas

(rear wall, ceiling, hinged front door). Each chamber was illuminated by a single 100-mA house light located in the top center of one wall. Auditory stimuli were delivered via a speaker connected to a programmable audio generator (ANL-926, Med-Associates) located in the top-left corner. Inside each chamber was a stainless steel grid floor (19 4.8 mm rods spaced 1.5 cm apart) wired to an electrical source for the delivery of footshock. The chambers were situated on a load-cell platform that recorded chamber displacement in response to the rats' motor activity as output digitized every 200 ms using the Threshold Activity software (Med-Associates). Locomotor activity was quantified by the raw load cell values (range = 0-100), and freezing behaviour quantified by calculating the number of load cell values below a threshold determined by comparing load cell output with an observer's ratings of freezing behaviour, i.e. the cessation of all movement with the exception of respiration-related movement. In the current study, this threshold was a load cell output of 5. To exclude momentary bouts (i.e. < 1 s) of inactivity, freezing was only scored after five or more contiguous observations below the freezing threshold (for further detail, see also [59, 60]). The time spent freezing displayed by rats was scored on subsequent days in (1) the training context, (2) a novel context in which rats had never been shocked, and (3) the training context a second time. During all conditioning and testing sessions, the rats' activity was monitored continuously using live video feed, via a small surveillance camera mounted above each chamber, and the data acquisition system described above. We confirmed the accuracy of threshold output of freezing behavior by manually scoring videotaped sessions, during which freezing was defined as the cessation of all movement with the exception of respiration-related movement.

The percentage of time spent actively exploring the chamber was quantified by calculating the number of load cell values above an 'activity threshold' determined by comparing

load cell output with an observer's ratings of exploratory behaviour. "Exploratory behaviour" included moving around the chamber and rearing, and excluded freezing, standing still, and grooming. In the current study, this threshold was defined as a load cell output of 10.

Procedures

Pain Sensitivity

Acute estradiol administration can affect nociception (e.g. [61, 62]), and this could act to affect performance on a fear conditioning task by changing the saliency of the shock administered. Therefore, three hours prior to training in the operant chambers (Fig. 1), each rat was subjected to a thermal pain sensitivity test [63-65]. Briefly, all male rats and half of the female rats were placed individually on a hotplate at 50°C, boxed in on all four sides by a cardboard wall 7.6 cm high. The length of time taken for each rat to lick one of its paws was recorded, and the rat immediately removed from the hotplate back to its home cage. Any rat remaining on the hotplate after 120 seconds was removed manually and its score recorded as 120 seconds (one oil-treated male and one estradiol-treated male). This was done to determine whether any treatment effects or sex differences found in performance on the fear conditioning tasks could be accounted for by differences in nociception.

Contextual fear conditioning

To examine the effects of estradiol on contextual conditioning we used a procedure used by Rudy et al. [56]. Briefly, rats were transported in clear cages to a holding room prior to coming into the testing room. Rats were exposed to conditioning chambers with two distinct odour cues (almond and orange, or cinnamon and rum extract), each presented on the end of a

cotton swab affixed to opposite corners of each chamber. To ensure that the specific odour combination used did not influence group results, each combination was used for half the rats in each group, and the odour combination switched for each rat during exposure to the 'novel' context (described below). One house light was presented on either the left or the right side of the box, and either the back wall or the front door to the box was covered with brown wax paper to further distinguish the training and novel contexts.

In the afternoon of the day following the last injection of estradiol or oil (day 16, beginning at 1400h), rats were transported from the holding room to the conditioning chamber. Each rat was lifted from the transport bin to the conditioning chamber, allowed to explore the chamber for 5 minutes, placed back into the transport bin and returned to its home cage. Seven minutes after the end of its first exposure to the conditioning chamber, each rat was again returned to the chamber to explore for an additional 5 minutes. This second pre-exposure was added to ensure good retention of the association between transport cues and the training context, so that rats could form an association between a shock given immediately in the training context and that context [66]. After two exposures to the chamber, each rat was returned to its home cage and transported back to the colony room.

The next day (day 17), 24h after initial exposure to the conditioning chamber, each rat was placed in the chamber and given an immediate footshock (2s, 1.80 mA) through the chamber floor. Each rat was returned to its home cage immediately upon conclusion of the footshock. Rats were tested for their response to the conditioning context 24h after they received a footshock (day 18). Each rat was placed in the chamber for 2 minutes, and the percentage of time spent freezing recorded as an indication of fearfulness in response to the context

(conditioning chamber). Performance on this task depends on the integrity of the hippocampus [56, 58].

Novel context and re-exposure to context chamber

In the morning of day 19 (beginning at 0730h), each rat was transported in an empty opaque polyurethane bin (48 x 27 x 20 cm) to a different conditioning chamber, with different odour distribution and types, and lighting and wall coverings distinct from the context in which they had been shocked. Each rat was permitted to explore the novel chamber for 2 minutes, and its freezing behaviour recorded as described above. Immediately following the session in the novel chamber, each rat was again transported in clear Plexiglas bins to the chamber in which it had been previously shocked for 1 minute, and its freezing behaviour scored. Rats were returned to the colony room until 1400h, when cued fear conditioning training began.

Cued Fear Conditioning

The afternoon of day 19 (starting at 1400h), rats (all males and half of the females) were transported to a conditioning chamber distinct from any to which they had been previously exposed. This chamber had no added odour cues, the outer box doors were shut (so the testing room was not visible to the rat), and the inner chamber was lit by one of the 'stimulus' lights on one wall of the chamber. Over the course of a 7-minute session, each rat received 3 pairings of a 10s tone (2 kHz, 80 dB) and a 2 s, 1.10 mA footshock delivered during the final 2s of the tone. The following day (day 20), each rat was placed in a conditioning chamber made distinct by variations in lighting and textures within the box. The freezing behaviour of each rat in this chamber was recorded over the course of 7 minutes, including three 15 second long tones (15

sec, 2 kHz; 80 dB) that matched the tones previously paired with footshock. Performance on this task is dependent on the integrity of the amygdala [56, 67].

Behavioural Recordings

For all procedures we used the Threshold Activity Software version 3.10 (SOF-806, Med Associates Inc.) for behavioural data recording (i.e. percentage of time spent freezing) during testing sessions. The subjects were tested and scored for the first two minutes of each training and testing session, and the time spent freezing converted to percentage of total time analyzed. Rats were excluded from analyses if they froze for more than 10% of the time during pre-exposure sessions (one oil-treated female, one estradiol-treated female, and one oil-treated male).

Data Analyses

For the pre-exposure condition dependent variables were analyzed using a repeated-measures ANOVA with sex (male, female) and treatment (oil, EB) as between-subjects factors, and session (first and second exposure) and time (first and second minute) as within-subjects factors. Percentage of time spent freezing and overall activity were each analyzed using a repeated-measures ANOVA with sex (male, female) and treatment (oil, EB) as between-subjects factors, and time (first and second minute of each test) as the within-subjects factor for the contextual (test 1), novel and cued conditions. For the second exposure to context a repeated-measures ANOVA with sex (male, female) and treatment (oil, EB) as between-subjects factors was conducted on the percentage of time spent freezing. Statistica 8.0 (Statsoft, Tulsa, OK, USA) was used for all analyses, and a significance level of $\alpha = 0.05$ was used. Post-hoc comparisons utilized the Newman-Keuls procedure. Based on our findings in a previous study

on hippocampal neurogenesis [20] we expected to find treatment effects on female behaviour in the current study. We therefore ran additional *a priori* comparisons of estradiol- and oil-treated females within each phase of training and testing. The significance level for the a priori tests was set using a Bonferroni correction for each analysis, as specified below.

Results

Pain sensitivity

To determine whether there were group differences in pain sensitivity that might affect our results, we first subjected rats to a thermal pain sensitivity test. Males took longer than females to escape the hotplate ($F(1,26) = 5.04$, $p \leq 0.03$). There was no significant main effect of treatment ($p \leq 0.71$) and no significant sex x treatment interaction effect ($p \leq 0.69$) (Table 1). Because of the sex effect on pain sensitivity we used the time taken to escape the hotplate as a covariate (continuous predictor) in all subsequent analyses involving freezing.

Pre-exposure Freezing Behaviour

Repeated-measures ANOVA on the percentage of time spent freezing during pre-exposure sessions revealed that rats exhibited more freezing behaviour in the second session than in the first (main effect of session: $F(1,41) = 6.49$, $p \leq 0.002$) (Table 2). Rats also froze more during the second minute of sessions than during the first (main effect of time: $F(1,41) = 4.05$, $p \leq 0.05$). There were no other significant main effects of sex ($p \leq 0.71$) or treatment ($p \leq 0.55$). Several interaction effects also approached significance, including sex x session x treatment ($F(1,41) = 3.10$, $p \leq 0.09$), sex x time ($F(1,41) = 3.67$, $p \leq 0.06$), time x treatment ($F(1,41) =$

2.96, $p \leq 0.09$), and sex x time x treatment ($F(1,41) = 3.40$, $p \leq 0.07$); all other interaction effects were not significant (p's > 0.13). After including the time to escape the hotplate as a covariate, repeated measures ANOVA showed no significant main or interaction effects (covariate effect: $F(1,25) = 0.44$, $p \leq 0.51$; sex x treatment interaction effect: $F(1,25) = 3.03$, $p \leq 0.10$; session x sex x treatment interaction effect: $F(1, 25) = 3.35$, $p \leq 0.08$; all other main and interaction effects $p > 0.14$).

Repeated-measures ANOVA on the percentage of time spent actively exploring the chamber during pre-exposure sessions revealed a significant main effects of session ($F(1,41) = 57.2$, $p \leq 0.0001$) and a significant sex x session x time interaction effect ($F(1,41) = 10.2$, $p \leq 0.003$) (Table 3). There was no significant main effect of sex ($p \leq 0.14$) or treatment ($p \leq 0.44$), and no other significant interaction effects (all p's > 0.15). Post-hoc testing revealed that in general rats were more active during the first pre-exposure session than the second, but this was true for females only in the second minute and for males only in the first minute (females: first minute: $p \leq 0.055$, second minute: $p \leq 0.0002$; males: first minute: $p \leq 0.0001$, second minute: $p \leq 0.17$).

Contextual Fear Conditioning

During the first exposure to the training context after footshock, males froze more than females (main effect of sex: $F(1,25) = 11.0$, $p \leq 0.02$), and rats froze more during the second minute of the contextual test than during the first (main effect of time: $F(1,25) = 26.1$, $p \leq 0.0001$) (Fig. 2A). There were no other significant main or interaction effects (all p's > 0.38). Including time to escape the hotplate as a covariate did not change these results (covariate effect: $F(1,25) = 5.80$, $p \leq 0.02$; main effect of sex: $F(1,25) = 8.92$, $p \leq 0.006$; main effect of time:

$F(1,25) = 14.9$, $p \leq 0.0007$; all other effects $p > 0.27$). A priori comparisons within females revealed no significant effect of treatment on freezing behaviour during the first context test (first minute: $p \leq 0.99$, second minute: $p \leq 0.99$).

Repeated-measures ANOVA on the percentage of time spent actively exploring the chamber during the first contextual testing session revealed that exploration was decreased in the second minute relative to the first ($F(1,41) = 21.1$, $p \leq 0.0001$), but there were no other significant main or interaction effects (all p's ≤ 0.11) (Fig. 2B).

During the second exposure to the training context after footshock, males froze more than females (main effect of sex: $F(1,41) = 20.9$, $p \leq 0.0001$) (Fig. 2C). This effect was not significant after including time to escape the hotplate as a covariate (covariate effect: $F(1,25) = 0.13$, $p \leq 0.73$; main effect of sex: $F(1,25) = 3.33$, $p \leq 0.08$). There was no significant effect of treatment ($p \leq 0.26$), and no sex x treatment interaction effect on the percentage of time spent freezing ($p \leq 0.99$). A priori comparisons within females revealed no significant effect of treatment on freezing behaviour during this second context test ($p \leq 0.30$).

During the second contextual testing session, males were less active than females ($F(1,41) = 5.12$, $p \leq 0.03$), but there were no other significant main or interaction effects (all p's > 0.18) (Fig. 2D).

Novel exposure

When exposed to the novel chamber, males froze more than females (main effect of sex: $F(1,41) = 16.4$, $p \leq 0.0002$) and this effect remained significant after including time to escape the hotplate as a covariate (covariate effect: $F(1,25) = 0.37$, $p \leq 0.55$; main effect of sex: $F(1,25) = 4.53$, $p \leq 0.04$) (Fig. 3A). There was also a main effect of time on the percentage of time spent

freezing ($F(1,41) = 4.48$, $p \leq 0.04$), and a significant sex x time interaction effect ($F(1,41) = 5.02$, $p \leq 0.03$), but these effects were not significant after including time to escape the hotplate as a covariate (both p's > 0.14). There were no other significant interaction effects on freezing behaviour (all p's > 0.37). Post hoc analysis revealed that males froze more than females (first minute: $p \leq 0.0002$, second minute: $p \leq 0.0002$), and that males froze more during the second minute than the first ($p \leq 0.004$) but females did not change their behaviour over time ($p \leq 0.93$) (Fig. 3A). A priori comparisons within females revealed that estradiol-treated females spent less time freezing than their oil-treated counterparts during the second minute of testing ($p \leq 0.01$), but not the first ($p \leq 0.99$).

Repeated-measures ANOVA on the percentage of time spent actively exploring the chamber during exposure to a novel context revealed significant effects of sex ($F(1,41) = 5.15$, $p \leq 0.03$) and time ($F(1,41) = 10.8$, $p \leq 0.002$), but not treatment ($p \leq 0.67$) (Fig. 3B). There was a significant sex x time interaction effect ($F(1,41) = 5.26$, $p \leq 0.03$), but no other interaction effects (all p's ≤ 0.49). Post hoc analysis revealed that males during the second minute of this test were less active than females at any time (both p's ≤ 0.01), and less active than males during the first minute ($p \leq 0.0004$; all other p's > 0.48).

Cued Fear Conditioning

To distinguish between effects of estradiol specific to the hippocampus and any effects on the amygdala, we also tested animals on a cued fear conditioning task. There were no significant main or interaction effects of sex or treatment on the percentage of time spent freezing during testing with the cue (all p's > 0.29) (Fig. 4A). These findings were not altered after including pain sensitivity as a covariate (covariate effect: $F(1,25) = 0.13$, $p \leq 0.73$). A

priori comparisons within females revealed no significant effect of treatment on the percentage of time spent freezing in response to the auditory cue ($p \leq 0.54$).

ANOVA on the percentage of time spent active during exposure to the auditory cue revealed that males were more active than females during this test ($F(1,26) = 7.26$, $p \leq 0.01$) (Fig. 4B). There was no significant effect of treatment ($p \leq 0.98$), and no interaction effect on the activity level of the rats ($p \leq 0.92$).

Discussion

Gonadectomized male rats froze more than females during exposure to both training and novel contexts after pre-exposure contextual fear conditioning, regardless of treatment (Figs. 2A-B, 3A). This suggests that overall males may have formed a stronger association of the training context with an aversive footshock. Prior exposure to estradiol resulted in significant behavioral effects in female rats only, with estradiol-treated females showing less freezing behavior than oil-treated females during post-training exposure to a novel context (Fig. 3A), but not during any other phase of training or testing. This suggests that previous repeated estradiol administration may enhance the ability of female rats to distinguish a novel context from a familiar one with which they have associated an aversive stimulus (e.g. footshock). These findings were not explained by differences between groups in pain sensitivity (Table 1) or exploratory behaviour (Figs. 2C-D, 3B, 4B).

Sex differences in freezing behaviour using a contextual pre-exposure training paradigm

After training in a contextual fear conditioning task relying on pre-exposure to the training context, male rats froze more than female rats, regardless of treatment group (gonadectomized or gonadectomy + estradiol). This is similar to the differences seen in intact rats, as intact male rats outperform intact females in contextual fear conditioning task without context pre-exposure [9]. In our study we found that long-term gonadectomized rats demonstrated a persistent sex difference favoring males. Ovariectomy enhances contextual fear conditioning relative to intact females (10 days after surgery), improving the performance of females to match that of intact males [11]. On the other hand, castration 4-6 weeks prior to testing impairs contextual fear conditioning in males relative to intact males [68]. Taken together, these previous data suggest that ovariectomized female rats may outperform gonadectomized males in a conditioned fear task. However, in the present study we found that castrated males outperformed ovariectomized females, suggesting that longer-term ovariectomy results in worsened performance of female rats. The varying results between studies may be related to the specifics of the training protocols used or to the strain of rat involved. First, as suggested above, our rats were gonadectomized prior to training for considerably longer than the rats used by Gupta et al. (24 days in the present study compared to 10 days in the previous study) [11]. This extended period after gonadectomy may have permitted activation of compensatory responses to the absence of gonadal hormones, or stabilization of the systems and responses affected by gonadectomy, for which 10 days may be insufficient. For example, circulating estrogen concentrations in female rats, though low, gradually increase over time after ovariectomy [69] and the effects of ovariectomy, and estrogen replacement, to influence the hippocampus vary with time since after ovariectomy [17, 35, 70, 71]. Second, other studies [11, 68] have used Long Evans rats whereas we used Sprague-Dawley rats. As albino rats often have

poor eyesight relative to their pigmented counterparts [72], we included olfactory cues in the training and testing contexts to ensure the rats were capable of performing the task presented. Very few studies make use of odor as contextual information, so it is possible that the inclusion of olfactory cues affects the nature or demands of the task. For example, repeated exposure to low levels of formaldehyde fumes enhances fear conditioning to a different odor (orange oil) in male rats, but not females [73]. Thus it is possible that sex differences in the ability to use, and reliance on, odors may affect performance on a contextual fear conditioning task that includes olfactory cues. Third, the 'contextual pre-exposure' protocol we used is unique among contextual fear conditioning tasks, in that the task cannot be learned if the hippocampus is compromised at any one or multiple stages of training and testing [56]. The more common protocol used by Gupta et al. makes it possible to solve the task even if the hippocampus is taken off-line [66]. Although the amygdala is also involved in the contextual task we used, we found no significant difference between the sexes and no significant effect of estradiol treatment on performance in a cued fear conditioning task in which performance requires the integrity of the amygdala but not the hippocampus. This suggests that the sex differences we found in performance on the contextual fear task we used are a result of difference in hippocampal function.

There is evidence to suggest that the sex differences in performance on a fear conditioning task may be related to sex differences in general anxiety levels. For example, proestrous female rats tend to spend more time in the open arms of an elevated plus maze [74-76] or in contact with a conspecific in a social interaction test [75], and less time freezing following a footshock [75] than males. However, these sex differences in measures of anxiety are not necessarily maintained when females are in diestrus [74, 75, 77], suggesting that

activational levels of ovarian hormones modulate this effect. Estradiol administration to ovariectomized females can have variable effects on measures of anxiety. For example, anxiolytic effects of estradiol have been shown in rats given systemic or intra-amygdala estradiol and tested in a open field [78, 79] or elevated plus task [80]. On the other hand, estradiol can reduce the anxiogenic actions of progesterone [16] or diazepam [80] and reduce activity (indicating increased anxiety) in an open field [81]. In the current study, female rats froze less than males after contextual fear conditioning, perhaps suggesting lower anxiety in females (Figs. 2-3). However, the initial response to the conditioning context did not differ between sexes or across groups (Tables 2-3), and previous estradiol treatment did not affect the amount of freezing exhibited in response to the training context after fear conditioning (Fig. 2). Thus it is unlikely that differences in overall anxiety levels can fully explain the differences observed.

Finally, we found that males took longer to respond to a mildly painful stimulus (hotplate) just prior to training (Table 1). Similar sex differences in nociception, with females being more sensitive to painful stimuli than males, have been reported by others in gonadally-intact rats [62, 65] and mice [82]. Consistent with our findings, these differences are maintained after gonadectomy [62, 83]. The amount of freezing displayed after contextual fear conditioning can be affected by the saliency of the stimulus [84-86], so it is important to consider potential group differences in nociception (i.e, perception of stimulus strength) in such a paradigm. In our study, sex or treatment differences in nociception could influence the reaction of rats to re-exposure to the training context. However, we found no estradiol-related differences in nociception, and while males in the current study were less sensitive than females to a painful stimulus, they exhibited more freezing after conditioning. Thus differences in pain sensitivity

alone cannot explain the sex differences seen in freezing behaviour, and in fact emphasize these differences.

Estradiol administered prior to training and testing has minimal effects on fear conditioning

Within each sex in the present study, treatment with estradiol was found to produce little difference in performance on the tests used. As estradiol seems to affect the male hippocampus only minimally [17-20, 87], this outcome was expected in the male rats. However, long-term estradiol administration has a variety of effects on the female hippocampus, including increasing cell proliferation while decreasing both overall cell death and young neuron survival [20]. In the current study, previous repeated exposure to estradiol did not significantly affect contextual fear conditioning using pre-exposure. This finding varies from the results of Gupta et al., who found that acute high levels of estradiol decreased the amount of time spent freezing in ovariectomized rats [11]. However, it should be noted that Gupta et al. administered estradiol to their rats 48 h and 4 h prior to conditioning, whereas we administered estradiol daily for much a longer period of time (15 d) and ceased administration 24 h prior to initial context exposure. The effects of estradiol and/or ovariectomy on a variety of neural parameters have been shown to vary with time [17, 35, 71]. Thus it is not surprising that different timeframes of estradiol administration produce different behavioural effects. Furthermore we have recently shown that there are sex differences in the concentration of estradiol in the hippocampus relative to intact rats after this same regimen of estradiol, with levels of estradiol higher in male rats than intact male rats, but similar to intact rats in females [34]. It is therefore possible that the sex difference in the effects of estradiol is a result of a difference in estradiol uptake into the hippocampus, or of a difference in metabolism of estradiol within the hippocampus.

Prior estradiol administration affects reaction of females to a novel context after fear conditioning

In the present experiment, when exposed to a novel context after training estradiol-treated rats displayed spent less time freezing than oil-treated females. The 'novel' context we used shared some of the individual features of the familiar training context (e.g. general size and shape). Therefore, post-training freezing in a novel context may indicate differences in generalization of the learned context-shock association, or in the ability to discriminate the training and novel contexts. We have previously shown that long-term estradiol administration to female rats increases cell proliferation in the dentate gyrus while decreasing the production of young neurons and overall cell death [20], suggesting that estradiol treatment increases cell turnover. This, coupled with our present results, suggests a role of cell turnover in the detection and encoding of subtle changes in the environment or in the recognition of novel stimuli. Indeed, it has been shown that the dentate gyrus of the hippocampus is particularly important for detecting both novelty and subtle changes in the environment [88, 89]. For example, normally rats will re-explore a familiar object if its position is changed, or if it is moved into a different context (e.g. round enclosure instead of square) [89]. Rats with lesions to the dentate gyrus fail to re-explore objects after either modification is made [89]. New cells produced in the dentate gyrus may play a key role in the ability to detect such changes, as rats demonstrating a higher degree of exploration of a novel environment also have high rates of cell proliferation [88]. Consistent with our results, the *survival* of these cells may be inversely related to exploratory behaviour, as rats that perform more exploratory behaviour in a novel environment have fewer cells overall in the granule cell layer of the dentate gyrus [88]. Network models of adult

neurogenesis suggest that for optimal learning, both young neuron proliferation and neuron death – i.e. neuronal turnover – should be increased in proportion to the 'novelty' of the new information [90]. In the current study, female rats given long-term estradiol treatment, which increases cell turnover [20], seemed better able to recognize a novel environment, as demonstrated post-training by reduced freezing in a novel chamber. It is important to note that training and testing began at the same time point that the measures taken of neurogenesis in our previous study were taken, 24 after the final injection of estradiol. Estradiol administration was not continued through training and testing, so that it was possible to test the effects of previous repeated prior exposure to estradiol in these animals. Although estradiol benzoate is cleared within about 24-48h from the circulation of rats after an acute injection [17, 92, 93], unpublished data from our laboratory suggest that if estradiol benzoate is administered over several days complete clearance does not occur until approximately 4 d after the final injection (see also [94]). Furthermore, estradiol administration can affect performance on learning and memory tasks days after administration (e.g. [94, 95]) and although cell proliferation and cell death rates may change post-estradiol treatment new neurons will likely survive as we have shown before [91]. Together this suggests that changes in new neuron density induced by repeated estradiol treatment may be related to behavioural differences even days after estradiol treatment has ceased. It is important to note that the paradigm we used does not necessarily rule out other estradiol-induced neuroplastic changes, such as synapse density (e.g. [18, 19, 96-100]), electrophysiological characteristics [101, 102], or changes in astrocyte number [103] as factors contributing to the reduction in freezing seen after exposure to the novel context after estradiol treatment.

It is possible that the differences seen in the novel chamber as a result of previous estradiol were the result of differences in extinction of the memory for the association between the conditioning chamber and footshock, that is that estradiol facilitated extinction in females. However, these rats froze to the same extent as their oil-treated counterparts when tested a second time in the training chamber, similar to the performance of rats in [13] on their first extinction trial. This suggests that the difference in the novel chamber was not due to differences in forgetting or extinction of the original learned context-shock association [104], which would be expected to carry over into the second contextual test. Others have also shown that estradiol given to ovariectomized rats 24 h before contextual fear conditioning does not affect the amount of freezing initially shown after training [13]. However, on subsequent extinction trials in the conditioning chamber 24 h apart, rats previously treated with estradiol freeze less than control rats up to 4 days after training, an effect mimicked by systemic or intrahippocampal infusions of an ERβ agonist (diarylpropionitrile, DPN) or an ERα agonist (propyl pyrazole triol, PPT) [13]. Interestingly, estradiol administered immediately after each extinction trial inhibits extinction [13], demonstrating that the effects of estradiol depend on whether it is present or not during testing, training, or both. Unpublished results from our laboratory suggest that in the current study, estradiol was present in moderate amounts in the serum throughout training and testing. Thus estradiol's extinction-enhancing effects (if given prior to extinction) and extinction-inhibiting effects (if given after each extinction trial) may have counteracted each other in this case.

Conclusions

In the present study, we found that gonadectomized male rats outperformed females in a contextual fear conditioning task that is dependent on the hippocampus, regardless of estradiol treatment. In addition, we have demonstrated that previous exposure to estradiol affects post-training performance of female rats, but not males, in response to a novel context by reducing the amount of freezing in the novel context. Along with previous data suggesting increased cellular turnover in response to estradiol treatment [20], this supports the hypothesis that adult neurogenesis in the hippocampus may be related to the recognition of novel contextual information. Finally, our results demonstrate a clear sex difference in performance on a fear conditioning task that relies on context pre-exposure [57, 58], a paradigm which in the future will allow for more detailed analysis of sex differences in hippocampal function.

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Figure Captions

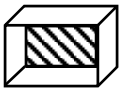
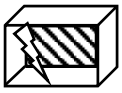
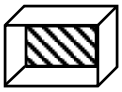
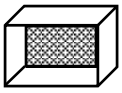
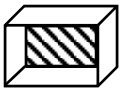
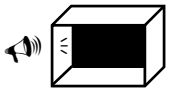
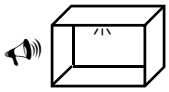
Figure 1: Experimental timeline. Rats were gonadectomized, allowed to recover, and then given daily injections of estradiol or vehicle (oil) for 15 days. The day following the last injection, rats were tested for thermal pain sensitivity, and began training in the conditioning chambers. The chambers were modified at each stage, with different features present in the chamber for the novel context exposure, and for the cued training and testing stages, as described in the text.

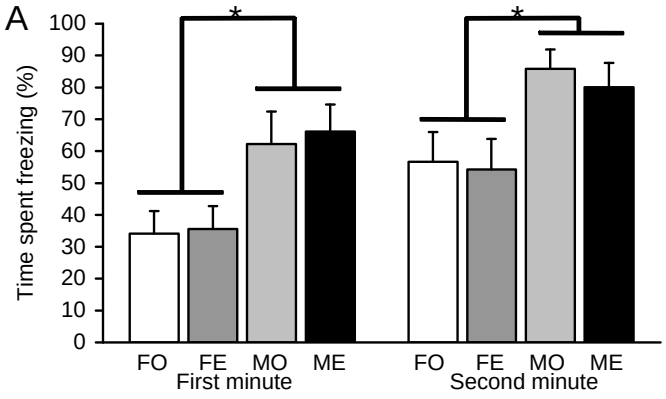
Figure 2: Freezing and exploratory behaviour of rats in a conditioning chamber after contextual fear conditioning of gonadectomized male (M) and female (F) rats previously given repeated injections of oil (O) or estradiol benzoate (E). Overall, gonadectomized male rats froze more than female rats during exposure to the training context, (A) 24h and (C) 36 h after a footshock was received in the same context. There was no significant sex difference in exploratory behaviour levels during (B) the first post-training exposure to the training context, but (D) males spent less time than females exploring during the second post-training exposure. Bars represent group means + standard error of the mean; lines above bars indicate statistically significant comparisons ($*p < 0.05$).

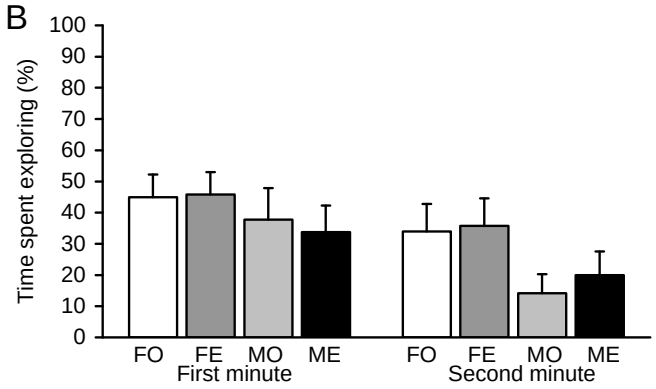
Figure 3: Freezing behaviour and activity levels of conditioned rats in a novel context. Gonadectomized male (M) and female (F) rats previously given repeated injections of oil (O) or estradiol benzoate (E) were placed in a novel chamber after exposure to the familiar training chamber. (A) Males froze more than females, regardless of treatment or time. When exposed to the novel chamber, female rats previously treated with estradiol froze less than their oil-treated

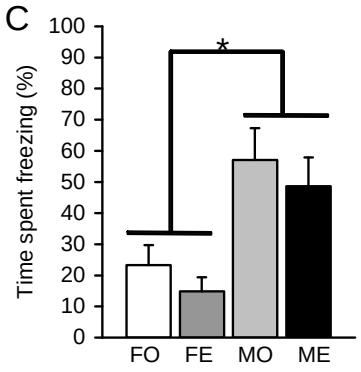
counterparts. (B) Males explored the chamber less than females during the second minute of this test. Bars represent group means + standard error of the mean; lines above bars indicate statistically significant comparisons (*$p < 0.05$).

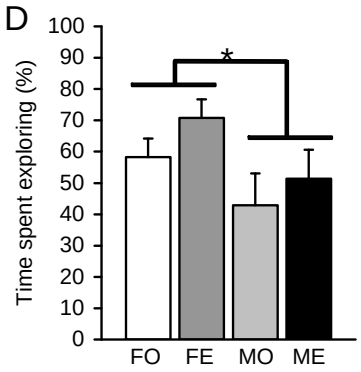
Figure 4: Freezing behaviour and activity levels of conditioned rats in response to an auditory cue that previously predicted footshock of gonadectomized male (M) and female (F) rats previously given repeated injections of oil (O) or estradiol benzoate (E). (A) When presented with the cue, all rats showed robust freezing, but there were no significant sex or treatment effects on this behaviour. (B) Males spent more time exploring the chamber than females during presentation of the auditory cue, but there was no significant effect of treatment on activity level. Bars represent group means + standard error of the mean; lines above bars indicate statistically significant comparisons (*$p < 0.05$).

Day	Treatment	Chamber exposure
Day -7	GDX	n/a
Days 1-15	E2 / oil injections	n/a
Day 16	Pain sensitivity test	n/a
	Pre-exposure	
Day 17	Imm. Shock	
Day 18	Context Test 1	
Day 19	Novel Context	
	Context Test 2	
	Cued Training	
Day 20	Cued Test	


A
Time spent freezing (%)
100
90
80
70
60
50
40
30
20
10
0
*
*
FO FE MO ME
First minute
FO FE MO ME
Second minute


B
Time spent exploring (%)
100
90
80
70
60
50
40
30
20
10
0
FO FE MO ME
First minute
FO FE MO ME
Second minute


C
Time spent freezing (%)
100
90
80
70
60
50
40
30
20
10
0
*
FO FE MO ME


D
Time spent exploring (%)
100
90
80
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60
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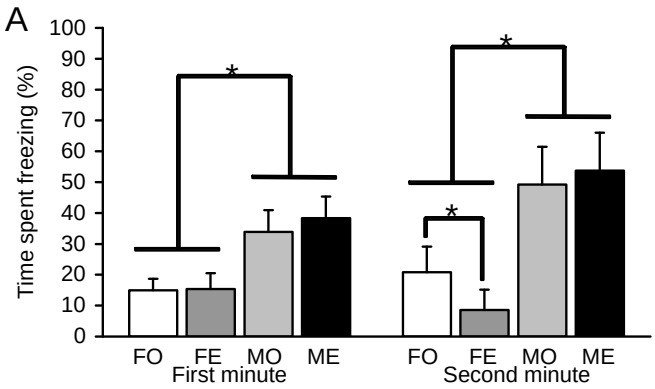
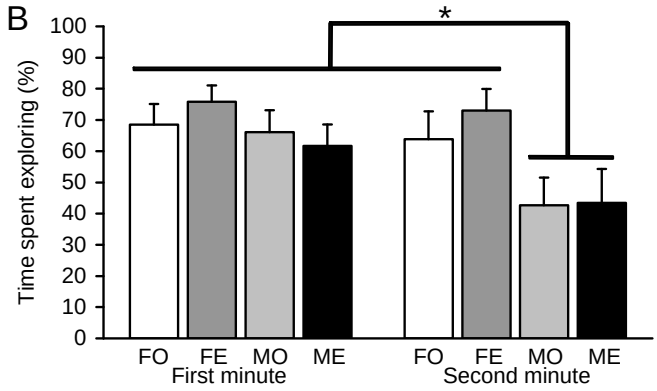
40
30
20
10
0
*
FO FE MO ME


A
Time spent freezing (%)
100
90
80
70
60
50
40
30
20
10
0
*
*
*
FO FE MO ME
First minute
FO FE MO ME
Second minute


B
Time spent exploring (%)
100
90
80
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60
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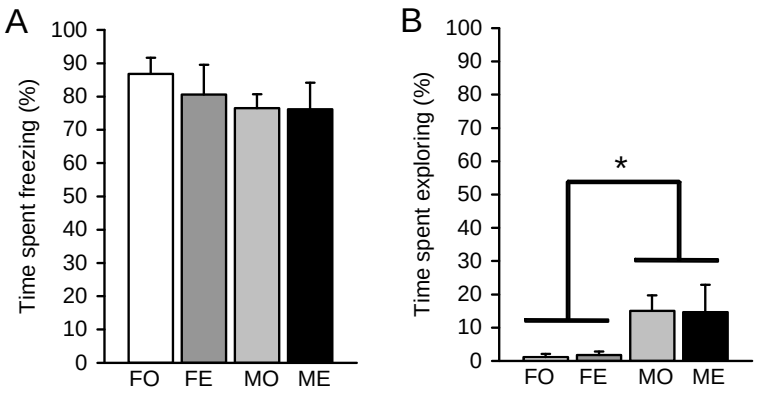
*
FO FE MO ME
First minute
FO FE MO ME
Second minute


A
Time spent freezing (%)
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Time spent exploring (%)
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ME

Table 1: Latency of rats to escape a painful thermal stimulus (50° hotplate) after repeated estradiol injections. Data are presented as mean ± SEM.

Sex	Treatment	Time to escape (s)
Female	Oil	23.0 ± 5.3
	Estradiol	23.3 ± 9.4
Male	Oil	55.7 ± 17.0
	Estradiol	45.9 ± 14.1

Table 2: Percentage of time spent freezing during pre-exposure tests. Data are presented as mean ± SEM.

		First pre-exposure		Second pre-exposure	
Sex	Treatment	First minute	Second minute	First minute	Second minute
Female	Oil	0.02 ± 0.02	0.00 ± 0.00	2.34 ± 1.41	2.19 ± 1.29
	Estradiol	0.15 ± 0.09	0.47 ± 0.34	1.24 ± 0.65	1.23 ± 0.64
Male	Oil	0.24 ± 0.24	0.39 ± 0.25	0.00 ± 0.00	0.42 ± 0.42
	Estradiol	0.00 ± 0.00	0.29 ± 0.19	0.16 ± 0.16	4.57 ± 2.17

Table 3: Percentage of time spent actively exploring chamber during pre-exposure tests.

Data are presented as mean ± SEM.

		First pre-exposure		Second pre-exposure	
Sex	Treatment	First minute	Second minute	First minute	Second minute
Female	Oil	93.7 ± 1.5	91.2 ± 1.7	83.2 ± 3.2	74.2 ± 3.7
	Estradiol	91.1 ± 2.8	91.9 ± 2.1	86.0 ± 3.7	79.1 ± 3.7
Male	Oil	98.2 ± 1.4	91.8 ± 2.7	82.8 ± 3.2	81.9 ± 9.0
	Estradiol	99.4 ± 0.3	94.1 ± 3.2	78.5 ± 3.7	91.0 ± 3.6