

## SEX DIFFERENCES IN CONTEXTUAL FEAR CONDITIONING ARE ASSOCIATED WITH DIFFERENTIAL VENTRAL HIPPOCAMPAL EXTRACELLULAR SIGNAL-REGULATED KINASE ACTIVATION

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**Abstract**—Although sex differences have been reported in hippocampal-dependent learning and memory, including contextual fear memories, the underlying molecular mechanisms contributing to such differences are not well understood. The present study examined the extent to which sex differences in contextual fear conditioning are related to differential activation of the extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK), a protein kinase critically involved in memory formation. We first show that male rats exhibit more long-term retention of contextual fear conditioning than female rats. During a tone test, females spent more time freezing than males, although both sexes exhibited robust retention of auditory fear learning. Using Western blot analysis, we then show that phosphorylated ERK levels in ventral, but not dorsal, hippocampus are higher in males than females, relative to same-sex controls, 60 minutes after fear conditioning. Post-conditioning increases in ERK activation were observed in the amygdala in both males and females, suggesting a selective effect of sex on hippocampal ERK activation. Together, these findings suggest that differential activation of the ERK signal transduction pathway in male and female rats, particularly in the ventral hippocampus, is associated with sex differences in contextual fear. © 2009 IBRO. Published by Elsevier Ltd. All rights reserved.

**Key words:** context fear, memory, extracellular signal-regulated kinase, ventral hippocampus, sex difference, rat.

The formation of memories involves the activation of protein kinase signaling cascades (Sweatt, 2004). One pathway critical for learning and memory is the extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) cascade (Adams and Sweatt, 2002). Learn-

ing in tasks such as fear conditioning, spatial water maze, and object recognition is accompanied by increased ERK activation (phosphorylation) in brain regions necessary for learning and memory (Atkins et al., 1998; Blum et al., 1999; Schafe et al., 2000; Kelly et al., 2003). Although both contextual and auditory fear conditioning are amygdala-dependent, the hippocampus is also involved in contextual fear conditioning (Fanselow and LeDoux, 1999; Sanders et al., 2003). Hippocampal lesions (Kim and Fanselow, 1992; Phillips and LeDoux, 1992; Young et al., 1994) and infusions of cholinergic antagonists, gamma-aminobutyric acid (GABA) agonists, or *N*-methyl-D-aspartate (NMDA) antagonists (Young et al., 1994; Bast et al., 2001; Wallenstein and Vago, 2001) severely impair contextual fear conditioning, while leaving auditory fear conditioning intact. ERK activation in the whole hippocampus has been observed in male rats 60 minutes after contextual fear conditioning (Atkins et al., 1998). Blocking ERK activation, by systemically administering an inhibitor of mitogen-activated protein kinase kinase (MEK, the kinase which directly phosphorylates ERK), impairs long-term memory (LTM) formation of contextual fear (Atkins et al., 1998; Selcher et al., 1999). Further, intraventricular infusions of MEK inhibitors selectively attenuate long-term, but not short-term, contextual fear memory (Schafe et al., 1999; Barrientos et al., 2002). These findings implicate hippocampal ERK activation in the formation of long-term contextual fear memories.

Thus far, however, the involvement of the hippocampal ERK pathway in fear conditioning has been studied solely in male rodents, despite reports that gonadally intact males acquire more contextual fear (as measured by % time spent freezing) than gonadally intact females (Maren et al., 1994; Anagnostaras et al., 1998; Lehmann et al., 1999; Pryce et al., 1999; Gupta et al., 2001; Wiltgen et al., 2001; Kudo et al., 2004). Several observations suggest that this sex difference is not the result of differences in non-mnemonic performance factors (such as activity level, expression of the fear response, or foot-shock sensitivity). For example, Kosten et al. (2006) reported that intact male rats acquire more contextual fear than intact females even when using a measure of fear (ultrasonic vocalizations) that is not dependent on activity. In terms of shock sensitivity, although some studies have reported heightened sensitivity in females (Beatty, 1979), this finding has not been consistent (Podhorna et al., 2002). Furthermore, despite the observations of reduced contextual freezing in females compared to males, sex differences in the duration of post-shock freezing (measured during conditioning) have not been reported (e.g. Gupta et al., 2001), suggest-

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**Abbreviations:** ANOVA, analysis of variance; CREB, CAMP-response element binding protein; DG-GCL, dentate gyrus-granule cell layer; ERK, extracellular signal-regulated kinase; GABA, gamma-aminobutyric acid; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IS, immediate shock; ITI, inter-trial interval; LTM, long-term memory; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; NMDA, *N*-methyl-D-aspartate; pERK, phosphorylated extracellular signal-regulated kinase; STM, short-term memory; tERK, total extracellular signal-regulated kinase.

ing that sex differences observed during context retention tests are not likely due to differences in processing of or sensitivity to the shock. Finally, several studies report that, although females exhibit less contextual freezing than males, they do not exhibit lower levels of auditory freezing (Maren et al., 1994; Anagnostaras et al., 1998; Lehmann et al., 1999). This finding also argues against non-mnemonic factors (such as expression of the fear response or shock sensitivity) accounting for the sex difference in contextual fear.

The notion that sex differences in contextual fear conditioning are mnemonic in nature is also supported by sex differences in hippocampal dendritic and synaptic morphology (Juraska et al., 1989; Madeira et al., 1991; Parducz and Garcia-Segura, 1993), granule cell number (Wimer and Wimer, 1985), and slice excitability (Smith et al., 2002). One study reported increased hippocampal phosphorylation of the transcription factor CAMP-response element binding protein (CREB) in male, but not female, rats following contextual fear conditioning (Kudo et al., 2004). Because ERK activates CREB, similar sex differences may also be observed in hippocampal ERK activity. Because fear conditioning occurs in a single session, alterations in ERK activation can easily be measured during the post-training period when memory formation occurs, thus allowing for identification of a potential mechanism by which previous observations of sex differences in hippocampal physiology and hippocampal-dependent memory formation arise.

The present study was designed to determine if sex differences in the formation of contextual fear memories are associated with sex differences in hippocampal ERK activation. We chose to compare gonadally intact male rats to gonadally intact female rats in order to aid in the comparison to previously published work (Maren et al., 1994; Anagnostaras et al., 1998; Lehmann et al., 1999; Pryce et al., 1999; Gupta et al., 2001; Wiltgen et al., 2001; Kudo et al., 2004). Further, previous observations indicate that there are no differences in contextual fear conditioning between intact and ovariectomized female rats (Gupta et al., 2001; Gresack and Frick, 2007), which suggests that circulating gonadal hormones such as estrogens and progestins do not influence this type of memory in females. As such, females in the present study were not ovariectomized or lavaged (to ascertain stage of the estrous cycle). In addition, when conditioning the rats, we used a moderate training protocol (consisting of three shock presentations, 0.5 mA, 1 s) that falls within the range of weaker (one shock, 0.4 mA, 1 s; Maren et al., 1994) and stronger (multiple presentations of longer, more intense shocks –1.0 mA, 2 s; Anagnostaras et al., 1998) protocols reporting sex differences in contextual fear. This moderate training protocol was used because ERK activation in contextual fear conditioning has previously been established in intact males using similar parameters (Selcher et al., 1999; Schafe et al., 2000). In the first experiment, retention of contextual and tone fear was measured by freezing in intact male and female rats approximately 1 and 24 hours after conditioning. Then,

in the second experiment, we used Western blotting to measure the magnitude of conditioning-induced ERK activation in whole, dorsal, and ventral hippocampus in both sexes. ERK activation was also measured in the amygdala to investigate the regionally specific effects of sex on fear conditioning-induced increases in ERK activity.

## EXPERIMENTAL PROCEDURES

### Subjects

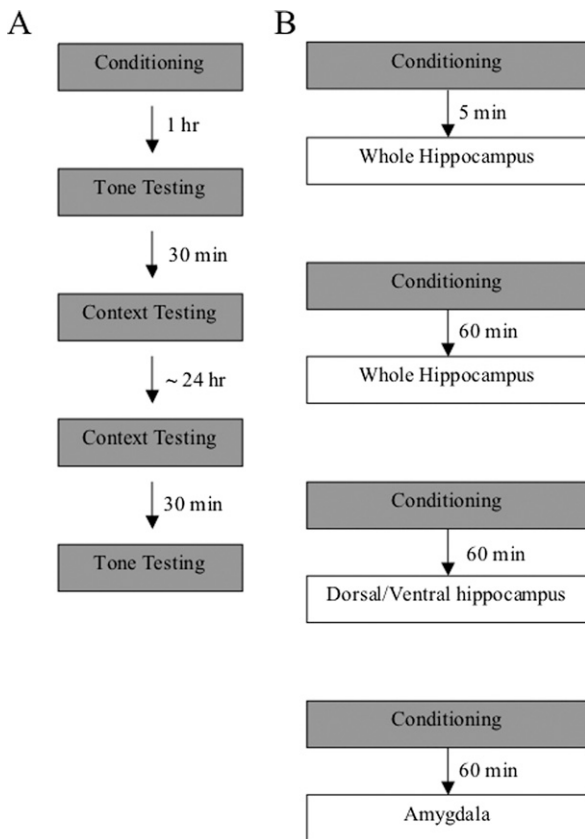
Naive male and female Long-Evans rats purchased from Harlan (Indianapolis, IN, USA) at 10 weeks of age were individually housed upon arrival. All rats were housed in a room with a 12-h light/dark cycle (lights on at 07:00 h). Food and water were available *ad libitum* for the duration of testing. Testing occurred at approximately 11 weeks of age. The number of animals used and their suffering was minimized in all experiments. All procedures followed the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and were approved by the Animal Care and Use Committee of Yale University.

### Behavioral procedures

**Apparatus.** For fear conditioning studies, training and testing were carried out in four identical chambers (12-inch W×10-inch D×12-inch H) each situated inside a sound attenuating box (Model H10-24A, Coulbourn Instruments, Allentown, PA, USA). Female rats were always run in chambers 1 and 2, whereas males were always run in chambers 3 and 4. A house light (Coulbourn Model H11-01R) illuminated the chamber during all conditioning sessions. A shock floor (Model H10-11R-TC-Sf) consisting of 18 stainless steel rods was wired to an animal shocker for foot-shock delivery. The floors were cleaned with 70% ethanol and the pans were washed with water prior to all conditioning and testing sessions.

**Fear conditioning.** The fear conditioning protocol was adapted from previously published protocols (Schafe et al., 1999; Selcher et al., 1999). A diagram outlining the fear conditioning procedure is shown in Fig. 1A. Subjects were randomly assigned to either a “Delay” group or an “Immediate Shock” (“IS”) group. On the day of conditioning, all rats were transported in their home cages from the colony room to a small hallway located adjacent to the testing room. Rats in the Delay group were placed in the conditioning chamber and, following an acclimation period (170 s), were presented with a tone (20 s, 75 dB, 5 kHz) that coterminated with a foot shock (1 s, 0.5 mA). A total of three tone–shock pairings were presented with an inter-trial interval (ITI) of 100 s. When 20 s had elapsed after each shock presentation, the post-shock freezing measurement began and continued for 20 s. Rats were removed from the chamber and returned to their home cage 40 s after the last shock.

Subjects in the IS condition received three foot shocks (0.5 mA, 1 s duration, separated by 500 ms) immediately following placement in the chamber. No tones were presented with the IS protocol and subjects were removed from the chamber 240 s after the final shock. Because the IS group was shocked immediately upon entering the conditioning chamber, they should not have had sufficient time to form a complete representation of the context and associate it with the shock. Thus, one would expect to observe minimal durations of freezing during context retention tests. This phenomenon, in which a very brief context exposure results in minimal conditioned fear, is known as the IS deficit (Fanselow, 1986, 1990). The IS group was included in the study to provide a



**Fig. 1.** (A) Timeline of context and tone tests following fear conditioning. Subjects in the Delay condition received three tone-shock pairings during conditioning and were tested for short-term and long-term tone and context retention at the time points indicated in the figure. Subjects in the IS condition received three shock presentations immediately following placement in the conditioning chamber. They were tested for short-term and long-term context retention tests only at the time points indicated in the figure. (B) Timeline of Western blotting assays following fear conditioning. In the Delay and IS conditions, ERK activation in the whole hippocampus was assayed 5 or 60 min after conditioning, and ERK activation in the dorsal hippocampus, ventral hippocampus, and amygdala were assayed 60 min after conditioning. ERK activation in the ventral hippocampus was also assayed in a second IS group (IS II) and a Tone group 60 min after conditioning.

basis for comparing the level of context fear memory in the Delay groups.

Shortly after conditioning, subjects were returned to the chamber for tone (Delay group only) and context (Delay and IS groups) retention tests designed to assess short-term memory (STM). The tone test occurred 1 hour after conditioning. The chambers were altered on several dimensions (tactile, odor, visual) in order to minimize generalization from the conditioning context. Specifically, the grid floor was covered with a piece of black Plexiglas. The Plexiglas was cleaned with peppermint soap prior to being placed in the chamber. Additionally, the house light was turned off for the duration of the tone retention test. Subjects were presented with three tones (20 s each, 140 s ITI) and freezing was scored during each tone presentation. Baseline freezing during a 20 s period prior to the first tone presentation was also measured to assay for generalization of fear. Subjects were returned to their home cage immediately following the final tone presentation. Thirty minutes after completing the tone test, subjects were returned to the conditioning chamber for the context test. The features of the chamber were identical to those used

during conditioning. Freezing was scored for a total of 1 min over three trials (20 s/trial, 140 s ITI).

The next day, subjects were given a second context and tone retention test to assess long-term memory (LTM). The context test was given 24 hours after conditioning (i.e., 22.5 hours after the context test on the previous day, Fig. 1A). The tone test was given 30 minutes after completion of the context test in order to counterbalance the order of retention test presentation, as in Schafe et al. (1999), and control for potential carryover effects. The protocol for both tests was identical to those described above, with the exception that the tone was presented 10 times during the tone test. Performance during the tone test was assessed during the first three tone presentations only in order to maintain consistency with the STM test.

**Behavioral data collection and analysis.** All conditioned stimulus (tone) and unconditioned stimulus (shock) presentations were controlled by the Graphic State Notation Software (Allentown, PA, USA) and delivered via the Habitest Linc system (Coulbourn Model H02-08). The conditioned fear response, freezing, was measured using an activity monitor mounted inside the fear conditioning chamber. Freezing was defined as the absence of any movement except that required for respiration. The activity monitor relied upon emitted infrared technology to continuously detect the subjects' movement throughout testing. Activity counts were automatically collected by the Graphic State program. The raw data counts were exported and transformed into percent freezing using the MATLAB (Natick, MA, USA) statistical program.

All data were analyzed with analysis of variance (ANOVA) using the SuperAnova (Berkeley, CA, USA) statistical program. Freezing during the acclimation (pre-shock) period of the conditioning session was analyzed using a one-way ANOVA with Sex as the independent variable. Freezing during the post-shock period was analyzed using a one-between (Sex) and one-within (Trial) repeated-measures ANOVA. During the context retention tests, freezing was analyzed using a 2 (Sex) × 2 (Condition: Immediate Shock, Delay) ANOVA. Follow-up *t*-tests were used to identify the source of significant interactions. For the tone retention tests, freezing prior to (Pre-tone) and during the tone presentations were analyzed separately using a one-way (Sex) ANOVA. Then, one-between (Sex) and one-within (Tone Exposure: Pre-tone, Tone) repeated-measures ANOVA was used to compare freezing in males and females prior to and during the tone presentation.

### Western blotting procedures

Using Western blotting procedures, sex differences in ERK activation were examined in new sets of male and female rats. Depending on the Western blotting study, subjects were assigned to the Delay condition, or to IS, Naïve, or Tone control groups. Fear conditioning in the Delay and IS groups occurred in the same manner described above. Briefly, rats in the Delay group received three tone-shock pairings (tone: 20 s, 75 dB, 5 kHz; shock: 1 s, 0.5 mA). When 20 s had elapsed after each shock presentation, the post-shock freezing measurement began and it continued for 20 s. Rats in the IS group received three shocks of the same duration and intensity as those given to the Delay group immediately following placement in the chamber. Rats in the Tone group were exposed to three tones (identical to those presented to the Delay group) but no shocks while in the chamber. The Tone group was included to control for observed increases in ERK activity due to tone presentation alone. Subjects assigned to the Naïve condition were not placed in the fear conditioning chambers at any point during conditioning. None of the subjects used in the Western blotting study could be tested for short-term or long-term memory retention because their use in the Western blot assay necessitated that they be decapitated at various time points post-condi-

tioning. Fig. 1B illustrates the time points at which various brain regions were collected after conditioning.

**Sample preparation.** Either 5 or 60 min after conditioning (Fig. 1B), subjects were briefly sedated with CO<sub>2</sub> and killed by decapitation. The brains were immediately removed and placed on ice. Depending on the experiment, the whole hippocampus, dorsal hippocampus, ventral hippocampus, or amygdala was bilaterally dissected on ice. Whole hippocampus was first exposed by removing the overlying cortex. For dorsal and ventral dissections, the whole hippocampus was scored with a surgical blade and visually divided into dorsal and ventral sections (1/3 dorsal, 2/3 ventral). All samples were weighed and stored at –80 °C until homogenization. For amygdala dissections, the entire brain was blocked on dry ice, immediately frozen, and stored at –80 °C until amygdala punches were collected. For punching, coronal sections (400 μm) were first obtained using a sliding freezing microtome. The amygdala was then localized and removed using a 0.5 mm punch tool (Fine Science Tools, Foster City, CA, USA). Tissue punches were confined to the lateral nucleus of the amygdala. All punches were stored at –80 °C until homogenization. Hippocampal and amygdala samples were homogenized in lysis buffer (hippocampal tissue homogenized at 1:50 w/v; amygdala tissue was homogenized in 200 μl of lysis buffer). Total protein amounts in each sample were determined using a Bradford protein assay (Bradford, 1976). Lysis and sample buffers were then added to each homogenate to equate total protein amounts in all samples, and all homogenates were boiled for 4 min prior to assay.

**Western blotting.** Samples from each individual rat were run separately and were electrophoresed on a 10% SDS-polyacrylamide gel, transferred to a PVDF membrane (Immobilon-P; Millipore, Burlington, MA, USA), and blocked in TTBS buffer containing 5% low-fat dry milk. Western blots were incubated with either an anti-phospho-p44/42 ERK/MAPK antibody (Thr202/Tyr204) (1:2000; Cell Signaling Technology, Beverly, MA, USA) or anti-total p44/42 ERK/MAPK antibody (1:1000; Cell Signaling Technology). Blots were incubated with an antirabbit secondary antibody conjugated to horseradish peroxidase (1:20,000; Cell Signaling Technology) and developed using enhanced chemiluminescence (Pierce Biotechnology, Rockford, IL, USA). Densitometry was conducted using Kodak 1 D 3.6 software (New Haven, CT, USA). When assessing changes in ERK activation, phosphorylated extracellular signal-regulated kinase (pERK) levels were first normalized to total extracellular signal-regulated kinase (tERK) levels. Normalized pERK levels in the IS conditions were first expressed as an immunoreactivity percentage relative to same-sex Naïve controls (100%). After determining that IS values did not statistically differ from Naïve values (see Results below), normalized pERK levels in the Delay conditions were expressed as an immunoreactivity percentage relative to normalized same-sex IS controls (100%).

**Data analysis.** Freezing in the Delay groups during the acclimation period was analyzed using a one-way ANOVA with Sex as the independent variable. Post-shock freezing was analyzed using a one between (Sex) and one within (Trial) repeated-measures ANOVA. In analyzing the Western blot data, densitometry was not conducted on p42 (ERK 2) and p44 (ERK 1) bands which only partially appeared on the blot. Statistical outliers (defined as two standard deviations above or below the mean normalized pERK and/or tERK values) were also excluded from analysis. In all hippocampal experiments, ERK immunoreactivity in IS and Naïve groups was first compared using a 2 (Sex)×2 (Condition: IS, Naïve) ANOVA. When examining the extent to which fear conditioning altered ERK expression, ERK immunoreactivity was analyzed using a 2 (Sex)×2 (Condition: Delay, IS) ANOVA. *t*-Tests were used to identify the source of significant interactions.

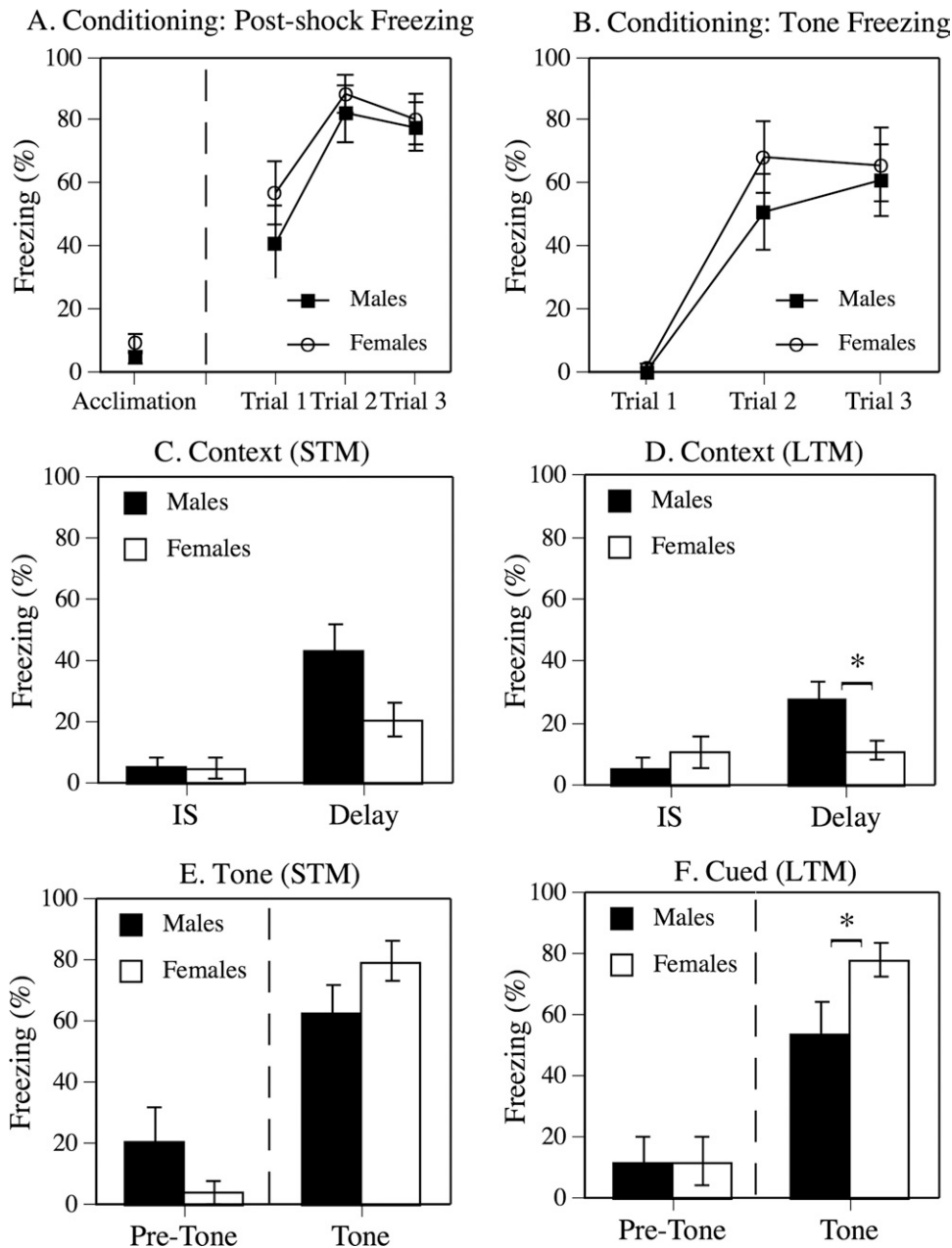
## RESULTS

### Sex differences in context and tone fear conditioning

In our first series of experiments, we examined whether sex differences existed in the retention of fear learning. We used a delay fear conditioning paradigm in which rats received three pairings of tone and shock. Rats were then tested for retention of contextual and tone (auditory) fear memory at two time intervals: approximately 1 and 24 h later. Thus, we were able to examine sex differences in context and tone fear shortly after fear acquisition (~1 h) and following the consolidation of fear learning (~24 h).

Mean percent freezing resulting from the fear-conditioning experiment is depicted in Fig. 2. Males and females did not differ in baseline freezing during the acclimation period prior to the first shock presentation (Fig. 2A). Post-shock freezing in both sexes increased across trials ( $F(2,48)=8.39$ ,  $P=0.007$ , Fig. 2A). The main effect of Sex and the Trial×Sex interaction were not significant, suggesting that both males and females were capable of processing the shock and similarly acquired context fear conditioning. Freezing during each tone presentation also significantly increased across trials in both sexes ( $F(2,48)=39.09$ ,  $P=0.0001$ , Fig. 2B). Neither the main effect of Sex nor the Trial×Sex interaction were significant, suggesting that both sexes processed the tone similarly and were able to form tone-shock associations. During the STM context test (~1 hour after conditioning), the Delay groups exhibited significantly more freezing than the IS groups ( $F(1,42)=20.37$ ,  $P=0.0001$ , Fig. 2C). One of the main differences between the Delay and IS groups was in the duration of context exposure prior to the shock presentation. The longer duration of freezing observed in the Delay group compared to the IS group may be attributed to a difference in context retention. Overall, the main effect of Sex was also significant ( $F(1,42)=4.02$ ,  $P=0.05$ ), with males freezing more than females. Inspection of Fig. 2C suggests that the Delay males, in particular, exhibited more freezing than the Delay females, although importantly, the Condition×Sex interaction did not reach significance, ( $F(1,42)=3.47$ ,  $P=0.07$ ). During the LTM context test (~24 hours after conditioning), the main effect of Condition was significant ( $F(1,42)=5.71$ ,  $P=0.02$ ), with the Delay group freezing more than IS group (Fig. 2D). Although the main effect of Sex was not significant, there was a significant Condition×Sex interaction ( $F(1,42)=5.48$ ,  $P=0.02$ ). *t*-Tests indicated that Delay males froze more than Delay females ( $t_{(24)}=2.51$ ,  $P=0.02$ , Fig. 2C).

The mean percent freezing of the Delay groups prior to and during tone presentations in the STM tone test is shown in Fig. 2E. Males and females did not differ in the percentage of time spent freezing prior to the first tone presentation (pre-tone). Freezing during the tone presentation was significantly higher in both sexes compared to pre-tone freezing ( $F(1,24)=62.47$ ,  $P=0.0001$ ). Minimal freezing observed during the pre-tone period suggests that the tactile, visual, and odor changes made to the chamber for the tone tests produced a reliable context shift and



**Fig. 2.** (A) Mean percent freezing ( $\pm$ SEM) in the Delay groups during the acclimation (pre-shock) and post-shock periods following the three tone-shock pairing trials. (B) Mean percent freezing ( $\pm$ SEM) during each tone presentation. (C, D) Mean percent freezing ( $\pm$ SEM) in the IS and Delay groups for males and females during STM (C) and LTM (D) context tests, \*  $P < 0.05$ . (E, F) Mean percent freezing ( $\pm$ SEM) for the Delay groups during the Pre-tone and Tone periods of the STM (E) and LTM (F) tone tests (Delay male,  $n = 13$ ; Delay female,  $n = 13$ ; IS male,  $n = 10$ ; IS female,  $n = 10$ ).

minimized generalization from the conditioning environment. As a result, freezing observed during the tone presentation can be attributed to the cue. Males and females did not differ in percent freezing during the tone presentation ( $F(1,24) = 2.39$ ,  $P = 0.14$ ) (Fig. 2E). As observed during the first test, there was no sex difference in baseline freezing during the pre-tone period in the LTM tone test (Fig. 2F). Freezing increased significantly during the tone presentation when compared to pre-tone levels of freezing ( $F(1,24) = 49.34$ ,  $P = 0.0001$ ), a finding which did not inter-

act with sex. However, during the tone presentation, percent freezing was greater in females than males ( $F(1,24) = 4.26$ ,  $P = 0.05$ ).

#### Hippocampal ERK activation after fear conditioning is specific to ventral hippocampus and is higher in males than in females

In our behavioral experiments, female rats exhibited a significantly shorter duration of freezing during the LTM context test compared to males. During the LTM tone

**Table 1.** Normalized hippocampal ERK phosphorylation (pERK) in the IS groups expressed as an immunoreactivity percentage relative to normalized pERK in the naïve groups (100%)

Sex, Isoform	% Immunoreactivity			
	Whole hip. 5 min	Whole hip. 60 min	Dorsal hip. 60 min	Ventral hip. 60 min
Male, p42	118.4 ( $\pm$ 21.5)	69.5 ( $\pm$ 14.8)	83.8 ( $\pm$ 9.1)	79.7 ( $\pm$ 6.6)
Female, p42	96.1 ( $\pm$ 19.3)	96.6 ( $\pm$ 19.7)	94.3 ( $\pm$ 15.8)	100.8 ( $\pm$ 6.9)
Male, p44	96.7 ( $\pm$ 13.9)	85.5 ( $\pm$ 19.6)	84.5 ( $\pm$ 11.3)	74.5 ( $\pm$ 10.7)
Female, p44	101.0 ( $\pm$ 40.4)	107.0 ( $\pm$ 23.7)	112.2 ( $\pm$ 21.9)	123.2 ( $\pm$ 30.5)

ERK phosphorylation in the whole, dorsal, and ventral hippocampi of the IS groups did not differ from phosphorylation in the Naïve groups (100%) at either time point for either isoform.

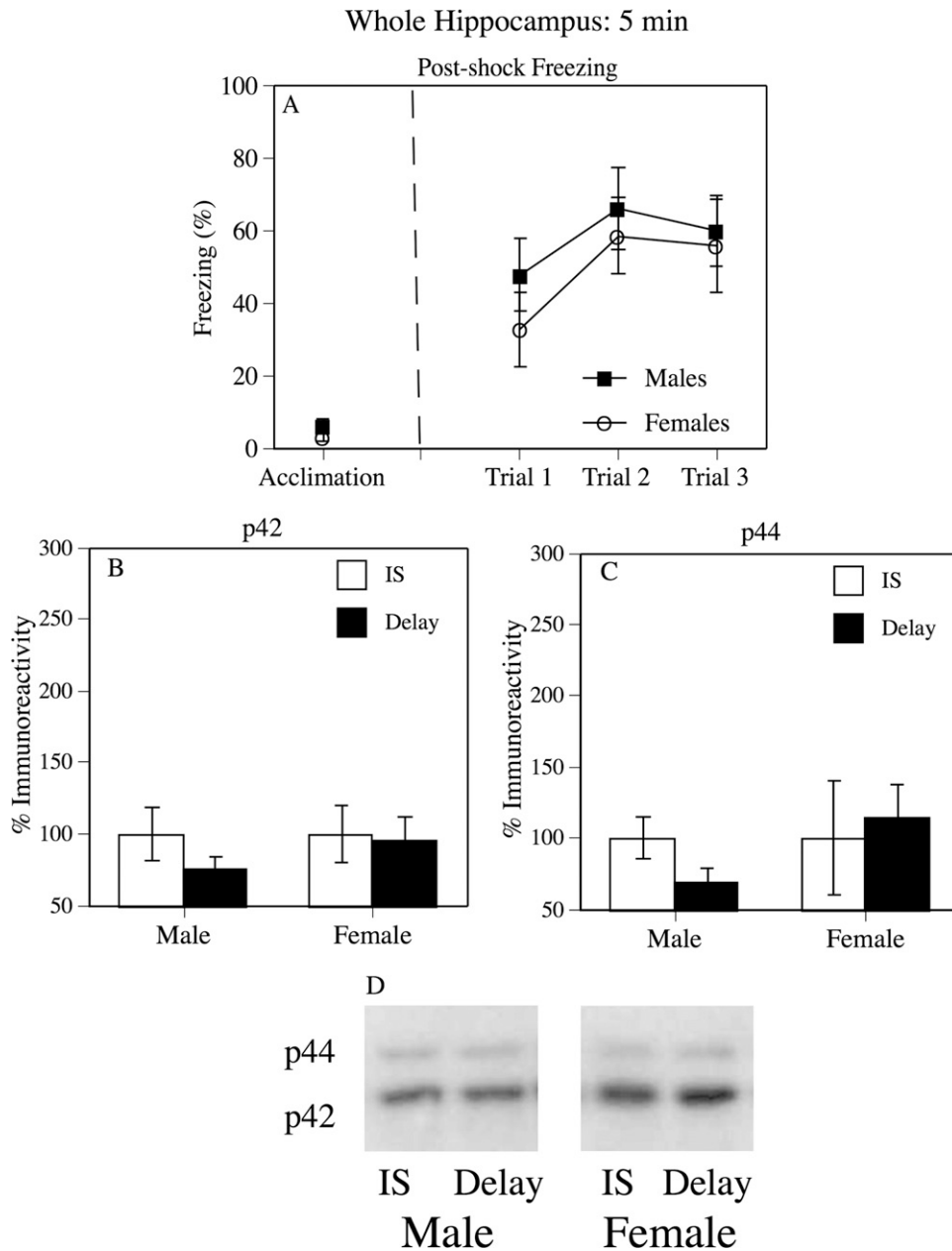
tests, although both sexes exhibited robust auditory fear retention, percent freezing was significantly higher in females than males. The finding of intact and enhanced auditory fear memory in the female rats suggests that their reduced contextual freezing does not reflect a general impairment in their ability to perform the freezing response or an altered sensitivity to the shock, but rather, may specifically reflect an impairment in context memory. Given the well-established role of the hippocampus in context fear learning (Kim and Fanselow, 1992; Wallenstein and Vago, 2001; Sanders et al., 2003) and of ERK activation in the formation of long-term contextual fear memories (Schafe et al., 1999), we next examined ERK activation in the hippocampus in male and female rats following fear learning. We first examined training-induced ERK activation in the whole hippocampus, followed by a more selective analysis of the dorsal and ventral hippocampus. In all hippocampal experiments, pERK levels in the IS control groups did not differ from levels in the Naïve groups in both sexes (Table 1), as suggested by the absence of significant main effects of Sex and Condition, and the Sex  $\times$  Condition interactions. These findings suggest that the handling associated with placement in the fear conditioning chambers and the shock presentations did not affect baseline levels of ERK activation. Therefore, in all subsequent analyses, ERK activation (phosphorylation) in Delay groups was compared to activation in the IS control groups, thus allowing changes in ERK immunoreactivity in the Delay groups to more clearly be viewed as a consequence of the context-shock association.

**ERK activation 5 min post-conditioning: whole hippocampus.** Subjects assigned to the Delay or IS groups were killed 5 min after fear conditioning, at which point whole hippocampus was removed. This time point was used to examine the immediate effects of conditioning on ERK activation. Although using these subjects for Western blot assays prevented their use in retention tests to measure fear memory, freezing during the acclimation (pre-shock) and post-shock periods in the Delay groups were recorded during the conditioning session (Fig. 3A). Males and females did not differ in baseline freezing during the acclimation period prior to the first shock presentation. Although post-shock freezing appeared to increase across trials, this change was not significant,  $F(2,46)=2.38$ ,  $P=0.10$ . Neither the main effect of Sex nor the Trial  $\times$  Sex interaction were significant, suggesting that both sexes

similarly processed the shock. The lack of a significant main effect of Condition for both p42 (Fig. 3B, 3D) and p44 (Fig. 3C, 3D) isoforms suggests that fear conditioning did not upregulate ERK phosphorylation 5 min after delay training in males and females (relative to same-sex IS controls). The main effect of Sex and the Condition  $\times$  Sex interaction were also not significant. Total p42 and p44 ERK levels were not significantly different between Delay and IS groups in either sex (Table 2).

**ERK activation 60 min post-conditioning: whole hippocampus.** Given that ERK activation was not altered 5 min after conditioning, ERK activation in the whole hippocampus was examined in a new group of Delay and IS subjects 60 min after fear conditioning. This time point was chosen because it coincides with the timing of learning-associated alterations in hippocampal ERK activation, as significant increases in hippocampal ERK activation following fear conditioning have previously been observed in male rodents 60 min after training (Atkins et al., 1998; Sananbenesi et al., 2003). Males and females in the Delay groups did not differ in baseline freezing during the acclimation period prior to the first shock presentation (Fig. 4A). Post-shock freezing increased across trials during conditioning ( $F(2,52)=7.34$ ,  $P=0.002$ ) (Fig. 4A). Neither the main effect of Sex nor the Trial  $\times$  Sex interaction was significant for freezing during the post-shock period. Although the analysis of post-shock freezing revealed no significant main effects or interaction, it did appear from Fig. 4A as if post-shock freezing was lower in females than in males. To address this issue, we examined freezing during each tone presentation to determine if this apparent difference could be due to sex differences in reactivity to the shock, processing of the tone stimulus, or formation of a tone-shock association. A repeated-measures ANOVA was conducted on tone freezing data for the three acquisition trials, which revealed no significant main effects of Sex or Trial, and no significant Sex  $\times$  Trial interaction (Fig. 4B), suggesting that both sexes processed the tone and shock similarly, and were both able to form a tone-shock association.

The main effects of Condition and Sex and the Condition  $\times$  Sex interaction (Fig. 5A, 5B) were not significant for the p42 (Condition:  $F(1,33)=1.16$ ,  $P=0.29$ ; Sex:  $F(1,33)=1.83$ ,  $P=0.19$ ; Condition  $\times$  Sex:  $F(1,33)=1.83$ ,  $P=0.19$ ) and p44 (Condition:  $F(1,31)=1.26$ ,  $P=0.27$ ; Sex:  $F(1,31)=4.05$ ,  $P=0.06$ ; Condition  $\times$  Sex:  $F(1,33)=4.05$ ,  $P=0.06$ ) isoforms, suggesting that training did not sex-



**Fig. 3.** (A) Mean ( $\pm$ SEM) percent freezing in Delay males and Delay females during the acclimation and post-shock periods following three tone-shock pairing trials. (B, C) Mean ( $\pm$ SEM) percent pERK 2 (p42) (B) and pERK 1 (p44) (C) Immunoreactivity from whole hippocampal dissections taken from rats in the IS (p42: males,  $n=8$ ; females,  $n=6$ ; p44: males,  $n=6$ ; females,  $n=5$ ) and Delay (p42: males,  $n=13$ ; females,  $n=11$ ; p44: males,  $n=12$ ; females,  $n=10$ ) conditions killed 5 min after conditioning. (D) Representative pERK blots from IS and Delay conditions at 5 min after conditioning.

selectively increase ERK phosphorylation. The lack of a significant interaction may have been driven in part by variability in the data, as inspection of Fig. 5A indicates a 2.2-fold increase in p42 % immunoreactivity in Delay males (relative to IS males) and a 0.86-fold decrease in Delay females (relative to IS females). For p44 activation, a 2.2-fold increase was observed in Delay males and a 0.67-fold decrease was observed in Delay females. The change in p44 ERK phosphorylation should be viewed with caution as a significant Condition $\times$ Sex interaction was

observed for tERK ( $F(1,31)=4.79$ ,  $P=0.04$ ) (Table 2). Although not statistically significant, follow-up  $t$ -tests revealed a trend towards lower levels of tERK in Delay males ( $P=0.08$ ), but not females ( $P=0.28$ ), relative to same-sex IS controls. Thus, the possible sex-specific changes in normalized p44 ERK phosphorylation in the Delay groups may in part be due to changes in total protein kinase levels. Significant main effects of Condition and Sex and their interaction were not observed for total p42 ERK immunoreactivity.

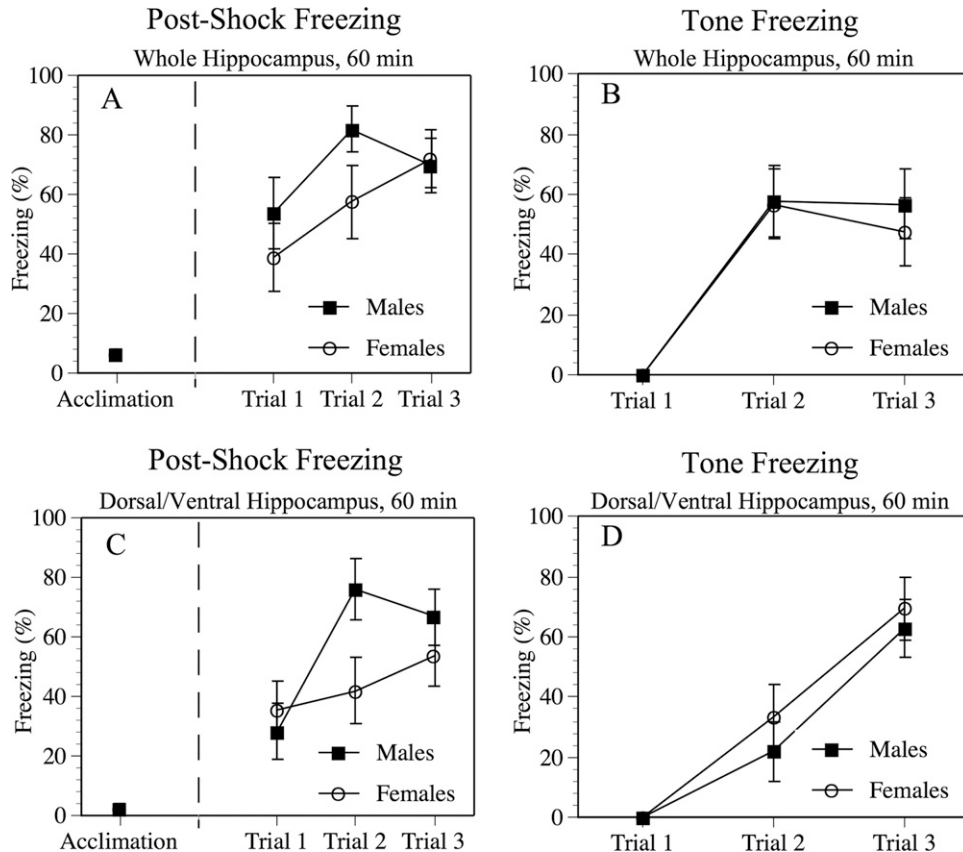
**Table 2.** tERK activation in the Delay conditions expressed as a percentage relative to IS controls (100%)

Sex, Isoform	% Immunoreactivity				
	Whole hip. 5 min	Whole hip. 60 min	Dorsal hip. 60 min	Ventral hip. 60 min	Amygdala 60 min
Male, p42	99.0 (±8.9)	75.1 (±11.3)	100.4 (±9.0)	91.2 (±5.6)	64.7 (±6.6)*
Female, p42	111.4 (±9.7)	106.7 (±10.2)	103.3 (±9.9)	108.1 (±8.3)	84.5 (±9.5)
Male, p44	99.9 (±11.2)	74.5 (±9.3)	103.5 (±11.9)	79.9 (±9.1)	65.0 (±8.1)*
Female, p44	113.3 (±15.6)	112.5 (±5.6)	110.9 (±11.4)	120.7 (±13.1)	71.8 (±9.1)

\*  $P < 0.05$ , relative to same-sex IS controls (100%).

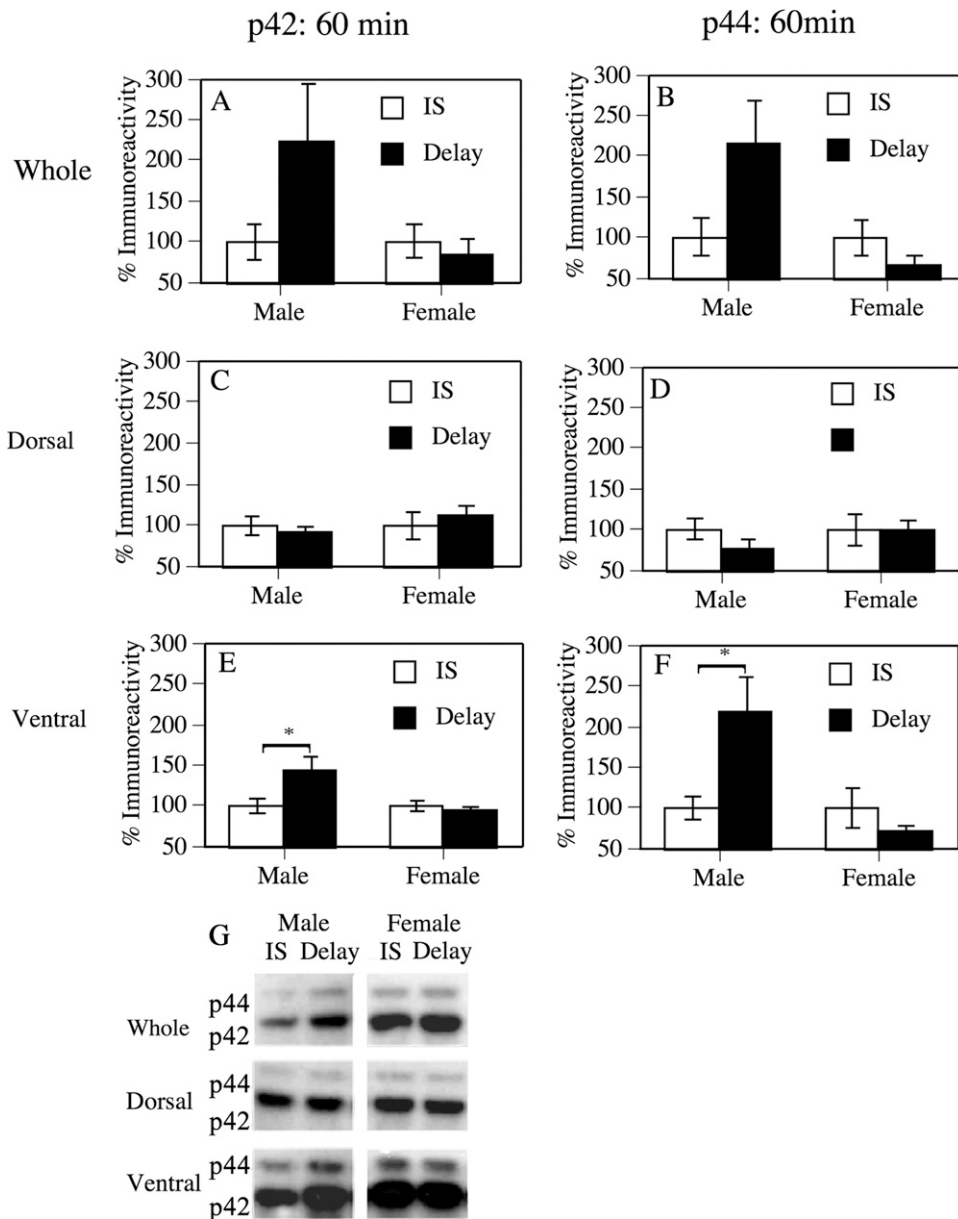
**ERK activation 60 min post-conditioning: dorsal hippocampus.** To determine if the variability in ERK activation observed in the whole hippocampus 60 min after fear conditioning could have been driven by activation in only a portion of the hippocampus, phosphorylated protein kinase levels were examined in dorsal and ventral hippocampal regions in a new group of Delay and IS subjects. Delay and IS subjects were conditioned, and both groups were killed 60 min after conditioning at which time the whole hippocampus of each rat was dissected into dorsal and ventral sections. Delay males and females did not differ in baseline freezing during the acclimation period (Fig. 4C). Post-shock freezing increased across trials during condi-

tioning ( $F(2,52) = 6.14, P = 0.004$ ). Neither the main effect of Sex nor the Trial×Sex interaction was significant for freezing during the post-shock period. Similar to the rats tested for analysis of the whole hippocampus, females tested for dorsal and ventral hippocampus assays appeared to exhibit somewhat less post-shock freezing than males, despite the aforementioned statistics to the contrary. Therefore, we conducted the same repeated-measures ANOVA on freezing during each tone presentation as described for rats collected for whole hippocampus assays, and again found no significant main effects of Sex and Trial, or significant Sex×Trial interaction (Fig. 4D). These data indicate that, among rats tested for dorsal and



**Fig. 4.** Mean (±SEM) percent freezing during conditioning in Delay males and Delay females. Specifically, freezing was measured during the acclimation period and during the post-shock periods following the three tone-shock pairing trials (A, C). Freezing was also measured during each tone presentation (B, D). Rats were killed 60 min after conditioning, at which point whole hippocampus (A, B) or dorsal and ventral hippocampus (C, D) were dissected for ERK analysis.





**Fig. 5.** Means ( $\pm$ SEM) for percent pERK immunoreactivity in whole (A, B), dorsal (C, D), and ventral (E, F) hippocampal dissections taken from male and female rats in the IS and Delay conditions killed 60 min after conditioning. pERK 2 (p42) immunoreactivity are presented in the left column (*Whole*: IS males,  $n=5$ ; Delay males,  $n=12$ ; IS females,  $n=8$ ; Delay females,  $n=12$ ; *Dorsal*: IS males,  $n=7$ ; Delay males,  $n=11$ ; IS females,  $n=8$ ; Delay females,  $n=13$ ; *Ventral*: IS males,  $n=7$ ; Delay males,  $n=11$ ; IS females,  $n=8$ ; Delay females,  $n=10$ ). pERK 1 (p44) immunoreactivity is presented in the right column (*Whole*: IS males,  $n=6$ ; Delay males,  $n=11$ ; IS females,  $n=8$ ; Delay females,  $n=10$ ; *Dorsal*: IS males,  $n=7$ ; Delay males,  $n=11$ ; IS females,  $n=8$ ; Delay females,  $n=13$ ; *Ventral*: IS males,  $n=7$ ; Delay males,  $n=12$ ; IS females,  $n=8$ ; Delay females,  $n=10$ ). (G) Representative whole, dorsal, and ventral hippocampal pERK blots from IS and Delay conditions at 60 min after conditioning. \*  $P=0.04$ .

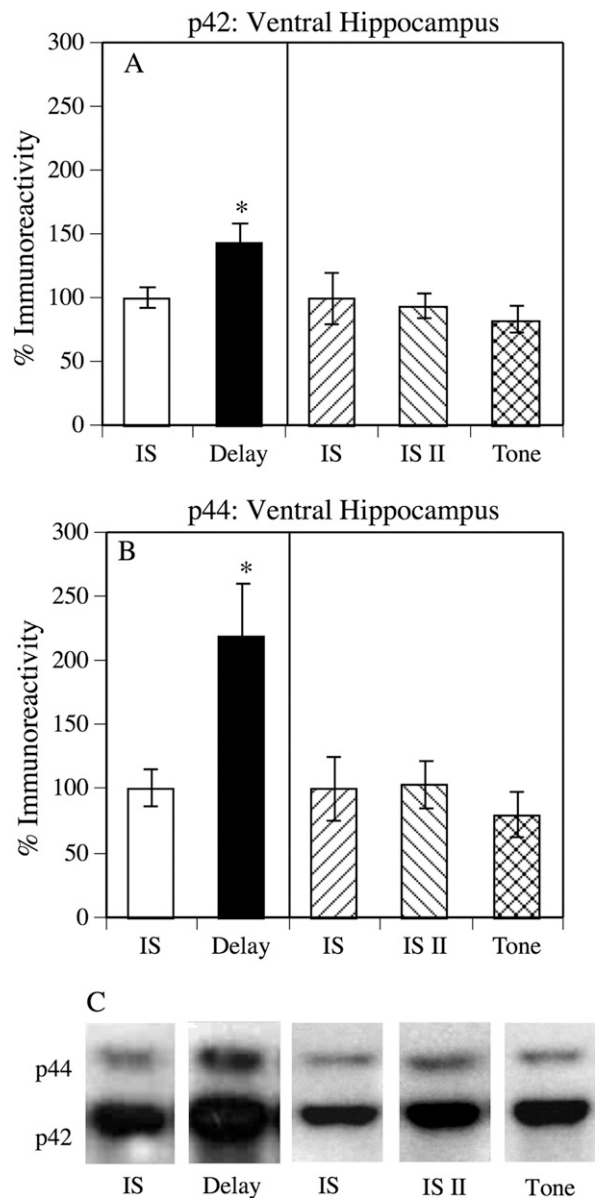
ventral hippocampus ERK assays, there were no sex differences in tone or shock processing or formation of a tone-shock association.

Fear conditioning did not increase dorsal hippocampal p42 (Fig. 5C, 5G) or p44 (Fig. 5D, 5G) ERK phosphorylation in either sex 60 min following conditioning. Accordingly, the main effects of Condition and Sex and the Condition $\times$ Sex interaction were not significant. Significant changes in total p42 and p44 ERK levels were also not observed (Table 2).

*ERK activation 60 min post-conditioning: ventral hippocampus.* Although not significant, ventral hippocampal p42 ERK phosphorylation in the Delay groups was slightly higher than in the IS groups, as indicated by the trend towards a significant main effect of Condition ( $F(1,32)=3.70$ ,  $P=0.06$ ) (Fig. 5E, 5G). Importantly, however, fear conditioning particularly increased p42 ERK phosphorylation in males, but not females, as suggested by the significant main effect of Sex ( $F(1,32)=5.19$ ,  $P=0.03$ ) and the Condition $\times$ Sex interaction ( $F(1,32)=5.19$ ,  $P=0.03$ ). *t*-Tests

used to identify the source of the interaction revealed that the percentage of pERK immunoreactivity was significantly higher in Delay males relative to IS males ( $P=0.04$ ). Significant differences were not observed between the female groups. This sex-specific upregulation in ERK phosphorylation was not due to changes in total protein kinase levels (Table 2). A similar pattern of sex-specific upregulation of pERK was observed when pERK was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH, loading control) (data not shown). Regarding p44 ERK phosphorylation, the main effect of Condition was not significant ( $F(1,33)=2.35$ ,  $P=0.13$ ), suggesting that ERK activation in the ventral hippocampus did not differ between Delay and IS groups 60 min following conditioning. However, as observed with ventral hippocampal p42 ERK phosphorylation, the main effect of Sex ( $F(1,33)=6.07$ ,  $P=0.02$ ) and the Condition $\times$ Sex interaction ( $F(1,33)=6.07$ ,  $P=0.02$ ) were significant, indicating a sex-specific effect of fear conditioning on ERK activation. Delay males exhibited a significant increase in p44 ERK phosphorylation relative to IS males ( $P=0.04$ , Fig. 5F, 5G), whereas the two female groups did not differ. The changes in normalized p44 ERK phosphorylation in males, but not females, were not due to changes in total protein kinase levels, as significant differences were not observed in either sex between the Delay and IS groups (Table 2). Further, similar results were obtained when pERK was normalized to GAPDH instead of tERK (data not shown).

Although the increase in ventral hippocampal ERK activation observed in the male Delay condition may be viewed as the result of the formation of a context-shock association, an alternative possibility may be that increases in pERK were a nonspecific result of the tone presentation. To control for tone-induced changes in ERK phosphorylation, a Tone only group was placed in the fear conditioning chamber. This group received the same protocol as those in the Delay group with the exception that these rats were never exposed to foot shocks. A second possibility may be that the significant increase in pERK was due to the slightly longer duration of time that the Delay group spent in the conditioning chamber compared to the IS group. Specifically, the delay fear conditioning protocol lasted approximately 9 min. Although the IS group was presented with the same number, duration, and intensity of shocks (thus, controlling for nonspecific effects of foot shock alone on ERK activation), the IS group spent approximately 4 min in the chamber. Therefore, to control for changes in ERK phosphorylation due to duration of time in the conditioning chamber, a second male IS group (termed IS II) was placed in the chamber. Males in the IS II group received the same protocol as the IS group, except that IS II subjects remained in the box for an additional 5 min following the last shock presentation. As a result, the total time the IS II group spent in the conditioning chamber was equal to the time spent by the Delay group in the chamber. ERK phosphorylation in the ventral hippocampus was measured 60 min after removal from the conditioning chamber. The Tone and IS II protocols did not



**Fig. 6.** Mean ( $\pm$ SEM) percent pERK 2 (p42) (A) and pERK 1 (p44) (B) immunoreactivity in ventral hippocampal dissections taken from male rats in the IS (p42,  $n=7$ ; p44,  $n=7$ ), IS II (p42,  $n=8$ ; p44,  $n=8$ ) and Tone (p42,  $n=8$ ; p44,  $n=7$ ) conditions killed 60 min after conditioning. For purposes of comparison, IS (white bar) and Delay (black bar) groups are replotted from Fig. 4E (p42) and 4F (p44). \*  $P=0.04$ . (C) Representative pERK blots from the IS, IS II, and Tone groups at 60 min after conditioning, with blots from IS and Delay groups shown for comparison.

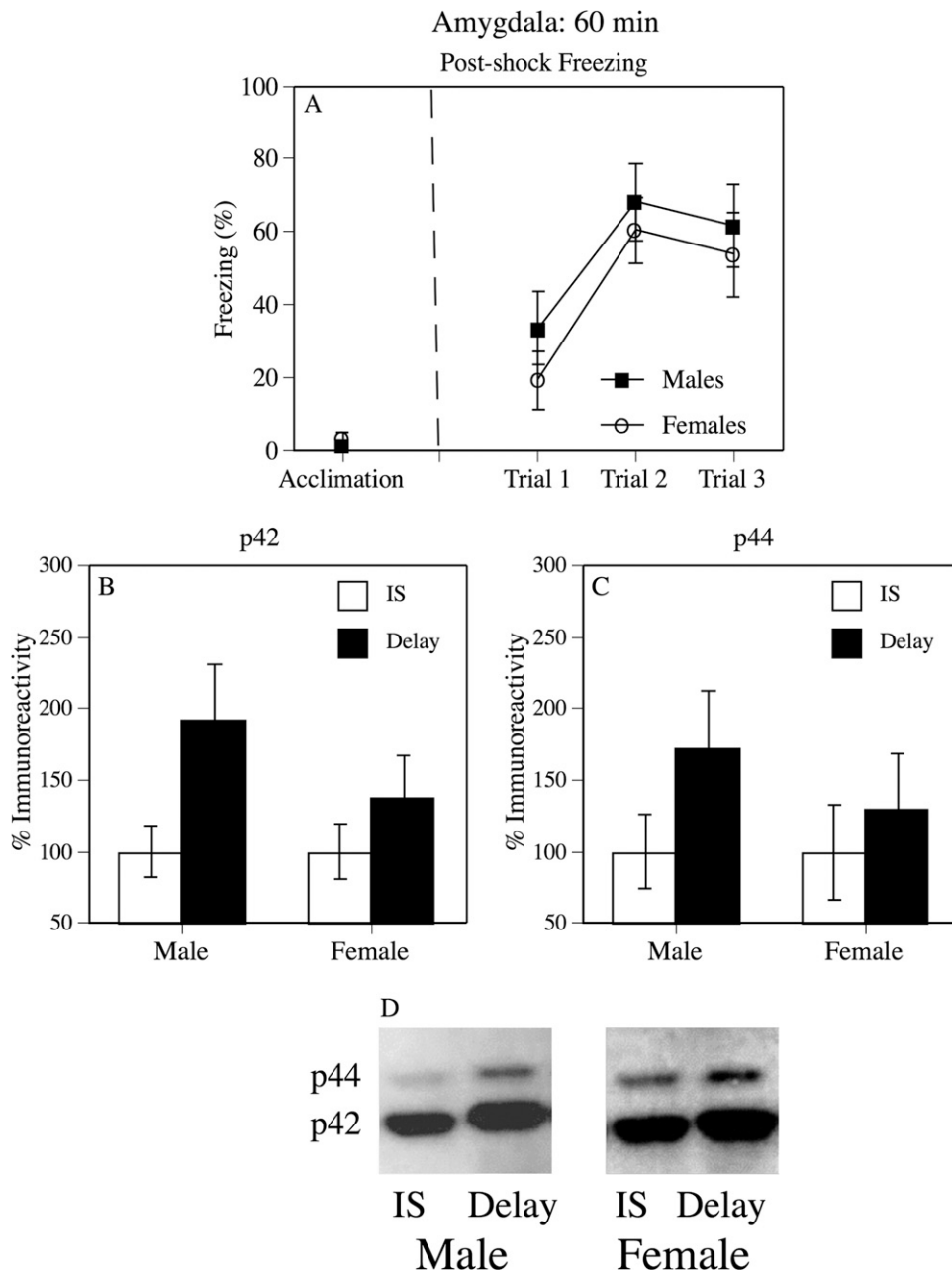
significantly increase p42 (Fig. 6A, 6C) or p44 ERK phosphorylation (Fig. 6B, 6C). Changes in tERK levels were also not observed (data not shown). These data further support the conclusion that the previously observed increase in ventral hippocampal ERK activation in Delay males was the result of the formation of the context-shock association, rather than exposure to the tone, shock, or context alone.

**ERK activation in the lateral amygdala after fear conditioning is similar in males and females**

The amygdala is critically involved in tone fear conditioning (Phillips and LeDoux, 1992; Fendt and Fanselow, 1999; LeDoux, 2000) and auditory fear conditioning has been shown to lead to an increase in p42 ERK activation in the lateral amygdala in male rats (Schafe et al., 2000). As demonstrated above, both sexes exhibit robust freezing to the tone one day after conditioning, with females freezing more than males (Fig. 2E). In the present experiment, ERK

activation in male and female rats was measured in the amygdala 60 min following fear conditioning. It was anticipated that ERK activation in the amygdala would be significantly increased in both Delay males and females relative to IS males and females and, potentially, that the magnitude of increased ERK activation would be higher in Delay females than Delay males.

Delay males and females did not differ in percent freezing during acclimation to the chamber (Fig. 7A). Post-shock freezing in the Delay groups increased across trials



**Fig. 7.** (A) Mean ( $\pm$ SEM) percent freezing in Delay males and Delay females during the acclimation and post-shock periods following the three tone-shock pairing trials. (B, C) Mean ( $\pm$ SEM) percent pERK 2 (p42) (B) and pERK 1 (p44) (C) immunoreactivity from amygdala punches taken from rats in the IS (p42: males,  $n=8$ ; females,  $n=8$ ; p44: males,  $n=8$ ; females,  $n=8$ ) and Delay (p42: males,  $n=10$ ; females,  $n=8$ ; p44: males,  $n=10$ ; females,  $n=8$ ) conditions killed 60 min after conditioning. (D) Representative pERK blots from IS and Delay groups 60 min after conditioning.

during conditioning ( $F(2,52)=12.60$ ,  $P=0.0001$ ). Neither the main effect of Sex nor the Trial $\times$ Sex interaction was significant for freezing during the post-shock period (Fig. 7A). In order to maintain consistency with the 60 min hippocampal analyses, we also conducted a repeated-measures ANOVA on tone freezing data for the three acquisition trials. This analysis also revealed no significant main effect of Sex or Trial, and no significant Sex $\times$ Trial interaction (data not shown), suggesting that both sexes processed the tone and shock similarly and were both able to form a tone-shock association.

A significant main effect of Condition was observed in p42 ERK activation ( $F(1,30)=4.76$ ,  $P=0.04$ ), as significant increases in pERK in the Delay condition relative to IS were observed regardless of sex (Fig. 7B, 7D). The main effects of Sex and the Condition $\times$ Sex interaction were not significant. Increases in ERK phosphorylation were not due to increases in total protein kinase levels, although a significant decrease in total p42 kinase levels was observed in the male Delay group (Table 2). Although not statistically significant, a moderate increase in p44 ERK phosphorylation was observed in Delay males and females ( $F(1,30)=3.51$ ,  $P=0.07$ ) (Fig. 7C). Neither the main effect of Sex nor the Condition $\times$ Sex interaction was significant. A significant decrease in total p44 kinase levels was again observed among the male Delay group (Table 2).

## DISCUSSION

The results of the present study indicated that male rats exhibit more long-term retention of contextual fear conditioning than female rats. During the LTM tone tests, although both sexes exhibited robust auditory fear retention, percent freezing was significantly higher in females than males, suggesting that the reduced contextual freezing observed among the females did not reflect a general memory impairment or a sex-difference in non-mnemonic performance factors. Additionally, we observed more ventral hippocampal activation of the protein kinase ERK in males than in females 60 min after fear conditioning, a time point that coincides with memory-related molecular alterations. The sex-specific increase in ventral hippocampal ERK immunoreactivity was not due to shock or tone presentation alone or to duration of time in the conditioning chamber, but rather, appeared specific to the formation of contextual fear memories. Post-conditioning increases in ERK activation were observed in the amygdala in both sexes, suggesting a selective effect of sex on hippocampal-mediated memory as indicated by our behavioral data.

Previous studies have shown that male rodents outperform female rodents in other hippocampal-dependent tests including the Morris water (Beiko et al., 2004), and radial arm (Gresack and Frick, 2003) mazes. Sex differences favoring males have also been reported in various aspects of hippocampal physiology. Males have a larger dentate gyrus–granule cell layer (DG-GCL) and CA1 region (Roof and Havens, 1992; Isgor and Sengelaub, 1998; Tabibnia et al., 1999), more dendritic branching in dentate and CA3 (Juraska, 1991; Isgor and Sengelaub, 2003), and

more mossy fiber synapses (Parducz and Garcia-Segura, 1993). The magnitude of long-term potentiation, the most promising current cellular model of synaptic plasticity leading to memory formation, induced at perforant path synapses in the DG is also greater in males than females (Maren et al., 1994). Given that synaptic plasticity is thought to underlie all new memories (Eichenbaum, 2002), the above findings support the notion that sex differences in the hippocampus contribute to mnemonic sex differences. Because the ERK pathway regulates plasticity-associated gene expression and protein synthesis, the present results extend the above findings by offering a possible mechanism through which the aforementioned sex differences in synaptic plasticity may arise, ultimately resulting in a sex difference in memory formation. In support of this idea, Kudo et al. (2004) also report increased hippocampal phosphorylation of the transcription factor CREB (a downstream effector of ERK) in male, but not female, rats following contextual fear conditioning. CREB activates immediate early genes such as *zif268* and increases the transcription of synapsin 1, a marker of synaptic growth and plasticity (Adams and Sweatt, 2002; Fournier et al., 2009). Determining whether fear conditioning-induced sex differences in ERK activation result in sex differences in synapsin-1 via differential CREB activation will be an important avenue to investigate in future studies.

In the present study, increased ERK activation in the hippocampus of males, but not females, was observed 60 min after fear conditioning, a finding that positively correlates with the sex difference observed in the retention of contextual fear. Neither sex exhibited elevated ERK activity in the hippocampus 5 min after conditioning, a finding which could reflect the relatively modest acquisition of fear conditioning (as reflected in the post-shock freezing values across trials, Fig. 3A). Both sexes did, however, exhibit significantly increased ERK activation in the amygdala 60 min after training, a result which likely reflects the fact that both sexes exhibited robust retention of auditory fear learning (Fig. 2). The time course of hippocampal ERK activation in males agrees with other reports of increased ERK activation in the whole hippocampus 60 min, but not immediately (5 min or less), after fear conditioning (Atkins et al., 1998; Sananbenesi et al., 2003; Runyan et al., 2004). Using immunohistochemistry, however, upregulation of pERK has been specifically observed in the CA1 subregion immediately following fear conditioning in male mice, although this is a transient effect whereby pERK levels return to baseline within 60 min (Trifilieff et al., 2006). A biphasic change in ERK activation in males, with increases observed at 5 and 60 min, has also been reported in the amygdala following fear conditioning (Paul et al., 2006). Thus, although learning consistently upregulates the ERK pathway regardless of sex (as evidenced, for example, by the amygdala findings in the present study), the time course and pattern of fear conditioning–induced increases in ERK activation may vary both within and between brain structures required for fear conditioning.

One potential concern with the ERK activation observed in the hippocampus at 60 minutes is that the post-

shock freezing of females depicted in Fig. 4A and 4C appears lower than that of males, despite the fact that statistical analyses in both experiments did not indicate any significant differences between the sexes. Lower post-shock freezing in females could indicate that sex differences in ventral hippocampal ERK activation resulted from a deficit not specifically associated with context conditioning. On the other hand, lower post-shock freezing in females could simply reflect the contextual fear deficit in females, since post-shock freezing is a form of short-term context memory. The tone freezing data illustrated in Fig. 4B and 4D support the latter interpretation, as both sexes froze similarly to the tone during training. Although tone freezing, as measured during acquisition, is confounded by learning of the context-shock association, it can nevertheless provide important information about how the rats learn to associate stimuli with shock. The fact that the sexes did not differ in tone freezing suggests similar acquisition of a tone-shock association, foot-shock sensitivity, and tone stimulus processing in males and females, and supports the conclusion that sex differences in ventral hippocampal ERK activation are associated with differences in contextual fear conditioning rather than a general memory impairment.

The increased hippocampal ERK activation in males, but not females, was specific to ventral hippocampus. The ventral hippocampus may be particularly involved in the formation of contextual fear memories because of its anatomical connections with the amygdala. The importance of the amygdala in both auditory and contextual fear conditioning is well established (Phillips and LeDoux, 1992; Fanselow and LeDoux, 1999; Huff and Rudy, 2004), as it is thought to support the formation of shock representations (Anagnostaras et al., 2001) and storage of auditory-shock associations, as well as modulate context memories (Huff and Rudy, 2004; Rudy et al., 2004). Interestingly, only the ventral region of the hippocampus maintains direct reciprocal projections to the amygdala (Van Groen and Wyss, 1990; Pitkanen et al., 2000). In contrast, the dorsal hippocampus receives inputs from the visual, auditory, and somatosensory cortices via the entorhinal and perirhinal cortices (Moser and Moser, 1998) and these inputs likely contribute to the importance of the dorsal hippocampus to spatial learning (Steffenach et al., 2005).

A greater involvement of ventral hippocampus in the formation of long-term fear memories has previously been implicated. Specifically, although ERK activation is increased in the whole hippocampus following contextual fear conditioning (Atkins et al., 1998), neither pre-training (Sananbenesi et al., 2003; Ahi et al., 2004) nor post-training (Giovannini et al., 2003) inhibition of ERK activation in the dorsal hippocampus impairs retention of contextual fear learning. These results imply that increased ERK activation in whole hippocampus may be due primarily to alterations in the ventral hippocampus, and the current findings support this notion. Previous studies report that post-training dorsal hippocampal inhibition of ERK activation impairs spatial navigation memory in male rats, and ERK activation during the post-training period is se-

lectively observed in dorsal, but not ventral, hippocampus (Blum et al., 1999). Together, these findings suggest that the involvement of hippocampal regions in memory formation may depend on the type of memory, with dorsal more involved in spatial memory and ventral involved in context representations related to fear memory. Numerous lesion findings also support the preferential involvement of ventral (Richmond et al., 1999; Bast et al., 2001; Zhang et al., 2001; Trivedi and Coover, 2004; although see Maren and Holt, 2004), but not dorsal (Maren et al., 1997; Frankland et al., 1998; Richmond et al., 1999; Rudy et al., 2002; Matus-Amat et al., 2004; Trivedi and Coover, 2004; Wiltgen et al., 2006) hippocampus in contextual fear and dorsal, but not ventral, hippocampus in spatial memory (Moser et al., 1993; Bannerman et al., 1999, 2002; Pothuizen et al., 2004).

Although correlational, the present results suggest that sex differences in ventral hippocampal ERK activation are involved in sex differences in contextual fear memory. These findings lay the groundwork for future studies investigating factors responsible for differential ERK activation in males and females. Many factors, including growth factors, calcium influx, and stimulation of dopaminergic, muscarinic, nicotinic, or serotonergic receptors activate the ERK pathway (Adams and Sweatt, 2002). Interestingly, we recently found that exogenous  $17\beta$ -estradiol also activates ERK (compared to vehicle-treated females), and this activation is necessary for estradiol to improve object memory in female mice (Fernandez et al., 2008). As such, the fluctuating estrogen and progesterone levels that females, but not males, are exposed to during the estrous cycle may influence the extent to which ERK is activated following learning, and have contributed to the sex differences in ERK activation and memory retention observed in the present study. This issue cannot be directly addressed by the present data because estrous cycling was not monitored in this study in order to prevent the stress associated with vaginal lavage in females from influencing performance in the conditioning task (see more on stress below). However, we feel it is unlikely that cyclic hormone fluctuations can primarily account for the results in the present study for a couple of reasons. First, Fig. 2 indicates that during all context and tone tests (and during the post-shock freezing period), the variability observed among the female rats was less than the variability observed in the males. If estrous cycle stage was a significant factor affecting behavior, then performance during the context and tone tests should be considerably more variable in females than in males. Second, if hormone fluctuations influenced the ERK signaling cascade, then one would expect to see increased variability in both ERK isoforms. For instance, although normalized p44 immunoreactivity was consistently more variable in females than in males (Table 1), this was not the case for p42, suggesting that hormone fluctuations likely did not contribute to the variability in p44 immunoreactivity. In addition, our previous work demonstrates that p42 in the hippocampus is more sensitive than p44 to the effects of exogenous estradiol (Fernandez et al., 2008), which does not support the suggestion that variability in p44 among females was primarily the result of the

estrous cycle. Third, as is often seen in other hippocampal-dependent tasks (e.g. Morris water maze; Frye, 1995; Healy et al., 1999; Frick and Berger-Sweeney, 2001), previous evidence suggests that the effects of estrous cycling on contextual fear conditioning are subtle and inconsistent. For example, female rats in proestrus (high hormone levels) reportedly exhibit less contextual freezing than females in estrus (low hormone levels) and to males (Markus and Zecevic, 1997; Cushman et al., 2006), suggesting that high circulating levels of estrogens impair female retention of contextual fear. However, another study found no differences in contextual fear conditioning between intact and ovariectomized female rats (Gupta et al., 2001), and we recently confirmed this finding using a conditioning paradigm identical to the one used in the present study (Gresack and Frick, 2007). These two reports suggest no effect of gonadal estrogens and progestins on contextual fear memory. Furthermore, estradiol replacement following ovariectomy has either no effect (Morgan and Pfaff, 2001; Day and Good, 2005) or improves (Jasnow et al., 2006) contextual fear conditioning relative to ovariectomized females, which contradicts the aforementioned estrous cycle findings. The inconsistencies in this literature highlight the need to assess the effects of endogenous and exogenous hormones on contextual fear conditioning, perhaps by directly comparing females in various stages of the estrous cycle with ovariectomized females, estradiol-treated females, and males. Such studies would help to clarify whether differences in sex-steroid hormone exposure at the time of testing contribute to the correlative sex differences in ERK activation and fear conditioning established in the present study.

Sex differences in hormone exposure during development may provide an alternative explanation accounting for the observed sex differences in ERK and context fear conditioning in adulthood. For example, during the perinatal period, male rodents are exposed to higher levels of estrogens than females, in part resulting from the aromatization of testosterone to estrogens (MacLusky and Nafzolin, 1981; Rhoda et al., 1984). These differences in developmental estrogen exposure have lasting effects in terms of “organizing” the structure and function of various brain regions (Breedlove and Hampson, 2002). Organizational effects often result in long-term hippocampal alterations that may account for sex differences in hippocampal-dependent learning and memory in adulthood. For example, prenatal administration of estradiol and testosterone, but not dihydrotestosterone (a form of testosterone which cannot be converted to estradiol) masculinizes CA1 volume and neuronal soma size, and these changes are consistent with male advantage in a spatial water maze task in adulthood (Isgor and Sengelaub, 1998). Further, Williams et al. (1990) have shown in adult rats that the male advantage in spatial working memory tested using the radial arm maze can be reversed if neonatal females are treated with estradiol and neonatal males are gonadectomized. In addition, neonatally gonadectomized males with bilateral estradiol implants in the hippocampus perform better on the radial arm maze in adulthood than

neonatally gonadectomized males receiving either no implants or implants in the hypothalamus (Williams and Meck, 1991, 1993), thus implicating the hippocampus as an important site of estradiol’s action for the organization of sex differences in spatial memory. The involvement of developmental estradiol in the organization of sex differences in other tasks involving the hippocampus, such as contextual fear conditioning, has not yet been investigated. Studies investigating the effects of developmental hormone exposure manipulation on sex differences in contextual fear in adulthood would help clarify this issue.

Aside from sex-steroid hormones, sex differences in the response to the shock stressor may also result in differential levels of ERK activation and contribute to sex differences in memory. For example, exposing rats to a different acute shock stressor increases hippocampal spine density in males, but decreases spine density in females (Shors et al., 2001), an effect that is dependent on NMDA receptor activation (Shors et al., 2004). Exposure to the same stressor facilitates eye-blink conditioning in males, but impairs eye-blink conditioning in females (Wood et al., 2001). Because NMDA receptors activate the ERK pathway, the sex-specific effects of stress on memory may occur via sex differences in ERK activation. Many disorders affecting cognition such as depression, anxiety, and Alzheimer’s disease are more prevalent in women than men (Mulnard et al., 2000; Altemus, 2006), and factors such as stress exacerbate these conditions. Identifying how factors such as estrogens and stress hormones either independently or conjointly contribute to the sex-dependent activation of the hippocampal ERK pathway following learning could provide a useful means of identifying the neural basis of sex differences in the prevalence of cognitive disorders.

## CONCLUSION

In conclusion, the present study demonstrates that male rats exhibit greater retention of contextual fear memory than females, a finding which correlates with sex differences in hippocampal ERK activation. Post-training ERK activation in males, but not females, was observed in ventral, but not dorsal, hippocampus, suggesting regional involvement of the hippocampus in the formation of fear memory. Together, the data indicate that sex differences in activation of a signal transduction pathway may contribute to mnemonic differences between males and females. Determining the extent to which molecular sex differences account for sex differences in memory will help to identify basic mechanisms underlying sex differences in cognition and may lay the groundwork for future investigation of sex differences in the etiology and treatment of cognitive-related mental disorders.

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