Stress enhancement of fear learning in mice is dependent upon stressor type: Effects of sex and ovarian hormones

Matthew J. Sanders,* Sheryl Stevens, Henry Boeh

Department of Psychology, Marquette University, Milwaukee, Wisconsin 53201

*Correspondence:
Matthew J. Sanders, Ph.D.
Department of Psychology
Marquette University
P.O. Box 1881
Milwaukee, WI 53201-1881
Electronic mail: matthew.sanders@marquette.edu
Tel: 414-288-2028
Fax: 414-288-5333
Abstract

In three experiments, chronic stress enhancement of subsequent fear learning was investigated in C57Bl/6 mice. The first experiment focused on the influence of stressor type on subsequent Pavlovian fear learning. Male mice were subjected to 7 d of either repeated restraint stress or chronic variable stress before undergoing a fear conditioning procedure with three tone-shock trials. Subsequent tests were conducted of contextual and tone fear, through measures of the freezing response. Repeated restraint altered pre-training activity and the unconditional response to shock, but was ineffective in influencing conditional fear. Chronic variable stress significantly inflated contextual fear without altering tone fear. In a second experiment, investigating potential sex differences in the fear-enhancing effects of stress, female mice were subjected to the very same procedures. Among females, chronic variable stress selectively altered tone fear, rather than contextual fear. A final experiment investigated the potential role of ovarian hormones by subjecting female mice to either ovariectomy or sham surgery before the stress procedures. Ovariectomy had no significant effect on the ability of stress to enhance fear in females. In sum, the experiments indicate that stressor type significantly influences subsequent fear learning, that males and females are differentially sensitive to fear enhancement by stress, and that the mechanisms mediating these sex differences lie outside of the immediate influence of ovarian hormones. The findings should allow for refinement of animal models of human psychiatric disorders and for further investigations into the genetic and molecular substrates of significant gender differences in fear and anxiety.

Keywords: Chronic stress; Pavlovian fear conditioning; Sex differences; Hormones
**Introduction**

Nearly 20% of Americans are affected by anxiety disorders each year (NIMH, 2007). Recent literature suggests that anxiety disorders are manifestations of dysfunctions in the stress response (Risbrough & Stein, 2006) which, while critical in responding to acute challenges, can become problematic when activated for extended periods of time (Campbell, Lin, DeVries, & Lambert, 2003). Anxiety disorders are often co-morbid with stress disorders and exposure to stress has been found to increase the likelihood of subsequent anxiety disorder formation (Alexander, Dennerstein, Kotz, & Richardson, 2007). The re-creation in animal models of human life experiences, which appear to influence susceptibility to later mental disorders, can be challenging. However, recent evidence indicates that the specific stressor type employed plays an important role in modeling adult pathology (Pohl, Olmstead, Wynne-Edwards, Harkness, & Menard, 2007). Animal studies have found, for example, that rodent models of childhood physical abuse lead to increases in anxiety, while models of childhood neglect are associated primarily with increases in depression-like behavior (Gibb, Butler, & Beck, 2003; Hankin, 2005; Levitan, Rector, Sheldon, & Goering, 2003; Pohl et al., 2007). Due to our underlying interest in the study of anxiety disorders specifically, we have utilized two stressor types (chronic variable stress and repeated restraint stress) to examine the effectiveness of different laboratory stressors on the enhancement of fear acquisition and expression.

It has been shown that women experience more chronic stress and respond more severely to stressful life events than men (Matud, 2004; Schmaus, Laubmeier, Boquiren, Herzer, & Zokowski, 2008). According to the National Institute of Mental Health and the
World Health Organization, women are twice as likely as men to suffer from anxiety disorders such as Generalized Anxiety Disorder and Post Traumatic Stress Disorder (Gater et al., 1998; NIMH, 2007). Close investigation of the gender differences observed in anxiety disorder occurrence has revealed that women only exhibit higher rates of anxiety and other mood disorders during the period between puberty and menopause (Kessler et al., 1994). Additionally, numerous studies have suggested that estrus cycle status may be a critical modulator of differences in the physiological responses of the two genders to stressful situations (Figueiredo, Dolgas, & Herman, 2002; Young, Altemus, Parkison, & Shastry, 2001). Combined, these findings suggest a vital role for reproductive hormones in modulating the stress response and thus in explaining gender differences in anxiety disorder rates.

In animal models examining the gender specific effects of stress, the chronic variable stress (CVS) and repeated restraint stress (RRS) procedures have demonstrated sex differences in behavioral outcome (Bowman, Zrull, & Luine, 2001; Luine, Villegas, Martinez, & McEwen, 1994; Pohl et al., 2007), thus presenting ideal models for the study of clinical anxiety disorders. Katz and colleagues (Katz, Roth, & Carrol, 1981) first presented the CVS procedure, in which animals are subjected to a variety of stressors such as shock, changes in housing conditions, and forced swim over a period of two to three weeks approximately thirty years ago. The current set of studies employs versions of both the repeated restraint and chronic variable procedures. Although a great deal is known about the learning impact of repeated restraint, and sex differences therein (Baran, Armstrong, Niren, Hanna, & Conrad, 2009), relatively little is known about these processes in the context of CVS. CVS is hypothesized to simulate the chronic,
unpredictable stress associated with human anxiety disorders and would be expected to produce a pattern of learning effects distinct from those of RRS.

In the laboratory setting, Pavlovian conditioning is an accepted representative model of clinical fear and anxiety (Phelps & LeDoux, 2005; Rau, DeCola, & Fanselow, 2005). Fear conditioning involves the pairing of a neutral stimulus with an aversive unconditional stimulus (US). Initially, the neutral stimulus does not elicit an emotional response, however following pairing with the US, the neutral stimulus becomes a conditional stimulus (CS). Thereafter, the CS induces fear/anxiety in anticipation of the aversive US. While normally fear learning is an adaptive process, problems can arise when learned fear is abnormally strong, persists for an abnormally long time, or is abnormally resistant to subsequent extinction. Each of these problems mark human anxiety disorders to some extent and can be experimentally investigated with laboratory fear conditioning procedures. The current set of investigations is part of a larger effort aimed at understanding the critical determinants of abnormal fear responses.

To our knowledge, no study has investigated the role of ovarian steroid hormones in behavioral changes following chronic variable stress exposure in mice. Among rats, chronic variable stress causes alterations in oxidative stress in the hypothalamus and this effect, in turn, is sensitive to ovarian steroids (Prediger, Gamaro, Crema, Fontella, & Dalmaz, 2004). Here, we investigated the importance of ovarian hormones in the behavioral response to chronic variable stress, by focusing on the relationship between stress exposure and fear processes. We hypothesized that different patterns of chronic stress would be differentially effective in inflating subsequent fear learning. Furthermore, we expected that female and male mice would differ in their sensitivity to the fear-
enhancing effects of the stressors. Finally, we postulated that ovarian steroid hormones would prove critical to any sex differences revealed in the stress enhancement of fear. Such findings would support further investigation of the role of female reproductive hormones in the development of anxiety disorders in the clinical setting.

Experiment 1

Materials and Methods

Subjects

Adult male mice (C57Bl/6 strain, aged 2-4 months) served as subjects. Mice were purchased from Charles-River (Portage, MI). Animals were housed in boxes of four, in the Marquette University Vivarium with free access to food and water under a 12:12h light:dark cycle (lights on 7:00 AM). All experimental procedures occurred in the light portion of the cycle. All procedures were approved by the Marquette University IACUC and conducted in accordance with the U.S. Public Health Service “Policy on Humane Care and use of Laboratory Animals.”

Apparatus and Procedures

Repeated Restraint Stress

Animals in the Repeated Restraint Stress group (RRS, n = 8) were subjected to restraint once per day for a period of 7 d. Animals were tailmarked each day with Sharpie pens, before being transported to the laboratory. In a room distinct from those used for fear conditioning and testing, animals were placed in wire-mesh tubes for a period of 1 h. The cylinders were composed of a double layer of nylon wire “screen” mesh (10cm X 10cm) attached at one end to a cylindrical PVC cap (3.5cm dia X 3.5 cm). Each animal was placed in the cylinder with its head at the PVC end. A reversible cable-
tie was placed around the other end of the cylinder and drawn shut, to prevent the animal from backing out of the restrainer. Animals were then removed from the tubes and transported back to the home cage. Control animals (CON, n = 8) were tailmarked and transported in a fashion identical to the stressed animals but otherwise remained in the home cage.

Chronic Variable Stress

Animals in the Chronic Variable Stress group (CVS, n = 7) were exposed to two stressors each day, one in the AM and then one in the PM, for a period of 7 d. Two of the PM treatments were conducted overnight, from the afternoon of the designated day until the following morning. Each day, animals were tailmarked prior to the AM treatment with Sharpie pens and then transported to a suite of rooms used for the stress procedures, in a separate laboratory from that used for fear conditioning and testing. Stress treatments were established in a semi-random order such that two different conditions were experienced each day and each condition was experienced twice, throughout the 7 d of stress treatment. Briefly, the stressors were:

Swim: swim in room temperature water for either 5 min or 10 min.

Vibration: placement on a laboratory shaker for 10 min or 30 min.

Restraint: placement in wire mesh restrainers for 30 min.

Cold: placement in a cold room (4 degrees C) for 30 min.

Ultrasound: placement in a bucket 40cm below an ultrasound emitter for 10 min.

Crowding: placement of two home cages of animals in a single cage overnight.

Isolation: placement of each animal in a separate cage overnight.
Most of the treatments required no specialized apparatus. Only the Vibration and Ultrasound Exposure conditions required specialized equipment. The Vibration condition was established with a Dubnoff metabolic shaking incubator (GCA Precision Scientific, Chicago, IL). The Ultrasound Exposure was established with a Pest Chaser Ultrasonic Repeller (Lititz, PA). Control animals (CON, n = 8) were tailmarked and transported in a fashion identical to the stressed animals but otherwise remained in the home cage.

Fear Conditioning and Testing

Twenty-four h after the final stress treatment, animals were trained in a Pavlovian delay fear conditioning procedure. Animals were tailmarked in the Vivarium before transport to the laboratory. After transport to the laboratory, animals were placed in the conditioning chambers and allowed 2 min of exposure before the presentation of any stimuli. After 2 min, animals were exposed to three tone-shock pairings. Each tone was 28 s in duration, 2800 Hz in frequency, and 85 dB in intensity. The termination of each tone stimulus was contiguous with the onset of a single shock. Each shock was 2s in duration and 0.75 mA in intensity. The 3 tone-shock pairings were delivered with a 30s inter-trial interval. Animals were removed from the chamber and returned to the home cage 30s after the final shock. Animals were trained in squads of four, in four identical conditioning chambers (30cm X 24cm X 21cm; Med Associates Inc., St. Albans, VT). The chambers were installed on a stainless-steel rack in a brightly lit room (8 100-Watt overhead incandescent bulbs). The ceiling and back wall of each chamber were constructed of opaque white plastic. The sides of each chamber were constructed of aluminum. One sidewall of each chamber held a speaker through which tone stimuli
were delivered. The front wall/door was constructed of clear polycarbonate plastic. The floor of each chamber was constructed of a removable grid and wastepan. Before each squad of animals was conditioned, the chambers were cleaned with a 1% acetic acid solution (in tap water) and dried thoroughly with paper towels and a hair dryer. A thin film of the same solution was placed in the wastepan of each chamber as well. The grid floor was composed of 36 stainless steel rods (3mm dia, spaced 8mm apart center to center). The grid floor, when placed in the chamber, made contact with a printed circuit board through which shock was delivered. The presentation of all stimuli was programmed with a PC running MedAssociates software (Med Associates, Inc., St. Albans, VT). During training and testing, background white noise (60 dB) was provided by a standard HEPA air filter. Sound levels for the background white noise and tone stimuli were calibrated and monitored with a Radio Shack dB meter (A scale). Shock intensity was confirmed for each grid of each chamber before the introduction of each squad of animals, with a storage oscilloscope (B&K Precision Corporation, Yorba Linda, CA) and a 10KΩ resistor. Context testing took place in the same chambers and under the same ambient conditions as did training but in the absence of any tone or shock stimuli.

Twenty-four h after training, animals were returned to the training chambers for a test of context fear. Animals again were tailmarked in the Vivarium and transported to the laboratory. They were placed in the conditioning chambers for a 5-min period for observation, and then returned immediately to the home cage.

Forty-eight h after training, animals were subjected to a test of tone fear. Animals were tailmarked in the Vivarium, transported to the laboratory, and placed in a novel context. After an initial 2-min period, they were exposed to a 3-min tone (identical to
that used during training). Tone testing was conducted in a set of chambers distinct from those used for conditioning. The chambers were situated on a stainless steel rack in a dimly lit room (8 40-Watt overhead incandescent bulbs). The chambers had the same outside dimensions as those used during training but contained no grid floor or wastepan. Additionally, a curved vinyl sheet was inserted in each chamber to render the internal shape hemi-cylindrical. A sidewall of each chamber contained a speaker through which the tone stimulus was delivered. The tone testing chambers were cleaned with a 5% Simple Green solution before the introduction of each squad of mice. Background noise (50 dB), during tone testing, was supplied by a HEPA air filter.

Measures and Analyses

During all behavioral procedures, a single camera recorded behavior from all four chambers simultaneously. The video signal was sent to a DVD recorder for storage and records were digitized later to quicktime files on a Macintosh G5. For measures made during the conditioning session, cage crossovers, activity bursts, and freezing were observed later from the recordings. During the first 2 min of context exposure, the number of cage crossovers was recorded. We simply bisected the cage during video analysis and counted the number of times each mouse crossed the midline (whole body, excepting the tail). The unconditional response was operationalized as the distance traveled by the animal during the first shock. Video was digitized at 10 Hz and NIH Image was used to calculate a simple measure of travel, using the X,Y coordinates of each animal during each sample. These pixel distances then were expressed as cm distances (Anagnostaras, Josselyn, Frankland, & Silva, 2000). For freezing measures, video records were digitized at 1 Hz. A human observer counted the number of samples,
per minute, in each resulting video file during which the animal made any movement. Freezing behavior simply was quantified as the percentage of samples during which no movement was detected. During the training session, we measured freezing during each tone and after each shock. Freezing responses also were measured during the context and tone tests. Freezing percentage scores for the context test were averaged across the 5-min exposure. Freezing percentages for the tone test were averaged separately for the 2-min baseline and 3-min tone periods.

One-way analyses of variance (ANOVA) were computed for pre-training crossovers, the activity burst, and the context test, with stress treatment as the independent variable. The training session and tone test freezing measures were subjected to mixed ANOVAs with stress treatment as a between-groups factor and temporal period as a repeated measure. Statistical significance was established at the p<.05 level for all tests.

Experiment 1 was conducted in two iterations. The first investigated the effects of the repeated restraint stress treatment and the second investigated the effects of the chronic variable stress treatment. Each phase employed separate groups of experimentally naïve animals. Statistical analyses showed no differences between the control groups employed in the two iterations. Therefore, the control animals were combined to create a single group and then used in a set of analyses employing all three groups.

**Results**

**Conditioning**
During the conditioning session, we made measures of pre-training activity, freezing behavior as it evolved over the three trials, and the activity burst to first shock. The data from the training session are depicted in Figure 1. One mouse was eliminated from the CVS group as an outlier (Grubb’s test), in the measure of pre-training activity. Before eliminating the mouse from the analysis, we performed a square-root transformation of the data. Even after transformation of the data, the mouse remained an outlier and so was eliminated from the analysis of pre-training activity. Groups differed significantly in exploratory behavior measured before the first tone-shock pairing [Figure 1A; F(2,27) = 5.47, p < .05]. Little to no freezing behavior was evident during the first tone stimulus, but freezing behavior developed significantly across trials such that all groups demonstrated robust freezing during the final Tone and Post-tone periods. Analysis of variance revealed a significant effect only of trial [Figure 1B; F(5,140) = 40.79, p<.0001]. The effect of stress was not significant [F < 1] and did not interact with the effect of trial [F < 1]. The unconditional response to shock did differ among groups. The velocity of travel during the shock was dependent upon stress type [Figure 1C; F(2,28) = 4.66, p < .05] and post-hoc analyses indicated that the RRS group differed from both the CVS and CON groups [Fisher’s PLSD, p<.05]. Thus, short-term measures of fear revealed no effect of stress exposure on the development of freezing but a reduction in active defensive responding induced by repeated restraint stress.

**Context and Tone Fear**

Data from the context and tone tests are depicted in Figure 2. Twenty-four h after conditioning, mice were returned to the training context. Freezing during this test differed significantly among the groups [Figure 2A; F(2,28) = 4.48, p<.05]. Post-hoc analyses
revealed that the CVS group demonstrated significantly greater context freezing than did the CON group. Twenty-four h after the context test, animals were placed in a set of novel chambers for a test of tone fear. During this test, freezing was measured both during a baseline period and during a tone period. The effect of period was the sole factor significantly impacting freezing during this test [Figure 2B; F(1,28) = 125.29, p<.0001]. Stress groups did not differ [F < 1], nor did the stress factor interact with that of period [F < 1].

Experiment 2

The results of Experiment 1 support the assertion that the type of stressor exposure determines subsequent enhancement of conditional fear responses in mice. They further support the use of the chronic variable stress procedure as a model of the processes by which stressful life experiences might sensitize humans to developing anxiety disorders. Gender discrepancies in the clinic (NIMH, 2007), along with the powerful impact of sex in determining stress effects on fear in rats (Baran et al., 2009), motivated us to pursue the potential impact of sex on the stress-enhancement of fear in mice.

Materials and methods

Subjects

Adult female mice (C57Bl/6 strain, aged 2-4 months) served as subjects. Mice were purchased from the same source as those of Experiment 1 and were treated in an identical fashion.

Apparatus and Procedures
All apparatus and procedures were identical to those of Experiment 1. Female mice were subjected to one of three stress procedures: repeated restraint stress (RRS, n = 8), chronic variable stress (CVS, n = 8), or control procedures (CON, n = 9) before undergoing Pavlovian fear conditioning.

Results

Conditioning

Data from the training session are depicted in Figure 3. Stress treatment failed to impact pre-training exploratory behavior [Figure 3A; F(2,22) = 1.91, p = .17]. All groups showed little freezing to the first or second tone stimulus during training but showed significant increases in freezing across the training session. The effect of trials was significant [Figure 3B; F(5,110) = 29.83, p < .0001]. The effect of stress approached significance [F(2,22) = 3.34, p = .054] with a trend for repeated restraint stress to reduce freezing during training. The stress effect and trials effect did not interact [F(10,110) = 1.24, p = .27]. The activity burst was not effected by stress treatment [Figure 3C; F(2,22) = 1.98; p = .16].

Context and Tone Fear

Data from the context and tone tests are depicted in Figure 4. Stress treatment did not impact freezing during the context test [Figure 4A; F(2,22) = 1.52, p = .24]. During the tone test, a significant period effect indicated that all groups demonstrated a significant increase in freezing during the tone period [Figure 4B; F(1,22) = 97.59; p < .0001]. Stress significantly impacted tone freezing [F(2,22) = 4.70; p < .05] but the effect of stress did not interact with the effect of trials [F(2,22) = 1.48; p = .25]. Post-hoc analyses revealed that the CVS group demonstrated significantly more freezing than did
the other two groups [Fisher’s PLSD, p < .05] during the tone period but not during the baseline period.

**Experiment 3**

The results of Experiment 2 indicate that male and female mice respond very differently to the chronic variable stress procedure. Sex differences in the impact of chronic stress on neurobiology depend upon ovarian steroids and are significantly affected by ovariectomy (Garrett & Wellman, 2009; McLaughlin et al. 2009b). Therefore, our observed sex differences in fear-enhancing impact of stress may depend on ovarian steroids. We pursued the hypothesis that ovariectomy would abolish the impact of chronic variable stress on subsequent fear learning in female mice (seen in Experiment 2) and perhaps render their behavioral response to stress more similar to that of the males (seen in Experiment 1).

**Materials and methods**

**Subjects**

Adult female mice (C57Bl/6 strain, aged 2 months) served as subjects. Mice were purchased from the Research Models and Services division of Charles-River (Portage, MI). All surgical procedures therefore were performed before delivery, under the auspices of the Charles River Institutional Animal Care and Use Committee. Surgery was performed when the mice were one month old. Mice were anesthetized with a ketamine/xylazine mixture and subjected to either fallopian tube crush or sham surgical procedures. The details of the surgical procedure are published by the supplier (Charles River, 2005). Mice were allowed approximately five weeks of recovery from the surgery
prior to behavioral treatments. As in the first two experiments, all behavioral procedures were approved by the Marquette University IACUC.

Apparatus and Procedures

Based upon the data from Experiments 1 and 2, we focused on the impact of ovariectomy on the efficacy of chronic variable stress in altering conditional fear. Thus, we excluded the Repeated Restraint Condition in this experiment. Otherwise, the apparatus and procedures involved in stress and conditioning treatments were identical to those of the first two experiments. Ovariectomized (OVX) and control-surgery (SHAM) animals were subjected only to either chronic variable stress (CVS) or control procedures (CON). The design yielded four groups of mice: OVX-CVS (n = 11), OVX-CON (n = 12), SHAM-CVS (n = 11), and SHAM-CON (n = 12). In order to confirm the influence of ovariectomy and stress, and to monitor potential illness in the wake of surgery, we weighed mice during the stress exposure period of the experiment (with a standard laboratory scale during the daily tailmarking procedure).

Results

Conditioning

Data from the conditioning session are depicted in Figure 5. Pre-training exploration of the conditioning chamber (Figure 5A) was not influenced significantly by either surgical treatment [F(1,42) = 2.34, p = .13] or stress [F(1,42) < 1]. The interaction of the two factors failed to reach significance [F(1,42) = 3.00, p = .09]. Over the course of training, animals showed low levels of freezing during early trials but acquired significant levels of freezing by the end of the training session (Figure 5B). Freezing during the training session showed a large degree of variance, and outlier analyses
identified four animals as outliers (1 each from the OVX-CON and SHAM-CON groups and 2 from the SHAM-CVS group). In the face of having to eliminate so many animals from the analysis, we performed a square-root transformation of the data. The transformed data indicated no outliers and, therefore, we performed the statistical analyses of acquisition freezing scores on the transformed data. The effect of trial was significant \[ F(5,210) = 47.16, \ p < .0001 \]. Surgery did not impact the acquisition of freezing during conditioning \[ F(1,42) < 1 \]. Stress also failed to impact the course of freezing during the conditioning session \[ F(1,42) = 2.38, \ p = .13 \] and did not interact with surgery \[ F(1,42) = 1.36, \ p = .25 \]. The shock response (Figure 5C) was not influenced significantly by surgery \[ F(1,42) = 2.14, \ p = .15 \] or stress \[ F < 1 \], and the two factors did not interact \[ F < 1 \].

**Context and Tone Fear**

Data from the context and tone tests are depicted in Figure 6. Freezing during the context test (Figure 6A) was not influenced significantly by either surgery \[ F(1,42) = 1.01, \ p = .32 \] or stress \[ F < 1 \], and the two factors did not interact \[ F(1,42) = 2.59, \ p = .12 \]. During the tone test, a significant effect of test period was found \[ Figure 6B; F(1,42) = 137.53, \ p < .0001 \]. Neither the effect of surgery \[ F < 1 \] or stress \[ F < 1 \] was significant, but stress significantly interacted with the test period effect \[ F(1,42) = 20.15, \ p < .0001 \]. Post-hoc analyses revealed that tone onset caused a greater increase in freezing (over baseline) in the CVS group than in the CON groups [Fisher’s PLSD, \( p < .05 \)].

**Body Weight**
We used body weight to confirm the impact of ovariectomy surgery and stress (McElroy and Wade, 1987; McLaughlin, Baran, Wright, & Conrad, 2005). Ovariectomy resulted in significantly enhanced body weight [Figure 7, F(1,42) = 85.56, p < .0001] and the stress treatment significantly reduced body weight over the stress period [F(1,42) = 9.83, p < .01]. However, these effects must be appreciated in the context of a significant 3-way interaction among surgery, stress, and day. Ovariectomized mice started the experiment significantly heavier than their sham-operated counterparts. Stress caused a significant decrease in body weight only among OVX mice.

**Discussion**

**Summary**

In sum, the present studies reveal that exposure to chronic stressors alter subsequent defensive responses and fear learning in mice, in a manner that is dependent upon both the type of stress and the sex of the animal. Among male mice, chronic variable stress produced a significant inflation of contextual fear conditioning. Repeated restraint stress, while having no significant impact upon long-term fear memory, did alter exploratory behavior and the response to shock. Among female mice, chronic variable stress instead produced a specific inflation of tone fear conditioning. Repeated restraint stress, while having no impact on long-term fear memory, tended to inhibit slightly the short-term freezing response during conditioning. Finally, ovariectomy did not modify the impact of stress on conditioned fear. Even after ovariectomy, female mice demonstrated a tone-specific inflation of conditional fear after chronic variable stress. Confirmatory measures of bodyweight revealed a significant impact of ovariectomy and an influence of ovariectomy on the physiological response to chronic stress.
Nonetheless, the results of our final experiment do not support a role for ovarian steroids in sex differences in the fear-inflating capacity of chronic variable stress.

**Limitations**

Comparisons of results across experiments must be made with some caution. Each experiment was executed at a different time, and it is appropriate to consider statistical comparisons only within the context of each experiment. Whereas male mice demonstrated no significant impact of stress on fear acquisition, female mice in Experiment 2 showed a trend for restraint stress to impair fear acquisition. However, the number of mice per group differed from Experiment 1 to Experiment 2. Therefore, the trend of restraint to impair freezing during acquisition, selectively in female mice, may have proven significant if the power of Experiment 2 matched that of Experiment 1. The long-term impact of stress on context and tone fear, however, appears to be qualitatively different in female and male mice. Females showed no effect of stress on context and a significant effect on tone fear, the significance of which would not have changed if the power of Experiment 2 matched that of Experiment 1. In Experiment 2, females demonstrated inflation in tone fear after chronic variable stress, an effect not seen at all in males. Experiment 3 confirmed the inflation of tone fear in female mice, with additional sets of experimentally naïve subjects. While differing numbers of animals across groups prevent us from making direct statistical analyses across Experiments, they do not alter the conclusion that the pattern of behavioral response to stress differs in kind between male and female mice. The results also demonstrate conclusively that chronic variable stress significantly alters subsequent defensive behavior in both males and females.
Thus, both sexes experience significant stress, but they respond uniquely in the enhancement of particular fear memories.

Ovariectomy might be expected to alter basic pain processes that, in turn, manifest themselves in changes in fear conditioning. Ovariectomy causes hyperalgesia in response to mechanical and thermal stimuli in mice, and ovarian steroids are implicated in the effect (Sanoja & Cervero, 2008). However, the hyperalgesia seen after ovariectomy is specific to particular stimuli and body regions, thus footshock responses might not be similarly affected. While some investigators have found that ovarian hormones influence responses to low- but not high-intensity footshock (Drury & Gold, 1978), others have reported no significant impact of ovariectomy or estrus cycle (Beatty & Fessler, 1977). Our procedures involved shock intensity sufficient to condition fear responses, and our dependent measure of footshock reactivity showed no impact of ovariectomy surgery (Figure 5). In addition, ovariectomy had no impact on the amount of fear conditioned by the shock. While we cannot rule out an effect of ovariectomy on some aspect of shock processing, we have no evidence to support such an effect. We should note that the shock response that we employed was sensitive enough to demonstrate that repeated restraint inhibits the activity burst in males (Figure 1). Stress-induced analgesia among the males could explain the deficit, but we have no independent measure of pain processing to confirm or deny this possibility. Nonetheless, the activity burst measure is sensitive to stress in males and insensitive to ovariectomy in females.

One additional limitation of the current set of studies lies in the exclusive focus on the behavioral impact of stress exposure. No direct measures were made of the activation of the stress response, during the course of stressor exposure. The data indicate clearly
that the stressors were effective in altering subsequent behavior: Restraint stress was effective in altering the shock response in males. Chronic variable stress was effective in altering subsequent fear conditioning in both sexes. However, we did not make concurrent physiological measures of stress system activation, in Experiments 1 and 2. In Experiment 3, we measured body weight to confirm the effectiveness of the stress procedure and the ovariectomy surgery. Ovariectomy typically produces a gain in body weight in rodents (McElroy & Wade, 1987), and we observed an identical effect here (Figure 7). Body weight measures further confirmed the effectiveness of the chronic variable stress procedure among ovariectomized mice; replicating recent findings with ovariectomy and chronic restraint stress in rats (McLaughlin et al., 2005). Other recent reports support the contention that seven days of chronic variable stress is sufficient to activate the stress system (Tauchi, Zhang, D’Alessio, Seely, & Herman, 2008). In future studies of the behavioral impact of chronic variable stress, concurrent measures of corticosterone response, body weight, and other direct measures of the stress response, will help illuminate the relationship between the behavioral and systemic consequences of stress.

**Implications and Future Directions**

Whenever sex differences emerge in behavior, sex hormones obviously are implicated. We found robust sex differences in stress enhancement of fear, and we began Experiment 3 with the hypothesis that these differences were mediated by sex hormones. Among humans, ovarian hormones are implicated in the findings that women are both more susceptible to the negative effects of stress (Matud, 2004; Schmaus et al., 2008), and more likely to develop anxiety disorders (Gater et al., 1998; NIMH, 2007; Pigott,
In rodents, ovarian hormones determine the severity of the stress response (Young et al., 2001) and experimentally elevated levels of estrogen increase fear responses (Morgan & Pfaff, 2001). In our study, ovariectomy did alter the impact of stress on body weight measures, but no modulatory effect was seen regarding stress inflation of fear. Of note, a number of recent studies have begun to reveal that sex differences can be driven significantly by sex-linked genetic factors, rather than sex hormones (Arnold, 2009). In fact, sex chromosomes exert powerful control over sexually dimorphic behaviors in social interaction (McPhie-Lalmansingh, Tejada, Weaver, & Rissman, 2008), aggression (Gatewood et al., 2006) and habit learning (Quinn, Hitchcott, Umeda, Arnold, & Taylor, 2007). Mouse genetic manipulations now allow for the dissociation of gonadal and genetic influences over sex differences (Arnold, Rissman, & DeVries, 2003), and could prove invaluable in delineating the mechanisms by which sex differences in fear are influenced by sex differences in stress response.

Even in the absence of a critical role for sex hormones, sex differences in stress hormones might be responsible for the effects observed in our studies. There are sex differences even in resting levels of corticosterone (Atkinson & Waddell, 1997), and differences in corticosterone levels have been implicated in sex differences in stress responses (Dalla, et al., 2005). Thus, males and females may begin the fear conditioning session with different levels of circulating corticosterone. Prevailing levels of corticosterone at the time of conditioning determine the amount of conditional fear that is established (de Quervain, Aerni, Schelling, & Roozendaal, 2009). Future investigations should endeavor to measure changes in corticosterone across the stressor period and at the time of acquisition. A second possibility is that males and females have different
corticosterone responses to the fear conditioning procedure itself, and the resulting difference in hormone levels after conditioning is responsible for sex differences in behavior. Post-training levels of corticosterone are critical in determining the strength of memory consolidation (de Quervain et al., 2009) and thus might prove critical to the stress enhancement of fear seen here. The chronic variable stress procedure alters the density of corticosterone receptors involved in the stress response (Herman, Adams, & Prewitt, 1995; Herman, Watson, & Spencer, 1999) and would be expected to therefore alter responses to subsequent stressors (including those engendered by fear acquisition events) and consolidation processes. Again, future effort must be made to measure corticosterone levels and reactivity in the post-training period, in order to uncover possible sex differences therein. In the context of acquisition and consolidation, we should note the pattern of responses in the repeated restraint group of Experiment 1. The restraint group demonstrated “deficient” shock reactivity at the time of training but normal levels of conditional fear later on. This pattern suggests the possibility that repeated restraint both diminishes acquisition and enhances consolidation. Future investigations must be conducted to tease out the effects of stress on acquisition and memory processes, by interposing the chronic stress events between conditioning and testing.

The neural circuitry responsible for the current pattern of results remains unknown. Our studies demonstrate that males and females show qualitatively different patterns of fear inflation after stress exposure. The stimulus specificity of fear enhancement, as well as the sex differences therein, hint at the potential neurobiological mechanisms involved. Different neural systems underlie fear conditioning to different
stimuli and these mechanisms might be differentially engaged by stress in male and female animals. The hippocampus is critically important for contextual fear conditioning (Kim & Fanselow, 1992; Philips & LeDoux, 1992; Sanders, Wiltgen, & Fanselow 2003). Stress is a well-known modulator of hippocampal function and physiology. Chronic stress alters hippocampal neurophysiology (Conrad, LeDoux, Magarinos, & McEwen, 1999; Magarinos, McEwen, Flugge, & Fuchs, 1996; McKittrick et al., 2000; Vyas, Mitra, Shankaranarayana Rao, & Chattarji, 2002) and alters hippocampus-dependent learning (Baran et al., 2009; Conrad et al., 1999; Park, Campbell, & Diamond, 2001). The findings of Experiment 1 are consistent with the idea that stress affects hippocampal function and indicate that chronic variable stress may sensitize hippocampal processes involved in contextual learning. The enhancement of context fear is consistent with prior studies of chronic stress effects in fear (Conrad et al., 1999), but the effect runs counter to the typical finding in spatial tasks where chronic restraint typically causes deficits in learning and memory (see Conrad, 2009). The discrepancy likely lies with the particularly aversive nature of the fear conditioning task. The lack of an effect on context fear, in Experiment 2 however, indicates that hippocampal structures in males and females may be differentially sensitive to chronic variables stress. Sex differences in stress have been studied most extensively in the context of spatial learning, and the findings may help guide hypotheses regarding effects in fear learning. In maze tasks, chronic stress has very different neurobiological and behavioral effects in male and female rats (Bowman, Beck, & Luine, 2003; McLaughlin et al., 2009a). In male rats, stress causes retraction of dendrites in hippocampal CA3 and corresponding deficits in spatial learning (McLaughlin, Gomez, Baran, & Conrad, 2007). In females, stress
typically causes milder damage in CA3 (Galea et al., 1997) yet enhances spatial performance (Bowman et al., 2003). In light of these findings, our results may reflect a difference between the sexes in stress-related hippocampal changes. Males may suffer greater damage after chronic variable stress, and the relationship between such damage and resulting behavior may differ between the sexes as well. In deed, males may show greater hippocampal plasticity and greater involvement of the hippocampus in contextual fear conditioning (Maren, De Oca, & Fanselow, 1994). Finally, our finding of tone fear inflation in females suggests sex differences in stress effects outside of the hippocampus, because tone and context fear are mediated by dissociable brain mechanisms (Fendt & Fanselow, 1999). Future investigations must be aimed at the effects of stress, and the modulating effect of sex, on dendritic and other changes within fear conditioning circuits. By directly measuring changes in the brain areas responsible for fear, we can begin to illuminate the mechanisms where stress and fear processes interact to produce critical sex differences in behavior.

**Acknowledgments**

The authors would like to thank Jon Toppen and Bernard J.V. Rahming, for assistance with the stress procedures. We would like to thank Dr. Amy Van Hecke for advice on statistical analyses. We are grateful for the insightful comments, of two anonymous reviewers of a prior version of the manuscript. Excellent animal husbandry was provided by the Marquette University Animal Resource Center.
References


*Neuropsychopharmacology, 25*, 881-891.
Figure Captions

Figure 1. Behavioral observations during the training session, for Experiment 1.  A. Mean (+SEM) number of cage crossovers during the first 2 min of the training session. Values represent the number of times the mouse crossed from one side of the chamber to the other. *: RRS animals were significantly more active than CON animals. B. Mean (±SEM) freezing response during the conditioning trials. Values represent the mean percentage of video samples, during the tones and post-shock periods, scored as freezing by a human observer. C. Mean (+SEM) activity burst in each of the three groups. Values represent the mean velocity (cm/s) of the flight response during the 2-s shock. †: RRS animals showed significantly reduced bursts, compared to the other two groups.

Figure 2. Behavioral observations during the context and tone tests, for Experiment 1. A. Mean (+SEM) freezing during the context test. Values represent mean percentage of video samples, taken over a 5-min exposure to the training context, scored as freezing by a human observer. *: CVS mice showed a significant increase in context freezing. B. Mean (±SEM) freezing during the tone test, conducted 48 h after training. Values represent the mean percentage of video samples, taken during a 2-min baseline and a 3-min tone exposure, scored as freezing by a human observer.

Figure 3. Behavioral observations during the training session, for Experiment 2.  A. Mean (+SEM) number of cage crossovers during the first 2 min of the training session. Values represent the number of times the mouse crossed from one side of the chamber to the other. B. Mean (±SEM) freezing response during the conditioning trials. Values represent the mean percentage of video samples, during the tones and post-shock periods, scored as freezing by a human observer. C. Mean (+SEM) activity burst in each of the
three groups. Values represent the mean velocity (cm/s) of the flight response during the 2-s shock.

**Figure 4.** Behavioral observations during the context and tone tests, for Experiment 2. A. Mean (+SEM) freezing during the context test. Values represent mean percentage of video samples, taken over a 5-min exposure to the training context, scored as freezing by a human observer. B. Mean (±SEM) freezing during the tone test, conducted 48h after training. Values represent the mean percentage of video samples, taken during a 2-min baseline and a 3-min tone exposure, scored as freezing by a human observer. †: CVS mice demonstrated significantly greater tone freezing than did the other two groups only during the tone period.

**Figure 5.** Behavioral observations during the training session, for Experiment 3. A. Mean (+SEM) number of cage crossovers during the first 2 min of the training session. Values represent the number of times the mouse crossed from one side of the chamber to the other. B. Mean (+SEM) freezing response during the conditioning trials. Values represent the mean percentage of video samples, during the tones and post-shock periods, scored as freezing by a human observer. C. Mean (+SEM) activity burst in each of the three groups. Values represent the mean velocity (cm/s) of the flight response during the 2-s shock.

**Figure 6.** Behavioral observations during the context and tone tests, for Experiment 3. A. Mean (+SEM) freezing during the context test. Values represent mean percentage of video samples, taken over a 5-min exposure to the training context, scored as freezing by a human observer. B. Mean (±SEM) freezing during the tone test, conducted 48 h after training. Values represent the mean percentage of video samples, taken during a 2-min
baseline and a 3-min tone exposure, scored as freezing by a human observer. *: CVS mice demonstrated significantly greater tone freezing than did the other two groups.

**Figure 7.** Body weight over the course of stress exposure, for Experiment 3. Values represent the mean (±SEM) body weight in each group. *: OVX animals were significantly heavier than SHAM animals at the start of stress exposure. Surgery interacted with the stress, with only the OVX animals demonstrating significant weight loss over the stress period. †: OVX-CVS animals lost significant weight when compared to OVX-CON animals.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7