Neurobiology of Learning and Memory 94 (2010) 254–262

Contents lists available at ScienceDirect

Neurobiology of Learning and Memory

journal homepage: www.elsevier.com/locate/ynlme

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

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ARTICLE INFO

*Article history:*
Received 1 March 2010
Revised 2 June 2010
Accepted 8 June 2010
Available online 19 June 2010

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

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.



## 1. 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

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doi:10.1016/j.nlm.2010.06.003

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, Roth, and 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 2–3 weeks approximately 30 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.

## 2. Experiment 1

### 2.1. Materials and methods

#### 2.1.1. 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 12:12 h 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 US Public Health Service "Policy on Humane Care and use of Laboratory Animals."

### 2.2. Apparatus and procedures

#### 2.2.1. 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 (10 cm × 10 cm) attached at one end to a cylindrical PVC cap (3.5 cm dia. × 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.

#### 2.2.2. 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 °C) for 30 min.

*Ultrasound*: placement in a bucket 40 cm 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.

#### 2.2.3. Fear conditioning and testing

Twenty-four hours 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