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Sex-specific impairment and recovery of spatial learning following the end of chronic unpredictable restraint stress: Potential relevance of limbic GAD

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Abstract

Chronic restraint stress alters hippocampal-dependent spatial learning and memory in a sex-dependent manner, impairing spatial performance in male rats and leaving intact or facilitating performance in female rats. Moreover, these stress-induced spatial memory deficits improve following post-stress recovery in males. The current study examined whether restraint administered in an unpredictable manner would eliminate these sex differences and impact a post-stress period on spatial ability and limbic glutamic acid decarboxylase (GAD₆₅) expression. Male (n=30) and female (n=30) adult Sprague-Dawley rats were assigned to non-stressed control (Con), chronic stress (Str-Imm), or chronic stress given a post-stress recovery period (Str-Rec). Stressed rats were unpredictably restrained for 21 days using daily non-repeated combinations of physical context, duration, and time of day. Then, all rats were tested on the radial arm water maze (RAWM) for two days and given one retention trial on the third day, with brains removed 30 minutes later to assess GAD₆₅ mRNA. In Str-Imm males, deficits occurred on day 1 of RAWM acquisition, an impairment that was not evident in the Str-Rec group. In contrast, females did not show significant outcomes following chronic stress or post-stress recovery. In males, amygdalar GAD₆₅ expression negatively correlated with RAWM performance on day 1. In females, hippocampal CA1 GAD₆₅ positively correlated with RAWM performance on day 1. These results demonstrate that GABAergic function may contribute to the sex differences observed following chronic stress. Furthermore, unpredictable restraint and a recovery period failed to eliminate the sex differences on spatial learning and memory.

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Keywords

Chronic stress; spatial learning; GABA; hippocampus; amygdala; sex differences; recovery

1. Introduction

It is increasingly apparent that chronic stress impairs hippocampal-dependent spatial learning and memory in a sex-dependent manner. In males, chronic stress impairs performance on the radial arm maze (Luine et al., 1994; Sweis et al., 2013), Y-Maze (McLaughlin et al., 2007), Morris water maze (Sousa et al., 2000; Kitraki et al., 2004a,b; Radecki et al., 2005; Song et al., 2006; McFadden et al., 2011; Green and McCormick, 2013), and the radial arm water maze task (RAWM, Hoffman et al., 2011; Hutchinson et al., 2012; For review see: Conrad 2010; Popoli et al., 2012; McEwen et al., 2012). Conversely, several studies report that chronic stress enhances spatial memory ability in females on the radial arm maze (Bowman et al., 2001), Y-maze (McLaughlin et al., 2007), object placement task (Beck and Luine, 2002), and Morris water maze (Kitraki et al., 2004a,b; McFadden et al., 2011; reviewed in Luine et al., 2007; Simpson & Kelly, 2012). These studies suggest that the sex of an individual influences the underlying neural mediators of spatial ability.

A putative target for chronic stress actions is the hippocampus, as it is essential for spatial memory (O'Keefe and Dostrovsky, 1971; Moser et al., 1993; Eichenbaum 1999; Conrad 2010) and contains an abundance of receptors for glucocorticoid stress hormones (McEwen et al., 1968). In male rats, chronic stress leads to a simplification of apical dendritic arbors within the CA3 region of the hippocampus (Watanabe et al., 1992a, 1992b; Galea et al., 1997; Conrad, 2006) and reduced glucocorticoid receptor number (Sapolsky et al., 1984a, 1984b), both of which correspond to deficits in spatial tasks (Hoffman et al., 2011; Wright et al., 2006). When chronically stressed males are given time to recover following the end of chronic stress, spatial ability improves in parallel with the restoration of the CA3 dendritic architecture (Hoffman et al., 2011; Ortiz et al., 2014). In contrast, chronically stressed, gonadally-intact female rats show mild or negligible basal dendritic retraction in the CA3 region (Galea et al., 1997; McLaughlin et al., 2010), which does not necessarily correspond with spatial memory (McLaughlin et al., 2005). Mitigating variables that might contribute to chronic stress effects on the hippocampus and spatial memory in females include the type of stressor (Park et al., 2008; McCormick et al., 2008), task (Wood & Shors, 1998; McLaughlin et al., 2008; McCormick et al., 2010), estrogen status (Shansky et al., 2006; Conrad et al., 2012; Ortiz et al., 2013) and even experimenter handling of the rats (Dobráková et al., 1993; Bohacek and Daniel, 2007; Hoffman et al., 2010). Whether females would return to their previous status in spatial ability following a post-stress recovery period has not been investigated. One goal of the present study is to investigate the potential sex differences arising from the effects of chronic stress and a post-stress recovery period on spatial learning and memory. Successful spatial memory recovery from chronic stress has been documented in male rats using the radial arm water maze (Hoffman et al., 2011; Ortiz et al., 2014) and so a similar task will be used in the current study.

An important variable that could impact sex differences in spatial memory performance is the type of stressor used. Restraint stress is commonly used in rodents due to its relative ease

of use, however chronic restraint elicits detriments in spatial learning and memory in male (Sousa et al., 2000; Hoffman et al., 2011; Gomez et al., 2012; Ortiz et al., 2014), but not female rats (Kitraki et al., 2004a; McLaughlin et al., 2010). Stressors can be categorized as being physical, psychological, or both and chronic restraint is thought to include components of both physical and psychological stressors (Buynitsky and Mostofsky, 2009). In humans, psychological stressors produce a stress response when the stressor consists of any one of three characteristics: loss of control, novelty, and unpredictability (Mason, 1968; Cohen, 1980; Miller, 1981). In rodents, administration of chronic restraint stress might consist of a loss of a sense of control, but lacks components in the other categories (i.e. novelty and unpredictability). Furthermore, repeatedly administering restraint leads to predictability, as male rats demonstrate habituation of the stress hormone response (Galea et al., 1997). Therefore, one purpose of the present study was to determine whether enhancing the unpredictable nature of chronic restraint would lead to impaired spatial ability in both male and female rats.

Another purpose of this study was to examine the role of inhibitory tone as it pertains to chronic stress-induced changes in spatial learning and memory. Glutamate and γ -aminobutyric acid (GABA) neurotransmitters are primarily responsible for regulating inhibitory tone. In male rats, chronic stress leads to significant increases in hippocampal extracellular glutamate (Joëls et al., 2004) and decreases in hippocampal GABA (Grønli et al., 2007; O'Mahony et al., 2011). Hippocampal GABA levels in males might be lowered following chronic stress, in part, due to decreases in glutamic acid decarboxylase (GAD) levels, the synthesizing enzyme for GABA (El-faramawy et al., 2009). Furthermore, other limbic areas may display a dysregulated inhibitory tone following chronic stress, which may also impact spatial learning and memory. MeA input into the HPA axis is critical for HPA axis activation following restraint stress (Dayas et al., 1999). Cells in the MeA exhibit the greatest level of *c-fos* activation following a psychological stressor compared to other amygdala nuclei (e.g. central amygdala). These findings suggest that the MeA is implicated in the response to stressors. Taken together, these studies provide support for the notion that the spatial memory impairments induced by chronic stress are mediated by changes in inhibitory tone the limbic system. Whether these changes contribute to the sex differences observed in spatial learning and memory tasks following chronic stress are unknown and will be investigated.

The current study tested the hypothesis that the unpredictability of a chronic stress paradigm impacts the sensitivity of females to the immediate and long-term (i.e., recovery from) effects of chronic stress. The behavioral endpoint of spatial learning and memory was chosen due to the well-established immediate and long-term effects of chronic restraint on male rats. In addition, this study explored the effect of chronic unpredictable restraint stress on GAD expression in limbic regions, as a likely mediator of chronic stress effects and a potential mediator of sex differences.

2. Material and methods

2.1. Animals

Sixty young adult Sprague-Dawley male and female rats (males=30, females=30) approximately 2 months of age were purchased from Charles River Laboratories (Wilmington, MA) and were pair housed with a same-sex cage mate at Arizona State University housing facilities. Male and female rats were housed in separate rooms and control and experimental groups were additionally separated into different chambers on a reverse light cycle (12:12; lights off at 06:00am). Rats were given one week to acclimate before any behavioral procedures were performed. Food and water were available *ad libitum* except during the restraint procedure when both control and stressed animals did not have access. Rats were weighed once a week throughout the experiment. Behavioral testing was conducted during the dark phase of the light cycle. All procedures were conducted according to federal guidelines outlined in the Guide for the Care and Use of Laboratory Rats (Institute of Laboratory Animal Resources on Life Science, National Research Council, 1996) and institutional guidelines set forth by the Institutional Animal Care and Use Committee at Arizona State University.

2.2. Group Assignments

Rats were randomly divided into six groups (n=10/group): male and female non-stressed control (Con), male and female stressed rats that were tested immediately (Str-Imm), and male and female stressed rats that were given a 21 day post-stress recovery period (Str-Rec). The experimental groups and timeline are depicted in (Figure 1A).

2.3. Chronic Unpredictable Restraint Stress Procedure

Rats were transported to different rooms each day and were restrained using restrainers made from wire mesh (purchased from Flynn and Enslow, San Francisco, CA, USA) with the ends sealed with grip guard sealer (ACE Hardware). The ends were secured with black binder clips. The chronic unpredictable restraint (CUR) schedule was comprised of different start time periods (ranges: 05:00–07:00, 08:00–10:00, 11:00–13:00, 14:00–16:00, 17:00–19:00), physical contexts (four different rooms), extract odors (almond, banana, orange), and restraint durations (no restraint, 30 minutes, 60 minutes, 90 minutes, and 120 minutes). After acclimation, the Str-Rec group was unpredictably restrained for 21 days then given a 21 day recovery period from restraint before behavioral testing. Str-Imm, however, were left undisturbed during the first 21 days, then unpredictably restrained during the subsequent 21 days and behaviorally tested the day after restraint ended. Con rats were left undisturbed until behavioral testing. Since the past literature indicates that even the subtle manipulation of handling to determine estrous status can impact female performance (Dobráková et al., 1993; Bohacek and Daniel, 2007; Hoffman et al., 2010), the gonadally-intact male and female rats were not manipulated to determine female estrous stage status.

2.4. Radial Arm Water Maze (RAWM)

2.4.1 Apparatus—The RAWM was constructed of black polypropylene with eight symmetrical arms (27.9 cm long by 12.7 cm wide) protruding from a circular center (48 cm

diameter). The water (20°C–22°C) was made opaque with non-toxic powdered black tempera paint in order to obscure the platform. Males and females were tested in separate rooms. Each room contained salient cues along the walls of the room. Groups were counterbalanced across experimenters.

2.4.2. Procedure—A testing platform was placed at the end of one arm, 2.5 cm below the surface of the opaque water. The platform location was kept constant throughout the trials for each rat, but was counterbalanced between rats. Testing occurred over three consecutive days between 9:00am and 3:00pm. The first two training days consisted of eight trials, with one retention trial given on the third day. A trial began as soon as the rat was released into a non-platformed arm (start arm), which varied across trials so that a rat did not start in the same start arm on consecutive trials. Additionally, a trial was never initiated in an arm directly across from the platformed arm. Rats were given a maximum of three minutes to find the hidden platform. If a rat failed to find the hidden platform, the experimenter guided the rat to it. Rats remained on the platform for 15 seconds before they were removed and returned to the testing cage underneath heat lamps. After each trial, the maze was cleared of any debris, and the water was stirred to prevent subsequent rats from using odor cues. To avoid exhaustion, rats of different groups were tested in cohorts of six to eight at a time. After a trial was completed, rats were placed back into the testing cage to rest while the other rats in the cohort were tested. Time between each trial for an individual rat ranged from 5 minutes to 20 minutes. An entry into an arm was scored when the tip of the rat's nose passed 11 cm into an arm.

2.5. Brain Collection and Tissue Processing

Thirty minutes after the start time of the retention trial on the final day of testing, rats were transported to a necropsy room and deeply anesthetized with isoflurane and rapidly decapitated. Adrenal glands were dissected and weighed. Brains were removed, blocked for hippocampus and amygdala, flash frozen in 2-methylbutane chilled with dry ice, and stored in a –80°C freezer until shipped frozen to Dr. Lucas for *in situ* hybridization processing.

2.6. *In Situ* Hybridization

In situ hybridization was utilized to quantify GAD₆₅ mRNA expression in the cornu ammonis 1 (CA1) and dentate gyrus (DG) regions of the hippocampus, as well as the ventromedial hypothalamus (VMH) and medial amygdala (MeA). Brains were maintained at –70°C until transfer to cryostat for sectioning (at –20°C) and subsequently kept at –20°C until used in the *in situ* protocol. Twenty micrometer coronal brain sections were thaw-mounted on to glass microscope slides (Superfrost Plus, VWR, West Chester PA) on a cryostat (Leica).

Antisense mRNA was generated from a cDNA plasmid (generously provided by Dr A.J. Tobin, UCLA) linearized with HdIII and transcribed with RNA Polymerase T3 (Promega) with [35-S]-UTP (Perkin-Elmer) to a specific activity of 4.5×10^8 in a 550 nucleotide mRNA fragment. Unincorporated nucleotides were removed by Rneasy® MinElute™ Cleanup Kit (Qiagen). Prehybridization of tissue sections included a brief drying at room temperature after removal from –20°C storage, postfixation in 4% formaldehyde/PBS,

rinsing in PBS, and acetylation in 0.25% acetic anhydride/0.1 M triethanolamine. Slides were passed through a dehydrating ethanol gradient and incubated with probe in hybridization solution at a saturated concentration (~ 28 Kcpm/ μ L) under glass coverslips at 45 °C for 18 h. Coverslips were removed in 4 \times standard sodium citrate and non-specifically bound probe was removed by treatment with RNase (Sigma-Aldrich) for 30 min. Sections were run through stringency washes of 1 \times SSC and 0.5 \times SSC at 37 °C, and 0.1 \times SSC at 45 °C. Sections were then dehydrated, air-dried and exposed to Kodak BioMax X-ray film for 3 days along with microscale 14 C standards (Amersham Biosciences). Autoradiographic images were transferred to a desktop illuminator (Kaiser Fototechnik, Buchen, Germany) with a CCD video camera (Hamamatsu C8484) with a MacroNikkor lens (Nikon) attached. X-ray film brain sections were digitized using computer assisted densitometry (Compix Imaging Systems, Sewickly PA). Background illumination was recorded (and digitally subtracted from subsequent images) and optical density was plotted as a function of microscale calibration values. Regions of interest in the CA1, DG, VMH, and MeA were selected among 4 to 6 coronal sections bilaterally, digitized and a calibrated mean optical density value was obtained for each location resulting in one averaged value of each region per animal.

2.7. Statistical Analyses

Statistical analyses were performed using SPSS (version 22). Physiological and behavioral data were analyzed with multifactorial or repeated measures analyses of variance (ANOVAs). Main effects and interactions were followed by Fisher's LSD post hoc tests when $p < 0.05$. Pearson's correlations were used to help determine the relationship between GAD₆₅ expression and RAWM performance. Data are represented as means \pm S.E.M. Results were considered statistically significant if $p < 0.05$.

3. Results

3.1 Physiological Measures

3.1.1 Body Weight—Attenuation of body weight gain throughout the study confirmed the effectiveness of CUR. Before experimental manipulation, the weight of male rats ranged from 282 grams to 332 grams, with an average weight of 317.5 grams. Female rats weight ranged from 194 grams to 230 grams, with an average weight of 210.3 grams. A multifactorial repeated measures ANOVA for stress history and sex over the two treatment periods (weeks 1–3: restraint on Str-Rec; weeks 4–6: restraint on Str-Imm) revealed significant main effects of stress ($F_{(2,54)} = 11.26$, $p < 0.001$) and sex ($F_{(1, 54)} = 194.69$, $p < 0.001$) and an interaction between treatment period and stress ($F_{(2, 54)} = 15.48$, $p < 0.001$). Unstressed males gained significantly more weight than did stressed males during CUR administration (weeks 1–3: Con > Str-Rec, $p < 0.01$, Str-Imm > Str-Rec, $p < 0.05$; weeks 4–6: Con/Str-Rec > Str-Imm, $p < 0.001$; Figure 1B). In addition, during the recovery period the Str-Rec males exhibited weight gain similar to Con males (Figure 1B). Interestingly, the same pattern was not evident in females. Unstressed females did not gain significantly more weight than stressed females during the restraint periods (Figure 1B). However, during the recovery period (weeks 4–6), the Str-Rec females exhibited a significant increase in weight gain compared to both Con ($p < 0.05$) and Str-Imm ($p < 0.01$) females.

3.1.2 Adrenal weight—Restraint stress produced a significant increase in adrenal weights relative to body weight in males, but not in females (Figure 1B). Adrenal weights (mg) were analyzed per 100 g body weight. A 2×3 ANOVA for sex and stress revealed a significant main effect of sex ($F_{(1, 50)} = 401.134$, $p < 0.001$) and a tendency for an effect of stress history ($F_{(2,50)} = 2.53$, $p = 0.09$) on adrenal weights, with no significant interaction of sex by stress ($F_{(2,50)} = 2.17$, $p = 0.125$). This analysis was followed up with separate one-way ANOVAs in males and females to examine stress history. In males, a significant effect of stress was found ($F_{(2, 26)} = 4.872$, $p < 0.05$; Figure 1B), while the females failed to show an effect of stress on adrenal weight (Figure 1B). Post hoc analyses in male rats revealed a significant increase in relative adrenal weight in both Str-Rec ($p < 0.05$) and Str-Imm ($p < 0.01$) groups compared to Con males (Figure 1B).

3.2 Radial Arm Water Maze

3.2.1 Acquisition—Significant acquisition effects of CUR were observed across training and this interacted with stress history. A repeated-measures ANOVA for stress history and sex across training blocks (two trials each, blocks 1–8) revealed a significant effect of block ($F_{(7,364)} = 32.41$, $p < 0.001$) on entry errors. Errors decreased as trials progressed, indicating that all rats learned the task (Figure 2). In addition, an interaction between stress history and block was detected ($F_{(14,364)} = 1.84$, $p < 0.05$), indicating that not all groups performed similarly across time and this was probed further by day, as described next.

Training Day One: To reduce the impact of variability during the information gathering trials, the first block of day 1 was parsed out and analyzed separately. A two-way ANOVA for stress history and sex on the first block (2 trials) of training revealed no main effects of treatment or sex on entry errors (Figure 2A and 2B), confirming that all groups made a similar number of entry errors at the beginning of training. The subsequent blocks on day 1 (blocks 2–4) reflect behavior impacted by information gathered in the first and subsequent blocks. A two-way repeated measures ANOVA across blocks 2–4 on entry errors revealed a significant interaction between stress history and sex ($F_{(2,52)} = 4.72$, $p < 0.05$) with no significant main effects of stress or sex. To further probe the sex-specific effects of stress history on acquisition, subsequent analyses assessed day 1 performance in males and females separately. In males, a repeated measures ANOVA on blocks 2–4 revealed a significant main effect of stress history ($F_{(2,26)} = 7.58$, $p < 0.01$). Post hoc analyses revealed that Str-Imm males made more errors than did Con ($p < 0.05$; Figure 2A) and Str-Rec males ($p < 0.001$; Figure 2A). Con and Str-Rec males performed similarly on blocks 2–4. In females, no main effect of stress history was detected on blocks 2–4 (Figure 2B).

Sex Differences on Day One: Additional ANOVAs were used to probe the stress by sex interaction on blocks 2–4. An effect of sex was probed for each stress cohort separately. A significant effect of sex was found between Str-Rec males and females on blocks 2–4 ($F_{(1,18)} = 8.57$, $p < 0.01$), such that Str-Rec females made more errors than did Str-Rec males (2.17 ± 0.34 vs. 1.1 ± 0.12 ; Figure 2C). For the Str-Imm comparison, a tendency for an effect of sex was also identified ($F_{(1,17)} = 3.00$, $p = 0.1$), such that Str-Imm females made fewer errors than did Str-Imm males (1.72 ± 0.33 vs. 2.67 ± 0.45 ; Figure 2C). No difference

in performance between Con females and males was evident (2.09 ± 0.46 vs. 1.67 ± 0.22 ; Figure 2C).

Training Day Two: On the second day of training (8 trials; blocks 5–8), no significant main effects of stress or sex were identified with a repeated measures ANOVA on entry errors (Figure 3). However, an interaction between block and stress was observed ($F_{(6,156)} = 3.08$, $p < 0.01$). To further probe performance on day two, repeated measures ANOVAs were run separately for males and females. This analysis indicated that the stress effect was carried by the males (block by stress interaction; $F_{(6,78)} = 3.89$, $p < 0.01$) but not the females. Block by block analysis of male performance revealed a significant effect of stress on block 5 only. Post hoc analyses indicated that this effect was caused by the Str-Rec males, such that Str-Rec males made more errors on block 5 than both Con and Str-Imm males ($p < 0.05$; Figure 2A). No effect of stress history on day two was identified in females.

3.2.2 Retention—Twenty-four hours after the final day of training, all groups were given a single retention trial to investigate long-term memory. A two-way ANOVA for stress history and sex did not reveal a significant main effect of either stress history or sex on either memory domain (Figure 2). Further probing of the retention trial with separate ANOVAs for males and females did not reveal any sex-specific effects of stress history.

Sample Size: One female Con rat was a statistical outlier and excluded, and one male Str-Imm rat was excluded for health issues. The final sample sizes were as follows: Male Con: $n=10$, male Str-Rec: $n=10$, male Str-Imm: $n=9$, female Con: $n=9$, female Str-Rec: $n=10$, female Str-Imm: $n=10$.

3.3 GAD₆₅ expression

In a first pass analysis, an omnibus 2×3 ANOVA for the variables of sex and stress history on GAD₆₅ expression was performed on each brain region analyzed. There were no significant main effects for sex, stress history, or interaction in any of the four areas examined (Table 1). Since the variable of stress was expected to alter GAD₆₅ expression in limbic structures (El-faramawy et al., 2009), planned comparisons were performed. However, no significant effects of stress history on GAD₆₅ expression were revealed in any of the four brain regions analyzed in either males or females (Table 1).

We found a wide range of variability in GAD₆₅ expression across animals. We took advantage of this variability and conducted correlation analyses to determine whether individual differences in GAD₆₅ expression were associated with RAWM performance. Pearson's correlations were conducted to determine the extent to which GAD₆₅ expression in the CA1, dentate gyrus (DG), ventromedial hypothalamus (VMH) and medial amygdala (MeA) correlated with performance on the RAWM (total errors on each day of training). GAD₆₅ expression significantly correlated with total errors on Day 1 of training in a sex and region-specific manner. In males, a significant negative correlation was identified between GAD₆₅ expression in the MeA and total errors on day 1 of training ($r = -0.41$, $p < 0.05$; Figure 3), such that increased errors were associated with decreased GAD₆₅ expression. In females, a significant positive correlation was identified between GAD₆₅ expression in the

CA1 and total errors on day 1 of training ($r = 0.59$, $p < 0.01$; Figure 3), such that increased errors were associated with increased GAD₆₅ expression. In both males and females, no significant correlations were found between GAD₆₅ expression and day 2 of training in any of the brain regions analyzed.

4. Discussion

4.1 Summary of Findings

The present study administered chronic unpredictable restraint (CUR) stress to investigate whether CUR would impact spatial learning and memory in male and female rats similarly with regard to the immediate and long-term effects of chronic stress. The effectiveness of CUR in male rats was confirmed by attenuated body weight gain and adrenal hypertrophy. In male rats that received CUR followed by a recovery period (Str-Rec), body weight gain returned to normal, while adrenal weights did not. Str-Imm female rats were resistant to the measured physiological effects of CUR administration, though Str-Rec females gained significantly more weight than did Con and Str-Imm females during the recovery period. Male rats that were tested on the RAWM immediately after CUR administration (Str-Imm) exhibited significant impairments during the acquisition phase of the task, as demonstrated by increased total errors on day one of testing. However, male rats that were given a 21-day recovery period (Str-Rec) performed better than Str-Imm males and similarly to non-stressed control males. CUR administration did not affect acquisition performance in females tested immediately (Str-Imm) or after a recovery period (Str-Rec). For the retention trial phase of the RAWM (day 3), the effects of CUR did not affect performance in either males or females. Interestingly, RAWM acquisition correlated with GAD₆₅ mRNA expression in a sex- and region-specific manner. In male rats, GAD₆₅ expression in the MeA was negatively correlated with total errors on day 1 of RAWM training. Conversely, in female rats GAD₆₅ expression in the hippocampal CA1 region was positively correlated with total errors on day 1 of RAWM training.

Physiological effects, such as attenuated body weight gain and enlarged adrenal glands, are common metrics used to determine chronic stress effectiveness. In the current study, chronically stressed male rats displayed increased relative adrenal weights compared to non-stressed controls, an effect that persisted even after a post-stress recovery period. In female rats, the adrenal to body weight ratio and body weight gain were unaltered by CUR. (Figure 1B). This contrasts with other studies showing that chronic restraint stress (McLaughlin et al., 2005; Conrad et al., 2012) and chronic unpredictable stress (McFadden et al., 2011) attenuate body weight gain in female rodents. Thus, it appears that females are less sensitive to CUR when compared to other stress paradigms. The predictability of chronic restraint stress administration was decreased in order to increase the effectiveness of the stressor on altering spatial performance, because repeated exposure to the same stressor leads to habituation of the HPA axis (Viau and Sawchenko, 2002). Habituation to a stressor depends on an organism's ability to recognize the familiarity of stressor and if an animal encounters a novel stressor then the response of the HPA axis can be enhanced (Bhatnagar and Dallman, 1998). Indeed, some studies have shown that the physical context in which a repeated stressor is administered affects subsequent HPA responses to the stressor (Grissom et al.,

2009). The present data suggest that manipulating the predictability in which a stressor is delivered, without changing the stressor itself per se, has little impact on altering body weight, adrenal size and spatial ability in female rats, but it does affect male rats in a manner consistent with other chronic stressors.

An extensive literature reveals that the effects of chronic stress on spatial learning and memory are determined by the sex of the animal (for reviews, see Conrad, 2010; Conrad and Bimonte-Nelson, 2010; McLaughlin et al., 2009). In males, some reports show that chronic stress impairs the learning aspect of spatial tasks (Luine et al., 1994; Sousa et al., 2000; Wright and Conrad, 2008), while others show that chronic stress affects only the memory for the task (Hoffman et al., 2011; Ortiz et al., 2014) or both (Rao and Raju, 2000; Radecki et al., 2005). Despite these discrepancies, the data reported here corroborate the established pattern of chronic stress having detrimental effects on learning in males, as administration of CUR to male rats produced deficits during the acquisition of the RAWM. Consistent with the literature, the CUR stressor in this study did not lead to learning and memory deficits in female rats (Kitraki et al., 2004a; McLaughlin et al., 2010). Although no significant effects were found, if any pattern was to be generously discerned, CUR may have even potentiated spatial learning in female rats, as documented in other reports on learning and memory (see Bowman et al., 2001; Kitraki et al., 2004b; Conrad et al., 2003; McLaughlin et al., 2005; Conrad et al., 2012). Thus, it appears that when restraint stress was made to be unpredictable, a non significant effect on spatial learning was observed in female rats. The common theme of these studies is that female rats appear to be resilient to the effects of chronic stress, especially when compared to male rats.

While CUR impaired spatial ability in the males, we found that a post-stress recovery period following chronic stress reversed the spatial learning deficits. The improvement of spatial ability in males following a post-stress recovery period is corroborated by other studies (Luine et al., 1994; Sousa et al., 2000; Hoffman et al., 2011; Bian et al., 2012; Ortiz et al., 2014), with outcomes that correspond to a restoration of hippocampal CA3 apical dendritic complexity following a post-stress recovery period (Conrad et al., 1999; Sousa et al., 2000; Hoffman et al., 2011). Unexpectedly, Str-Rec males showed impaired performance on day 2 (trial 1), implying that they did not retain the previous day's information, an outcome that contrasts with our previous findings (Hoffman et al., 2011, Ortiz et al., 2014) and others (Sousa et al., 2000). However, the Str-Rec male performance on day 2 is confounded by acquisition differences occurring on day one. Specifically, Con and Str-Imm males made more arm entries during acquisition on day one than did Str-Rec males, which means that all male groups had different arm entry exposures on day 1. Thus, these *a priori* behavioral differences on day 1 confound any interpretations for performance on day 2. Despite these discrepancies, we add to the growing body of literature to show that allowing a post-stress recovery period following chronic stress allows for the return of spatial learning and memory.

The mechanisms underlying chronic stress-induced spatial learning deficits, and subsequent recovery in males, are unclear, but may involve altered inhibitory tone in limbic brain regions. GABA, the main inhibitory neurotransmitter, works in concert with glutamate to maintain a balanced excitatory/inhibitory tone in many brain circuits (Herman et al., 2004).

Disruption of the delicate balance of these tones, as seen following chronic stress, is associated with impaired learning and memory and compromised neuronal integrity (Magariños et al., 1999; Watanabe et al., 1992b; Yoon et al., 2008). In the current study, GAD₆₅ expression was measured in the CA1 subfield of the hippocampus, the medial amygdala, the dentate gyrus, and the ventromedial hypothalamus; brain regions known to display sexual dimorphisms (Matsumoto et al., 1983; Cooke et al., 1999; Madeira et al., 2001; MacLusky et al., 2004), to be influenced by chronic stress (Alfarez et al., 2003; Heine et al., 2004; Jankord and Herman, 2008; Radahmadi et al., 2014) and/or to mediate spatial learning and memory (Moser et al., 1993; Whitlock et al., 2006; Hunsaker et al., 2007; Goodrich-Hunsaker et al., 2008). We predicted that CUR would impact GAD₆₅ expression in a region and sex dependent manner. However, we found no significant main effects of CUR or sex in GAD₆₅ expression in any of the brain regions analyzed. We followed up with exploratory correlational analyses to determine the extent to which individual differences in GAD₆₅ expression contributed to performance on the RAWM. It was observed that GAD₆₅ expression in the medial amygdala (MeA) of males negatively correlated with spatial learning: increased errors were associated with less GAD₆₅ expression in the MeA. This brain region is intriguing because it is responsible for emotional regulation (Phelps and Ledoux, 2005) and is impacted by stress (Prewitt and Herman, 1997; Dayas and Day, 2002; Vyas et al., 2002, 2004; Rosenkranz et al., 2010). It is fascinating that the distribution of the data show the Str-Imm males clustered together and with low GAD₆₅ expression. In contrast, spatial learning in females positively correlated with GAD₆₅ expression in the CA1 region of the hippocampus: increased errors correlated with higher CA1 GAD₆₅ expression and the groups (Con, Str-Imm, Str-Rec) are dispersed throughout the graph. The lack of a correlation between spatial learning and amygdalar GAD₆₅ expression in females is consistent with CUR not impacting spatial ability in females. However, the CA1 region is involved with spatial ability and shows plasticity with spatial learning (Moser et al., 1993; Whitlock et al., 2006). In sum, it appears that GAD₆₅ expression in stress-sensitive limbic regions may be associated with sex differences on spatial ability tasks. A caveat in the current study is that animals underwent three days of RAWM testing, which can increase GAD expression in of itself (Bianchi et al., 2007; de Groote and Linthorst, 2007). Additionally, estradiol can regulate the inhibitory tone in the hippocampus of female rats through GAD₆₅ and GAD₆₇ mRNA expression (McCarthy et al., 1995), without necessarily affecting on GABA_A receptor subunit mRNA expression (Weiland and Orchinik, 1995). Thus, inhibitory tone in limbic regions is affected by a number of factors, with chronic stress being one, but with other mediators (e.g. RAWM testing and sex hormones) that were present in the current study also regulating GAD₆₅ expression. Future studies are needed to elucidate the influence that inhibitory tone, chronic stress, and a post-stress recovery period have on spatial learning between the sexes.

5. Conclusions

In this study, administration of CUR induced spatial learning deficits in male rats and this negatively correlated with GAD₆₅ mRNA expression in the MeA an area important for emotional regulation. Conversely, administration of CUR led to no effects on spatial learning in female rats. Consequently, increasing the unpredictability of chronic restraint

stress did not impact the resilience of female rats to chronic stress on these outcomes. Nevertheless, female rat performance on RAWM correlated with GAD₆₅ mRNA expression in the CA1 region, an area that is highly plastic and responsive to ovarian hormones (McLaughlin et al., 2010; Conrad et al., 2012). The present findings represent an important first step in identifying the brain regions and neural mechanisms that contribute to sex differences in behavior and plasticity in response to chronic stress. Further investigations are necessary to determine how inhibitory tone in the amygdala and hippocampus contribute to the stress-induced behavioral differences observed between the sexes.

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Abbreviations

ANOVA	analysis of variance
CA1	cornu ammonis 1
CA3	cornu ammonis 3
Con	control
CUR	chronic unpredictable restraint
GABA	γ -aminobutyric acid
GAD₆₅	glutamic acid decarboxylase
RAWM	radial arm water maze
Str-Imm	Stress-Immediate
Str-Rec	Stress-Recovery

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Highlights

- In males, chronic unpredictable restraint impaired spatial learning.
- Stress-induced spatial learning deficits in males improved after a recovery period.
- Spatial learning negatively correlated with medial amygdala GAD₆₅ in males.
- In females, chronic unpredictable restraint had no effect on RAWM performance.
- Female RAWM spatial learning positively correlated with hippocampal CA1 GAD₆₅.

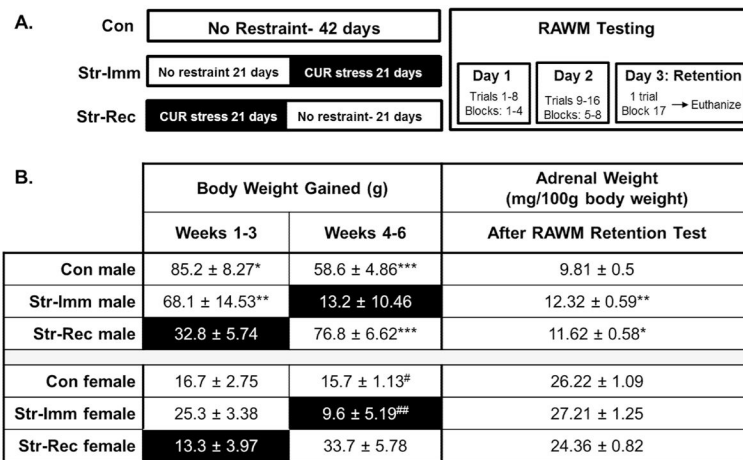


Figure 1. Experimental Timeline and Physiological Data

A. Experimental Timeline. Male and female rats were assigned to a chronic unpredictable restraint (CUR) stress for 21 days and then given a 21 day post-stress recovery period (Str-Rec) before radial arm water maze (RAWM) testing, or tested the day after restraint ended (Str-Imm), or left undisturbed (Con). All rats underwent 3 days of training and testing on the RAWM and were then sacrificed 30 minutes after the last testing session. Note that darkened blocks are shown to indicate when CUR stress occurred. **B. Stressor Effectiveness.** In males, CUR significantly attenuated body weight gain. Unstressed males gained significantly more weight than stressed males during CUR administration. During weeks 1–3, Str-Rec rats gained significantly less weight than both Con (* $p < 0.05$) and Str-Imm (** $p < 0.01$) males. During weeks 4–6, Str-Imm males gained significantly less weight than Con (***) $p < 0.001$) and Str-Rec (* $p < 0.001$) males. In females, CUR did not significantly attenuate body weight gain. However, during recovery (weeks 4–6), Str-Rec females exhibited significantly increased body weight gain compared to both Con (# $p < 0.05$) and Str-Imm (## $p < 0.01$) females. In males, CUR significantly increased relative adrenal weight in both Str-Imm (** $p < 0.01$) and Str-Rec (* $p < 0.05$) groups compared to Con males. In females, CUR had no effect on adrenal weight. Note that darkened blocks are shown to indicate when CUR stress occurred.

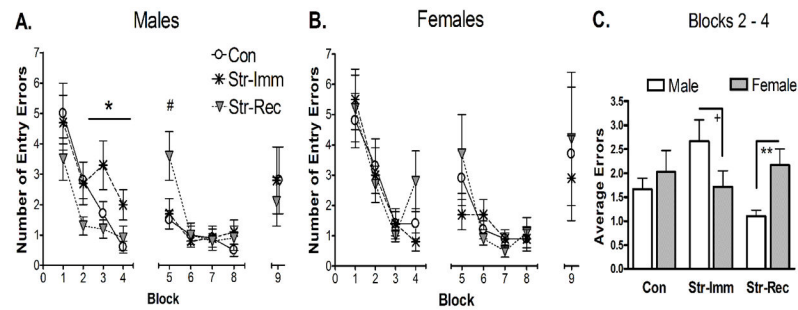


Figure 2. Radial Arm Water Maze

A and B. All groups demonstrated improved performance during acquisition (Day 1: blocks 1–4, Day 2: blocks 5–8). Data points represent mean entry errors \pm SEM for each block. **A.** In males, significant acquisition effects were found on day 1. After the first block of information gathering (block 1), Str-Imm males made significantly more errors compared to both CON and Str-Rec males (* $p < 0.05$, blocks 2–4). Str-Rec and Con males showed statistically similar number of errors on day 1. However, at the beginning of the second day of training, Str-Rec rats made more errors than both Con and Str-Imm males (# $p < 0.05$, block 5), possibly indicating greater overnight forgetting. No other effects of stress history were found in males on day 2 of training (blocks 5–8) or on the retention trial (block 9). **B.** In females, no main effect of history was detected on any day or block. **C.** Sex differences were evident on day 1, blocks 2–4, where males demonstrated significant effects of stress history. Str-Rec females made significantly more errors than Str-Rec males (** $p < 0.01$) while Str-Imm females demonstrated a pattern of making fewer errors than Str-Imm males (+ $p = 0.1$). Con males and females did not differ. Bars represent mean \pm SEM entry errors on blocks 2–4.

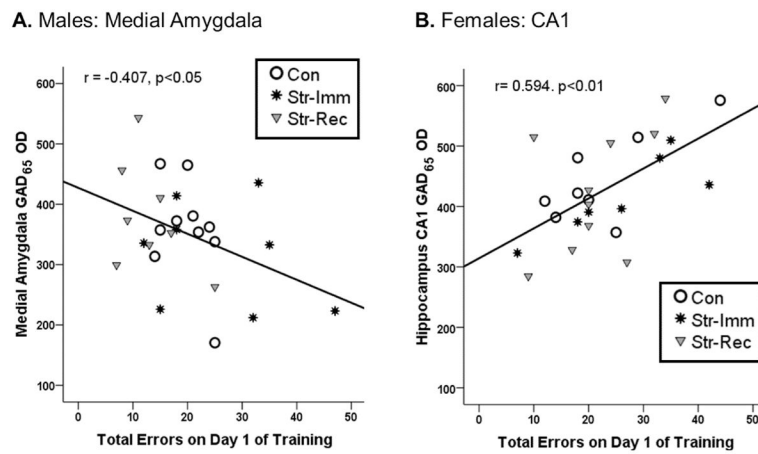


Figure 3. Correlation between GAD₆₅ expression and Acquisition on the RAWM

A. In males, GAD₆₅ expression in the medial amygdala was negatively correlated with total errors on day 1 of training ($r = -0.407, p < 0.05$), such that increased errors were associated with decreased GAD₆₅ expression. **B.** In females, GAD₆₅ expression in the CA1 was positively correlated with total errors on day 1 of training ($r = 0.594, p < 0.01$), such that increased errors were associated with increased GAD₆₅ expression.

Table 1**Mean GAD₆₅ expression**

Calibrated optical densities from *in situ* hybridization results of GAD₆₅ mRNA (nCi/g) expression from all brain regions analyzed. No differences between sex or stress conditions were found to be significant in any of the brain regions analyzed. Data are represented as mean \pm SEM.

	Mean GAD ₆₅ expression (nCi/g)			
	CA1	MeA	DG	VMH
Con male	437.7 \pm 33.07	358.0 \pm 26.16	533.6 \pm 27.01	355.8 \pm 44.34
Str-Imm male	459.5 \pm 20.86	317.1 \pm 31.00	538.4 \pm 15.25	356.9 \pm 57.69
Str-Rec male	438.7 \pm 19.63	378.8 \pm 31.78	553.0 \pm 26.08	335.1 \pm 37.90
Con female	444.1 \pm 26.00	334.6 \pm 31.45	542.1 \pm 9.66	345.3 \pm 56.82
Str-Imm female	423.8 \pm 32.28	333.0 \pm 24.28	543.1 \pm 21.28	241.6 \pm 38.16
Str-Rec female	415.8 \pm 24.24	335.1 \pm 28.12	555.6 \pm 22.89	258.2 \pm 30.39