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Chronic restraint stress enhances radial arm maze performance in female rats

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ABSTRACT

Effects of chronic restraint stress (21 and 28 days) on physiological and behavioral parameters in female rats were examined. Total (bound and free) and free corticosterone (CORT) levels were measured at different time points during the stress period. Higher total CORT levels were observed in stressed rats during the stress period but returned to baseline at 15 days post-stress. Additionally, free CORT levels decreased across the stress period. Estrous cyclicity was monitored daily in all animals. Stress had no apparent effects on estrous cyclicity, in rats with either normal length or elongated estrous cycles, but stressed females gained less weight than controls. Following the stress period, subjects were tested for open field activity and radial arm maze (RAM) performance. Females stressed for 21 days showed enhanced spatial memory performance on the RAM. A longer period of restraint, 28 days, also led to less weight gain by stressed subjects and unaltered estrous cycle lengths, but was not associated with enhanced RAM performance. Further analysis indicated that RAM performance was influenced by specific estrous cycle day, particularly during proestrus. Following 21 days of restraint stress all animals in proestrus, regardless of treatment, showed impaired acquisition. After 28 days, stressed females in proestrus performed better than proestrus controls. These results are discussed in relation to previously reported effects of stress in male rats.

INTRODUCTION

When homeostasis is threatened by stress, the body undergoes a myriad of changes. While stressful events take many forms, their consequences can be classified as either acute (i.e., short-term and adaptive) or chronic (i.e., long-term and maladaptive). One primary physical change that occurs in response to stress is the release of glucocorticoids (GC) by the adrenal glands [22], which is regulated by the hypothalamo–pituitary–adrenocortical (HPA) axis. Short-term GC changes, in response to stress, are adaptive. However, prolonged exposure to stress and subsequent sustained elevated GC levels has an adverse effect. The hippocampus has the highest density of GC target receptors in the brain and has been implicated in the regulation of the HPA stress axis and the behavioral response to stress [12]. Thus, the hippocampus provides a convenient model for studying the neurobiological effects of increased levels of GC associated with stress.

In rats, chronic physical stress (e.g., foot shock or cold water swim) or psychosocial stress (e.g., restraint or placement in a novel environment), affects hippocampal morphology [30] and function. Structural changes in the male rat hippocampus following stress include decreases in both apical dendritic branching as well as total dendritic length [41]. The observed hippocampal atrophy is mediated by increased GC levels and N-methyl-Image -aspartate receptor-mediated excitatory input and can be prevented by inhibiting GC secretion [31]. Stress and subsequent elevated CORT levels have been shown to block hippocampal long-term potentiation [17] and primed burst potentiation [11]. Both of these physiological processes are involved in the regulation of learning and memory formation. Following 21 days of chronic restraint, male rat performance is impaired on the radial arm maze (RAM), a test of spatial learning and memory [28]. Stressed male rats make their first mistake sooner and have less correct responses in their first eight choices as compared to control males. The changes in spatial memory performance following chronic stress are reversible and appear to be temporally constrained [28]. For example, 14 days of chronic stress improves radial arm performance in male rats [25] and behavioral deficits are not observed until 21 days of stress [28]. Prolonged elevated CORT levels are also associated with impaired performance on a number of other tasks that require the use of spatial memory including the Y-maze [10] the Morris water maze [8] and the Barnes maze [32]. These mazes, like the radial arm maze, assess rodent spatial learning and memory.

Most research investigating the influence of stress on male and female rats has focused on the effects of acute stress and limited work has examined the effects of chronic stress. It has been shown that behavioral sex differences exist in non-spatial, non-reward tasks following 3 weeks of chronic stress: males are impaired while females are not impaired in the object recognition task [24]. Interestingly, these sex differences extend beyond behavioral parameters and can be observed in both neural and endocrine dimensions following 21 days of chronic stress [19]. Female rats show less atrophy of apical dendritic branches than males after chronic stress. Furthermore, a significant decrease in the number of branch points within the basal dendritic area is observed in stressed females when compared to controls [19]. Stressed females also show higher levels of plasma CORT and take longer to habituate to the stress than males [19]. In comparison to males, female rats show a different pattern of neurochemical changes following chronic stress [6 and 24].

Studies in this and other laboratories have investigated the effects of stress on male spatial memory performance utilizing the RAM [25 and 28] and the Y-maze [10]; however, the effects of chronic stress on female RAM performance have not been investigated. Based on the previously reported sex differences in behavioral, anatomical, neuroendocrine, and neurochemical responses to chronic stress [3, 5, 19, 24, 25 and 28], we were interested in examining the effect of chronic stress on female RAM performance. In the present studies, the effects of chronic restraint stress (21 and 28 days) were examined in intact, cycling female rats at the neuroendocrine and behavioral levels. The estrous cycle was monitored to determine whether the chronic stress paradigm altered the mean length of the estrous cycles. Bound and free serum CORT levels were measured in stressed animals to assess stress-induced changes. The open field test was used to provide an overall indication of locomotor activity and the eight-arm radial arm maze was used to measure spatial memory performance.

METHODS

Experiment 1

Subjects

Forty female Sprague–Dawley rats (Harlan Sprague–Dawley, Indianapolis, IN), aged 55–60 days (upon arrival) served as subjects. All animals were maintained on a 14/10-h light–dark cycle (lights on at 05:00 h) and in accordance with the NIH Guide for Care and Use of Animals and allowed to acclimate to the housing environment for 2 weeks. During the acclimation period and the 21-day stress period, all subjects were double housed in plastic tubs. Animals were singly housed during behavioral studies since food deprivation was necessary for the behavioral task. Throughout the acclimation period, stress period, and behavioral testing, all animals received daily vaginal lavages to determine their estrous cycle phase (14:00–15:00 h). Briefly, the vagina is gently lavaged with physiological saline on a Q-tip and smeared on a slide. The slides are rinsed, stained with Coomassie Blue, rinsed, dried and examined under a microscope. The estrous cycle day was determined based upon the presence of leukocytes, nucleated epithelial or cornified epithelial cells. Rats that exhibited all phases of the estrous cycle, across a 2-week period, were randomly assigned to either a stressed (n=15) or control (non-stressed, n=13) condition. Other intact females, that exhibited all phases of the estrous cycle (n=12) were assigned to a quiet control group, which did not receive stress or behavioral testing. The quiet control females were double housed, had free access to food and water, and received daily vaginal lavages during the acclimation period. The quiet controls were used to obtain basal CORT levels on day 1 of the stress paradigm and were not used during the remainder of the experiment.

During the 21-day stress period, stressed animals were placed in Plexiglas restrainer tubes manufactured by Harvard Apparatus for 6 h (always beginning between 08:00 and 09:00 h). The tube has air holes for ventilation and the animals have limited room for movement. Rats were placed in an isolation chamber with ventilation and maintained at room temperature. The

chamber was located in a separate room, adjacent to the animal colony, which allowed for the separation of stressed and control animals during the stress period.

Immediately following the last stress day, both stressed and non-stressed subjects were transferred to single housing and placed on food restriction. Because of the food-reward nature of the radial arm maze, food deprivation is necessary to reduce animals to 90% of their normal body weight. All subjects had free access to water and were weighed regularly. Subjects were given daily food rations (two to three rat chow pellets) depending on their weight following behavioral testing. All subjects were sacrificed by rapid decapitation 14 days post-stress (cohort 1) and 15 days post-stress (cohort 2).

Corticosterone radioimmunoassay (RIA)

Blood samples were collected from the tail during the first hour of restraint stress on days 1, 7, 14, and 21 from the stress animals. Briefly, the tail was soaked in warm water and the tip quickly removed via a straight blade razor. The tail was palpated to collect the blood sample and then treated with antibiotic spray. Blood was sampled on day 1 from the quiet control animals, to provide basal CORT levels, by briefly restraining them (for approximately 2 min). All quiet controls (stress day 1) and stressed females (stress days 1, 7, 14, and 21) had blood samples taken; however, to control for CORT levels across the estrous cycle [3, 34 and 39], only quiet control females in proestrus on stress day 1 (n=5) and stressed females in proestrus (n=5, stress day 1, and n=6, stress days 7, 14, and 21) were selected from the overall data set and only these samples were measured. Total CORT levels were measured by radioimmunoassay using the Coat-A-Count® assay kit available from Diagnostic Products Corporation (Los Angeles, CA; catalog number TKRC1). Free CORT was assessed using a modification of Edwards et al. [13] technique. Briefly, Concanavalin-A conjugated to Sepharose-B™ (Sigma, St. Louis, MO) was added to the serum sample to precipitate the corticosterone that is bound to corticosterone binding globulin protein. The samples were centrifuged and the supernatant, containing free CORT, was measured by RIA. Samples were analyzed in duplicate. In order to have sufficient volume to run the assay, serum samples were diluted to 300 µl (using the zero calibrator provided with the assay kits, as specified by the vendor) and calculations were made accordingly. All samples were run in one assay and the detection limit was 5.7 ng/ml, as defined by the 95% confidence limits of the zero standard.

An additional CORT RIA was performed on the trunk blood of the stressed and non-stressed females following sacrifice at the end of the behavioral trials. The animals were sacrificed by rapid decapitation post stress day 14/15 and both total and free CORT levels were assessed in the same manner as previously described (except no dilution with the zero calibrator was necessary). CORT levels are expressed as ng/ml.

Behavioral measures

Open field

On day 1 post-stress, subjects were tested on the open field, which provides an overall measurement of locomotor activity. In random fashion, subjects were placed singly in the center of a 4×4 enclosed area with the floor marked out into 9″ squares. All subjects remained on the open field for 6 min, during which time behaviors for the first 3 min and the second 3 min were tallied for each animal. These behaviors included sector visits (movement across squares), rears (raising up on haunches with forelimbs 3–4 cm off the floor), wall climbs, grooms, and defecations.

Radial arm maze

Spatial memory was assessed using the eight-arm radial maze. All subjects were trained on post-stress days 3–6 (two training trials per day). During training, the rats' behavior is gradually shaped so that the animal goes to the end of the arms for a food reinforcer (1/4 peanut). Training begins with peanuts concentrated in the center of the maze. Across the training sessions, the number of peanuts on the maze is decreased and gradually moved closer to the ends of the arms. After the seventh training session, the task was identical to regular trials: rats were placed in the center of the maze and given a maximum of 10 min to visit all eight arms to receive the peanut rewards at the end of each arm. An arm was counted as being visited if the rat either traversed two-thirds of the arm's length, if the arm was entered and the peanut eaten, or if the arm was entered but the peanut was not eaten. Errors were scored as reentries into arms previously visited within that same session. On post-stress days 7–10 subjects received eight regular trials (two trials/day) and on post-stress days 11–13 subjects received delay trials. During delay trials, the rat is removed from the maze after making the initial four choices, placed in their home cage for a predetermined amount of time, and then returned to the maze to complete their last four remaining choices. Performance is scored by three choice accuracy measures: number of correct choices in first eight arm visits; choice where the first mistake was made; and total number of choices to complete the task.

Data analysis

Measurements of animal weight gain, estrous cyclicity, and serum CORT level data were from a split-plot factorial design with treatment (between-subjects) and time (within-subjects or repeated) as design factors. A two-factor ANOVA was used to test for statistically significant differences in weight gain between stressed and non-stressed animals across the 21-day stress period. Mean estrous cycle lengths for the stressed and non-stressed animals during pre-stress, stress, and post-stress time periods were examined for statistically significant differences using ANOVA as well. Initially, an independent samples *t*-test was used to examine differences in serum CORT levels between stressed and quiet control animals on Day 1 of the stress paradigm. Subsequently, variance in CORT levels of the stressed animals across the 21 days

was partitioned using a two-factor (treatment×stress day) ANOVA, and Dunnett's post-hoc procedure was used to compare CORT values for stressed females across the stress period to values obtained for these animals on day 1 for statistically significant differences. Finally, a dependent samples *t*-test was used to test for statistically significant differences in CORT levels obtained from the trunk blood of stressed and non-stressed females upon sacrifice after behavioral testing.

The behavioral data were also from a split-plot factorial design. Open field measures were analyzed using a two-factor ANOVA with treatment (stressed, non-stressed) and time (first 3 min, second 3 min of the task) as between-subjects and within-subjects, or repeated, design factors, respectively. While radial arm maze data were from a three-factor design, only the between-subjects factor (treatment) and one repeated factor (time) were experimenter-controlled. The second within-subjects design factor, estrous cycle, was subject-dependent and was not completely crossed with the time factor. Because time and estrous cycle formed an unbalanced, partial, and thus confounded interaction both relative to treatment and relative to the error terms necessary to use a three-factor ANOVA accurately, stress treatment effects were analyzed initially across the regular and delay trials using a 2×4 (treatment×time [RAM testing day]) ANOVA without regard to estrous cycle of the animals. Subsequently, treatment and time effects were examined for each specific estrous cycle using 2×4 ANOVAs to provide a better description of treatment, time and treatment×time effects.

Animal weight was measured in grams, and descriptive statistics for weight and behavioral data are expressed as mean±S.E.M. Regular RAM trials (two per day) were averaged for each day. For purposes of determining statistical significance of effects, Type I error rate was set at 0.05 for individual global and families of post-hoc tests.

Experiment 2

Subjects

Twenty-four female Sprague–Dawley rats (Harlan Sprague–Dawley) aged 55–60 days served as subjects. Group assignment, daily vaginal lavages, periodic weighing, and behavioral assessment were as described in Experiment 1. The stress period was extended to 28 days.

General procedure

All post-stress procedures, including food restriction, open field assessment, and RAM testing were identical to those described in Experiment 1.

RESULTS

Experiment 1

Physiological measures

Weight

Animals' weights were monitored during the acclimation, stress and behavioral testing periods. There was no main effect of stress on weight gain. There were significant effects of time ($F_{3,115}$=13.05, P<0.0001) and the treatment×time interaction ($F_{3,115}$=8.07, P<0.0001). Stressed animals weighed less than the controls from stress day 13 (260.08±3.63 and 248.93±3.24, control and stress, respectively) through the end of the stress paradigm (265.15±4.20 and 255.44±3.29, control and stress, respectively).

Estrous cyclicity

Stressed females did not statistically differ from controls with respect to their average estrous cycle length. The average cycle length during the pre-stress period was 8.7±0.45 and 7.9±0.59 days (mean±S.E.M.) for control and stressed animals, respectively. Cycle length during the stress period was 7.6±0.46 days (controls) and 7.2±0.34 days (stressed) and the post-stress period was 8.2±0.39 and 6.8±0.47 days (control and stressed, respectively). A separate analysis was performed on the females that exhibited more typical estrous cycle lengths, 4–5 days. This subgroup of stressed females did not differ from controls with respect to their mean estrous cycle length. During the pre-stress period the mean cycle length was 5.5±0.5 and 5.7±0.37 days (mean±S.E.M.) for control and stressed animals respectively. Average cycle length during the stress period was 5.5±0.5 for controls and 5.6±0.4 for stressed and during the post-stress period was 5.35±0.35 and 5.34±0.38 days (control and stressed, respectively).

Corticosterone levels

Serum corticosterone (CORT) was measured in the stressed animals following 1 h of restraint on days 1, 7, 14, and 21 of the stress period. A companion group of quiet control animals, neither stressed nor control, was sampled only on day 1 to provide baseline CORT measurements. Serum measurements of total CORT, as well as free CORT, were obtained. Significant differences between the stressed and quiet control animals in total CORT (t=2.43, P<0.03), as well as free CORT (t=3.13, P<0.02) were found on day 1 (see Table 1). In addition, a significant effect of day of stress on levels of both total CORT ($F_{3,19}$=3.82, P<0.02) and free CORT ($F_{3,19}$=5.87, P<0.005) in the stressed females over days 1–21 of stress was observed using ANOVA. Dunnett's test post-hoc analysis, with Type I error controlled at 0.05 for the family of tests, identified significant differences in total CORT levels in stressed females on day 1 from levels on days 7, 14, and 21 (critical DIFFERENCE=184.28) and free CORT levels in

stressed females on day 1 from those obtained on days 7, 14, and 21 (critical DIFFERENCE=144.54) (see Table 1).

Table 1
Comparison of total (bound and free) and free serum corticosterone levels between stressed and quiet control female rats[a]

	Quiet control day 1	Day of chronic stress			
		1	7	14	21
Total serum corticosterone levels	232±14	504±112	310±53	215±36	291±31
Free serum corticosterone levels	147±14	404±82	236±38	151±45	150±26

[a] Total and free serum corticosterone (CORT) levels are expressed as ng/ml. Entries are mean±S.E.M. To control for CORT levels across the estrous cycle, RIA analysis was performed only on females in proestrus (n=5, quiet controls, stress day 1; n=5, stressed group, stress day 1; and n=6, stressed females, stress days 7, 14, and 21). A two-sample t-test compared the means of stressed and quiet controls on day 1 of the stress paradigm and indicated that stressed animals had higher levels of total and free CORT than controls. ANOVA assessed serum CORT levels in the stressed animals across 1–21 days of chronic restraint indicating a main effect of stress day on total CORT ($F_{3,19}$=3.82, P<0.02) and free CORT ($F_{3,19}$=5.87, P<0.005), Dunnett's post-hoc analysis indicated that both total CORT and free CORT measurements on day 1 were different from levels on days 7, 14, and 21.

The non-stressed and stressed behaviorally analyzed females were sacrificed by rapid decapitation 14–15 days after the stress period and their trunk blood was obtained and subjected to standard RIA methods; no significant difference was observed in stressed animals versus non-stressed controls (data not shown).

Behavioral measures

Open field

Open field measurements were quantified for the first 3 min and second 3 min of the task. There was a significant difference between groups in outer sector crossings ($F_{1,27}$=4.20, P=0.0504), with stressed females making fewer overall visits (52.25±2.99) than controls (61.38±3.31). The total number of outer sector visits decreased over time for both groups ($F_{1,27}$=19.19, P<0.0002). No differences were observed between groups in total number of wall climbs; however, the number of wall climbs decreased across time for both groups ($F_{1,27}$=20.87, P<0.0001). No significant differences were observed for grooming, rearing, or inside sector crossing between the groups.

Radial arm maze

Daily restraint stress for 21 days led to a small, but significant, difference in performance on the RAM. As seen in Fig. 1A, stress enhanced performance as measured by the total number of visits required to complete the task. Stressed females completed the task in fewer visits than the controls ($F_{1,26}$=9.64, P<0.005). Both groups took fewer visits to complete the task across time ($F_{3,160}$=4.52, P<0.005), indicating that acquisition of the task was occurring. Additionally,

stressed females made more correct choices in the first eight visits of the task as compared to the control animals ($F_{1,26}$=5.19, P<0.03) (see Fig. 1B). There were no significant treatment effects for the visit in which the first error occurred across regular RAM trials.

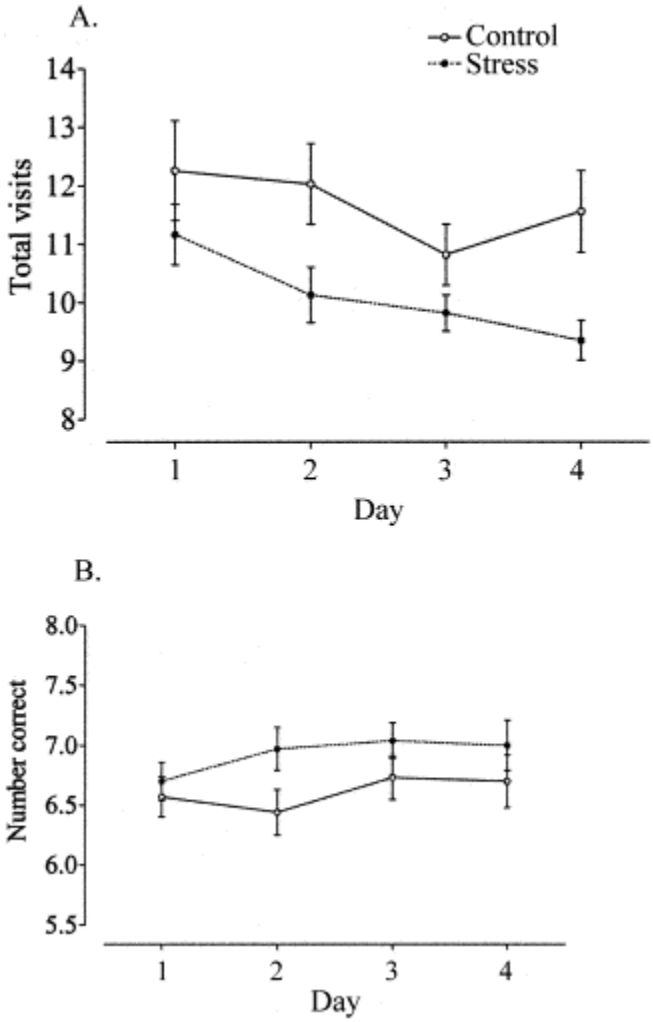

Fig. 1. Twenty-one days of chronic restraint stress enhances female radial arm maze performance. (A) Entries are the mean±S.E.M. A two-factor mixed ANOVA (treatment×time) indicated that all animals improved over time with regards to the total visits required to finish the task ($F_{3,160}$=4.52, P<0.005); however, overall stressed females required fewer total visits (10.26±0.24) to complete the task than controls (11.68±0.36) ($F_{1,26}$=9.64, P<0.005). (B) Entries are the mean±S.E.M. A two-factor mixed ANOVA (treatment×time) indicted that stressed females (6.91±0.09) had more correct choices in the first eight visits of the task as compared to controls (6.59±0.11) ($F_{1,26}$=5.19, P<0.03).

In order to better describe and understand the main treatment and treatment×time effects, treatment effects were examined separately for females in proestrus, estrus, and diestrus. Additional behavioral differences between treatment groups were observed when estrous cycle

day was held constant. While all stressed females required significantly fewer total visits to complete the task, the effect reached significance only for stressed subjects in diestrus (treatment×time, holding constant cycle) ($F_{1,19}$=5.09, P<0.04). Additionally, animals in proestrus, both non-stressed and stressed, showed impaired RAM acquisition as evidenced by the visit in which the first mistake occurred (treatment×time, holding constant cycle) ($F_{2,42}$=3.20, P=0.0507) (see Fig. 2). Animals in estrus ($F_{2,30}$=1.17, P>0.34) and diestrus ($F_{3,47}$=1.34, P>0.27) did not show this acquisition effect (see Fig. 2). Stress effects on other choice accuracy parameters during specific estrous cycle days were non-significant.

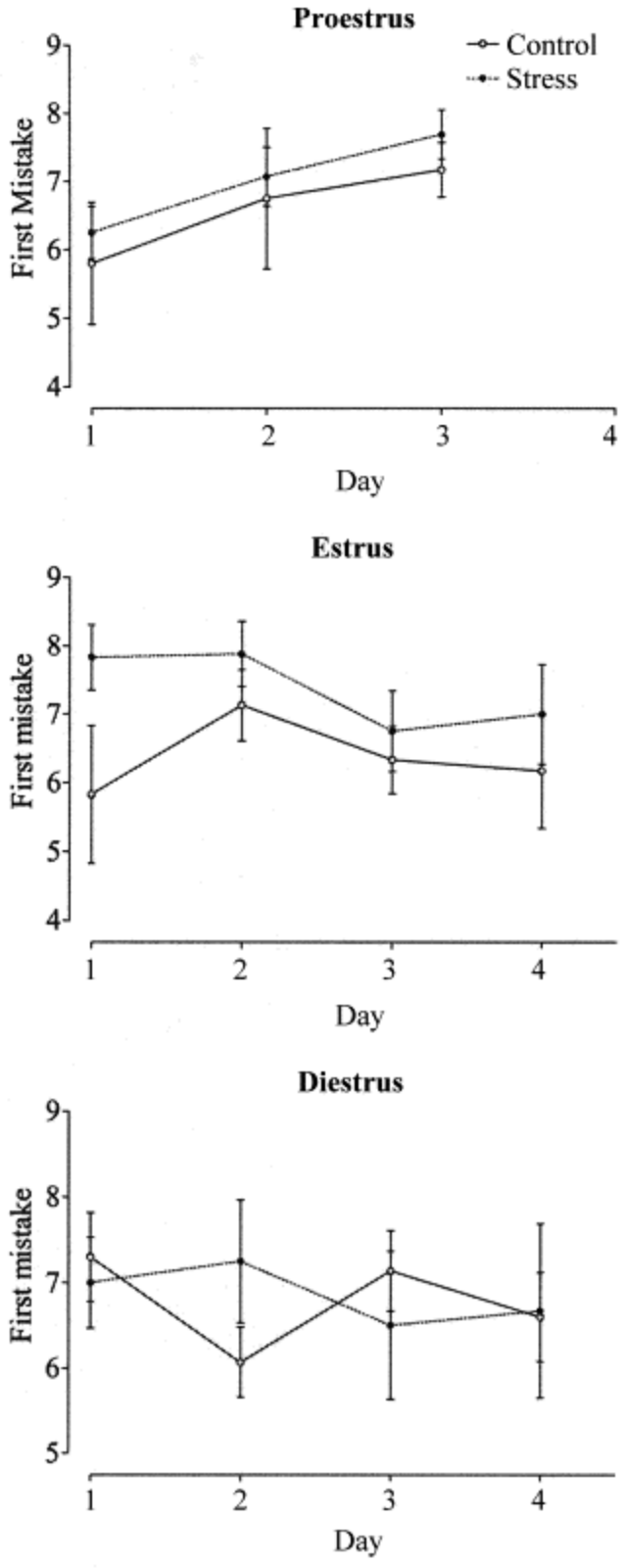

Fig. 2. Effects of 21 days of stress on RAM performance in females at various days of the estrous cycle. Entries are the mean±S.E.M. Two-factor mixed ANOVAs (treatment×time, holding

constant estrous cycle day) were used for analyses. Subjects in proestrus, regardless of treatment, improved over time on the visit in which the first mistake occurred ($F_{2,42}$=3.20, P=0.0507; no animals were in proestrus during behavioral testing on day 4). This improvement indicates that females in proestrus require longer acquisition of the task, while animals in estrus ($F_{2,30}$=1.17, P>0.34) and diestrus ($F_{3,47}$=1.34, P>0.27) did not show this acquisition effect.

No significant differences in performance between control and stress females, or during any estrous cycle stage, were observed during RAM trials with a 15-min, 1-, 2-, or 4-h delay between the fourth and fifth choice (data not shown).

Experiment 2

Results from Experiment 1 showed that performance on a spatial memory task is enhanced in female rats following 21 days of chronic restraint stress. In contrast, previous work in our laboratory has shown that male rats are impaired following this 21-day stress paradigm [28]; however, males that are stressed for 14 days show enhanced RAM performance [25]. Taken together, current results in females and previous results in males [25 and 28] suggest that females may require longer periods of stress than males to show impairments. Alternatively, females may not be impaired by chronic restraint stress. To test these possibilities, a second experiment was conducted in which the stress period was extended to 28 days.

Physiological measures

Weight

Following 28-days of chronic restraint stress, there was no main effect of stress on weight gain. There were significant effects of time ($F_{4,75}$=36.94, P<0.001) and a significant treatment×time interaction ($F_{4,75}$=8.58, P<0.001): stressed females (211.58±3.66) weighed less than controls from stress day 7 (225.75±4.35) through stress day 21 (223.58±4.09 and 238±3.49, stressed and control, respectively); however, by stress day 28 both stress and control animals weighed approximately the same (248.66±2.0 and 244±5.72, stressed and control, respectively).

Estrous cycle

There were no effects of 28 days of chronic restraint stress on average cycle length. During the pre-stress period, the average cycle lengths were 8.5±0.37 and 7.6±0.22 days for controls and stressed animals, respectively (mean±S.E.M.). During stress, the lengths were 8.6±0.30 and 8.5±0.28 days (controls and stressed, respectively) and during the post-stress period cycle lengths were 9.1±0.38 and 8.0±0.43 days (controls and stressed, respectively). One stressed animal exhibited constant diestrus during the stress period and two controls exhibited constant

estrous during the behavioral period. These three animals were not included in the estrous cycle means. A separate analysis was performed on the females that exhibited more typical estrous cycle lengths, 4–5 days. This subgroup of stressed females did not differ from controls with respect to their mean estrous cycle length. During the pre-stress period the mean cycle length was 4.6±0.3 and 5.0±0.1 days (mean±S.E.M.) for control and stressed animals, respectively. Average cycle length during the stress period was 4.8±0.4 for controls and 5.1±0.1 for stressed and during the post-stress period was 5.5±0.3 and 5.5±0.3 days (control and stressed, respectively).

Behavioral measures

Open field assessment

Following 28 days of chronic stress, there were no differences between the stressed and non-stressed subjects on any performance parameters on the open field task. All subjects showed habituation during the second half of the task, making more visits to the inner sectors ($F_{1,22}$=5.42, P<0.03) and fewer wall climbs ($F_{1,22}$=6.26, P<0.02).

Radial arm maze

There were no overall differences between stressed and control females on any RAM choice accuracy parameter following 28 days of stress (see Fig. 3). All subjects improved over time on the number of correct choices in the first eight visits ($F_{1,148}$=3.04, P<0.03), indicating an acquisition effect.

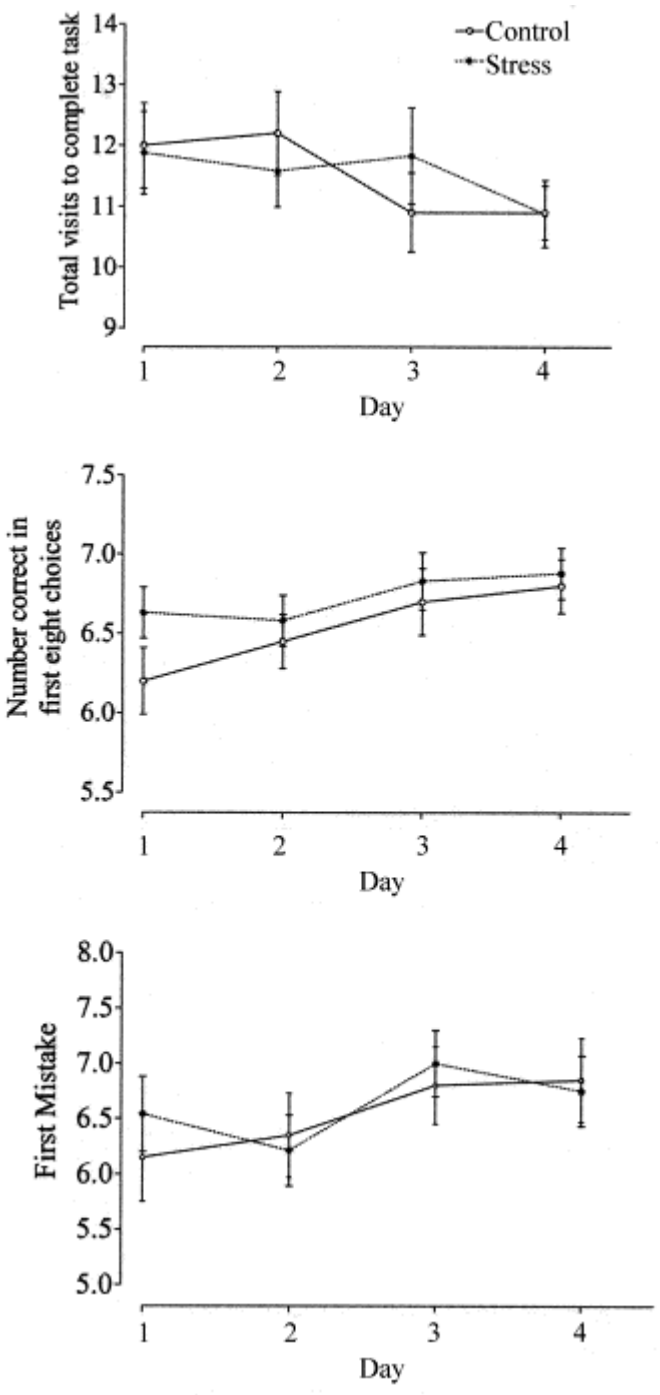

Fig. 3. Twenty-eight days of restraint stress did not alter female RAM performance. Entries are the mean±S.E.M. There were no differences between the treatment groups on any RAM choice accuracy parameter including total visits to complete the task ($F_{1,27}$=0.01, P>0.92), number correct in first eight visits ($F_{1,27}$=2.75, P>0.10), and the choice on which the first mistake occurred ($F_{1,27}$=0.15, P>0.70).

However, there were small, but statically significant, differences among the treatment groups when evaluated at specific days during the estrous cycle. Stressed animals in proestrus had better choice accuracy rates on the number of correct choices in first eight visits (6.77±0.18) as compared to controls (6.28±0.09) ($F_{1,17}$=4.74, P<0.04). As in Experiment 1, which assessed effects following 21 days of stress, chronic stress did not affect female RAM performance during delay trials (data not shown).

DISCUSSION

Body weight

Female rats used in these experiments gained weight across time; however, stressed rats weighed less than their counterpart controls across 21 days of stress. This result is consistent with previous studies in male rats in which both restraint stress and CORT administration causes a decrease in body weight in an indirect fashion [1, 6 and 32]. That is, restraint stress leads to increased levels of CORT, which in turn leads to an overall decrease in weight gain. This would be comparable to observations in which other stressors (e.g., cold stress) have been shown to interact with CORT on signals of energy balance [1]. Interestingly, by 28 days of restraint, stressed female subjects in this study did not weigh less than controls. This result suggests that females may respond differently to males in relation to effects of stress on weight gain, but further experiments are necessary to substantiate this observation.

Corticosterone levels

CORT exists in two forms in plasma, free or bound, with total CORT levels, as measured by RIA, being the sum of free and bound. Typically, studies have only investigated the effects of stress on total CORT levels, with little attention being paid to levels of circulating free CORT [3, 9 and 19]. Bound CORT is generally considered to be biologically inactive, while free CORT exerts the effects of this hormone [16]. To determine the amount of biologically active CORT, we removed the bound fraction (see Methods). We were interested in assessing free CORT levels to determine if they could account for the difference in male and female sensitivity previously observed in anatomical and behavioral responses to chronic stress [19, 24, 25 and 28]. Chronic restraint stress leads to elevated levels of both free and bound CORT (see Table 1) and the levels of total CORT observed in the current study are consistent with those previously reported [3, 9, 34 and 39]. The levels of total CORT in females is higher than that reported for males [19] and reflects the sexual dimorphic CORT release of the HPA axis following stress [3]

On stress day 1, total CORT levels were higher than those observed for stress days 7, 14, and 21, suggesting that the stressed females had habituated to the stress paradigm by day 7. Free CORT levels on stress day 1 were also higher than those observed on days 7, 14 or 21. This result suggests that other stress-induced mechanisms may be involved in regulating spatial memory enhancement, as no significant difference in the amounts of biologically active (free) CORT exists by the end of the stress period (21 days). However, this observation does not

exclude the possibility that the initial increase in free CORT levels triggers a cascade of events that results in the observed enhancement. Interestingly, the ratio of free:bound CORT is approximately 80% on stress day 1 and falls to 50% by day 21. It is possible that declining free:bound CORT levels are partly responsible for females' resistance to the effects of chronic restraint stress. Currently, little is known about the active levels of CORT in male rats following stress; if higher free:bound CORT levels exist in males, this could account for the detrimental effects of restraint stress observed in male rats. Future studies should investigate the profile of free:bound CORT ratios in males in response to stress.

Estrous cycle

We examined estrous cycle patterns in rats stressed for 21 or 28 days. Results indicate that neither 21 nor 28 days of chronic restraint stress leads to significant differences in estrous cycle length, in females with either normal length (4–5 days) or elongated cycles (up to 9 days), when compared to control females. While average cycle lengths were longer than that usually reported (4–5 days) for Sprague Dawley or other strains, they are not inconsistent with previously reported observations [23]. This increase in cycle length could be attributed to the subjects or methodology used. The females used in the current experiments were young, 10–12 weeks, and possibly had yet to establish mature cycling patterns. Alternatively, constant vaginal smearing could be responsible for this temporal increase in cyclicity.

Stress has been reported to cause alterations in reproductive function in humans, non-human primates, and rats. In humans, stressors such as psychological distress [21], environmental toxin exposure [37], and strenuous exercise [7] lead to aberrations in menstrual cycles and/or amenorrhea. In rats, chronic exposure to physical stressors (forced swimming, foot shock, temperature extremes) leads to estrous cyclicity alterations [4, 20 and 35]. However, less severe stressors do not appear to disrupt estrous cycling in rats. Anderson et al. [2] showed that a 14-day sustained stress paradigm in which rats received around-the-clock, signaled, intermittent foot shock and could either avoid/escape the shock or had no control over the shock did not experience estrous cycle alterations. Additionally, mild food deprivation (85% body weight) did not disrupt normal estrous cycle patterns in Sprague–Dawley rats [38]. It appears that stressors of various severities have differential effects on the estrous cycle. Stressors that disrupt the rat estrous cycle are generally more severe and physical in nature. Restraint stress is believed to be a psychosocial stressor [29] and subsequently did not appear to disrupt estrous cyclicity of female rats in this study that exhibited either normal length or elongated cycles.

Stress and memory

Previous studies in this and other laboratories have investigated stress-effects on male RAM performance, and therefore, we were interested in whether similar behavioral effects would be present in stressed females. The eight-arm radial maze was used to evaluate the effect of

chronic stress on female spatial memory. The current experiments are, to the best of our knowledge, the first investigations of the effect of chronic stress on spatial memory, specifically using the radial arm maze, in female rats. Our data indicates that 21 days of chronic restraint stress enhances spatial memory in females. That is, stressed female rats scored better than controls on both the total visits to complete the task, as well as, the number of correct choices in the first eight visits (see Fig. 1A,B). This result is different from data previously reported from this laboratory for male rats, which are impaired following 21 days of stress [28]. Specifically, stressed males make their first mistake sooner and have less correct responses in their first eight choices as compared to controls following 21 days of chronic restraint stress [28]. In addition, prolonged elevated CORT levels (i.e., chronic stress or exogenous administration of CORT) have been shown to impair male rat performance on other tasks that require the use of spatial memory including the Y maze, the Morris water maze, and the Barnes maze [8, 10 and 32]. The dimorphic behavioral response to chronic stress is consistent with the sexual dimorphism observed in morphology [19] and chemistry [5, 6 and 24] following restraint stress. As previously reported, in contrast to males, female rats do not show atrophy of apical dendritic branching following 21 days of restraint stress [19]. Female rats also show a different pattern of neurochemical changes following stress than males, with neither frontal cortex dopamine or amygdala norepinephrine being affected [5, 6 and 24]. Both males and females show changes in hippocampal amino acid levels, but male changes are centered in CA3, and female changes are centered in CA1 regions [24].

The sex difference on RAM performance following 21 days of chronic stress may be the result of influences exerted by gonadal hormones, specifically estrogen, on the stress response. Interestingly, following 21 days of restraint stress, estrogen levels were decreased in female rats [19]. Thus, this decline in estrogen could be partly responsible for the change from a stress-dependent enhancement of RAM performance at 21 days of stress to a lack of enhancement in RAM following 28 days of stress. We speculate that estrogen results in female resistance to stress-induced impairments by exerting either a direct protective effect on the hippocampus or by modifying the HPA cascade in females (e.g., estrogen could be influencing CORT release or GC receptor density).

It has been well established that estrogens play a beneficial role in learning and memory in both humans and rats. Both estrogen-treated gonadectomized males [27] and estrogen-treated ovariectomized females [26] perform better during delay RAM trials than controls. Estrogen enhancements have also been reported on numerous other spatial memory tasks including two-way active avoidance paradigm [36] and T-maze acquisition [14]. It is unclear at what level estrogen is exerting its moderating effect on the HPA stress response and whether the effect is activational or organizational. As stated, estrogen could be exerting a direct effect on behavior or could be affecting the CORT response at the neurochemical or molecular level (e.g., estrogen could be altering glucocorticoid receptor levels [15]).

Alternatively, estrogens could be exerting an effect on spatial learning and memory by increasing spine density in the CA1 hippocampal cells [33]. As previously mentioned, elevated CORT levels, in female rats, do not lead to apical hippocampal atrophy in CA3 neurons (as compared to males); however, there is resulting basal remodeling [19]. Possibly, this remodeling

in the female hippocampus following stress and the enhancing effects of estrogen on the CA1 pyramidal cells, are working together to lead to stress-dependent enhancements in females.

Interestingly, during delay trials no effects of stress on female performance were noted and this result is consistent with previous results in male rats [25]. This result suggests that stress-dependent enhancements in spatial memory are possibly not sufficient to overcome the high cognitive demand of delay trials. Alternatively, no effects of stress on female performance during delay trials could be because stress-dependent enhancements are temporally constrained. Luine et al. has shown that male rats stressed for 21 days, but not tested on the RAM until 18 days post-stress, show no effects of stress on performance [28]. Additionally, Magarinos and McEwen have shown that stress-induced dendritic atrophy are reversed after termination of the stress paradigm (by 5–10 days, as referenced in Luine [29] and Galea et al. [19]). This temporally dependent reversibility of stress effects makes examining spatial memory using the radial arm maze problematic because it requires 4–5 days of training prior to regular trials. Thus, it is possible that potential stress effects could attenuate. Nonetheless, it is noteworthy that both enhancements and impairments of RAM spatial memory performance (depending on stress duration) have been reported in male rats [25 and 28] and that we have shown female enhancement on the RAM following 21 days of stress. This sexually dimorphic pattern of results is similar to other work in our lab where stress effects were evaluated beginning 3 days following stress termination [24]. Male and female rats were stressed for 21 days and evaluated in the same experiment for object placement ability, a spatial memory task. Results showed that stressed males were impaired on the task, whereas stressed females were enhanced on the task [24]. Thus, it appears that chronic restraint stress impairs male and enhances female spatial memory from 3 to 10 days following termination of stress [24 and 28]

Female RAM performance, following stress, also appears to be influenced by the estrous cycle in the current studies. Following 21 days of stress, when treatment effects are examined at each day of the estrous cycle, there is a trend for all stressed animals to perform better than controls; however, performance of stressed females reached significance only during diestrus. It is unclear why females in diestrus, when hormone levels are low, are performing better. One possibility is that during diestrus, there are not sufficient levels of gonadal hormone to interact with the stress effect. Additionally, proestrus females, regardless of treatment, had impaired RAM acquisition following 21 days of stress. This proestrus-associated impairment is similar to previously reported results which showed impaired spatial memory acquisition during proestrus when gonadal hormones are at their highest [18 and 40]. Interestingly, acquisition is not impaired during proestrus following 28 days of stress, and stressed rats in proestrus perform better as compared to controls. Measurement of serum adrenal and gonadal hormones in females stressed for varying periods are clearly necessary and may provide information for understanding these complex neuroendocrine interactions on behavior.

Twenty-one days of chronic stress enhanced female RAM performance, while 28 days neither enhanced nor impaired performance. This pattern of results is different from male rats in which prolonged exposure to stress changes from enhanced spatial memory at 14 days of stress to maladaptive (RAM performance is impaired following 21 days of restraint) [25 and 28]. Stress exerts a biphasic effect on the central and peripheral nervous systems, with limited amounts of

stress being advantageous to an organism and prolonged stress being deleterious. Based on the current results, it does not appear that stress exerts the same time course of action on memory function in female rats. It remains to be investigated if extremely prolonged periods (e.g., 35 days) of restraint stress leads to impairments in female rats.

In summary, the current studies provide novel information about the effects of chronic restraint stress on intact female rats. Unlike previously reported work from this and other laboratories that have investigated stress effects of RAM performance in males, female rats are enhanced on RAM following 21 days of chronic restraint stress, but these enhancements do not extend to 28 days of stress. We speculate that these differences are due, in part, to moderating effects of gonadal hormones (estrogen in particular) on the HPA response and/or hippocampus sensitivity to chronic stress. Current studies are investigating the effects of stress on memory in females with varying amounts of circulating estrogen.

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