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Differential effects of mild repeated restraint stress on behaviors and $GABA_A$ receptors in male and female rats

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Abstract

We previously reported that the very mild stress of individual housing influenced seizure risk and γ-amino butyric acid ($GABA_A$) receptor activity differentially between male and female rats. The aim of the present set of studies was to assess sex differences in behavioral responses to a more pronounced type of stressor, repeated restraint stress. We also wanted to determine the role of $GABA_A$ receptors in effects of this stressor. Our data suggest that repeated restraint stress afforded short-term protection against seizure induction in both male and female rats. Moreover, this protection was more persistent in female than male rats. This stress paradigm also elicited a reduction in general activity in male rats, whereas female rats displayed prolonged increased activity following the repeated restraint stress exposure. However, there were limited effects on anxiety-like behaviors, as determined by time spent in the open arms on the elevated plus maze. Sex differences in stress-induced increases in plasma corticosterone levels were observed, which generally correlated with sex differences in behavioral measures. There were no significant effects of the repeated restraint stress exposure on benzodiazepine/$GABA_A$ receptor density or affinity nor on receptor function. Taken together, these findings provide additional evidence to support the important influences of sex in responding to stress and highlight the need to consider this context when addressing the role of stress in health issues for women and men.

Keywords: Repeated stress; Rats; Behaviors; Sex differences; Seizures; Bicuculline; $GABA_A$ receptor and chloride uptake

1. Introduction

Repeated stress or stressful events can lead to the development of a number of medical and psychopathological disorders, including anxiety, depression and enhanced seizure risk (Kamarack and Jennings, 1991; Kessler, 1997; Lancman et al., 1993). The nature of the stress response and its impact on health depends on the type of stressor as well as the sensitivity of the organism to the stress. Several factors contribute to stress responses. One's sex has been identified as one important factor that influences perception of and responses to stress. A significant body of evidence implicates interactions between stress and sex on behaviors, endocrine responses and brain adaptations (for example, see Bujas et al., 1997; Kant et al., 1983; Troisi, 2001). Recently, clinical assessment of stress responses showed that while men tend to flee and seek isolation in response to a stressful situation, women seek each other for social support and evince "tend and befriend behaviors" (Taylor et al., 2001). In addition, women are affected more by anxiety and also have a higher incidence of mood or affective disorders than men (Myin-Germeys et al., 2004; Sonne et al., 2003). Laboratory animal studies show that females are generally more sensitive to stress than males (Chadda and Devaud, 2004; Kennett et al., 1986; Perrot-Sinal et al., 2004; Rivier, 1993; Wilson and Biscardi, 1994). For example, one study reported that repeated restraint stress increased defensive behaviors in response to cat odor in female rats whereas it had no effect on male rats (Perrot-Sinal et al., 2004). Sex differences are also observed in stress-induced changes in learning and memory, with male rats exhibiting reduced memory performance whereas female rats exhibit improved

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learning and memory following stress exposure (Bowman et al., 2001; Holscher, 1999; Kitraki et al., 2004). Furthermore, stress-induced increases in locomotor activity and anxiety were attenuated by group housing in female, but not male rats (Andrade and Guimaraes, 2003; Westenbroek et al., 2003). In our previous study, we showed that addition of a single restraint stress increased seizure risk specifically in individually housed males and group housed females, supporting the evidence of significant sex differences in response to stress that varies depending on the type of stressor and the environmental context (Chadda and Devaud, 2004).

There is also a growing literature on sex differences in neurochemical alterations in response to different types of stressors. Females generally show a potentiated activation of the hypothalamic-pituitary-adrenal (HPA) axis in response to stressful stimuli compared to males. For example, female rats showed a greater and more prolonged elevation in plasma corticosterone levels compared to males following a single, acute challenge with a physical or chemical stressor (Figueiredo et al., 2002; Rivier, 1993; Wilson and Biscardi, 1994). Recent clinical studies found that women had a greater release of cortisol in response to a social stressor compared to men (Stroud et al., 2002) and a correlation between increased cortisol and memory deficits in women compared to men (Wolf et al., 2001). Stress exposure has also been found to elicit sex differences in neurochemical responses involving various neurotransmitter systems, (Acosta and Rubio, 1994; Akinci and Johnston, 1993a; Blanchard et al., 2001; Galic et al., 2004; Palanza et al., 2001), with considerable evidence supporting the involvement of $GABA_A$ receptors (Akinci and Johnston, 1993b; Chadda and Devaud, 2004; Gruen et al., 1995; Verkuyl et al., 2005; Wilson and Biscardi, 1994; Wilson et al., 2004).

The aim of the present study was to further explore sex and brain interactions in response to a repeated mild type of stressor, restraint stress, to determine how the type of stressor would impact sex differences in behavioral, endocrine and neurochemical responses. Females operate under a different hormonal milieu than males, which can influence many responses. Therefore, a subset of these studies included ovariectomized female rats to allow us to begin to directly determine the importance of ovarian steroids in impacting stress responses.

2. Methods

2.1. Animals

Male and female Sprague–Dawley rats (Simonson Labs, Gilroy, CA) were 50±2 days old and weighed 150–190 g at the beginning of experiments. Bilateral ovariectomies were performed under ketamine/xylazine anesthesia in several subsets of female rats at 38–43 days of age. Sham surgeries were performed on the remaining female rats in these studies. Animals were allowed to recover from the surgery for at least 7 days prior to initiation of experimental procedures. Animals were housed 3 per cage. All animals were handled briefly each day and provided lab chow and water ad libitum. Stage of estrus was monitored in the intact female rats by histological examination of vaginal smears collected daily. Experiments were scheduled for when cycling females were in estrus or diestrus1 (when estrogen and progesterone levels are low). Preliminary studies found no effects of sham surgeries on responses of intact females; therefore, groups were combined for data collection and analysis. All experimental procedures were conducted according to I.S.U. Institutional Animal Care and Use Committee approved protocols following NIH guidelines. All experiments were conducted during the early part of the light period (lights on at 7:00 am; off at 7:00 pm).

2.2. Stress paradigm

Groups of rats were exposed to restraint stress for 30 min twice a day (at an interval of 5 h) for 10 days with the final stress exposure during the morning of the 11th day. Animals were placed in individual compartments of a transparent plexiglass 'pie-cage' container, which impeded, but did not prevent, movement. Testing was conducted at 10 min, 2 h and 24 h after the last stress exposure with control (unstressed but handled daily) animals tested at each time point. An additional subset of rats was exposed only to a single acute restraint stress for 30 min the morning of the 11th day and tested at 10 min after the single restraint stress exposure.

2.3. Drugs

Bicuculline was purchased from Sigma (St. Louis, MO). Both ^{36}chloride and [^{3}H]flunitrazepam were purchased from Perkin-Elmer/NEN (Boston, MA).

2.4. Bicuculline seizure thresholds

Seizure threshold determinations were made by constant tail vein infusion of bicuculline as previously described (Chadda and Devaud, 2004; Devaud and Chadda, 2001). The endpoint of the measure was taken as time to the first myoclonic twitch of the face and/or neck. Seizure thresholds were calculated from the time of infusion (min) × concentration of bicuculline (0.05 mg/ml)/ body weight (kg). Decreased seizure thresholds are equivalent to increased seizure susceptibility. All seizure threshold determinations were made between 8:00 am and 12:00 noon.

2.5. Open field activity

Locomotor activity in the open field was measured as previously described (Devaud et al., 2002; Devaud, 2003).

The open field apparatus consisted of a plexiglass box measuring $40 \times 40 \times 40$ cm with an 8-cm grid marking the floor into 72 squares. Each animal was placed in the center of the open field and allowed to explore for a 10-min session under ambient fluorescent room light. The open field was cleaned and dried after each test. Total number of line crossings were scored and summated in three bins (0–1 min, 1–5 min and 5–10 min).

2.6. Elevated plus maze

Anxiety-like behaviors were measured using the elevated plus maze. The maze was elevated 1 m above the floor and consisted of four 51-cm-long, 11.5-cm-wide arms arranged at right angles. The closed arms had translucent walls 30 cm high extending the length of the arm. The open arms had a low (1.5 cm) edge. At the time of the test, each animal was placed in the center of the maze facing an open arm and allowed to explore for a 5-min session under ambient room light. The plus maze was cleaned and dried between animals. The number of open and closed arm entries along with the time spent in each arm were scored and summated. A rat was considered to be within an arm when all its paws had crossed into the arm. Changes in the percent time or percent entries in the open arm are believed to reflect anxiety while the total number of arm entries indicate general activity (Handley and McBlane, 1993; Pellow et al., 1985; Pellow and File, 1986).

2.7. Corticosterone radioimmunoassay

At the same time points of behavioral testing after the final stress exposure, separate groups of animals were rapidly decapitated for collection of trunk blood in tubes containing ethylenediamine tetra-acetic acid at the same time points as for the behavioral experiments. Tubes were centrifuged at low speed and supernatant was collected. Plasma samples were stored frozen at −70 °C until the time of assay. Corticosterone assays were conducted in the laboratory of Dr. Deborah Finn (Oregon Health and Sciences University, Portland, OR). The RIA procedure used ^{125}I-CORT from ICN Pharmaceuticals (Costa Mesa, CA) and antisera from Ventrex (Portland, ME). Plasma (5 μl) was diluted with 100 μl of sterile water. Samples were immersed in boiling water for 5 min to denature corticosterone-binding globulin. Counts per minute were normalized and fit to a least-squares regression equation produced by log–logit transformation of the standards. Mass of samples was calculated by interpolation of the standards. The detectable range of the assay was from 0.1 to 400 μg of CORT per 100 ml of plasma. Intra- and inter-assay coefficients of variation were <10%. The specificity of the assay is very high, with only 4% cross-reactivity to deoxycorticosterone.

2.8. [^{3}H]Flunitrazepam binding assay

Frozen cerebral cortical tissue harvested from animals across all treatment conditions was homogenized in 50 volumes ice-cold wash buffer (50 mM Tris–HCl, pH 7.4) followed by centrifugation at $30{,}000 \times g$ for 20 min. Pellets were resuspended in 50 volumes wash buffer and centrifuged again at high speed for 20 min. The resulting pellets were frozen at −70 °C until time of assay. At the time of assay, homogenates were resuspended in 100 volumes of wash buffer and centrifuged again at high speed for 20 min. The tissue was washed once more. The final pellets were resuspended in 80 volumes of ice-cold assay buffer (50 mM Tris–HCl, pH 7.4 at 4 °C with 120 mM HCl, 1 mM EDTA and 5 mM KCl). A concentration range of 0.21–24 nM [^{3}H] flunitrazepam was used. GABA (5 μM final) was added to all tubes. Diazepam (10 μM) was used to define non-specific binding. Radioactivity was added last to initiate the reaction. All samples were run in triplicate. Tubes were incubated for 60 min at 4 °C. Incubation was terminated by rapid vacuum filtration followed by three 5-ml washes with ice-cold assay buffer over GF/C filters. Retained radioactivity was measured by liquid scintillation spectroscopy.

2.9. GABA-gated ^{36}chloride uptake assay

Fresh cerebral cortical tissue was rapidly harvested over ice, with tissue from two to three rats pooled for each treatment group per experiment. Tissue was added to 30 volumes assay buffer (20 mM HEPES, 118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSo_4$, 2.5 mM $CaCl_2$, pH to 7.4 at 25 °C with Tris base), then homogenized using a glass–glass dounce. The homogenate was filtered through three layers of nylon mesh and centrifuged at $1000 \times g$ for 15 min at 4 °C. The pellet was resuspended in 30 volumes assay buffer and centrifuged again. The final pellet was resuspended in 5 volumes assay buffer. Tubes were preincubated at 30 °C for 12 min. At this time, 200 μl of ^{36}Cl (0.2 μCi/tube)±varying concentrations of GABA in assay buffer were added and incubated for 4 s. Incubation was terminated by the addition of 4 ml of ice-cold assay buffer containing picrotoxin (100 μM) followed by vacuum filtration over S&S #32 filters pre-soaked in 0.1% BSA using a Hoefer single manifold apparatus. The tissue was washed twice more with ice-cold assay buffer. All samples were run in triplicate. Retained ^{36}chloride was assayed by scintillation spectroscopy. Total chloride uptake was calculated according to percent ^{36}chloride out of total chloride present in the assay buffer. Basal chloride uptake (in the absence of GABA) was subtracted from all values.

2.9.1. Statistics

Behavioral data and CORT levels were compared using two-way analysis of variance (ANOVA) with sex and treatment (stress at different time intervals or no stress) as

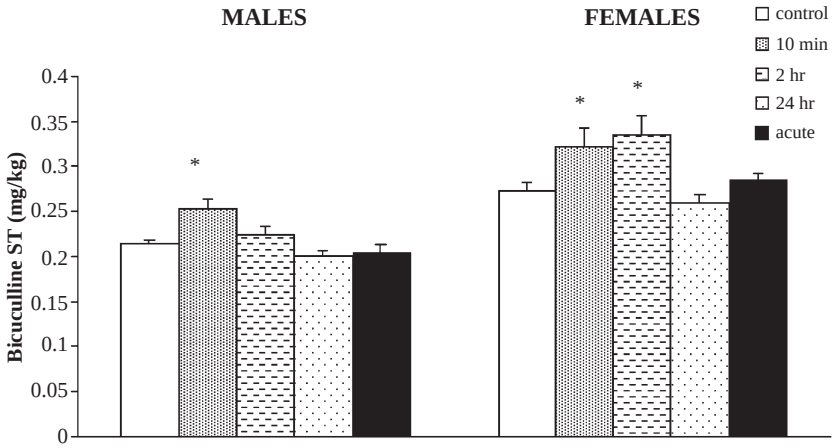

Fig. 1. Effects of repeated restraint stress on bicuculline seizure thresholds in male and female rats. *$P<0.05$, $N=9-37$ per group with control conditions collapsed across all testing time points.

factors. Treatment groups within males and females were compared using one-way ANOVA. Once significant main effects were seen, specific differences between groups were analyzed using the post-hoc Tukey's test. Significance level was set at $P<0.05$. Parameter estimates for saturation binding analyses as well as EC_{50} and E_{max} values for GABA-gated chloride uptake were generated using Prism 4 (Graph Pad, San Diego, CA). Animals were used once only for any determination to avoid the confounds of repeated testing. Separate sets of control animals were tested at each time point.

3. Results

3.1. Seizure threshold

As seen in Fig. 1, there were interesting sex differences in seizure risk following exposure to repeated restraint stress. There were main effects of both sex [F (1,158)=10.3, $P<0.001$] and stress timing [F (4,158)=111.3, $P<0.001$] on bicuculline seizure thresholds. Consistent with previous experiments, basal bicuculline seizure thresholds were significantly higher in females than males. Repeated restraint stress led to a significant increase (18%) in bicuculline seizure threshold in male [F (4,86)=6.5, $P<0.001$] and female rats [F (4,72)=5.6, $P<0.001$] at 10 min after the final stress exposure (Fig. 1). Interestingly, this effect persisted through 2 h only in female rats. Seizure thresholds remained significantly increased by 18%, from 0.274±0.008 to 0.323±0.02 mg/kg bicuculline in females, but not males (0.214±0.005 to 0.225±0.008 mg/kg).

3.2. Plus maze activity

We next determined the effects of repeated restraint stress on anxiety-like measures and general activity. In general, this repeated stress exposure paradigm had minimal effects

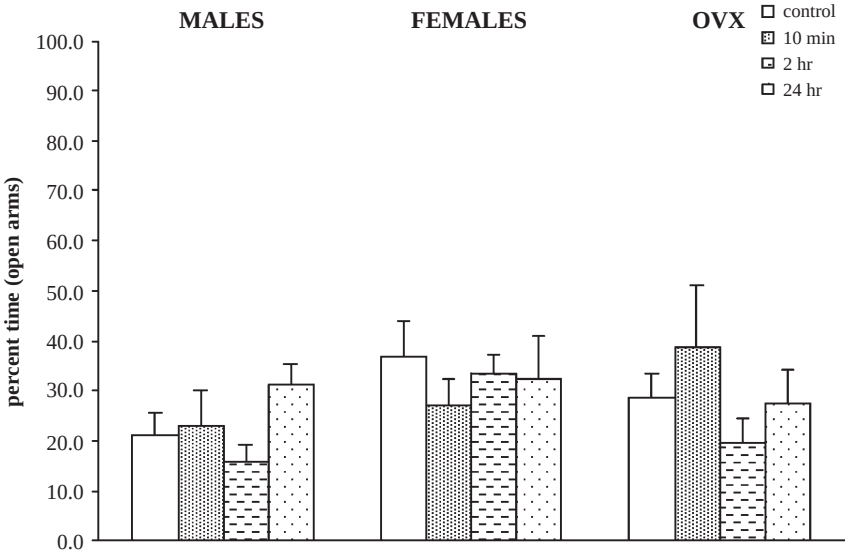

Fig. 2. Effects of repeated restraint stress on % of open arm time in an elevated plus maze in male and female rats. $N=7-10$ per treatment condition.

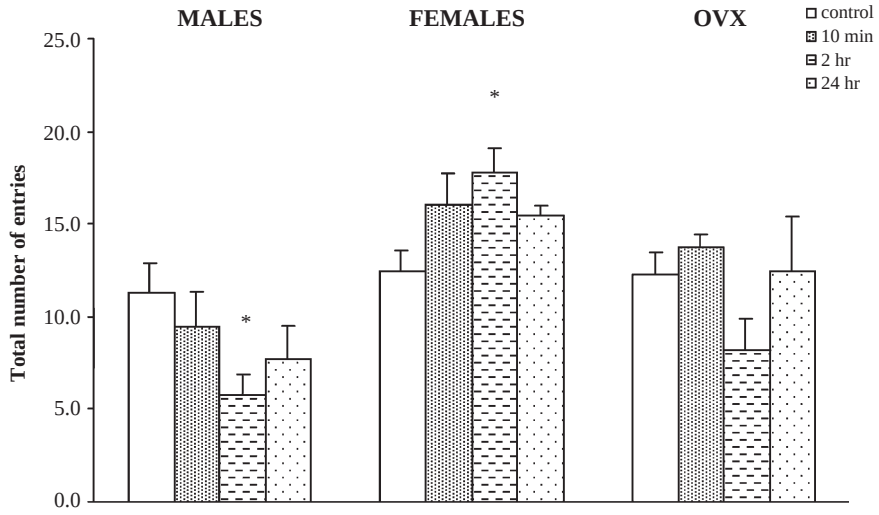

Fig. 3. Effects of repeated restraint stress on total number of entries in an elevated plus maze in male and female rats. *$P<0.05$, $N=7-10$ per treatment condition.

on anxiety-like measures as assessed with the elevated plus maze (Fig. 2). The percent time spent in closed or open arms was not significantly altered by repeated restraint stress at any time point, although there was a trend toward a decrease in open arm times at 10 min for intact females but only at 2 h for males and OVX females. However, there was a significant effect on general activity, represented by total arm entries (Fig. 3), which followed the trend towards open arm times. Sex [F (2,68)=14.7, $P<0.001$] had a significant main effect on total number of entries, with intact females displaying a greater number of entries across all treatment times compared to males and OVX females. For example, at 2 h after the final repeated restraint stress, there was a 42% increase in the total number of entries, from 12.5±1.1 to 17.8±1.3, in female rats. In contrast, there was a marked, significant 50% decrease, from 11.3±1.6 to 5.8±1.1, in the total number of entries in male rats. OVX female rats also displayed a 34% decrease in the total number of entries, from 12.2±1.2 to 8.1±1.7 at this time point. Total number of arm entries remained elevated in females and decreased in males through 24 h but this effect did not persist in the OVX female group.

3.3. Open field behaviors

Open field behaviors were used to assess the effects of repeated restraint stress on general exploratory activity (Fig. 4). Sex had an overall main effect on locomotor activity [F (2,70)=8.6, $P<0.001$] at 0–1 min and [F (2,70)=7.9, $P<0.001$] at 1–5 min. General activity was minimally affected by the repeated restraint stress exposure in male rats, in a dramatic contrast to female rats. The major effect

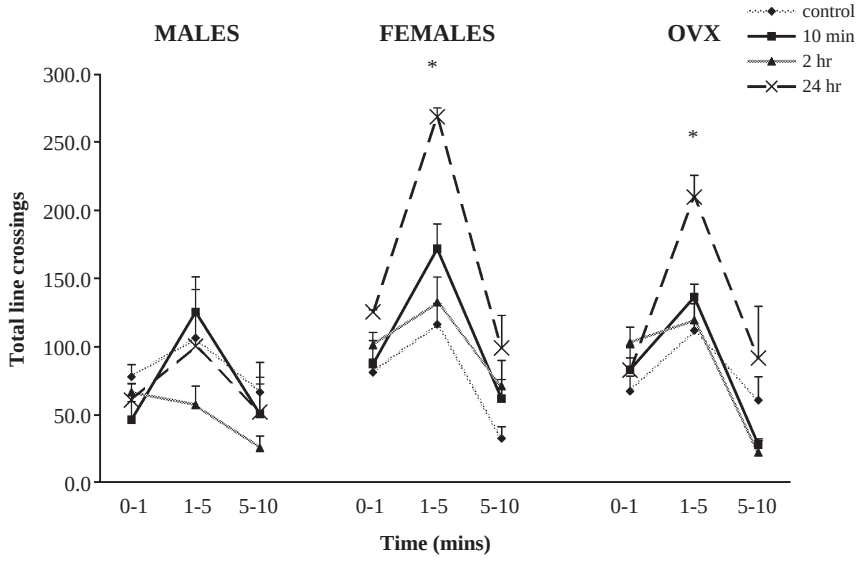

Fig. 4. Effects of repeated restraint stress on general activity in an open field box in male and female rats. *$P<0.05$, $N=5-6$ per treatment condition.

in male rats occurred at the 2-h test time, when there was a decrease in locomotion, most marked at the 1–5 min, compared to control levels. Activity was increased in intact, but not OVX females at all time points. A significant increase in locomotor activity at 24 h after the last repeated restraint stress was seen in both groups of females (from 116.3±15.3 to 268.5±18.5 line crossings in intact females and from 111.9±26.3 to 209.8±15.8 line crossings in ovariectomized females) compared to non-stressed controls, especially during the 1–5 min bin.

3.4. Corticosterone levels

Plasma CORT levels were assessed as an endocrine measure indicative of stress-induced activation of the HPA axis. There were significant main effects of sex [F (2,163)=17.5, $P<0.001$] and stress [F (4,163)=12.6, $P<0.001$] on CORT levels (Fig. 5). At 10 min after the final repeated stress exposure, CORT levels were significantly elevated in all rats compared to non-stressed but handled controls. However, the increase in CORT levels (from 5.5±1.3 to 21.4±2.8 μg/dl) in repeatedly stressed male rats was smaller compared to the increase in CORT levels (to 26.0±8.2 μg/dl) in male rats exposed to a single acute stress. Likewise, the increase in CORT levels (from 0.97±0.4 to 18.7±4.7 μg/dl) in OVX rats at this time was smaller compared to CORT levels (40.2±6.9 μg/dl) in OVX rats only exposed to a single acute stress. In contrast, increases in CORT levels (from 24.0±4.9 to 54.7±8.3 μg/dl) in intact female rats at 10 min after the final repeated stress was greater compared to the increase in CORT levels (41.9±7.8 μg/dl) in female rats exposed to a single acute stress. Stress-induced increases in CORT levels were lost by 2 h and remained at basal levels through 24 h (data not shown).

Table 1
Saturation binding estimates for [^{3}H]flunitrazepam in cortex following repeated mild stress in male and female rats

	K_D (nM)	B_{max} (fmol/mg protein)
Males		
Control	1.3±0.18	2525±34
10 min	1.4±0.11	2555±100
2 h	1.6±0.2	2450±107
Acute	1.6±0.27	2248±100
Intact females		
Control	1.34±0.14	2410±86
10 min	1.56±0.04	2826±68
2 h	1.32±0.05	2300±104
Acute	1.45±0.12	2685±168
OVX females		
Control	1.1±0.02	2320±36
10 min	1.1±0.2	2460±122
2 h	1.2±0.2	2460±91
Acute	1.0±0.02	2511±145

Saturation binding estimates were determined using Prism 4 and are the average from triplicate determinations over two independent experiments.

3.5. $GABA_A$ receptor measurements

To determine the role of $GABA_A$ receptors in mediating sex differences in behavioral responses to repeated restraint stress, we analyzed the [^{3}H]flunitrazepam binding to the benzodiazepine site of the $GABA_A$ receptor complex and $GABA_A$ receptor-mediated chloride uptake in cerebral cortical tissue. As shown in Table 1, binding estimates for [^{3}H]flunitrazepam binding were quite similar among all treatment conditions. There was an interesting trend towards and increased affinity, but not density, of binding for OVX females compared to intact females and males, but this did not reach significance. GABA-gated chloride uptake was

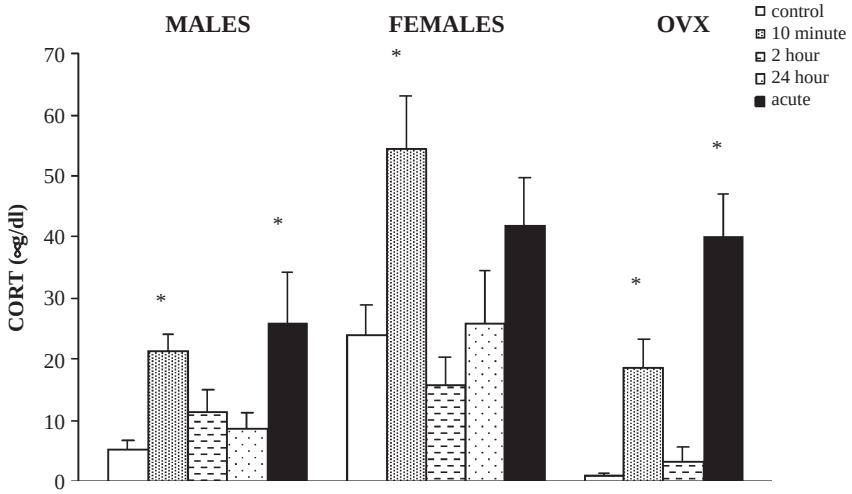

Fig. 5. Effects of repeated restraint stress on plasma corticosterone levels in male and female rats across different time points. *$P<0.05$. $N=6–26$ per treatment condition with control values collapsed across treatment conditions. The OVX group has no 24-h data because as we saw, no effect at 24 h in initial experiments with males and intact females, we did not test this time point in additional experiments that included OVX females.

Table 2
GABA-gated chloride uptake in male and female rat cortical synaptoneurosomes across several time points, after the final stress exposure

	EC_{50} (nM)	E_{max} (nmol/mg protein)
Males		
Control	21.22±1.14	28.76±1.24
10 min	19.43±1.19	27.52±1.16
2 h	14.07±1.16	19.82±1.14
24 h	14.18±1.15	16.47±1.04
Acute	17.66±1.24	17.09±0.99
Females		
Control	21.87±1.24	20.1±1.03
10 min	22.81±1.13	18.39±1.06
2 h	22.63±1.21	20.2±1.17
24 h	23.55±1.25	17.76±1.03
Acute	19.48±1.24	16.59±1.03

Data presented are the mean±S.E.M. generated by Prism 4 from determinations run in triplicate across GABA concentrations from 0 to 600 μM.

conducted only in intact male and female animals (Table 2). While there was a trend toward a chronic stress-induced increase of response (approximately 30%) only in males, this did not reach statistical significance (no overall main effect of sex or treatment times, $P>0.05$). Neither acute or repeated restraint stress altered maximal GABA-stimulated potentiation of chloride uptake.

4. Discussion

The intent of the present study was to gain a better understanding of the importance of sex in influencing responses to a repeated, mild type of stressor as this has relevance to everyday life. We found that the repeated restraint stress transiently reduced seizure risk in both male and female rats. This finding suggested that this type of stress exposure activated coping mechanisms, serving to protect the animals against seizure induction. Several earlier reports found that acute and more severe types of stressor also reduced risk for seizures (Drugan et al., 1985; Galic et al., 2004; Pericic and Bujas, 1997; Pericic et al., 1999, 2000). With the current milder type stress paradigm, this protection against seizure induction was also significant and showed sex differences in responses. This suggests there must be some significant sex differences in compensatory mechanisms underlying the reduced risk for seizures following stress exposure. In contrast, a single acute restraint stress exposure did not alter seizure susceptibility in either male or female rats. This showed that the single stress was not severe enough to cause significant changes, but the repeated nature of the stress resulted in an enhanced, rather than reduced, response.

Stress has a variety of effects on any number of measurable behaviors, in addition to seizure risk. Similar to reports for seizure risk, stress can either induce or alleviate anxiety-like behaviors in rats, depending on the type and severity of the stressor (D'Aquila et al., 1994; Ducottet and Belzung, 2004, 2005; Van Gaalen and Steckler, 2000). Although there were limited effects of the repeated restraint stress exposure on plus maze behaviors, we did find bidirectional sex differences in total activity on the plus maze. Male and OVX female rats exhibited decreased total activity (number of arm entries) following the stress exposure whereas female rats showed an increased number of entries. This finding was in agreement with other studies suggesting strain- and sex-selective effects of stress on general activity (D'Aquila et al., 1994; Van Gaalen and Steckler, 2000). These results also support an earlier study showing that plus maze behaviors are mostly characterized by activity in female rats but by anxiety in male rats (Fernandes et al., 1999). The sex differences in stress-induced changes in activity generalized to a second measure, locomotion in the open field. Line crossings increased in both male and female rats immediately following the last stress exposure compared to control levels, indicative of an enhancement of general activity in response to novelty. However, this increased activity persisted in females, but not males. We again observed a bidirectional response, with males displaying a decrease in activity at the 2-h time point, similar to observations made with the elevated plus maze. In contrast, female rats displayed a rebound increase in activity that was particularly dramatic and persisted through 24 h after the final stress exposure. These findings suggest that the repeated mild type of stress resulted in adaptations that differed both by degree and persistence between male and female rats. In these measures, the OVX female behaviors appeared more similar to males than intact females, suggesting that ovarian steroids play an activational role in moderating activity responses to the mild stressor. There have been contradictory reports concerning the effects of chronic stress on activity in rats. A physical stress consisting of 5 days of foot shock decreased locomotor activity and increased immobility in male rats (Pijlman et al., 2003). However, a second study found that chronic exposure to foot shock caused an increase in locomotor activity in both male and female rats (Westenbroek et al., 2003). This highlights the dependence of stress responses on the context of the challenge, both extrinsic (environmental) and intrinsic (hormonal or neurobiological). In the present study, we found marked sex differences in the persistence and extent of behavioral responses to our stress paradigm. This may arise from an overshoot in compensatory neurochemical and or neuroendocrine responses that is enhanced by the presence of ovarian steroids.

Investigation of stress hormone responses elicited by the repeated stress paradigm showed increases in plasma CORT levels in male and female rats shortly after the final repeated restraint stress exposure. It was unexpected to see the persistent activation of the HPA axis following the repeated mild stress exposure, as a number of reports have shown habituation of the stress endocrine response following a

repeated stress, suggesting acclimatization. There were also significant sex differences in this measure. The stress-induced elevations in plasma CORT at 10 min in male rats showed some habituation because there was a smaller increase in CORT levels following the repeated restraint stress exposure compared to the response following the single, acute restraint stress. In contrast, female rats displayed sensitization to the repeated stress, by a larger increase in CORT levels in response to the repeated stress compared to the acute stress exposure. These findings support a previous report, which found that female rats released more CORT and for a longer period compared to males following 21 days of repeated restraint stress (Galea et al., 1997). It is likely that this enhanced CORT response in females contributed to mechanisms underlying the repeated stress-induced sex differences in the behaviors measured in this study.

As GABAergic neurotransmission has been implicated in having a role in stress responses, we next investigated the effects of repeated restraint stress on $GABA_A$ receptor levels and function. We found that neither the density nor affinity of the benzodiazepine binding site of $GABA_A$ receptors was effected by the repeated restraint stress exposure. Several previous reports showed major changes in $GABA_A$ receptor binding parameters following stress exposures, but this was in response to a more severe stressor. Changes in GABAergic neurotransmission may also result from alterations in the subunit expression of $GABA_A$ receptors (which likely confer different functional properties), without observable changes in receptor density or affinity. Several studies found alterations in $GABA_A$ receptor subunit levels in specific brain regions in response to acute or repeated stress (Gruen et al., 1995; Martijena et al., 2002; Montpied et al., 1993; Orchinik et al., 1995). However, the present study found no significant repeated stress-induced alterations in GABA-gated chloride uptake, suggesting that it is unlikely that properties of cortical $GABA_A$ receptors are altered by the repeated mild stress paradigm. These studies do not negate the involvement of GABAergic neurotransmission in neurochemical responses to stress. It may be selective to other brain regions (such as the hippocampus, prepiriform cortex or amygdala); additional brain areas that that are believed to be involved in expression of seizure risk and/or anxiety-like behaviors. Conducting dose–response curves for $GABA_A$ receptor-mediated chloride uptake requires a fair amount of tissue and so the standard use of cerebral cortex; future studies could address the regional aspects by running single point analysis of more discrete brain areas.

This study did find sex differences in effects of the repeated mild stress on plasma corticosterone levels that were consistent with behavioral measures and with other reports of an enhanced HPA axis response to stress by females compared to males. It was interesting to note the clear sex differences in adaptations to the repeated stress compared to an acute stress—with males showing some habituation while females displayed sensitization. This differential response could play an important role in risk for health problems and may provide some basis for the observed differences in prevalence for a number of mental disorders (such as anxiety and depression) between men and women. As prolonged increases in CORT levels reduce GABA-mediated inhibitory neurotransmission (see Dallman et al., 2004 for review), this would provide a link between current findings and the role of $GABA_A$ receptors in regulating brain excitability, supporting the need to continue to address this interaction.

The sex differences observed in behaviors and corticosterone measures in response to stress may involve either organizational or activational influences. Organizational effects arise from sexual dimorphism that occurs during development whereas activational influences occur because of the direct effects of circulating gonadal steroids in brain. Ovarian steroids, primarily 17β estradiol and progesterone, have been shown to exert a variety of effects in the CNS following stress exposure (Barbaccia et al., 1996; Maggi et al., 2004; Morrow et al., 1995; Purdy et al., 1991; Shors et al., 2001). In particular, a number of progesterone derivatives, termed neuroactive steroids, modulate $GABA_A$ receptor activity (see Paul and Purdy, 1992 for review), which may contribute to sex differences in stress responses. Therefore observed sex differences may partly arise from differential levels of endogenous modulators of $GABA_A$ receptors (Belelli and Gee, 1989; see Reddy, 2002 for review).

Several studies have reported alterations in steroid levels by stress (Higashi et al., 2005; Serra et al., 2000). A recent report studied the effect of acute and chronic stress on 17β-estradiol-replaced OVX rats. Although 17β-estradiol treatment did not alter acute stress responses, chronically stressed rats exhibited reductions in anxiety-like behaviors (Lunga and Herbert, 2004). In the present set of studies, stress-induced responses varied across sex treatment groups, with OVX females responding like males in some measures but more like intact females in others. This suggests that both organizational and activational influences have a role in conferring sex differences in response to the repeated restraint stress.

In summary, the present study found significant sex differences in response to a repeated, mild restraint stress. Responses to stress involve a cascade of events, which can vary depending on the type, severity and length of the stressor. These findings highlight the influence of internal context (being male or female) as an important determinant of responses. The value of comparisons across sex groups lies in the implications for sex differences in a number of stress-related health issues, including anxiety and seizure risk.

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