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# Sex differences in effects of mild chronic stress on seizure risk and $GABA_A$ receptors in rats

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**Abstract**

Social stress is a common occurrence in our society that can negatively impact health. Therefore, we wanted to study the effects of a mild stressor designed to model social stress on seizure susceptibility and $GABA_A$ receptors in male and female rats. The mild chronic stress of individual housing consistently decreased bicuculline (but not pentylenetetrazol, PTZ) seizure thresholds by 10–15% in both sexes. Housing conditions did not alter the anticonvulsant activity of diazepam or ethanol, although the anticonvulsant effect of ethanol was significantly greater against PTZ-induced seizures. Experiments testing the addition of an acute restraint stress unmasked sex differences in seizure induction. The acute stress also selectively decreased the potency of GABA to modulate $GABA_A$ receptor-mediated chloride uptake in group-housed females. There were additional sex differences by housing condition for $GABA_A$ receptor-gated chloride uptake but no differences in [$^3$H]flunitrazepam binding. We also found significant effects of sex and housing on ethanol-induced increases in corticosterone (CORT) levels. In summary, there were complex and sex-selective effects of mild chronic stress on seizure induction and $GABA_A$ receptors. Gaining a better understanding of mechanisms underlying interactions between sex and stress has important implications for addressing health concerns about stress in men and women.


*Keywords:* Sex differences; Stress; Seizure risk; $GABA_A$

## 1. Introduction

Stress plays an important, often overlooked, role in human health. Stress can operate at many levels and be acute/chronic or mild/severe, depending on how it is applied as well as by how it is perceived. In humans, stress has been suggested to contribute to risk for seizures (Lancman et al., 1993), heart disease (Kamarck and Jennings, 1991), high blood pressure (Snider and Kuchel, 1983), and emotional disorders, such as depression (Kessler, 1997). Several research laboratories have been investigating the effects of stress in a variety of animal models. Our interest has focused on social stress, as it is the form of stress most commonly experienced by humans in day-to-day living. Social stress generally results from a sense of lack of control over one's environment and interactions.

Also of interest is the increasing evidence that men and women may respond differently to stressors. Recent findings showed that women might react to stressful situations by "tend-and-befriend" behaviors (Taylor et al., 2001; Troisi, 2001). In contrast, men may display more of the "fight-and-flight response" when in stressful situations. Sex differences have also been observed in several animal models of stress (Blanchard et al., 2001; Brown and Grunberg, 1995; Palanza et al., 2001). For example, Brown and coworkers found that overcrowded living conditions decreased corticosterone (CORT) levels in female rats, whereas this increased CORT levels in male rats, suggesting that crowding stresses males but not females. In female mice, individual housing decreased the tendency to explore (i.e., increased anxiety), whereas in male mice it was group housing that decreased exploratory behaviors (Palanza et al., 2001). These results suggest that even something as simple as housing condition can model social stress and have a sex-selective impact on behaviors.

There is evidence that one's sex may influence basal brain excitability, differentially increasing the risk for harm from stress in males and females. Studies measuring seizure susceptibility found subtle sex differences in basal seizure

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risk between males and females as well as differences within females across the estrus cycle (Finn and Gee, 1994; Kokka et al., 1992; Wilson, 1992). In contrast, we found minimal sex differences in basal bicuculline seizure susceptibility when testing intact females in estrus or diestrus compared with ovariectomized females (Devaud et al., 2000). Studies with humans have found that men and women can display gender differences in expression of epilepsy. Seizure risk and/or severity have been found to vary with the menstrual cycle in some women and this is called *catamenial epilepsy* (Schachter, 1988).

As levels of ovarian steroids, including progesterone, estradiol, and some of their neuroactive metabolites, fluctuate across the estrus/menstrual cycle, it is possible that they influence basal seizure susceptibility. However, the interplay among being female, risk for seizures, and the influence of stress on this risk has not been explored. Therefore, the aim of the present study was to determine whether use of the mild chronic stressor, individual housing, exerted sex-selective actions on seizure thresholds (seizure susceptibility) in intact male and female rats compared with the grouped, but not crowded, housing condition.

We also investigated the role of $GABA_A$ receptors in these responses as a previous report showed that swim stress preferentially affected $GABA_A$ receptor binding in whole brain from male but not female mice (Akinci and Johnson, 1993). A large number of studies have implicated $GABA_A$ receptors as being involved in responding to stress. We have previously reported sex-selective alterations in seizure risk, $GABA_A$ receptor function and level of expression of several $GABA_A$ receptor subunits in our studies of ethanol dependence and withdrawal (Devaud et al., 1996; 1999, 2000, 2003). As ethanol dependence and withdrawal are stressful, observed alterations in $GABA_A$ receptors could include effects of stress. In the present study, we directly tested the effects of a mild type of social stress in male and female rats. Furthermore, we tested the effects of several additional, acute stressors added on to the differential housing conditions. We found interesting and significant sex-selective effects that appeared to depend, at least in part, on the convulsant agent used. We also found significant chronic mild stress-induced differences in $GABA_A$ receptor function between male and female rats.

## 2. Methods

### 2.1. Animals

Male and female Sprague–Dawley rats (Harlan, Indianapolis, IN) were 50±2 days old and weighed 150–190 g at the beginning of experiments. Group-housed animals were placed three per cage, whereas individual housing was one animal per cage. Housing conditions were maintained for 10 days, with all animals in the same room. Animals were handled daily with lab chow and water provided ad libitum. Female rats were monitored for stage of estrus by histological examination of daily vaginal smears. They routinely had 4- to 5-day cycles. Most of the females (approximately 80%) were in estrus or diestrus I at the time of testing, with variability in stage of estrus kept equivalent across treatment groups on any day of testing. We chose to test all treatment conditions for both males and females on the same day to control for possible day-to-day variations in seizure thresholds, rather than strictly controlling for stage of estrus. Therefore, each experiment was conducted over several days with an equivalent number of animals tested under all conditions on any one day.

Additional experiments were conducted to assess interactions between exposure to an additional single acute stressor with the two housing conditions. Individual or group-housed rats were exposed to a restraint stress for 30 min following 10 days of housing and at 2 h prior to seizure threshold determinations. All experiments were conducted according to our Institutional Animal Care and Use Committee approved protocols following NIH guidelines.

### 2.2. Drugs

Bicuculline and pentylenetetrazol (PTZ) were purchased from Sigma (St. Louis, MO). Ethanol was diluted to 18% v/v in saline for intraperitoneal administration. Both $^{36}Cl$ and [$^3$H]flunitrazepam were purchased from Perkin-Elmer/NEN (Boston, MA).

### 2.3. Seizure threshold determinations

Seizure threshold determinations were made by constant tail vein infusion of the chemoconvulsant. Animals were gently restrained for insertion of a 25-gauge butterfly needle into a lateral tail vein. The needle was taped in place and the animal was freed from the hold and held lightly by the tip of the tail, which allowed free movement. Bicuculline was dissolved in 1 N HCl, the pH adjusted to neutral by the addition of 1 N NaOH, then diluted to a final concentration of 0.05 mg/ml. PTZ was diluted in normal saline to a final concentration of 5 mg/ml. Convulsants were infused at a rate of 1.6 ml/min. 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 chemoconvulsant/body weight (kg). Decreased seizure thresholds are equivalent to increased seizure susceptibility. Ethanol was administered at a final dose of 2.5 g/kg by intraperitoneal injection 30 min prior to seizure threshold determinations. Diazepam was dissolved in 10% β-cyclodextrin and administered at a concentration of 5 mg/ml with a 1-ml/kg volume of injection 30 min prior to seizure threshold determinations. All seizure threshold determinations were made between 8:00 a.m. and 12:00 noon. Animals were used only once for any determination. All experiments were repeated at least once to increase sample

size for statistical reliability and to verify reproducibility of response.

### 2.4. CORT radioimmunoassay

Animals were sacrificed at two time points for collection of plasma and assay of CORT after 10 days of housing. In one set of experiments, blood was collected following 10 days of the different housing conditions and at 30 min after the acute ethanol injection, but without additional testing. In the second set of experiments, blood was collected from ethanol-treated animals immediately following seizure induction. Animals were rapidly decapitated for collection of trunk blood in tubes containing ethylenediamine tetraacetic acid (EDTA). All tubes were centrifuged at low speed and supernatant was collected. Plasma samples were stored frozen at −70 °C until time of assay. CORT assays were conducted using radioimmunoassay (RIA) in the laboratory of Dr. Deborah Finn (Oregon Health and Sciences University, Portland, OR). The RIA was adapted from a previously reported procedure (Keith et al., 1978) and 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 CORT-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 interassay coefficients of variation were <10%. The specificity of the assay is very high, with only 4% cross-reactivity to deoxycorticosterone.

### 2.5. GABA-gated $^{36}$Cl uptake assay

Following 10 days of the experimental housing conditions and 2 h after application of an acute restraint stress, fresh cerebral cortical tissue was rapidly harvested over ice, with tissue from two to three rats pooled from each treatment group. Tissue was gently homogenized in 30 volumes assay buffer (20 mM HEPES, 118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, and 10 mM glucose, pH 7.4) using a glass–glass dounce. The homogenate was filtered through three layers of nylon mesh and centrifuged at 1000×*g* for 15 min at 4 °C. The pellet was gently resuspended in assay buffer and washed twice more. The final pellet was resuspended to a protein concentration of approximately 5 mg/ml. Aliquots of tissue were preincubated for 12 min at 30 °C. At this time, 200 μl of the $^{36}$Cl (0.2 μCi per tube)±varying GABA concentrations (0–600 μM) were added and incubated with tissue for 5 s. Incubations were terminated by the addition of 4 ml of ice cold assay buffer followed by vacuum filtration over S&S 32 filters presoaked in 0.1% BSA. The tissue was rinsed twice more with ice-cold assay buffer. All samples were run in triplicate. Retained $^{36}$Cl was assayed by scintillation spectroscopy and total $GABA_A$ receptor-mediated chloride uptake was calculated according to percent of $^{36}$Cl/total chloride present in the assay buffer. Basal chloride uptake (without addition of GABA) was subtracted for specific determination of $GABA_A$ receptor-gated chloride uptake.

### 2.6. [$^3$H]Flunitrazepam binding assay

Frozen cerebral cortical tissue was homogenized in 50 volumes ice-cold wash buffer (50 mM Tris–HCl, pH 7.4) followed by centrifugation at 30,000×*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 washed twice more. The final pellets were resuspended in 80 volumes of ice-cold assay buffer (50 mM Tris–HCl, 120 mM NaCl, 1 mM EDTA, and 5 mM KCl, pH 7.4). A concentration range of 0.2–24 nM [$^3$H]flunitrazepam was used to determine saturation binding estimates. GABA (5 μM final) was added to all tubes. Diazepam (10 μM) was used to define nonspecific 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 determined by liquid scintillation spectroscopy.

#### 2.6.1. Statistical analysis

Parameter estimates for saturation binding analyses as well as GABA-gated chloride uptake were generated using Prism 3 (GraphPad, San Diego, CA). Seizure threshold data were analyzed for significance by ANOVA with housing, sex, and treatment (ethanol, diazepam, and restraint stress) as independent factors and the *t* test was used for post hoc analysis.

## 3. Results

Individually housed male and female rats showed small, but consistent decreases in bicuculline seizure thresholds compared with group-housed animals (Fig. 1). Statistical analysis verified that housing conditions had significant effects on bicuculline seizure thresholds [$F(1,64)=7$, $P<.01$]. Seizure thresholds decreased by 13%, from 0.33±0.01 mg/kg for group-housed to 0.29±0.01 mg/kg bicuculline, in individually housed male rats, and by 10%, from 0.44±0.02 to 0.39±0.02 mg/kg bicuculline, in individually housed female rats. There was an additional main effect of sex [$F(1,64)=42.5$, $P<.001$] with bicuculline seizure thresholds in female rats being approximately 35% higher than male rats for group or individual housing.

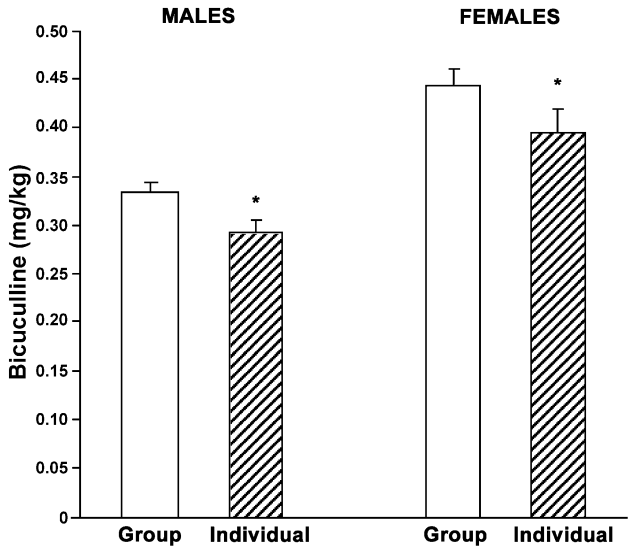


Fig. 1. Bicuculline seizure thresholds were significantly reduced in individually housed compared with group-housed male and female rats. All female values were significantly higher compared with male values. *$P$<.05 compared with group housing within sex ($n$=16–18 per treatment group per sex across three independent experiments).

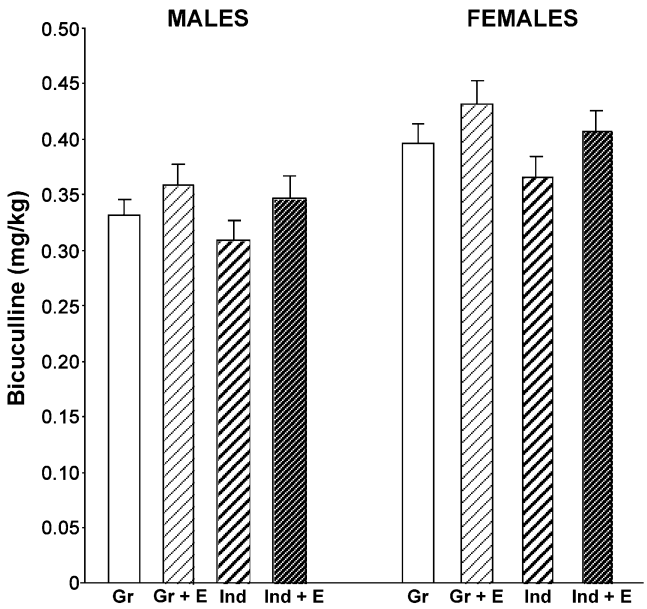


Fig. 3. Ethanol (2.5 g/kg) administration did not significantly elevate bicuculline seizure thresholds in either group- or individually housed male and female rats. Gr=group, Gr+E=group housed with ethanol treatment, Ind=individual, Ind+E=individual housing with ethanol treatment. All the female values were significantly higher compared with male values. *$P$<.05 compared with control housing conditions within ($n$=10–12 per treatment group per sex across two independent experiments).

Next, the anticonvulsant action of diazepam and ethanol were studied under these different housing conditions (Fig. 2). The main effect of sex on seizure thresholds was again observed [$F$(1,79)=55.9, $P$<.001]. In addition, diazepam significantly increased seizure thresholds to a similar extent regardless of sex or housing conditions, except for a smaller effect (28% rather than nearly 40% increase) in individually housed female rats [$F$(1,79)=72.6, $P$<.001]. As shown in Fig. 3, when ethanol was tested for its anticonvulsant effect against bicuculline-induced seizures, there was no interaction between ethanol and housing, nor did ethanol exert a significant anticonvulsant effect when analyzed within sex. Seizure thresholds were slightly increased from 0.33±0.01 to 0.36±0.02 mg/kg bicuculline in group-housed male rats and from 0.32±0.02 to 0.35±0.02 mg/kg bicuculline in individually housed male rats following the ethanol treatment (2.5 g/kg). Acute

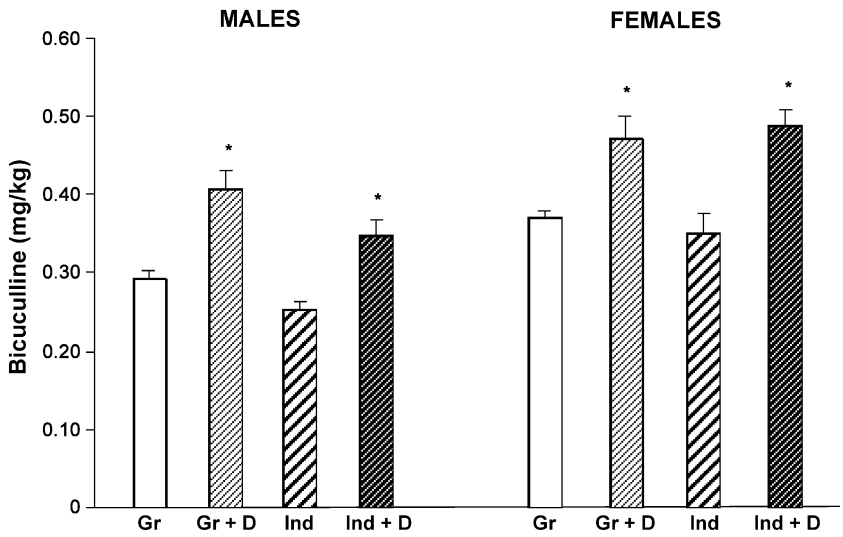


Fig. 2. Diazepam administration (5 mg/kg) significantly increased seizure thresholds in both group- and individually housed male and female rats. Gr=group, Gr+D=group housed with diazepam treatment, Ind=individual, Ind+D=individual housing with diazepam treatment. All female values were significantly higher compared with male values. *$P$<.05 compared with control housing conditions within sex ($n$=9–13 per treatment group per sex).

administration of ethanol also slightly increased seizure thresholds in female rats, shifting values from 0.40±0.02 to 0.43±0.02 mg/kg bicuculline in group-housed and from 0.37±0.02 to 0.41±0.02 mg/kg of bicuculline, in individually housed female rats. Sex again had a significant main effect on bicuculline seizure thresholds [$F(1,77)=23.3$, $P<.001$].

In marked contrast to our findings with bicuculline, responses to seizure induction by PTZ did not show an effect of housing conditions on basal seizure susceptibility (Fig. 4). Nor was there a main effect of sex. However, ethanol displayed a significant anticonvulsant effect [$F(1,36)=199.2$, $P<.001$] and a significant Sex×Ethanol interaction was observed [$F(1,36)= 10.6$, $P<.002$]. Pretreatment with ethanol increased PTZ seizure thresholds by more than 50%, from 29.4±3.5 to 46.8±1.6 mg/kg for group-housed male rats and from 30.8±0.9 to 46.4±2.3 mg/kg for individually housed male rats. PTZ seizure thresholds were also significantly elevated, by nearly 75%, in female rats; from 30.8±1.4 to 54.0±1.9 mg/kg PTZ in group-housed and from 31.3±2.2 to 54.5±3.9 mg/kg PTZ in individually housed female rats.

To test for an association between the effects of stress on seizure thresholds and hormonal indices of stress, CORT levels were analyzed from animals after 10 days of group or individual housing (Fig. 5). Housing [$F(1,29)=10.3$, $P<.003$], sex [$F(1,29)$, $P<.007$, and ethanol [$F(1,29)=150.6$, $P<.001$] all had significant main effects on CORT levels. In addition, there were significant Housing×Sex interaction [$F(1,29)=4.6$, $P<.04$], Housing×Ethanol [$F(1,29)=7.7$, $P<.01$], and Housing×Sex×Ethanol [$F(1,29)=7.3$, $P<.01$] interactions. Acute ethanol administration (2.5 g/kg) dramatically increased CORT levels from 2.3±2.2 to 54.1±11.2 μg/dl in group-housed male rats and from 0.07±0.03 to 45.5±8.7 μg/dl in individually housed male rats. Acute ethanol administration also significantly increased CORT levels, from 3.2±1.6 to 97±8.03 μg/dl, in group-housed female rats and from 1±0.4 μg/dl to 51.97±10.4 μg/dl in individually housed female rats. As shown in Fig. 5B, we repeated this experiment, with blood collected for CORT assay immediately after seizure induction; we found significant elevations in CORT levels induced by the seizures across housing conditions. Furthermore, only ethanol [$F(1,52)=11.6$, $P<.001$] had a significant effect on CORT levels after seizure induction, although the Housing×Sex interaction [$F(1,52)=8.5$, $P<.005$] also reached significance. The elevation in CORT levels induced by acute ethanol administration was lost following seizure induction in individually housed male rats. In contrast, the ethanol-induced response was exacerbated in individually housed female rats following seizure induction.

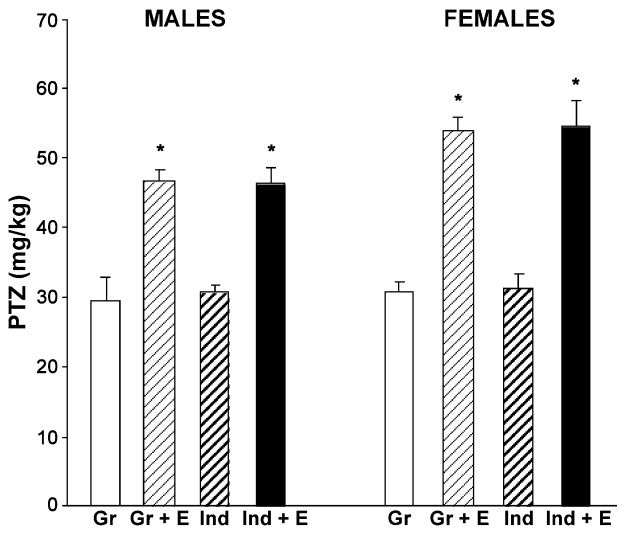


Fig. 4. When PTZ was used as the convulsant, acute ethanol (2.5 g/kg) administration elevated seizure thresholds in male and female rats regardless of housing conditions. Gr=group, Gr+E=group housed with ethanol treatment, Ind=individual, Ind+E=individual housing with ethanol treatment. *$P<.01$ compared with control housing conditions within sex ($n$=4–6 per treatment group per sex).

To further explore the effects of stress on seizure risk, additional experiments were conducted that included a single acute restraint stress applied to group- and individually housed male and female rats after 10 days of experimental housing conditions. Housing [$F(1,55)=16.8$, $P<.001$], sex [$F(1,55)=4.2$, $P<.001$], and restraint stress [$F(1,55)=6.8$, $P<.012$] all showed significant main effects on bicuculline seizure thresholds (Fig. 6). As before, we observed a main effect of sex on bicuculline seizure thresholds with female rats displaying a higher seizure threshold (reduced seizure susceptibility) across all treatment conditions compared with males. Within sex comparisons of treatments also showed that seizure thresholds were significantly reduced in individually housed animals. The addition of an acute stressor had no effect on group-housed male rats; seizure thresholds were 0.23±0.01 mg/kg without and 0.22±0.01 mg/kg bicuculline with the acute stress. However, the addition of this single acute stress further decreased seizure thresholds from 0.20±0.01 to 0.18±0.01 mg/kg bicuculline, in individually housed males. In contrast, the single restraint stress significantly reduced seizure thresholds by 20%, from 0.29±0.01 to 0.25±0.01 mg/kg for group-housed female rats, but did not alter thresholds in individually housed females (0.24±0.01 vs. 0.23±0.01 mg/kg bicuculline).

Finally, we expanded our study of the effects of a single, acute restraint stress on seizure risk to determine if PTZ-induced seizures were affected similarly to bicuculline-induced seizures (Fig. 7). In contrast to the earlier study when we did not see a major effect of sex when using PTZ as the convulsant, we did observe a main sex effect [$F(1,86)=146.4$, $P<.001$] as well as significant main effects of housing [$F(1,86)=13.0$, $P<.001$], restraint stress [$F(1,86)=5.0$, $P<.03$], and Housing×Sex interaction [$F(1,86)=6.1$, $P<.02$]. There was a 13% decrease in seizure thresholds from 23.1±0.4 to 20.2±0.5 mg PTZ with chronic, individual housing in male rats whereas

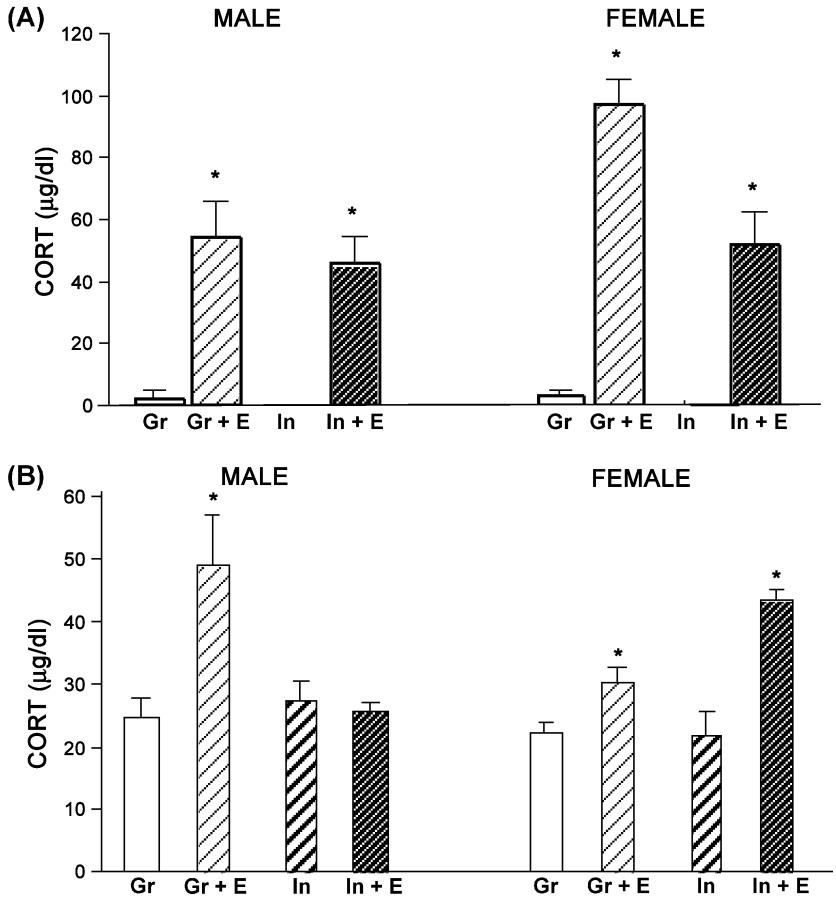


Fig. 5. Acute ethanol administration (2.5 g/kg) selectively increased CORT levels in group-housed male and female rats compared with individually housed male and female rats. Gr=group, Gr+E=group housed with ethanol treatment, In=individual, In+E=individual housing with ethanol treatment. *$P$<.05 compared with group housing conditions ($n$=4–8 per treatment per group per sex). (A) CORT levels across treatment conditions without seizure induction; (B) CORT levels after seizure threshold determinations.

there was no effect of housing in females. The addition of the acute stress only slightly reduced PTZ seizure thresholds to 21.9±0.7 mg/kg PTZ in group-housed and to 19.4±0.3 mg/kg PTZ in individually housed male rats. The acute restraint stress had no effect on group-housed female rats (26.6±0.7 without acute stress and 26.7±0.7 mg/kg PTZ with acute stress). However, individually housed female rats responded with a significant decrease in PTZ seizure thresholds from 27.5±1.2 to 25.1±0.5 mg/kg PTZ after the addition of the acute stress.

To explore possible association between sex-selective changes in seizure thresholds and cellular adaptations following chronic mild stress, we assayed [$^3$H]flunitrazepam binding to the benzodiazepine site on $GABA_A$ receptors and $GABA_A$ receptor-mediated chloride uptake in cerebral cortical tissue. Binding estimates obtained for [$^3$H]flunitrazepam were nearly identical between group- and individually housed male and female rats (Table 1). In contrast, $GABA_A$ receptor-gated chloride uptake measurements showed differential effects of housing as well as sex on the potency of GABA to enhance chloride uptake via $GABA_A$ receptors (Table 2). $EC_{50}$ values were increased by 38% in individual- compared with group-housed male rats. Similarly, $EC_{50}$ values were increased by 41% in individual- compared with group-housed female rats. Furthermore, the addition of a single brief restraint stress further elevated $EC_{50}$ values in both group-housed and individually housed male rats, whereas it reduced $EC_{50}$ values (increased the potency of GABA) by 32% in individual but not group-housed female rats.

## 4. Discussion

Findings from the present study showed that a simple paradigm involving individual rather than group housing conditions to model mild "social stress" could influence seizure risk and $GABA_A$ receptor activity in male and female rats. We found that individual housing tended to reduce seizure thresholds similarly in male and female rats, suggesting a similar response to this stress between the sexes. We also observed higher basal bicuculline seizure thresholds in female compared with male rats across all treatment conditions, even with controlling for stage of estrus (and so circulating levels of ovarian steroids). This suggests that

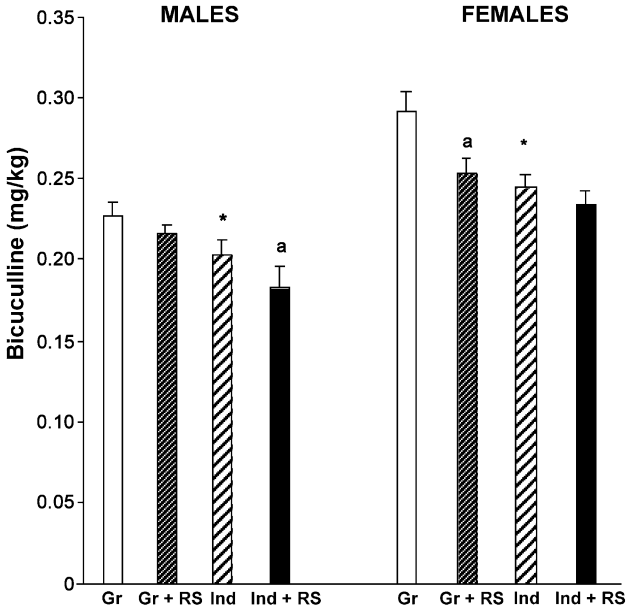


Fig. 6. The addition of an acute restraint stress significantly reduced bicuculline seizure thresholds only in group-housed female rats. Gr=group, Gr+RS=group housed with acute restrain stress, Ind=individual, Ind+RS=individual housing with acute restraint stress. All female values were significantly higher compared with male values. *$P$<.05 compared with group-housed values within sex. [a]$P$<.05 compared with nonacute stressed group ($n$=6–9 per treatment per group per sex across two independent experiments).

additional sex-selective factors, beyond circulating levels of ovarian hormones, influence seizure risk.

Of interest was the finding that the significant sex and housing differences in seizure thresholds observed with

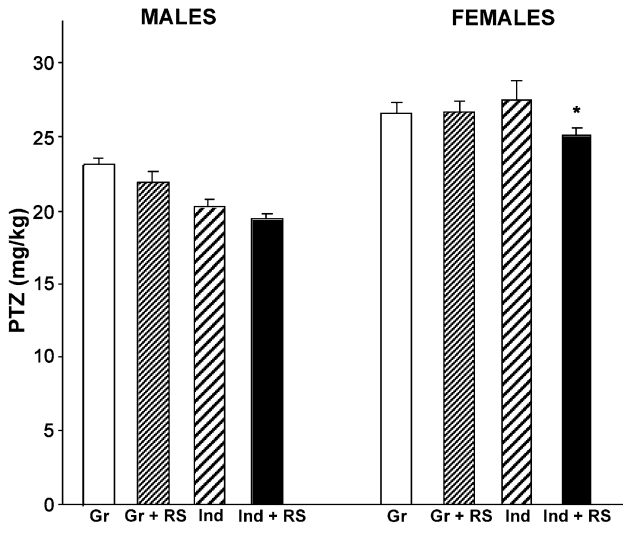


Fig. 7. The addition of an acute restraint stress did not alter PTZ seizure induction in any treatment groups except for individually housed female rats. Gr=group, Gr+RS=group housed with acute restrain stress, Ind=individual, Ind+RS=individual housing with acute restraint stress. *$P$<.05 compared with individually housed values within sex ($n$=10–13 per treatment per group per sex across two independent experiments).

Table 1
Saturation binding parameters for [$^3$H]flunitrazepam in cerebral cortex from group-housed or individually housed male and female rats

| | $K_d$ (nM) | $B_{max}$ (fmol/mg protein) |
|---|---|---|
| *Males* | | |
| Group | 1.57±0.01 | 2716±348 |
| Individual | 1.60±0.02 | 3033±117 |
| *Females* | | |
| Group | 1.58±0.06 | 3529±112 |
| Individual | 1.60±0.48 | 2590±472 |

Saturation binding estimates were determined using Prism 3 and are the average from triplicate determinations over two independent experiments.

bicuculline-induced seizures were not generally observed when PTZ was used as the convulsant. This could be because the precise mechanisms of action for seizure induction by these convulsants differ. Whereas bicuculline is a highly selective and potent $GABA_A$ receptor antagonist, PTZ can alter membrane excitability by effecting potassium, calcium, and sodium currents, in addition to blocking the chloride channel of $GABA_A$ receptors (Klee et al., 1973; Mashimo and Sekiya, 1981). This very interesting divergence in response between bicuculline and PTZ may be because seizure induction can involve different sequelae of seizure initiation and expression, depending on the trigger. Initiation, generalization, and overt expression of seizures require recruitment of several sites in brain, with initiation primarily targeted at forebrain sites and generalization involving spread to the contralateral hemisphere and hindbrain sites (MacDonald and Greenfield, 1997; McNamara, 1994). Studies in animal models have shown that GABAergic neurotransmission is intimately involved in the genesis of seizures (Gale, 1992). Therefore, stress-induced alterations in seizure threshold likely involve modulation of

Table 2
$GABA_A$ receptor-gated chloride uptake in group- and individually housed male and female rat cortical synaptoneurosomes

| | $EC_{50}$ (nM) | $EC_{max}$ (nmol/mg protein) |
|---|---|---|
| *Males* | | |
| Group | 32.8±2 | 49.8±1.7 |
| Group+RS | 23.2±3.2[a] | 34±2.1[a] |
| Individual | 45.4±2.4* | 46.3±1.6 |
| Individual+RS | 29.5±1.2 | 34.8±1.8[a] |
| *Females* | | |
| Group | 23.9±3.8[b] | 45.4±3.3 |
| Group+RS | 43.2±3.0[a,b] | 39±1.7 |
| Individual | 33.9±3*[,b] | 47.5±2.5 |
| Individual+RS | 29.3±2.4* | 35±1.6[a] |

Data presented are the mean±S.E.M. generated by Prism 3 from determinations run in triplicate across a dose response curve for GABA from 0 to 600 μM. RS=with acute restraint stress.

[a] $P$<.05 compared with without acute stress values.

[b] $P$<.05 compared with male values.

* $P$<.05 compared with group-housed values.

$GABA_A$ receptors. In support of this, we found that individual housing decreased the potency of GABA to enhance chloride flux in both male and female rats.

Sex differences in basal seizure susceptibility and variability across stage of estrus have been reported previously (Finn and Gee, 1994; Kokka et al., 1992; Wilson, 1992). A recent study also reported that stage of estrus effected responses to individual housing differentially in female mice (Palanza et al., 2001). However, we controlled for stage of estrus throughout the course of these experiments, including designing testing such that all treatment conditions were tested on the same day. During both estrus and diestrus I, progesterone and estradiol levels are low, which should reduce any direct effect of ovarian steroids on seizure risk. We did observe some variability in basal seizure thresholds and in the extent of sex differences across the series of experiments. This shows that although we controlled for stage of estrus, additional factors can affect seizure risk and either enhance or reduce the sex differences generally observed. Basal $EC_{50}$ values for $GABA_A$ receptor-gated chloride uptake were consistently lower (GABA was more potent) in females than males, regardless of housing condition, suggesting the possibility of basal differences in activity of $GABA_A$ receptors between male and female rats. We also found that GABA was more potent in stimulating chloride uptake through $GABA_A$ receptors in a previous study (Devaud et al., 2003).

Additional sex differences in response to mild chronic stress were found following exposure to a brief restraint stress 2 h prior to seizure threshold determinations. Bicuculline seizure thresholds were decreased in individually housed male rats more than in group-housed male rats. Conversely, bicuculline seizure thresholds were significantly decreased in group-housed female rats compared with individually housed female rats following the acute restraint stress. These data extend previous findings reporting sex differences in response to stress by several other behavioral approaches (Blanchard et al., 2001; Palanza et al., 2001; Wilson and Biscardi, 1994), with female generally showing enhanced behavioral and CORT responses compared with males. For example, group-housed female mice showed increased exploratory behavior compared with individually housed female mice whereas individually housed male mice exhibited an increased exploratory behavior compared with group-housed male mice (Palanza et al., 2001). Our data extend these findings to suggest that sensitivity to seizure induction by bicuculline is differentially altered between male and female rats following exposure to varying stressors and that stress activates $GABA_A$ receptors in a manner that differs between males and females. Therefore, complex interactions between sex and stress may influence brain activity at the level of $GABA_A$ receptors. This implies that basic brain excitability may be differentially impacted by stress in males and females.

We also explored the effects of a chronic mild stressor on seizure thresholds in male and female rats on responses to acute administration of two anticonvulsants, diazepam and ethanol. Diazepam was a potent anticonvulsant in our hands, but displayed minimal differences in its actions regardless of sex or housing conditions. The anticonvulsant effects of ethanol depended on the convulsant used, with little effect against bicuculline-induced seizures and prominent actions against PTZ-induced seizures. Furthermore, female rats displayed a greater response to ethanol than males, regardless of housing conditions. Several previous reports have also found important, sex-selective interactions between PTZ and ethanol. When using PTZ as a discriminative stimulus, males were found to be more sensitive to the stimulus than females during ethanol withdrawal (Jung et al., 1999; 2000; 2002). Female rats showed an increased sensitivity to diazepam during ethanol withdrawal (Jung et al., 1999), additional evidence for sex-selective responses to stress involving $GABA_A$ receptors. Our data also suggest complex and, possibly, sex-selective actions of ethanol that require further evaluation.

While ethanol can display anticonvulsant effects, it also acts as a stressor, causing significant increases in CORT levels with acute administration. For example, the effects of ethanol on CORT levels were greater and more persistent in female than male rats (Rivier, 1993). Our assessment of CORT levels following the chronic mild stress of individual housing showed that basal CORT levels were minimal in both males and females, regardless of housing condition, suggesting habituation to housing conditions. Acute ethanol administration caused dramatic increases in CORT levels in both male and female rats. Group-housed females seemed to be most vulnerable to the stressor effect of ethanol, with levels increasing more than 50-fold. When ethanol was administered prior to seizure induction and CORT levels were assayed from blood collected just after seizure determinations, the anticonvulsant activity of ethanol was much greater than its stressor effect, with ethanol-induced significant increases in PTZ seizure thresholds in both male and female rats. At this time, we found a different pattern of sex differences in CORT levels. These data showed that the interaction between the anticonvulsant and stress effect of ethanol as well as the stress of seizure activity resulted in sex-selective outcomes. Furthermore, these data suggested a disconnect between effects of stress on brain excitability and alterations in CORT levels. This is a particularly interesting observation, as a number of investigators have found that, in general, CORT administration increases brain excitability (seizure risk). For example CORT acted as a proconvulsant, significantly increasing severity of seizures in mice undergoing withdrawal from ethanol, pentobarbital, or diazepam (Roberts et al., 1994) or exacerbated kainic acid-induced seizure activity (Talmi et al., 1995). However, another report found that while CORT administration increased anxiety (a consistent finding), it did not alter PTZ-induced convulsions (Andreatini and Leite, 1994).

Assessing the direct effects of specific steroids on neuronal activity is difficult because many steroids are rapidly metabolized to various steroid intermediates, such as neuroactive steroids, that have direct effects on neurons, primarily via actions at $GABA_A$ receptors (see Paul and Purdy, 1992; Mellon, 1994; Stone, 1996, for reviews). For example, progesterone is converted to pregnanolone or allopregnanolone, which are positive modulators of $GABA_A$ receptors and have been shown to possess anxiolytic and anticonvulsant activity (Bitran et al., 1991; Finn and Gee, 1994). Furthermore, CORT is converted to 3α21-dihydroxy-5α-pregnan-20-one, another potent positive modulator of $GABA_A$ receptors with anxiolytic and anticonvulsant activity (Stone, 1996; Devaud et al., 1996). Several reports showed that stress elevates brain and plasma levels of these GABAergic neuroactive steroids (Barbaccia et al., 1996; Reddy and Rogawski, 2002). Effects of CORT on brain excitability may be counterbalanced by actions of neuroactive steroids (derived from CORT or as a result of stress). Moreover, as females have higher circulating levels of progesterone (and neuroactive progesterone derivatives), this could confer inherent differences in susceptibility and responses to stress and/or seizure risk due to the differing hormonal milieu.

Interactions among sex, stress, and seizure risk could also be influenced by additional external factors that many not be only difficult to control but may not even be recognized. One environmental factor, handling, can be perceived as a mild stressor (Weinberg et al., 1978). All the animals in our studies were handled daily to allow them to habituate to this procedure, leading us to assume that handling for testing was not stressful. The low CORT levels we detected across housing conditions verified this assumption. A report published several years ago found seasonal variations in severity of PTZ-induced seizures in male mice, even with stringent control of environmental conditions (Loscher and Fiedler, 1996). Therefore, additional factors could have a role in some of the sex and stress differences we found in this series of experiments.

We did not observe sex or stress differences in cortical benzodiazepine binding to $GABA_A$ receptors, in contrast to a previous report studying the effects of a more severe, acute stressor. Swim stress was found to differentially affect binding parameters in whole brain from male compared with female mice (Akinci and Johnson, 1993). Several reasons for this apparent discrepancy could include species and tissue differences, with rat cerebral cortical homogenates used in our studies. We also employed a different stressor paradigm and it may be that our stress model was not severe enough to result in persistent, significant changes in the density or affinity of $GABA_A$ receptors. Our study conditions involved a chronic stress, rather than simply acute challenge. We did find a higher basal sensitivity to activation of $GABA_A$ receptors in female compared with male rats. Also, we observed a sex-selective effect of acute restraint stress on $GABA_A$ receptor function. The addition of a brief restraint stress increased the potency of GABA in both group- and individually housed male rats and individually housed female rats. However, it significantly decreased the potency of GABA to stimulate chloride uptake only in group-housed female rats. These findings showed that sex-selective changes in function of $GABA_A$ receptors could be observed even without concomitant changes in receptor number or affinity.

It has been well established that pharmacological properties of $GABA_A$ receptors are influenced by subunit composition (see Rudolph et al., 2001, for a review). Therefore, if chronic stress results in expression of $GABA_A$ receptors having a different subunit assembly than in the unstressed state, this could result in the changes in the potency of GABA to enhance $GABA_A$ receptor-mediated chloride uptake, and, ultimately, susceptibility to seizures. In our previous investigations of the effects of chronic ethanol exposure, we found selective alterations in expression of several $GABA_A$ receptor subunits (Devaud et al., 1995; 1998) and that there were significant sex differences in ethanol-induced adaptations at the whole animal as well as receptor level (Devaud et al., 1996; 1999; 2000; 2002; 2003; Devaud and Chadda, 2001). Our present findings further support the suggestion that stress-induced alterations in $GABA_A$ receptor function can influence the risk for seizures in a sex-selective manner.

Taken together, this study of social stress in an animal model uncovered important sex differences in behaviors, $GABA_A$ receptor function, and hormonal responses to acute stress. If these findings extrapolate to humans, they suggest that stressors, both social and nonsocial, may differentially influence the risk for seizures in men and women. Continuing studies will further elucidate basic mechanisms underlying stress to better delineate the importance of sex and stress interactions in increasing risk for a number of diseases, including epilepsy, mental disorders, and cardiovascular disease.

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