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A comparison of female and male rats' ETOH-induced ataxia and exploration following restraint or swim stress

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Abstract

Animal models of stress reactivity are often employed in developing treatments for humans. Many studies use shock stress, and most use male rats. These experiments compare female and male rats exposed to either restraint stress (RS) or ambient-temperature swim stress (SS), using two durations of each stressor and naive controls. The ataxic effects of a 0.6 g/kg ip dose of ethanol (ETOH) were measured. Females exhibited less ataxia than males following ETOH administration. There were no significant effects of stress on ETOH-induced ataxia. Exploration was also measured in an open-field test (OFT) both pre- and poststress. In the prestress OFT, females were more active than males. For the no-stress groups and the shorter-duration stress groups, exploration decreased between the first and second OFTs. However, the groups exposed to the longer-duration stress did not show this expected decrease in exploration. A key finding of this research is that while sex differences may be present at baseline, the sexes may react similarly to stress. These data extend knowledge on sex differences in stress, alcohol reactivity and exploratory behavior.

Keywords: Sex differences; Restraint; Forced swim; Exploration; Alcohol; Rat; Activity; Stress response; Behavior

1. Introduction

Animal models of stress reactivity are often employed in basic research intended to increase understanding of the physiological and behavioral effects of stress in humans. Stress has been linked to both depression and anxiety disorders (Anisman and Zacharko, 1982; Leonard and Song, 1996), and animal models of stress have been used to investigate the neurochemical mechanisms of these disorders (File, 1996). Many epidemiological studies have reported significant sex differences in the incidence of mood disorders (Culbertson, 1997; Klerman and Weissman, 1989; Nolen-Hoeksema, 1990). Despite this, until recently, the majority of animal models of the stress response were developed using male organisms.

Studies using animal models have shown both behavioral and biochemical differences in the stress response. Evidence for sex differences in stress-related behavior includes the finding that females have a shorter latency to escape in the shuttlebox task following uncontrollable stress than males (Steenbergen et al., 1990), and that female rats drink less than males in the punished phase of the thirsty-lick conflict task (Johnston and File, 1991). Stress-induced neurochemical sex differences have been shown, with females producing higher concentrations of corticosterone in response to both restraint stress (Livezey et al., 1985) and cold-water swim stress (Wilson and Biscardi, 1994) than males. Sex differences also exist in adrenocorticotropic hormone secretion (ACTH; Kitay, 1961) and catecholamine release (Livezey et al., 1985; Weinstock et al., 1998). Certain neurotransmitter systems show sex differences both constitutively and following stressful situations, such as gamma aminobutyric acid (GABA) (Wilson, 1992), which has also been implicated in mood disorders (Breier and Paul, 1990; Petty, 1995).

We are interested in the functional significance of these differences between males and females. For the purposes of the present study, functional relevance is defined as changes in reactivity to drugs administered poststress, as well as behavioral reactivity to stress. The central question in this research is whether the effects of restraint stress and swim stress on females and males are similar when measured through ethanol (ETOH) reactivity and exploration behavior.

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Research comparing different stressors has been conducted (i.e. Heinrichs et al., 1994; Martijena et al., 1997; Odio and Maickel, 1985), and this work suggests that the different types of stress may have different effects. In addition, the effect of duration and other parameters of stress have been found to be significant (Bodnar, 1990). Behavioral and biochemical responses may differ according to the type of stressor applied (Armario et al., 1991; Heinrichs et al., 1994). Finally, the time course of the stress response has been found to depend on the type of stress used (Odio and Maickel, 1985). The relationship between duration of stress exposure and the behavioral effects of stress may not be linear. When plus-maze behavior is assessed following 15 min of either forced ambient-temperature swim stress or 15 min of restraint, the restrained rats are more likely to avoid the open arms than the nonstressed controls, while swim-stressed animals resemble controls (Martijena et al., 1997). Yet, when 2 min of forced swim are compared to 60 min of restraint in the same behavioral measure, responses to the two stressors are not different (Heinrichs et al., 1994). This raises the question of whether or not the effects of duration of stress exposure on behavior are linear. Does shortening swim time and lengthening restraint time produce changes that make the behavioral responses to the two different stressors similar? In response to this question, the present study protocols include a 15-min exposure to either swim or restraint stress, compared to a 75-min exposure to restraint or a 5-min exposure to swim.

In the present paper, we evaluate both pharmacological and behavioral responsivity to restraint stress and swim stress, using two durations of each stressor. We measure the effects of restraint stress and swim stress on ETOH-induced ataxia in both females and males. We also examine both pre- and poststress open field behavior to determine how sex differences in baseline responses may influence stress-induced responsivity.

2. General method

2.1. Subjects

Male and female Sprague–Dawley rats obtained from Charles River Laboratories (Stoneridge, NY) were used. Animals weighed between 181 and 200 g on arrival and were allowed to acclimate to laboratory conditions for at least 7 days prior to testing. Animals were housed by sex, with two to four animals per cage in polyethylene tub cages and maintained on a 12-h light/dark cycle (lights on 06:00–18:00 h). All experiments were performed between 06:30 and 14:00 h. Free access to standard lab chow and water was provided at all times except during testing. Nonstress controls were held in the vivarium during stress exposure and were deprived of food and water for intervals identical to those of the stress-exposed animals.

2.2. Apparatus

Restraint stress was administered using clear Plexiglas semicylinders (16.51 cm long × 7.62 cm wide × 5.398 cm high) attached to flat bases. The tubes restricted gross motor movement, without inhibiting breathing. As an added precaution against the animals shifting position, tails were taped down to the bases using cloth athletic tape. Swim stress was administered using clear Plexiglas cylinders 20.32 cm in diameter × 45.08 cm high filled to a depth of 29 cm with ambient-temperature water (24 ± 2 °C).

ETOH reactivity was measured as motor ataxia using a rotarod (RR) treadmill (model no. 7700, Ugo Basile Biological Research Apparatus, 21025 Comerio, Varese, Italy). This apparatus is a horizontal cylinder 6 cm in diameter, 35 cm long, divided into four equal sections. It operates in a manner similar to a treadmill, connected to a motor that rotates the rod at 10 rpm. Exploration was measured in an open-field test (OFT). The field was square, 121.92 cm^2, with sides 30.48 cm high, constructed of plywood painted black.

2.3. Procedure

Each experiment employed both female and male subjects assigned randomly to one of three groups: long-duration stress, short-duration stress or handled control, resulting in six groups, three of each sex. Males and females were run on alternate days, and all equipment was washed with a 25% solution of 95% ETOH and allowed to dry between runs.

Fig. 1 shows the general experimental procedure for both Experiments 1 and 2. In the morning, animals were weighed, assigned randomly to groups and housed individually in holding cages. Following weighing, the first OFT was performed with all animals. All trials were videotaped. Activity was coded from videotapes using an overlay grid dividing the field into 36 equal squares of 24.38 cm^2. Activity was defined as the number of squares entered; entry into a square was counted when the animal's front paws and shoulders crossed a line into a new square. Activity counts were obtained for each 1-min period of the 10-min exposure. This coding was performed by experimenters blind to group membership, Pearson's r for inter-observer reliability was $> .99$.

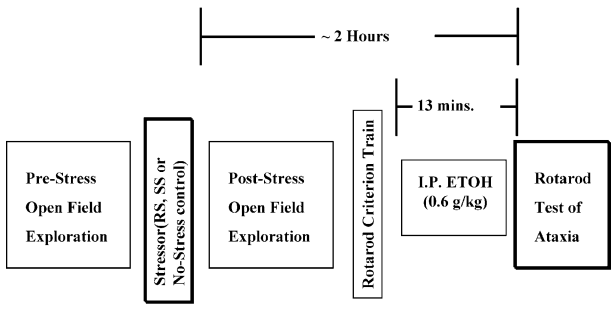

Fig. 1. General behavioral protocol for examining the impact of sex and stress (RS = restraint stress; SS = swim stress) on alcohol-induced motor ataxia and open-field exploration.

The animals assigned to the stress groups were then exposed to the stressor, and the no-stress control subjects were returned to the vivarium. At this time, an assistant coded the animals and all further tests were performed with the investigator blind to group membership. Following this coding, the poststress OFT provided a second measure of exploration for all six groups. Immediately following this poststress OFT, the protocol for testing stress-related changes in alcohol-induced incoordination using the RR treadmill commenced. This device is commonly used in studies of drug-induced ataxia (e.g. Dar, 1990; Miller et al., 1987), and the protocol used was identical to that of Drugan et al. (1996). Prior to intraperitoneal (ip) administration of alcohol, all animals were tested to a criterion of 2 min of continuous running on the RR. Using this criterion after stress exposure and before drug administration established that any deficits in performance found following drug absorption were due to the effects of the drug, not due to other factors such as fatigue. The 2-min criterion is relatively simple for the animals to reach, with more than 98% of subjects meeting criterion, as in prior studies (Drugan et al., 1996).

Approximately 2 h after the termination of stress exposure, the animals were injected with 0.6 g/kg ip of 95% ETOH, in a 20% vol/vol solution of distilled water. The 2-h poststress time point was chosen based on earlier work showing that bicuculline-induced seizure susceptibility (Drugan et al., 1985), benzodiazepine-induced ataxia (Austin et al., 1999) and ETOH-induced ataxia (Brown et al., 2001) are altered 2 h after stress. The 0.6 g/kg dose of ETOH in males is effective for discriminating between animals naive to stress and those exposed to uncontrollable tailshock, while higher doses impair both nonstressed and stressed rats (Drugan et al., 1996). Following an absorption period in a holding cage (mean time = 13 min), animals were tested for the amount of time that they remained on the rod. At most, three successive trials were conducted. Maximum trial length was 300 s. If the animal remained on the RR for at least 180 s on the first trial and the full 300 s on the second, no further trial was conducted. These cutoff times were established to allow for testing of all subjects at or near the 2-h poststress time point. Rotarod balance score (RRB) was computed as the average time of the two or three trials.

3. Experiment 1. The effects of 0, 15 or 75 min of restraint stress on exploration behavior and reactivity to alcohol in male and female rats

Restraint stress has been shown to affect rats both biochemically and behaviorally, and many of these effects have been shown to vary between females and males (Albonetti and Farabollini, 1992, 1995; Farabollini et al., 1993). In this experiment, we compared female and male rats exposed to long-duration (75 min) and short-duration (15 min) restraint stress with handled controls, resulting in six different groups.

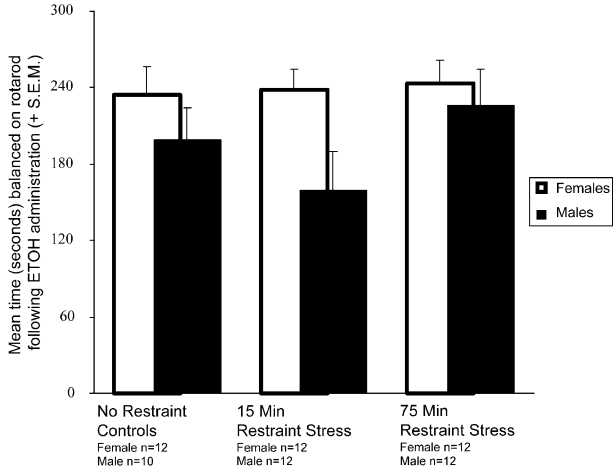

Fig. 2. Mean (+S.E.M.) RRBs for males and females exposed to restraint stress or no stress.

3.1. Results

The effects of sex and exposure to restraint stress on motor incoordination due to alcohol were assessed by a two-factor ANOVA with sex (female, male) and level of stress (0, 15 or 75 min) as the factors. This analysis revealed that the sexes differed in ETOH-induced motor incoordination, with females remaining on the RR longer overall [$F(1,64) = 5.04$, $P < .05$]. There was no effect of restraint stress ($P > .05$), nor was there an interaction of sex by restraint stress ($P > .05$). Results for the six groups are presented in Fig. 2.

The number of squares entered in the OFT for each of the six groups during each minute of both 10-min exposures is presented in Fig. 3. Females were more active than males in the OFT prior to restraint stress [$F(1,68) = 7.422$, $P < .01$].

The existence of this sex difference prior to any exposure to stress necessitated the use of difference scores (poststress minus prestress activity) as a way to control for baseline sexual dimorphism. The difference scores for exploration are presented in Fig. 4. Sexual dimorphism is not evident in the difference scores ($P > .05$). However, the length of exposure to restraint stress (0, 15 or 75 min) did affect the change in exploration behavior [$F(2,69) = 12.733$, $P < .001$], with the longer-duration stress groups exploring nearly as much or more in the second OFT exposure as in the first. Seventy-five minutes of restraint stress reduced the difference between pre- and poststress activity in the OFT as compared with both the 0-min restraint stress group ($P < .001$) and the 15-min restraint stress groups ($P < .001$), when collapsed across sex.

4. Experiment 2. The effects of 0, 5 or 15 min of ambient-temperature swim stress on exploration behavior and reactivity to alcohol in both male and female rats

The effects of swim stress in rats have been well investigated. There is a limited amount of research available

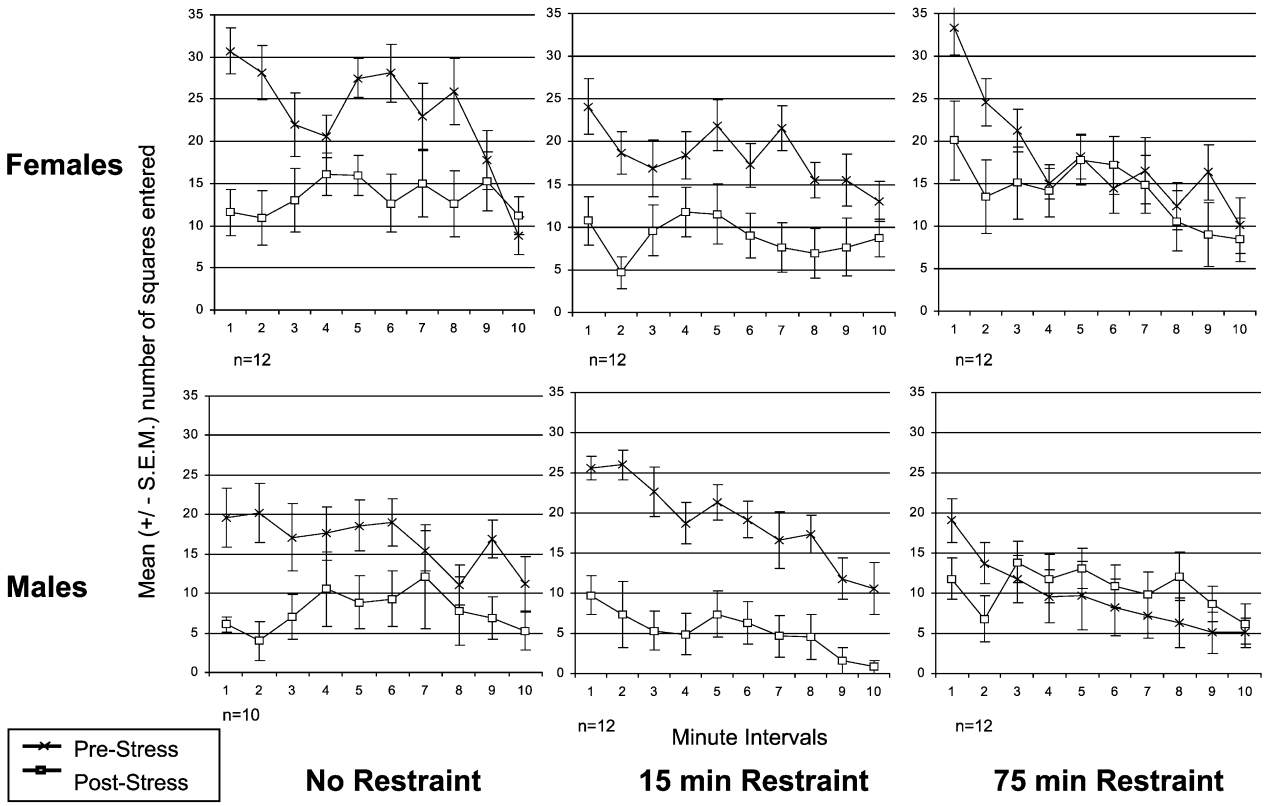

Fig. 3. Mean (±S.E.M.) number of squares entered for each group of restraint-stressed or nonstressed controls for each of the 10 min of the two open-field exposures.

on sex differences in response to this stressor (Akinci and Johnston, 1993; Alonso et al., 1991), though evidence for a greater corticosterone response and a greater increase in benzodiazepine receptor numbers due to swim stress in females as compared to males has been presented (Wilson and Biscardi, 1994). It has also been shown that the [^{3}H]GABA binding properties of the mouse forebrain are affected by swim stress, that this effect varies by sex and that the sex difference may be attributable to endogenous modulators of the GABA receptor (Akinci and Johnston, 1993). This experiment examined the behavioral reactivity of males and females to 5 and 15 min of exposure to this stressor, comparing them to handled controls.

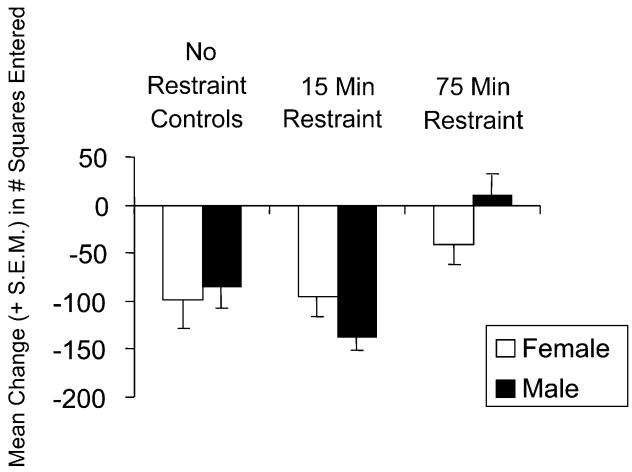

Fig. 4. Mean (+S.E.M.) difference scores in open field exploration (poststress minus prestress) for restraint-stressed and nonstressed control groups. Negative difference scores indicate decreased exploration between the prestress and poststress exposures.

4.1. Results

Similar to the results of Experiment 1, female rats exhibited less motor incoordination than males following ETOH exposure [$F(1,65) = 9.23$, $P < .01$]. There was no significant effect of swim stress level ($P > .05$), nor was there an interaction of sex by stress level in motor incoordination ($P > .05$). Results for the six groups are presented in Fig. 5.

Consistent with the main effect of sex mentioned above, females had higher balance scores than males, irrespective of stress exposure. Although the data from the nonstress control group in this experiment appears to be different from that of Experiment 1, the difference in means did not approach significance, but it reflects the variability to be expected in this measure. The use of concurrent control groups for all experiments assured that environmental conditions did not produce systematic variability.

The number of squares entered in the open field for each of the six groups during each minute of the two 10-min

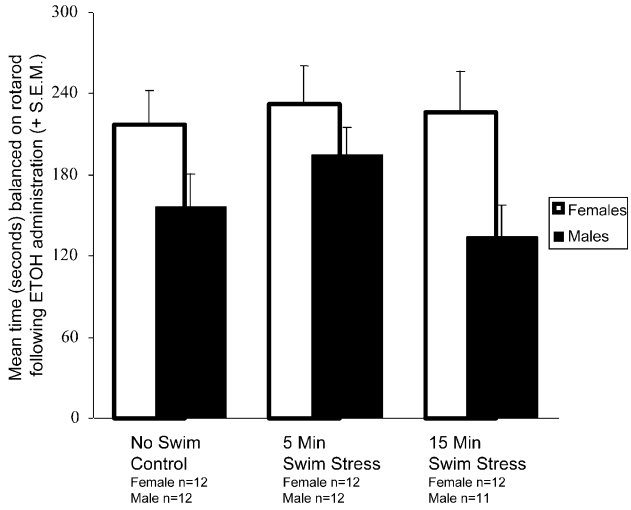

Fig. 5. Mean (+S.E.M.) RRBs for males and females exposed to swim stress or no stress.

exposures is presented in Fig. 6. As in Experiment 1, greater activity in the open field by females was established by comparing the total number of squares entered in the first 10-min exposure by one-way ANOVA with sex (female, male) as the factor [$F(1,70) = 12.619$, $P < .01$].

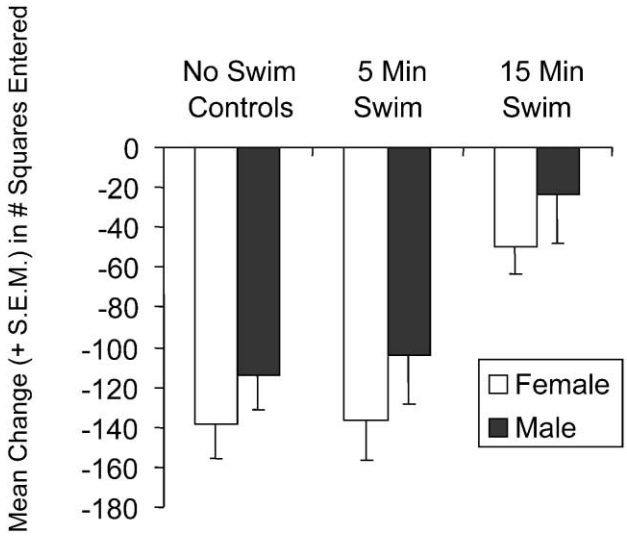

Fig. 7. Mean (+S.E.M.) difference scores in open field exploration (poststress minus prestress) for swim-stressed and nonstressed control groups. Negative difference scores indicate decreased exploration between the prestress and poststress exposures.

The existence of this sex difference prior to any exposure to stress again necessitated the use of difference scores

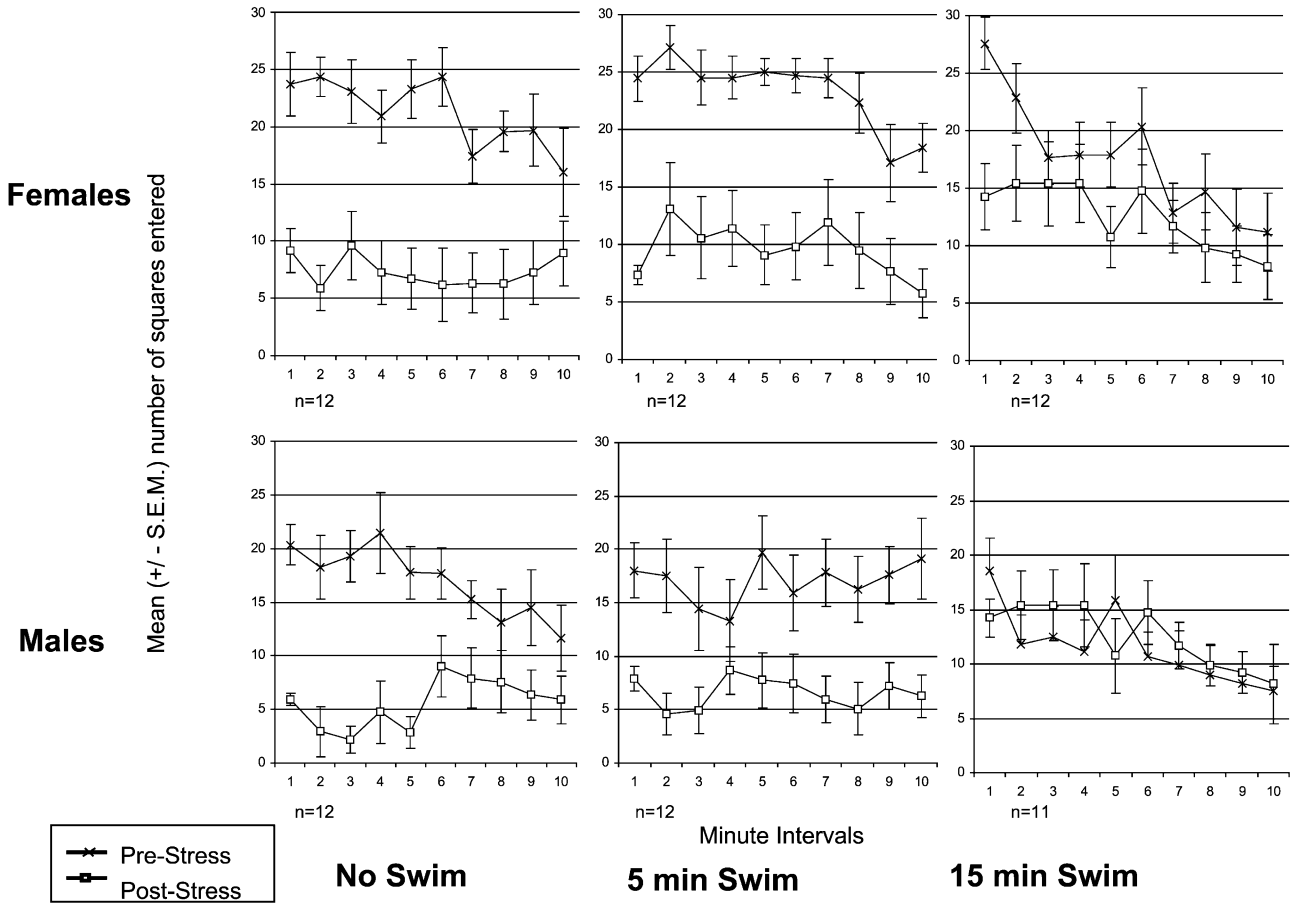

Fig. 6. Mean (±S.E.M.) number of squares entered for each group of swim-stressed or nonstressed controls for each of the 10 min of the two open-field exposures.

(poststress minus prestress activity) as a way to control for baseline sexual dimorphism. The difference scores for exploration are presented in Fig. 7. Sexual dimorphism is not evident in the difference scores ($P > .05$). However, the length of exposure to swim stress (0, 5 or 15 min) did affect the change in exploration behavior [$F(2, 66) = 10.732$, $P < .001$], with the subjects exposed to the longer duration of stress again exploring nearly as much in the poststress OFT as in the prestress exploration test. Fifteen minutes of swim stress reduced the difference between pre- and post-stress activity in the OFT as compared with both the 0-min swim stress group ($P < .05$) and the 5-min swim stress groups ($P < .001$), when collapsed across sex.

5. General discussion

These studies examined the effects of restraint stress and ambient-temperature swim stress in rats, with a focus on ETOH reactivity and exploration as behavioral endpoints. The broad goals of this research were to examine whether or not laboratory stressors commonly used in models of stress result in comparable behavioral effects, and whether female and male rats are different in their baseline behaviors and/or in their reactions to stress. The findings presented here are consistent with previous work, in that they show sex differences at baseline in all measures, though they extend other work by suggesting that there may be similarity between the sexes in stress reactivity, once baseline differences are taken into account.

The behavioral indices used in this research, exploratory behavior (Haleem et al., 1988; Meng and Drugan, 1993) and ETOH-induced ataxia at a dose of 0.6 g/kg (Austin et al., 1999; Drugan et al., 1996), are influenced by uncontrollable stress in male rats. However, neither restraint stress nor swim stress influenced alcohol-induced ataxia in the present study. In contrast, restraint and swim stress influenced exploratory behavior in the OFT. The failure to detect any difference in ataxia due to stress in the present research suggests that exposure to uncontrollable restraint or swim stress for the durations used in the present study may be fundamentally different from other stressors, including uncontrollable intermittent tailshock stress (Drugan et al., 1989).

The suggestion that the type of stress may be relevant to the behavioral effects of stress is consistent with previous results in which varying the duration or pattern of stress exposure can produce divergent effects (Bodnar, 1990; Bodnar and Sikorszky 1983; Terman et al., 1984, 1986). More recently, this has been well described for restraint stress (Glavin et al., 1994). The results presented here, as well as in previous works, showing that parametric alterations of a stressor modify subsequent behavioral and physiological responses (Bodnar, 1990; Terman et al., 1984), highlight the continuing need for studies comparing different stressors and durations of exposure. For instance, the ETOH dose employed here was selected based on prior research demonstrating 0.6 g/kg to be the optimal dose for selectively impairing male rats exposed to uncontrollable tailshock stress, but not those naive to stress or exposed to controllable stress (Drugan et al., 1996). Specifically, the rats were exposed to either no stress, uncontrollable tailshock or controllable tailshock using a triadic design and then were administered a 0.6 g/kg dose of ETOH. It was anticipated that the present work would extend this finding beyond shock stress, identifying a stressor and dose of ETOH that would create deficits analogous to those caused by uncontrollable tailshock stress. However, alcohol did not affect stressed animals any differently than nonstressed animals in the present study. Further research might attempt to identify the minimally effective dose of ETOH to observe these differences in females and males.

While we did not demonstrate any effects of stress on ETOH reactivity, a clear sex difference was observed. Males were found to be significantly different from females in the present experiment, with females exhibiting less motor incoordination in response to ETOH. These findings demonstrate that there are fundamental sex differences in this task, independent of stress. One possible explanation is that males and females differ in body weight, and that the task is therefore easier for females, independent of the effects of ETOH. Subsequent unpublished investigations (Crompton et al., 1999) using vehicle injections in both males and females exposed to 10 min of ambient-temperature swim stress and nonstressed controls confirm that this is not the case. Table 1 shows these data illustrating that nonstressed males and females injected with vehicle do not differ in the amount of time they remain on the RR.

Further, although the pharmacokinetics of alcohol distribution and its metabolism and elimination have long been known to differ between males and females, recent research examining alcohol levels in the brain has found that blood alcohol levels do not accurately represent alcohol levels in the brain. Despite some differences between male and female rats in blood alcohol concentrations during the 2 h following a 0.8 g/kg ip injection of alcohol, ETOH concentrations in the brain perfusate were not sexually dimorphic (Crippens et al., 1999). Therefore, in the time course employed here, brain concentrations of ETOH may be assumed to have been similar between males and females.

Table 1
Effects of sex and stress on RR performance following vehicle injection

Sex and treatment	*n*	Mean RRB	Standard error of the mean	t(df)	*P*
Male no-stress	9	242.37	27.15	$t(16) = -0.079$	n.s.
Female no-stress	9	245.18	22.83		
Male swim stress	9	218.74	33.66	$t(16) = -0.151$	n.s.
Female swim stress	9	225.28	27.27		

Mean RRBs, standard errors and sex comparisons for vehicle-injected males and females, both no-stress control and 15-min ambient-temperature swim stress.

The observed sex differences may instead reflect interactions of alcohol with neurotransmitter systems. In addition to its nonspecific effects on neural membranes (Beauge et al., 1984; Goldstein, 1987), this drug has been shown to have effects at the serotonin and NMDA/glutamate receptors (Grant, 1994). The GABA–benzodiazepine receptor complex is also a mechanism in the ataxic effects of ETOH, as demonstrated by the ability of the partial inverse benzodiazepine agonist RO15-4513 to reverse ETOH-induced ataxia (Suzdak et al., 1986; Dar, 1992). Given demonstrated sex differences in the actions of benzodiazepines (Fernandez-Guasti and Picazo, 1990), the possible role of GABA in mood disorders (Petty, 1995) and the previously discussed role of stress in the sex-dependent incidence of these disorders, in addition to the tendency of humans to self-medicate with alcohol, it would seem worthwhile to continue to investigate sex differences in the ataxic effects of alcohol and stress responsivity as part of the continuing effort to understand the effects of stress.

The results of the OFT confirm that the stress protocols employed here produced significant behavioral change. Animals exposed to the longest durations of stress (75 min of restraint or 15 min of swim) behaved similarly in the pre- and poststress open field exposures, while the handled controls and the animals exposed to the shorter-duration stress showed a marked decrease in exploration between the first and second exposures. The decrease in exploration shown by the control and short-duration stress groups may be interpreted as habituation based on familiarity with the field. In this case, the fact that the longer-duration stressed animals failed to show the characteristic decrease might suggest that these groups are failing to habituate.

An inability to habituate following restraint/swim stress is particularly striking in light of other research showing that the exploration behavior of male rats decreases with exposure to both external aversive stimuli and anxiogenic compounds (Meng and Drugan, 1993). Given this pattern, we would expect to see at least the male rats showing reduced exploration following the stress exposure, a result strikingly absent in the case of the longer-duration stressed animals. Both males and females in the longer-duration stress condition showed no evidence of habituation as evidenced by reduced exploration behavior. One possible explanation for this is that the longer-duration stress might have induced some change in biochemical state, and that in this state, the OFT was again novel to the animals even on the second exposure.

Isolating these stress effects on habituation in males and females required a statistical control for the baseline sex difference by using difference scores in the statistical analysis. This observation may be the most provocative contribution of the present study. When comparing males and females, many sex differences have been found, including the observation that females tend to be more active than males in the open field (Archer, 1975; Johnston and File, 1991). By controlling for this sex difference, the fact that males and females are similar in their reactions to stress emerges.

Taken together, the findings from these two experiments suggest experimental design considerations for future research. With the recent surge in emphasis on investigation of females as well as males, researchers must be sensitive to the fact that baseline sex differences may obscure similarities in the way that animals respond to stress. Many studies use the method of comparing stressed animals to nonstressed controls to identify the effects of stress. This between-groups design can be a powerful way to obtain information regarding anatomical or biochemical changes due to stress. However, by controlling for individual differences, the within-groups design may have greater statistical power and can be a useful tool for detecting relatively subtle changes. Measuring open-field behavior both before and after stress, as well as using nonstressed control groups, for example, enabled us to measure changes in individual behavior due to stress exposure in addition to between-groups differences. Given the amount of variability found in behavioral data regarding stress reactivity, and the degree of overlap between the male and female groups, the within-subjects design was especially powerful. Analyzing the results in terms of difference scores revealed the similarity between sexes by removing the sex difference at baseline while simultaneously controlling for individual differences at baseline. This suggests that investigation of the effects of stress on males and females might benefit from the use of prestress measures as a way to isolate the effects of sex on behavior.

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