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Sex Differences in Effects of Opioid Blockade on Stress-Induced Freezing Behavior

LAURA COUSINO KLEIN,* E. JON POPKE,† AND NEIL E. GRUNBERG‡

*Department of Psychology, University of California–Los Angeles, 5586 Franz Hall, Box 951563, Los Angeles, CA 90095-1563
†Division of Neurotoxicology, National Center for Toxicological Research, 3900 NCTR Road, Jefferson, AR 72079-9502
‡Department of Medical and Clinical Psychology, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, MD 20814-4799

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KLEIN, L. C., E. J. POPKE AND N. E. GRUNBERG. *Sex differences in effects of opioid blockade on stress-induced freezing behavior.* PHARMACOL BIOCHEM BEHAV **61**(4) 413–417, 1998.—The present experiment examined the effects of naloxone on freezing behavior in male and female rats following stress and no-stress conditions. Twelve male and 12 female Wistar rats were exposed to 10 min of mild, unpredictable footshock stress and to a comparable no-stress condition. Immediately following stress or no-stress conditions, subjects were injected with naloxone or saline, and two independent observers measured freezing behavior. In male rats, naloxone potentiated freezing following stress but had no effect on freezing following no-stress. In females, naloxone did not affect freezing regardless of stress conditions. These results reveal a sex difference in effects of naloxone on freezing behavior and suggest that sex differences may exist with respect to the role of endogenous opioids under stress. © 1998 Elsevier Science Inc.

Freezing Endogenous opioid peptides Naloxone Sex differences Stress Footshock

FREEZING behavior is an "immobilized" rodent response to the environment that is part of the repertoire of species-specific defense reactions in rats (2). When exhibited after stress, this behavior has been interpreted as evidence that fear has been conditioned to the context surrounding the stressful event (6,9,14,19). Endogenous opioids, released in response to stress, may play an important role in modulating this freezing response. Specifically, reports indicate that administration of opioid antagonists (e.g., naloxone, naltrexone) increases the amount of time rats will freeze in response to a stressor (6,8,14,19). Despite reports that male and female rats differ with respect to endogenous opioid responses to stress (1,10), there have been no direct comparisons of male and female freezing responses following stress and the role that opioids may play in modulating this behavior. The literature suggests that female rats display greater amounts of hormonal responses (e.g., glucocorticoids, corticosteroids) to stress (5,15, 17), whereas male rats display greater behavioral responses (e.g., defecation, decreased exploration, freezing) to stress (11, 12,20). To better understand sex differences in mechanisms that may underlie freezing behavior, we examined the effects of an opioid antagonist, naloxone, on freezing following exposure to a mild, footshock stressor.

METHOD

Subjects

Subjects were 12 male and 12 female Wistar rats (Charles River Laboratories, Wilmington, MA) that weighed 360g and 250 g, respectively, at the beginning of the experiment. Animals were experimentally naive and were housed individually in standard rat shoebox cages (35.6 × 15.2 × 20.3 cm) with wire grid floors over absorbent hardwood-chip contact bedding (Pine-Dri). The housing room was maintained at 23°C, 50% relative humidity, on a 12 L:12 D cycle (lights on at 0700 h). Subjects had continuous access to tap water and standard laboratory food pellets (Agway Prolab 3200) throughout the experiment.

Requests for reprints should be addressed to Dr. L. Cousino Klein, University of California–Los Angeles, Department of Psychology, Box 9515663, 5586 Franz Hall, Los Angeles, CA 90095-1563.

Drugs

Endogenous opioid antagonism was induced by intraperitoneal (IP) injection of 1.5 mg/kg naloxone hydrochloride (HCl; DuPont Pharmaceutical, Wilmington, DE). This dosage of naloxone-HCl effectively induces opioid withdrawal behaviors in opioid-dependent male and female rats (18,22). Naloxone-HCl was suspended in 0.86% NaCl solution in a concentration of 0.4 mg/ml.

Stressor

During each stress session, subjects were exposed to 10 min of inescapable, unpredictable electric footshock that was delivered, on average, every 40 s (range 10–70 s). Each shock stimulus lasted for 200 ms. The stimuli ranged from 0.1 to 0.8 mA across sessions and were delivered through a scrambler to the grid floor of a sound-attenuated operant chamber (ENV-001, Med Associates, East Fairfield, VT). This stimulus delivery schedule resulted in a total of 3 s of footshock over the 10-min stress period. The maximum amount (i.e., 0.8 mA) and duration of footshock has been used in previous research as a mild stressor in male and female rats and results in a significant increase in corticosterone, a biochemical index of stress (18,22). The operant chambers were illuminated with a houselight during the 10-min stressor period. The operant chambers were connected to a power supply (SG600/C, Med Associates, East Fairfield, VT) and were controlled by MED-PC computer software (23), which was programmed in Turbo Pascal (version 6.0).

Procedure

Subjects were gentled by daily handling for 3 days at the start of the experiment. After 3 days of gentling (i.e., experimental day 4), subjects were placed in the operant chambers for 10 min, without footshock, and episodes of grooming, rearing, vocalization, and teeth-chattering were recorded by two independent observers. The purpose of this procedure was to assess baseline occurrences of these behavioral indices of stress. These behaviors were selected on the basis of published reports of stress-related behaviors in male and female rats during exposure to painful (e.g., intermittent footshock) and nonpainful stressors (e.g., novel environment, conditioned fear, restraint) (3,4,14,21). Beginning on experimental day 5 and continuing through experimental day 14, subjects received 10 min of daily exposure to mild, unpredictable, inescapable footshock stress as described above. The shock level was set at 0.1 mA on the first stress day and was increased daily to a maximum shock level of 0.8 mA. During each 10-min stressor period, subjects were observed to assess effects of stress on episodes of grooming, rearing, vocalization, and teeth-chattering as described above. Immediately following these 10-min behavioral observations on days 4 (no shock), 8 (0.8 mA footshock), 14 (0.8 mA footshock), and 15 (no shock), each animal received a single IP injection of either naloxone (1.5 mg/kg) or saline (0.90% NaCl solution) and were transferred to their homecage. Subjects were randomly assigned to drug conditions and injection volumes of saline or naloxone ranged from 0.8–1.0 ml.

Freezing behavior was defined as the lack of all observable body skeleton movement with the exception of those movements related to respiration. Freezing behavior was assessed in the home cage and began 5 min after naloxone injection to provide time for drug distribution within the body. Subjects were observed in groups of four over a 20-min observation period. Specifically, each rat was observed for a 30-s window during each 1-min interval by one observer. This time-sampling procedure resulted in a total observation period of 10 min (i.e., 20, 30-s observations) for each animal. Total seconds of freezing behavior was timed during each observation window by a battery-operated, quartz stopwatch, and was evaluated after stress and no-stress conditions.

Treatment of Data and Statistical Analyses

To examine the behavioral responses to the footshock stress, total episodes of grooming, rearing, vocalization, and teeth-chattering were recorded by two independent observers during stress and no-stress conditions (interrater reliability coefficient: Pearson product-moment correlation = +0.96 in the present experiment). The number of these behaviors recorded by each observer was averaged to derive a behavioral stress index (BSI) for each animal. Repeated-measures ANOVA was used to compare the mean BSI obtained during the stress conditions (days 8 and 14) with the mean BSI obtained during the no-stress conditions (days 4 and 15). One-way ANOVA was used to examine sex differences in BSI under no-stress conditions. Because males had a significantly greater BSI under no-stress conditions than did females, analysis of covariance (ANCOVA), using no-stress BSI values as covariates, was used to examine sex differences in the BSI under stress conditions.

Repeated-measures ANOVA was used to compare mean seconds of freezing behavior observed following 10 min of stress (days 8 and 14) with mean seconds of freezing behavior observed following 10 min without stress (days 4 and 15). Separate one-way ANOVAs were used to examine the effects of sex and naloxone on freezing behavior following no-stress conditions. One-way ANOVA also was used to examine effects of naloxone on freezing behavior following stress conditions. Analysis of covariance (ANCOVA), using no-stress freezing values as the covariate, was used to examine effects of sex on freezing following stress conditions. All significance tests were two-tailed and were evaluated at $\alpha = 0.05$ to determine significance.

RESULTS

Figure 1 presents the behavioral response to no-stress and stress conditions displayed by male and female rats. Repeated-measures ANOVA revealed significantly more stress-related behaviors during stress than during no-stress conditions, $F(1, 22) = 188.14$, $p < 0.05$. This effect of stress occurred in males, $F(1, 10) = 137.99$, $p < 0.05$, and in females, $F(1, 10) = 67.22$, $p < 0.05$. Under conditions of no-stress, males exhibited significantly more stress-related behaviors than did females, $F(1, 22) = 10.57$, $p < 0.05$. Consistent with previous reports with rats, males displayed significantly more stress-related behaviors during stress than did females, $F(1, 20) = 5.08$, $p < 0.05$.

Figure 2 presents effects of naloxone on the average amount of time spent freezing (seconds) by males (see Fig. 2A) and females (see Fig. 2B) following stress and no-stress conditions as observed for 30 s during every minute over a 20-min observation period. Following stress, there was a significant drug by sex interaction, $F(1, 19) = 9.27$, $p < 0.05$, with naloxone potentiating freezing behavior in males, $F(1, 10) = 8.95$, $p < 0.05$, and having no effect in females. In addition, males displayed greater amounts of poststress freezing behavior than did females, $F(1, 19) = 24.88$, $p < 0.05$, and animals exposed to naloxone froze significantly more following stress

FIG. 1. Effects of 0.8 mA-footshock on behavioral stress responses of male (□) and female (■) rats. Total stress index was calculated as the mean number of stress behaviors (grooming, vocalizations, teeth-chattering, and defecation) observed by two independent observers during 10 min of stress (days 8 and 14) and no-stress (days 4 and 15). Error bars are equal to the standard error of the mean.

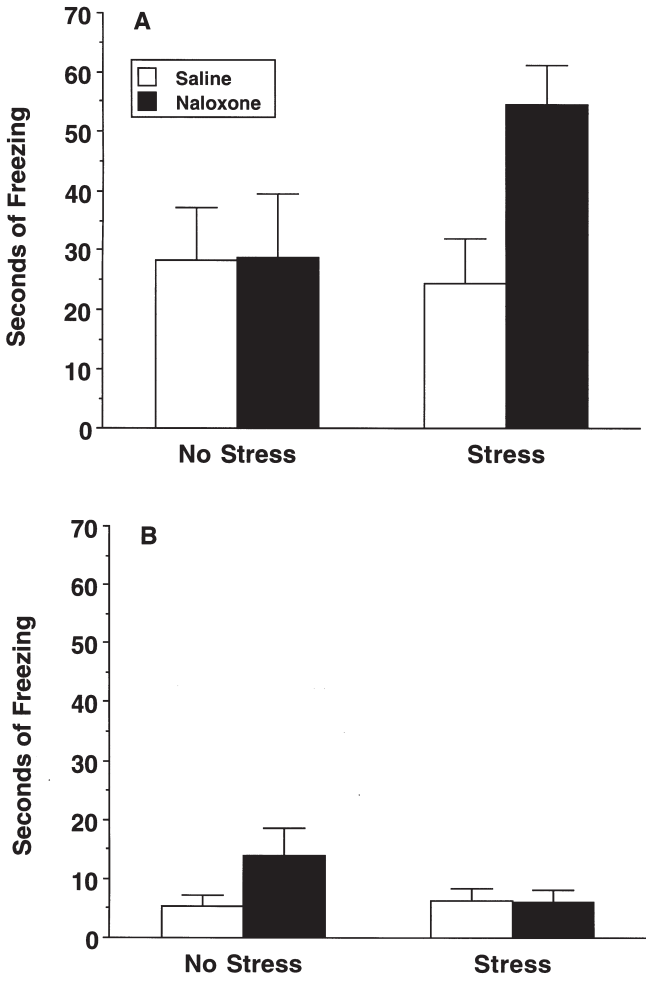

FIG. 2. Seconds of freezing behavior observed in male (A) and female (B) rats in response to saline (□) or naloxone (■) following stress and no-stress conditions as observed for 30 s during every minute over a 20-min observation period. Error bars are equal to the standard error of the mean.

than did animals exposed to saline, $F(1, 19) = 7.21$, $p < 0.05$. Further analyses indicated that there were no significant differences in poststress freezing between male and female rats exposed to saline (24.25 ± 7.57 s vs. 6.17 ± 2.09 s, respectively). In contrast, male rats exposed to naloxone froze significantly longer following stress than did female rats exposed to naloxone [54.33 ± 6.63 s vs. 5.92 ± 2.17 s, respectively; $F(1, 9) = 39.77$, $p < 0.05$].

Similarly, males froze significantly more than did females following the no-stress period, $F(1, 20) = 6.36$, $p < 0.05$. In contrast to conditions of stress, there was no effect of naloxone on freezing behavior in males following a period without stress (saline: 28.17 ± 9.05 s vs. naloxone: 28.67 ± 10.72 s). Among females, apparent effects of naloxone on freezing behavior following no-stress were not statistically significant (saline: 5.33 ± 1.18 s vs. naloxone: 13.92 ± 4.71 s).

DISCUSSION

The present experiment examined effects of naloxone on freezing behavior, an adaptive response to environmental threat or stress, in male and female rats. For males, naloxone potentiated freezing behavior following stress but had no effect on freezing behavior following a period without stress. In contrast, naloxone had no effect on freezing behavior in females, regardless of whether administration followed stress or no-stress conditions. This sex difference in the effects of naloxone on freezing behavior suggests that sex differences may exist in endogenous opioid modulation of this adaptive rodent response. The finding that males also displayed significantly more stress-related behaviors during stress than did females is consistent with previous reports that sex differences exist with respect to behavioral responses to stress (11,12,20). Because males displayed an increase in poststress freezing following naloxone injection, it is possible that opioid modulation of freezing may be a more important factor for males than it is for females. In contrast, these results also may suggest that female rats are less sensitive to opioid-mediation of poststress freezing or that they have a qualitatively different response to stress than do males. One possible explanation may be the role that corticosterone may play in behavioral responses to stress. Earlier studies suggest that females display greater hormonal responses (e.g., glucocorticoids, corticosteroids) to stress (5,15,17). Therefore, it also is possible that corticosteroids or glucocorticoids may be more relevant to poststress freezing for female rats than they are for male rats, suggesting a differential biochemical response to stress in males compared with females. An earlier report suggests that female rats will shift stress-related behavior patterns towards male patterns when administered a drug that alters corticosteroid responses to parallel male corticosteroid responses to stress (13). It is unknown whether there were, in fact, sex differences in corticosteroid, glucocorticoid, or endogenous opioid levels among the animals in the present experiment. Future experiments should examine peripheral and central nervous system endogenous opioid peptide and hormonal responses to

stress to further determine how these factors might influence poststress freezing in males and females.

The present findings with female rats also are consistent with an earlier report that naloxone administered to female rats at the time of stress exposure has no effect on freezing behavior immediately after stress (19). It is possible that the differences in freezing behavior following naloxone injection were a result of sex differences in sensitivity to opioids. Specifically, it is possible that the female rats were less sensitive to the effects of opioid antagonists or endogenous opioid agonists. For example, a recent report suggests that female rats self-administer significantly more opioids than do male rats following exposure to 0.8 mA of intermittent footshock stress, a level similar to that used in the present experiment (18). In that same experiment, however, male rats displayed significantly more opiate withdrawal behaviors following 1.5 mg/kg injection of naloxone despite lower amounts of opioid consumption. Results from the present experiment, as well as these reported sex differences in opioid consumption and withdrawal, may reflect differences in the endogenous opioid peptide system of male and female rats. Future studies should evaluate these potential differences in opioid sensitivity to help clarify sex differences in behavioral effects of naloxone under stress and poststress conditions.

It is important to note the role that a lack of a stress effect on freezing behavior might have played in the present experiment. In light of previous reports, this finding could be expected for two reasons. First, previous studies suggest that poststress freezing behavior is dramatically decreased when animals are observed in an environment that is different from where the stressor originally occurred [e.g., (7)]. In the present experiment, poststress freezing behavior was assessed in the home cage, a separate environment from where the stress procedure actually occurred. As a result, the present results may reflect a significant decrease in the amount of freezing behavior than would have been observed had the animals been placed back in the operant chamber where the shock actually occurred. Second, it is possible that elevations in no-shock freezing were observed on the last day because of the within-subject design used in the present experiment. Specifically, no-stress observations of freezing on the last day occurred after animals had been stressed on the prior day. As a result, it is possible that animals displayed elevated levels of freezing 24 h following stress conditions. This pattern of 24-h delayed postshock freezing has been reported in the literature (7) and suggests that generalized fear may have been associated between the shock chambers and the home cage, resulting in increased freezing. Given these two explanations, it is particularly noteworthy that the present experiment provides evidence of naloxone-induced freezing following stress compared with no-stress conditions despite this lack of a stress effect on freezing behavior. Future experiments should incorporate a between-subject design, and perhaps test subjects in the same and separate environments in order to delineate the possible effects of contextual stimuli on poststress freezing.

Endogenous opioids play an important role in pain and stress responses, and the present findings are consistent with other reports of sex differences in behavioral and neuroendocrine responses to stress (1,5,10–12,15,16). These results suggest that sex differences may exist with respect to opioid-mediated behaviors, and may indicate that males and females differ in their behavioral and biological responses to environmental threat.

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