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Chronic restraint stress causes a delayed increase in responding for palatable food cues during forced abstinence via a dopamine D₁-like receptor-mediated mechanism

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Abstract

Relapse to unhealthy eating habits in dieters is often triggered by stress. Animal models, moreover, have confirmed a causal role for acute stress in relapse. The role of *chronic* stress in relapse vulnerability, however, has received relatively little attention. Therefore, in the present study, we used an abstinence-based relapse model in rats to test the hypothesis that exposure to chronic stress increases subsequent relapse vulnerability. Rats were trained to press a lever for highly palatable food reinforcers in daily 3-hr sessions and then tested for food seeking (i.e., responding for food associated cues) both before and after an acute or chronic restraint stress procedure (3 h/day $\times$ 1 day or 10 days, respectively) or control procedure (unstressed). The second food seeking test was conducted either 1 day or 7 days after the last restraint. Because chronic stress causes dopamine D₁-like receptor-mediated alterations in prefrontal cortex (a relapse node), we also assessed dopaminergic involvement by administering either SCH-23390 (10.0 μ g/kg; i.p.), a dopamine D₁-like receptor antagonist, or vehicle prior to daily treatments. Results showed that chronically, but not acutely, stressed rats displayed increased food seeking 7 days, but not 1 day, after the last restraint. Importantly, SCH-23390 combined with chronic stress reversed this effect. These results suggest that drugs targeting D₁-like receptors during chronic stress may help to prevent future relapse in dieters.

Keywords

ABSTINENCE; CUE; DOPAMINE; RELAPSE; SCH-23390; STRESS

1. INTRODUCTION

Obesity, a relatively common condition in industrial societies, is linked to a number of negative health consequences (e.g., type 2 diabetes, cardiovascular disease, cancer) [1] and is

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due, at least in part, to the rise in consumption of highly palatable foods [2]. Although many overweight individuals attempt to lose weight by reducing their intake of unhealthy foods, long-term success of dietary treatments is low because most individuals relapse to unhealthy eating habits within months of starting treatment [3, 4]. Although relapse is driven by several factors, clinical data suggest that stress is a common trigger [5, 6].

To study the mechanisms of stress-induced relapse experimentally, many researchers have begun using the reinstatement model [7], an animal model of relapse. The prototype α -2 adrenoceptor antagonist yohimbine has been the stressor of choice in contemporary reinstatement studies because of its robust and reliable effects on reinstatement of food seeking when administered acutely [8]. Moreover, we recently showed that rats with a history of *chronic* yohimbine exposure showed greater acute yohimbine- and food-priming-induced reinstatement of food seeking several days after the cessation of chronic yohimbine treatment [9, 10].

Although yohimbine has several practical and translational advantages [11], stressful experiences produce a variety of neurochemical and neurohormonal changes, and a pharmacological manipulation targeting one subset of these changes may not adequately model real-world stress in humans. Moreover, recent evidence suggests that some of the effects of yohimbine on operant responding may not be related to stress-like effects and may be independent of the history of contingent self-administration of food [12]. Therefore, a main goal of the present study was to extend our previous findings by determining whether the effects of chronic yohimbine on palatable food seeking generalize to a non-pharmacological stressor. Specifically, we tested the effect of chronic restraint stress on responding for palatable food-associated cues during forced abstinence. We chose restraint because it is a well-established procedure that produces significant increases in plasma corticosterone levels [13, 14]. We chose an abstinence-based, as opposed to an extinction-based, model because we also wanted to determine whether chronic stress had any effect on “incubation of craving,” or the time-dependent increase in responding for food-associated cues [15], a phenomenon primarily assessed using an abstinence-based model.

Another goal in the present study was to determine the role of dopamine D₁-like receptors (D₁Rs) in any chronic restraint stress-related effects on food seeking because D₁Rs play a critical role in several types of food-seeking behaviors. For example, yohimbine-, cue-, food priming-, and context-induced reinstatement of food seeking are all blocked by the D₁R antagonist SCH-23390 [16–18]. SCH-23390 also blocks sucrose seeking following forced abstinence [19]. Many other stress-related neurobehavioral effects are mediated by D₁Rs as well. For example, acute stress causes cognitive deficits [20–22] that are reversed by prior administration of drugs that either block stress-induced increases in prefrontal cortex (PFC) dopamine or antagonize D₁Rs [21, 23]. Chronic stress, moreover, has been shown to cause changes in mesocortical dopamine transmission [24] and D₁R signaling [25, 26]. Particularly relevant to the present study, we found that SCH-23390, when combined with chronic yohimbine injections, reversed or attenuated all effects of yohimbine on later food seeking after extinction [10]. Thus, in the present study, we tested the possibility that blockade of D₁Rs with SCH-23390 during repeated restraint stress would attenuate any effects of this treatment on later food seeking after abstinence.

2. MATERIALS AND METHODS

2.1. Subjects and Apparatus

Adult male, Sprague-Dawley rats ($n = 97$) weighing 250–350 g at the commencement of experiments were obtained from Harlan Sprague-Dawley (Indianapolis, IN). Seven rats were excluded from the study due to poor health or unreliable food-reinforced responding during training. Rats were housed individually under standard laboratory conditions (12-hr light cycle from 7:00 AM to 7:00 PM) with *ad libitum* access to standard chow and water in their home cages for the duration of the study. Rats were weighed daily. All testing was conducted between 7:00 AM and 7:00 PM and occurred in standard modular operant conditioning chambers (Coulbourn Instruments, Whitehall, PA) that were housed in sound-attenuating, ventilated cubicles and connected to a PC with the Graphic State software interface system (Coulbourn Instruments). Each chamber is equipped with an active and an inactive response lever. Responses on the inactive lever are recorded, but have no programmed consequences. Chambers also include a house light, a row of multicolored LED cue lamps (above active lever), a tone generator, and a food tray (between the two response levers).

2.2. Food Self-Administration

Rats were trained to press the lever for food reinforcers contingent upon a fixed-ratio (FR)-1 schedule of reinforcement over the course of 1 to 3 days (this training period ended when 80 reinforcers were earned). During these training sessions, if 15 min elapsed without an active lever response a reinforcer was administered non-contingently. Following initial training, rats responded on an FR-1 schedule during daily 3-hr sessions for 12 days. Each lever press resulted in delivery of a 45-mg food pellet containing 12.7% fat, 66.7% carbohydrate, and 20.6% protein (Catalogue # 1811155, TestDiet). This pellet type was chosen based on pellet preference tests conducted by Pickens et al. [27], in which it was determined to be the most preferred pellet. Delivery of the pellet was accompanied by a tone + flashing cue light conditioned stimulus (CS) presented for 5 s, which was followed by a 20-s time-out period signaled by illumination of the house light. See Fig. 1 for schematic of experimental design.

2.3. Extinction Test 1

On the day following the last self-administration session, a 30-min extinction test was conducted for all rats. Contingencies during the extinction tests were identical to self-administration sessions except that the primary reinforcer (palatable food pellet) was not delivered.

2.4. Stress Manipulation and Extinction Test 2

2.4.1. Experiment 1: Chronic Stress—On the day following the 30-min extinction test, stressed rats in Exp. 1 were exposed to daily restraint stress. Each day, rats were placed in plastic semi-cylindrical restrainers (8.57 cm diameter x 21.59 cm length; Braintree Scientific) in an isolated room separate from other rats for 3 h for 10 consecutive days. Chronic restraint stress is a well-established procedure that produces significant increases in plasma corticosterone levels [13, 14]. To assess dopaminergic involvement in chronic stress

effects, rats received injections of SCH-23390 (0.0 or 10.0 $\mu\text{g/kg}$; i.p.), a D_1R antagonist, immediately prior to placement in restrainers. SCH-23390 was dissolved in physiological saline at a concentration of 10.0 $\mu\text{g/ml}$, and the dose is based on previous research [9, 10, 16, 18]. Unstressed rats were injected daily with SCH-23390 or vehicle and then returned to their home cages.

Following the treatment period, rats underwent a second extinction test. The contingencies were identical to the first extinction test, but it was 3 h in length. To examine any time-dependent effects of stress, the second extinction test occurred either 1 day (1D) or 7 days (7D) after the last day of treatment in separate groups of rats. Thus, rats were randomly assigned to one of eight treatment conditions: Vehicle + Unstressed/1D ($n = 6$), SCH-23390 + Unstressed/1D ($n = 6$), Vehicle + Stressed/1D ($n = 6$), SCH-23390 + Stressed/1D ($n = 7$), vehicle + Unstressed/7D ($n = 6$), SCH-23390 + Unstressed/7D ($n = 6$), Vehicle + Stressed/7D ($n = 5$), SCH-23390 + Stressed/7D ($n = 6$).

2.4.2. Experiment 2: Acute Stress—Rats in Exp. 2 also were randomly assigned to one of eight treatment conditions: Vehicle + Unstressed/1D ($n = 5$), SCH-23390 + Unstressed/1D ($n = 5$), Vehicle + Stressed/1D ($n = 5$), SCH-23390 + Stressed/1D ($n = 5$), Vehicle + Unstressed/7D ($n = 5$), SCH-23390 + Unstressed/7D ($n = 5$), Vehicle + Stressed/7D ($n = 6$), SCH-23390 + Stressed/7D ($n = 6$). However, rats received their assigned drug and stress treatment only once on Day 11 of abstinence (the day that corresponds with the 10th day of chronic treatment in Exp. 1). On Days 2 through 10 of abstinence rats were left undisturbed in their home cages except for daily weighing.

2.5. Adrenal Gland Removal

Approximately 24 h after the 3-h extinction test, rats were overdosed with a commercial euthanasia solution containing pentobarbital sodium (390 mg/ml) + phenytoin sodium (50 mg/ml) and adrenal glands were removed and weighed.

2.6. Statistical Analyses

Data from Exps. 1 and 2 were analyzed separately using mixed factorial ANOVAs. The main dependent variable was lever pressing during extinction tests. For Exp. 1, change in body weight across treatment days and adrenal weight also were used as dependent variables. Within-subjects factors included abstinence day (extinction sessions 1 and 2) and lever (active and inactive). Between-subjects factors included stress condition (stressed or unstressed), SCH-23390 dose (0.0 or 10.0 $\mu\text{g/kg}$), and number of days after treatment (1 day or 7 days). All ANOVAs were followed by Bonferroni post-tests.

3. RESULTS

3.1. Self-Administration

Fig. 2a shows lever pressing and pellets earned across the 12 days of self-administration in all rats. All rats displayed a clear preference for the active lever. Fig. 2b shows body weight in all rats across self-administration days.

3.2. Experiment 1: Chronic Stress

3.2.1. Effects of Chronic Stress on Body and Adrenal Weight—As shown in Fig. 3a, chronic stress significantly attenuated weight gain [main effect of stress; $F(1, 44) = 307.67, p < .001$]. There also was a main effect of day [$F(9, 396) = 13.23, p < .001$] and a Day x Stress interaction [$F(9, 396) = 87.92, p < .001$] in that stressed rats lost weight quickly over the first several days of treatment, but then began to gain weight around Day 9 of treatment. Although SCH-23390 treatment somewhat attenuated weight loss in stressed rats, this effect was not statistically significant.

Relative adrenal weight was significantly increased in stressed compared to unstressed rats [Fig. 3b; main effect of stress, $F(1, 40) = 7.08, p < .05$]. Conversely, relative adrenal weight significantly *decreased* in SCH-23390-treated compared to -untreated rats [main effect of SCH-23390, $F(1, 40) = 4.25, p < .05$]. Bonferroni post-tests showed that relative adrenal weight was significantly greater in the Vehicle + Stressed group compared to the SCH-23390 + Unstressed group 2 days after chronic stress.

3.2.2. Within-Subjects Analysis of Extinction Responding—For this analysis, lever pressing during the entire 30-min of Extinction Test 1 was compared to the first 30 min of Extinction Test 2. Although there was no significant change in lever pressing from Extinction Test 1 to 2 in any treatment group, it is noteworthy that the SCH + Unstressed group was the only treatment group to show an increase in active lever pressing from Test 1 to 2 when Test 2 occurred 1 day after treatment (see Fig. 4a), and the Vehicle + Stressed group was the only treatment group to show an increase in active lever pressing from Test 1 to 2 when Test 2 occurred 7 days after treatment (see Fig. 4b). For both abstinence timepoints, ANOVA results showed a main effect of lever [$F(1, 19) = 94.66, p < .001$ and $F(1, 19) = 173.75, p < .001$ for abstinence days 12 and 18, respectively] and a Day x Lever interaction [$F(1, 19) = 6.25, p < .05$ and $F(1, 19) = 7.17, p < .05$ for abstinence days 12 and 18, respectively] in that overall (i.e., collapsed across treatment groups) active lever pressing decreased modestly from Test 1 to 2, while inactive lever pressing increased modestly.

3.2.3. Between-Subjects Analysis of Extinction Responding—For this analysis, lever pressing during Extinction Test 2 was used as the dependent variable. ANOVA results showed a main effect of lever [$F(1, 40) = 135.40, p < .001$], a Stress x Day interaction [$F(1, 40) = 10.64, p < .01$], and a Lever x SCH-23390 x Stress x Day interaction [$F(1, 40) = 4.40, p < .05$]. Bonferroni post-tests revealed that rats in the Vehicle + Stressed group tested 7 days after chronic stress displayed significantly more active lever pressing compared to rats in the same treatment group tested 1 day after chronic stress (see Fig. 5).

3.3. Experiment 2: Acute Stress

3.3.1. Within-Subjects Analysis of Extinction Responding—For this analysis, as in Exp. 1, lever pressing during the entire 30-min of Extinction Test 1 was compared to the first 30 min of Extinction Test 2. There was no significant change in lever pressing from Extinction Test 1 to 2 in any treatment group regardless of whether Extinction Test 2 occurred 1 day or 7 days after treatment (see Fig. 6). For both abstinence timepoints, ANOVA results showed a main effect of lever [$F(1, 16) = 323.50, p < .001$ and $F(1, 18) =$

247.97, $p < .001$ for abstinence days 12 and 18, respectively], and for abstinence day 12, there also was a Day x Lever interaction [$F(1, 16) = 12.94$, $p < .01$] in that overall active lever pressing decreased modestly from Test 1 to 2, while inactive lever pressing increased modestly.

3.3.2. Between-Subjects Analysis of Extinction Responding—For this analysis, as in Exp. 1, lever pressing during Extinction Test 2 was used as the dependent variable. ANOVA results showed a main effect of lever [$F(1, 34) = 314.22$, $p < .001$] and a Lever x Day interaction [$F(1, 34) = 7.79$, $p < .01$] in that overall active lever pressing was modestly higher 7 days compared to 1 day after treatment, while inactive lever pressing was modestly lower at the later timepoint (see Fig. 7).

4. DISCUSSION

We tested the hypothesis that exposure to chronic restraint stress would cause an increase in vulnerability to relapse as assessed using an abstinence-based animal model. Results supported our hypothesis by showing that chronically, but not acutely, stressed rats displayed increased responding for food-associated cues 7 days, but not 1 day, after the last restraint. Moreover, SCH-23390 combined with chronic stress reversed this behavioral effect, highlighting the important role of D_1R signaling in the effects of chronic stress on palatable food-seeking.

Because the effect of chronic stress on food seeking was blocked by SCH-23390 administration, it is possible that D_1R antagonism reduced the hypothalamic-pituitary-adrenal (HPA) axis response to stress. This interpretation, however, is unlikely given that a) there was a robust decrease in weight gain in the SCH + Stressed group, similar to that observed in the Vehicle + Stressed group, b) the difference in adrenal weights 2 days after stress in the SCH + Stressed versus SCH + Unstressed groups is very similar to that seen in the Vehicle groups, and c) there is evidence that intra-medial PFC injections of SCH-23390 do not alter the HPA axis response to restraint stress as measured by plasma corticosterone concentrations [28]. Thus, it appears that SCH-23390 did not reduce the “stressfulness” of the manipulation.

These results support and extend our previous findings with the pharmacological stressor yohimbine [9, 10] by showing that a non-pharmacological stressor, restraint, also produces lasting increases in palatable food-seeking behavior. We hypothesize that this is due to engagement of a common pathway involved in food seeking. Indeed, both yohimbine and non-pharmacological stressors strongly activate [29, 30] and selectively increase dopamine release [12, 31] in PFC. The release of dopamine in PFC and the activation of D_1Rs in general appear to be critical mechanisms underlying food-seeking behavior [16, 17, 19]. Results showing that SCH-23390 combined with yohimbine [9, 10] and restraint stress (present study) blocked the effects of both stressors on food seeking provide further support for a shared mechanism.

Another main finding was that chronic, but not acute, restraint increased later food seeking. These behavioral findings are in line with differential neuropharmacological effects of acute

vs. chronic stress. Thus, although acute stress induces increases in extracellular dopamine in PFC, these increases are transient—PFC dopamine levels return to baseline relatively rapidly following the acute stress exposure [32, 33]. On the other hand, chronic stress has been shown to *reduce* both the spontaneous electrophysiological activity of ventral tegmental dopamine neurons [24] and basal levels of dopamine in PFC [25, 26]. The reduction in PFC dopamine levels, moreover, lasts for at least 3 months following the cessation of chronic stress [26]. Blockade of D₁Rs during restraint stress in the present study may have blocked stress-induced changes in dopaminergic transmission, thereby reversing the effects of chronic stress on later food-seeking behavior.

In our previous studies with yohimbine, we found that the effects of chronic yohimbine on palatable food seeking were evident at least 1 week later [9, 10]; however, the time-course of chronic stress effects on food seeking has not been systematically studied. In the present study we found that increased food seeking was observed 7 days, but not 1 day, following the cessation of chronic stress. Although this is a novel finding with regard to chronic stress effects on food seeking, this is not the first report of a post-stress incubation period. For example, in the single prolonged stress (SPS) animal model of post-traumatic stress disorder (PTSD), in which rats are subjected once to three consecutive stressors (restraint, forced swim, ether), the PTSD-like phenotype manifests only after a 7-day stress-free period [34–36]. The delayed effects of SPS include alterations in HPA axis function and deficits in retention of extinguished fear. Although it is not known whether the delayed effects of SPS involve D₁R signaling, it is interesting to speculate that D₁Rs may be a common pathway involved in maladaptive conditioned responses to both aversive and appetitive cues (e.g., PTSD and relapse to unhealthy eating behavior). In future studies, it would be worthwhile to study in more detail the time-course and longevity of stress effects on food seeking.

Along with neurochemical changes, chronic stress is associated with several structural changes in medial PFC, including dendritic retraction in pyramidal cells [37, 38]. In a recent report by Lin et al [28], microinjections of SCH-23390 into the prelimbic region of medial PFC during chronic restraint reversed this dendritic retraction. Given that medial PFC is a critical node in the circuitry of relapse to palatable food seeking [16, 39] it is possible that D₁R-mediated structural changes in this region due to chronic stress contributed to the increased food seeking observed in the present study. Importantly, however, the effects of chronic stress on medial PFC morphology are evident immediately after the termination of stress, a time-point at which we did not observe increased food seeking. Although the medial PFC recovers by 3 weeks after the termination of stress [40], a detailed time-course of dendritic changes following stress is lacking. The question of whether longer-term structural changes in medial PFC following stress are related to behavioral plasticity regulating food seeking is worthy of direct testing in future studies.

It is important to point out that most of the research on chronic stress has been conducted using males. However, relatively recent evidence suggests that the effects of chronic stress are sex-dependent with respect to numerous neural and behavioral endpoints. Thus, in contrast to male rats, female rats display stress-induced dendritic *hypertrophy* in medial PFC pyramidal neurons [41]. Moreover, stress alters microglial cell activation patterns in the medial PFC of females, but not males [42]. With regard to prefrontal-mediated behaviors,

chronic stress is associated with deficits in spatial working memory tasks in males and improvements in females [43, 44]. Given that the proportion of women who seek dietary treatment is at least twice that of men [45, 46], a critical question to be addressed in future studies is whether chronic stress differentially affects food seeking in males vs. females.

Finally, it is noteworthy that unstressed animals did not show a time-dependent increase in responding for food-associated cues, or “incubation of craving” [15]. We observed incubation only in stressed animals. This was somewhat surprising given the large literature describing incubation of both drug and sucrose seeking [15, 47]; however, one potentially important methodological detail in our study is the use of a within-subjects design (i.e., incubation was assessed within the same animals tested twice). Studies reporting incubation of craving have primarily used between-subjects designs (i.e., separate groups of rats were tested in a single extinction test performed at different days during abstinence), which makes a direct comparison with the present study difficult. Nonetheless, given that incubation is a phenomenon that models some aspects of human drug addiction, such as the progressive increase in cue-induced drug craving during early abstinence [48] and relapse vulnerability even after prolonged abstinence [49], our findings could be interpreted to suggest that chronic stress promotes maladaptive, or addiction-like, food seeking under conditions that otherwise do not promote strong cue-induced craving. It would be worthwhile in future studies to determine whether chronic stress potentiates incubation of food seeking under conditions that promote incubation in unstressed animals.

In conclusion, our results suggest that chronic stress induces neural changes that manifest as increased food seeking after a stress-free period. Therefore, from a clinical perspective, individuals on a dietary treatment plan who also are exposed to chronic stress may be most vulnerable to cue-induced relapse at some point *after* the stress has subsided. Inasmuch as SCH-23390 during chronic stress blocked these delayed stress effects, drugs targeting D₁Rs during chronic stress may help to prevent future relapse in dieters

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Abbreviations

CS	conditioned stimulus
D₁R	D ₁ -like receptor
PTSD	post-traumatic stress disorder
FR	fixed ratio
PFC	prefrontal cortex

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Highlights

Relapse to unhealthy eating habits in dieters is often triggered by acute stress.

The role of *chronic* stress in relapse vulnerability, however, is less clear.

We assessed the role of chronic stress in relapse to palatable food seeking in rats.

Chronically stressed rats displayed increased food seeking 7 days after stress.

Blockade of D₁-like receptors during chronic stress reversed this effect.

Fig. 1.
Schematic representation of the experimental design for assessing effects of chronic (10 days) or acute (1 day) restraint stress on responding for palatable food-associated cues during forced abstinence.

Fig. 2.

(a) Responding in all animals across 12 days of self-administration. Rats responded on an FR-1 schedule of reinforcement in daily 3-h sessions. Delivery of the pellet was accompanied by a tone + flashing cue light conditioned stimulus presented for 5 s, which was followed by a 20-s time-out period signaled by illumination of the house light. (b) Body weight across the 12 days of self-administration in all animals. All data in figure are represented as mean $\pm$ SEM. Points without error bars indicate the SEM is too small to illustrate.

Fig. 3.

Experiment 1: Chronic Stress. (a) Weight change in chronically stressed and unstressed rats receiving vehicle or SCH-23390 injection (10.0 $\mu\text{g/kg}$; i.p.). Injections were given immediately prior to placement in restrainers (3 h/day) or transport back to home cages. (b) Adrenal weight in chronically stressed and unstressed rats receiving vehicle or SCH-23390 injection expressed in mg/100 g body weight. Adrenal glands were removed approximately 24 h after the 3-h extinction test occurring either 1 day or 7 days after the last chronic treatment. * $p < .05$ compared to SCH-23390 + Unstressed group on same day, Bonferroni post-test. All data in figure are represented as mean $\pm$ SEM.

Fig. 4.

Experiment 1: Chronic Stress. Within-subjects analysis of active and inactive lever pressing during extinction tests given before and after the 10-day treatment period. Contingencies during the extinction tests were identical to self-administration sessions except that the primary reinforcer (palatable food pellet) was not delivered. Extinction Test 1 occurred on Day 1 of abstinence for all rats, and Extinction Test 2 occurred either (a) 1 day or (b) 7 days after the last day of treatment, corresponding to either Day 12 or 18 of abstinence, respectively. Lever pressing during the entire 30-min of Extinction Test 1 was compared to the first 30 min of Extinction Test 2. All data in figure are represented as mean $\pm$ SEM.

Fig. 5.
Experiment 1: Chronic Stress. Between-subjects analysis of active and inactive lever pressing during 3-h extinction tests occurring 1 day or 7 days after the last chronic treatment. ** $p < .01$ compared to 1 day after treatment in same chronic treatment group, Bonferroni post-test. All data in figure are represented as mean $\pm$ SEM.

Fig. 6.

Experiment 2: Acute Stress. Within-subjects analysis of active and inactive lever pressing during extinction tests given before and after four different acute treatments. Extinction Test 1 occurred on Day 1 of abstinence for all rats, and Extinction Test 2 occurred either (a) 1 day or (b) 7 days after the acute treatment, corresponding to either Day 12 or 18 of abstinence, respectively. All data in figure are represented as mean $\pm$ SEM.

Fig. 7. Experiment 2: Acute Stress. Between-subjects analysis of active and inactive lever pressing during 3-h extinction tests occurring 1 day or 7 days after four different acute treatments. All data in figure are represented as mean $\pm$ SEM.