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ORIGINAL INVESTIGATION

# The $CRF_1$ receptor antagonist R121919 attenuates the neuroendocrine and behavioral effects of precipitated lorazepam withdrawal

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**Abstract**
*Rationale* Corticotropin-releasing factor (CRF) is the primary physiologic regulator of the hypothalamic-pituitary-adrenal (HPA) axis and serves to globally coordinate the mammalian stress response. Hyperactivity of central nervous system CRF neurotransmission, acting primarily via the $CRF_1$ receptor, has been strongly implicated in the pathophysiology of depression and anxiety. Furthermore, there is evidence of enhanced CRF transcription, release, and neuronal activity after the administration of and withdrawal from several drugs of abuse, including cannabis, cocaine, ethanol, and morphine. Treatment with CRF antagonists has been demonstrated to reduce the severity of certain drug withdrawal symptoms, implicating a specific role for activation of CRF neurons in mediating the anxiogenic and stress-like reactions observed after abrupt drug discontinuation.
*Objectives/Methods* To extend these findings, we investigated whether pretreatment with the selective $CRF_1$ receptor antagonist R121919 decreases the behavioral and neuroendocrine activation observed after the precipitation of benzodiazepine (BZ) withdrawal in BZ-dependent rats.
*Results* Pretreatment with R121919 attenuated the subsequent HPA axis activation, behavioral measures of anxiety, and expression of the CRF gene in the paraventricular nucleus of the hypothalamus, as measured by CRF heteronuclear RNA, which occurs after flumazenil-precipitation of withdrawal from the BZ, lorazepam.
*Conclusions* These results indicate that the activation of CRF neuronal systems may be a common neurobiological mechanism in withdrawal from drugs of abuse and moreover, that the $CRF_1$ receptor subtype plays a major role in mediating the effects of CRF on neuroendocrine and behavioral responses during BZ withdrawal. Therefore, $CRF_1$ receptor antagonists may be of therapeutic utility in the treatment of drug withdrawal syndromes.

**Keywords** Anxiety · Benzodiazepines · Corticotropin-releasing factor · HPA axis · Stress · Withdrawal

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The neuropeptide corticotropin-releasing factor (CRF), the major physiological regulator of the hypothalamic-pituitary-adrenal (HPA) axis, mediates the endocrine response to stress. Substantial evidence has accumulated to support the hypothesis that in extrahypothalamic limbic structures and brainstem nuclei, CRF additionally functions as a neurotransmitter and serves to mediate the behavioral, immune, and autonomic components of the stress response. In addition to this key role, there is considerable evidence implicating CRF in the pathophysiology of mood and anxiety disorders (Owens and Nemeroff 1991).

Preclinical data generated in laboratory animals demonstrates that either central administration of CRF or transgenic overexpression produces behavioral evidence of heightened anxiety (Dunn and Berridge 1990; Heinrichs et al. 1997a). In humans, elevated concentrations of CRF have been observed in the cerebrospinal fluid (CSF) of drug-free

patients suffering from posttraumatic stress disorder, obsessive–compulsive disorder, and alcohol withdrawal, conditions in which the patients demonstrate severe anxiety and increased vulnerability to stress (Altemus et al. 1994; Adinoff et al. 1996; Bremner et al. 1997; Baker et al. 1999). Studies of $CRF_1$ receptor knock-out mice, $CRF_1$ antisense oligonucleotides, and $CRF_1$-selective antagonists indicate that it is this receptor subtype that mediates the anxiogenic effects of CRF (Schulz et al. 1996; Heinrichs et al. 1997b; Griebel et al. 1998; Skutella et al. 1998; Smith et al. 1998; Timpl et al. 1998; Liebsch et al. 1999; Okuyama et al. 1999).

In contrast to the anxiogenic role of CRF, benzodiazepines (BZs) are a class of drugs that potentiate GABA-mediated $Cl^-$ current and have been clinically utilized as antianxiety agents for more than 40 years. They produce a number of effects that oppose those of CRF, including suppression of HPA axis activation (Krulik and Cerny 1971; Kalogeras et al. 1990; Rohrer et al. 1994; Cowley et al. 1995), attenuation of stress-induced CRF transcription and release (Imaki et al. 1995; Pich et al. 1995), and reduction in the anxiogenic effects of centrally administered CRF (Britton et al. 1985; Swerdlow et al. 1986; Dunn and File 1987). Moreover, our laboratory has previously shown that chronic treatment with the triazolobenzodiazepine alprazolam in rats produced dichotomous effects on CRF neuronal systems, with decreases in indices of CRF and $CRF_1$ receptor functioning and increases in indices of urocortin I and $CRF_2$ receptor functioning (Skelton et al. 2000).

Although BZs tend to exert their behavioral effects acutely, they are frequently administered to patients over long periods of time because of the chronic nature of anxiety disorders, producing a state of physiological dependence (Ballenger 1991; Romach et al. 1992). When chronic BZ usage is discontinued abruptly, a withdrawal syndrome is produced, consisting of a constellation of adverse physiological and behavioral responses. The anxiety, HPA axis activation, and autonomic activation that characterize the BZ withdrawal syndrome classically resemble the characteristics of the mammalian stress response, suggesting the involvement of central CRF neuronal systems. Our laboratory has previously documented increased HPA axis activity after withdrawal from chronic BZ administration (Owens et al. 1991). In fact, the acute phase of withdrawal from several drugs that produce dependence is associated with evidence of increased central CRF neuronal activity, including HPA axis activation (Keith et al. 1983; Roberts et al. 1992; Milanes et al. 1998), increased CRF messenger RNA (mRNA) transcription and/or peptide release within the amygdala (Pich et al. 1995; Rodriguez de Fonseca et al. 1997; Richter and Weiss 1999; Maj et al. 2003; Zhou et al. 2003), and/or increased CSF CRF concentrations (Adinoff et al. 1996) after withdrawal from cannabis, cocaine, ethanol, and morphine.

These results further suggest that the activation of CRF neuronal systems may be a common neurobiological factor in withdrawal from drugs of abuse. If heightened CRF activity is a causal element in the adverse symptoms of drug withdrawal, then blocking CRF neurotransmission with a CRF receptor antagonist should theoretically attenuate the withdrawal syndrome. This hypothesis is supported by reports that pretreatment with CRF antagonists (both nonselective and $CRF_1$-selective) significantly attenuated many of the behavioral and autonomic signs of precipitated morphine withdrawal in rats (Brugger et al. 1998; Iredale et al. 2000; Lu et al. 2000; Funada et al. 2001; McNally and Akil 2002). Additionally, withdrawal-induced anxiety, after chronic cocaine or ethanol administration, was diminished by pretreatment with a CRF antagonist or CRF antiserum (Baldwin et al. 1991; Rassnick et al. 1993; Sarnyai et al. 1995; Basso et al. 1999). These findings imply a specific role for activation of CRF neurons in mediating the anxiogenic and stress-like reactions during withdrawal from cocaine, ethanol, and morphine, which are likely also present during BZ withdrawal. In this study, we therefore sought to determine whether pretreatment with the selective $CRF_1$ antagonist R121919 decreases the behavioral and neuroendocrine activation observed after the precipitation of BZ withdrawal in BZ-dependent rats. Although abstinence-induced withdrawal is the more common etiology of withdrawal after chronic BZ use in humans, antagonist-precipitated withdrawal is more amenable to study in rats due to the temporally defined nature of the symptoms and immediacy of onset. Although differing in onset and duration, the constellation of symptoms produced during both abstinence-induced and antagonist-precipitated withdrawal, and thus, likely, the underlying pathophysiologic mechanisms involved in their mediation are quite similar.

## Materials and methods

### Animals

Male Sprague–Dawley rats (225–250 g on arrival; Charles River Laboratories, Raleigh, NC) were housed in pairs with water available ad libitum in an environmentally controlled animal facility with a 12-h light/dark cycle (lights on at 0730 h). The food supply consisted of standard rat chow available ad libitum. The animals were weighed and handled daily throughout the course of the experiment. All animal protocols were approved by the Emory IACUC and the “Guide for Care and Use of Laboratory Animals” (Institute of Laboratory Animal Resources, National Academy, 1996) was followed.

Experimental design and drug treatment

*Experiment 1—HPA axis response to precipitated lorazepam withdrawal* In this experiment, rats ($n$=8–10 per group) were rendered lorazepam dependent by the administration of 10 mg $kg^{-1}$ $day^{-1}$ of lorazepam in polyethylene glycol 400 for 12 days via two subcutaneously implanted Alzet osmotic minipumps (Alza, Palo Alta CA). The use of subcutaneously implanted osmotic minipumps to deliver lorazepam has been previously examined by Miller et al. (1988) and was found to yield fairly constant concentrations of the drug in the plasma and brain with little variability over 2–14 days of administration, with the 10 mg $kg^{-1}day^{-1}$ dose of lorazepam utilized in this study demonstrated to produce plasma lorazepam levels of ~250 ng/ml. A serum lorazepam concentration of 200 ng/ml has been reported to be the median effective dose for the control of generalized tonic–clonic seizures in a rat model of status epilepticus (Walton and Treiman 1990). The minipumps were implanted subcutaneously under methoxyfluorane anesthesia, as described in detail in Stout et al. (2002). Four days before the precipitation of withdrawal, the rats were implanted with a jugular venous cannula under aseptic conditions, as described in detail in Thrivikraman et al. (2002). The rats were thereafter returned to fresh cages and single housed. On the day of the experiment, each rat was assigned to one of four experimental groups as follows. At the beginning of the experiment, half of the rats were pretreated with the $CRF_1$ antagonist R121919 (5 mg/kg) while the other half received vehicle injections (50% PEG 400 in $ddH_2O$). Sixty minutes later, lorazepam withdrawal was precipitated in half of each of these groups by the administration of the BZ antagonist, flumazenil (10 mg/kg), while the other half received vehicle injection (70% PEG 400 in $ddH_2O$). The $CRF_1$ antagonist or vehicle was administered intravenously (i.v.) into the jugular cannula in an injection volume of 1 ml/kg followed by a heparinized saline flush, while injections of flumazenil or vehicle were administered intraperitoneally (i.p.) in an injection volume of 2 ml/kg. The i.v. route of administration was chosen for R121919 because of the ease of administration for the acute dosing of this medication, in a timed manner, to multiple rats with established i.v. access. Whereas this route of administration is not that which would be utilized for human studies of this compound, R121919 is a nonpeptide CRF1 antagonist, which can be administered orally to humans, and has already been demonstrated to have clinical efficacy in diminishing the symptoms of depression and anxiety (Zobel et al. 2000).

Blood samples (0.3 ml) were taken from the unrestrained rat and collected in EDTA-containing tubes just before the administration of the $CRF_1$ antagonist or vehicle ($t$=−60 min), just before the injection of flumazenil or vehicle ($t$=0 min), and at 10, 20, 30, 60, 90, and 120 min postinjection. The blood samples were centrifuged at 12,000×$g$ for 5 min (4°C) and the plasma collected and stored at −80°C until assay for adrenocorticotropic hormone (ACTH) and corticosterone levels.

*Experiment 2—behavior in the defensive withdrawal paradigm* As in experiment 1, rats ($n$=6–7 per group) received lorazepam (10 mg $kg^{-1}$ $day^{-1}$) via dual subcutaneously implanted osmotic minipumps for 12 days and were assigned to one of four experimental conditions. For this experiment, the $CRF_1$ antagonist or vehicle were administered subcutaneously (s.c.) in an injection volume of 1 ml/kg, 60 min before i.p. injections of flumazenil or vehicle (injection volume=2 ml/kg). Rats were then introduced into the defensive withdrawal paradigm 5 min after flumazenil or vehicle injection for a 15-min testing session. Ten minutes after the completion of the behavioral testing, the rats were killed by decapitation (at $t$=30 min after flumazenil or vehicle injection). Brains were immediately removed, frozen on dry ice and stored at −80°C until sectioned for analysis of CRF heteronuclear RNA (hnRNA) expression.

The dose of R121919 (5 mg/kg) utilized in this experiment was previously shown to produce ~75% occupancy of the $CRF_1$ receptor in the parietal cortex 75 min after s.c. injection. At the same timepoint, the antagonist did not appreciably occupy $CRF_{2A}$ receptors in the lateral septum, and thus exhibited the expected selectivity for the $CRF_1$ receptor subtype (Gutman et al. 2003).

ACTH and corticosterone radioimmunoassay analysis

Plasma ACTH was assayed in 100 μl samples using the Allegro™ HS-ACTH kit (Nichols Institute, San Juan Capistrano, CA) with 50 μl of iodonated tracer and one avidin coated bead per tube as previously described (Thrivikraman et al. 2000). Corticosterone was determined in 5 μl plasma samples using the ImmunoChem™ Double Antibody kit (ICN Biomedicals, Costa Mesa, CA) with half of the trace and antibody volume as previously described. The ACTH assay has a sensitivity of 2 pg/ml and an intra- and intercoefficients of variation of less than 6%. The corticosterone assay has a sensitivity of 1 ng/ml and an intra- and intercoefficients of variation of less than 10%.

In situ hybridization

Serial coronal brain sections (20 μm) were sliced on a cryostat at −17°C, thaw mounted onto SuperFrost Plus slides (Fisher, Pittsburgh, PA) under RNAse-free conditions, and stored with Humi-Cap desiccant capsules (Gibco BRL Products, Grand Island, NY) at −80°C until the assay.

In situ hybridization was performed according to previously published protocols (Skelton et al. 2000).

The CRF heteronuclear riboprobe was constructed from a 495-bp insert ligated into a pBluescript II SK+ plasmid (P. Sawchenko, Salk Institute for Biological Studies, La Jolla, CA). The insert includes sequence for the single intron found in the rat CRF gene.

### Image analysis

Images from the in situ hybridization and receptor autoradiography films were digitized with a Dage-MTI CCD-72 (Michigan City, IN) image analysis system equipped with a Nikon camera. Semiquantitative analysis was performed as described in detail in Skelton et al. 2000.

### Defensive withdrawal paradigm

The apparatus consisted of an open field (75×75 cm) painted in flat gray with 50 cm high walls made of white Plexiglass. The light intensity was approximately 400–500 lux throughout the field. Each trial began 5 min after injection of flumazenil or vehicle, as the rat was placed in front of a black PVC tube (10 cm in diameter×21 cm in length, closed at one end) and allowed to walk in under its own power. The tube was then placed into the field at a distance of 20 cm from a corner with the open end of the tube facing the corner. The behavior of the rat was videotaped for 15 min, and the following parameters are scored: (1) latency—the time from the placing of the tube into the field until the rat first places all four paws into the open field, (2) total time in tube—the total amount of time the rat spends withdrawn inside the tube, and (3) number of entries into the open field—the number of times the rat enters the open field (all four paws). Ten minutes after the completion of the trial ($t$=30 min after injection of flumazenil or vehicle), the rats were killed by decapitation, and the brains were collected on dry ice and stored at −80°C until cryostat sectioning.

To minimize the stress associated with the novelty of the open field, the rats were familiarized to the open field and the defensive withdrawal tube for 15 min on the 2 days before the experimental trial. All familiarization and testing was performed between 0900 and 1230 h.

### Drugs

Flumazenil was a gift from Hoffmann-LaRoche (Basel, Switzerland). Lorazepam was a gift from Wyeth-Ayerst (Princeton, NJ). R121919 was a gift from Neurocrine Biosciences and Janssen Pharmaceuticals (San Diego, CA).

### Statistics

For analysis of serial concentrations of ACTH and corticosterone in experiment 1, results were analyzed by three-way analysis of variance (ANOVA) (drug×drug×time) with repeated measures on the factor of time, while analysis of the 'area under the curve' for ACTH and corticosterone concentrations in this experiment was made by two-way ANOVA (drug×drug). In experiment 2, results were analyzed by two-way ANOVA (drug×drug) for the variable of 'latency' to emerge from tube. As the data for the variables of 'total time spent in tube' and 'number of transitions' were not normally distributed, these data were analyzed by nonparametric two-way ANOVA on ranks with post hoc pairwise comparisons by Tukey's HSD. Because only minimal CRF hnRNA expression was observed in the paraventricular nucleus of the hypothalamus (PVN) of rats that did not receive flumazenil, comparisons of this parameter were made between the flumazenil-treated rats only by means of a two-tailed $t$ test. Student–Newman–Keuls pairwise testing was utilized for post hoc analysis after ANOVA, except as noted above. All data are expressed as means±SEM.

## Results

### Experiment 1—HPA axis response to precipitated lorazepam withdrawal

Results obtained by three-way ANOVA with repeated measures on the factor of time indicated significant effects on ACTH concentrations for $CRF_1$ receptor antagonist (R121919) administration ($F_{1,34}$=9.70; $p$<0.01), flumazenil-precipitation of BZ withdrawal ($F_{1,34}$=4.06; $p$=0.05), and time ($F_{7,238}$=42.25; $p$<0.0001). In addition, there were significant interactions between the factors of R121919 administration×time ($F_{7,238}$=4.19; $p$<0.001) and flumazenil administration×time ($F_{7,238}$=5.05; $p$=<0.0001). Total area under the curve for ACTH concentrations integrated over the duration of the experiment demonstrated a significant effect for R121919 administration ($F_{1,34}$=9.79; $p$<0.01) by two-way ANOVA.

Just before infusion of R121919 or vehicle i.v. into the jugular cannula, plasma ACTH concentrations were low, indicating that the rats were not 'stressed' basally (ACTH concentrations for the groups ranged from 0.9–8.7 pg/ml; Fig. 1). In the context of this study, the operational definition of 'stress' we are utilizing refers to environmental or biological perturbations that disturb the rat's homeostasis, as evidence by an activation of the HPA axis, which is a core component of the classically defined stress response.

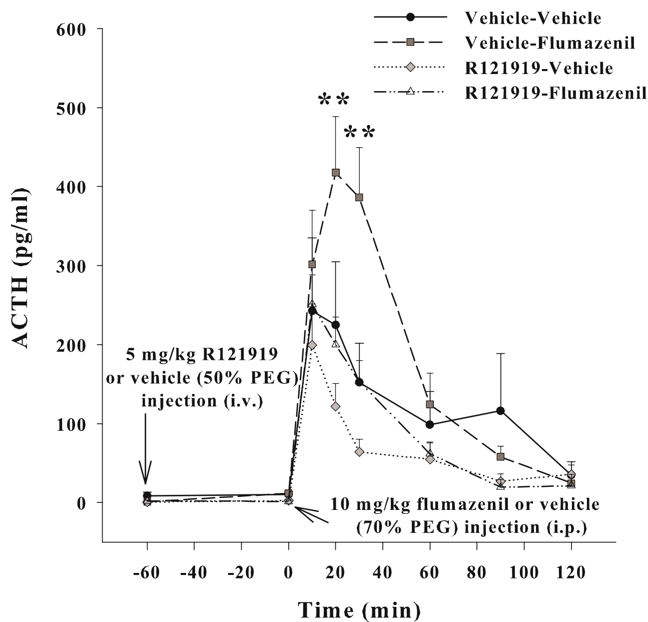


**Fig. 1** Pretreatment with the $CRF_1$ antagonist R121919 attenuates the peak ACTH response to flumazenil-precipitated lorazepam withdrawal. Plasma ACTH was measured in blood samples obtained from cannulae implanted in the jugular vein of male Sprague–Dawley rats administered lorazepam via subcutaneously implanted osmotic minipumps at a concentration of 10 mg $kg^{-1}$ $day^{-1}$ for 12 days. Rats received pretreatment with either vehicle or 5 mg/kg R121919, followed 60 min later by the administration of vehicle or 10 mg/kg flumazenil to precipitate withdrawal. Each *bar* represents mean±SEM; $n$=8–10 per group at each time point. *Double asterisks* $p<0.01$ vs all other groups at that time point by three-way ANOVA with repeated measured on the factor of time, followed by Student–Newman–Keuls post hoc pairwise analysis

In the blood sample taken 60 min later, before administration of flumazenil or vehicle, ACTH levels continued to remain low and were not significantly different from the previous sample, demonstrating that the $CRF_1$ antagonist did not alter the already low basal HPA axis functioning in these rats. Immediately after the i.p. injection of flumazenil, plasma ACTH concentrations rose sharply as a result of the precipitation of benzodiazepine withdrawal. Plasma ACTH concentrations also increased to a lesser extent after vehicle injection, as a result of the nonspecific stress of handling and injection. Ten minutes after the i.p. injection, plasma ACTH concentrations peaked in all rats except for the vehicle-pretreated/flumazenil-withdrawn group, in which ACTH concentrations continued to rise to a maximum at 20 min. Pretreatment with R121919 before flumazenil precipitation of lorazepam withdrawal significantly attenuated the ACTH response at both the 20- and 30-min time points as compared to the vehicle-pretreated/flumazenil-withdrawn rats (417.7±71.1 vs 199.8±35.4 pg/ml at 20 min, and 386.2±63.1 vs 153.4±26.6 pg/ml at 30 min; $p<0.01$; Fig. 1) and significantly decreased, by 46.6%, the area under the curve representing the ACTH response over the entire 120 min after precipitation of BZ withdrawal (21,199.1±3,107.5 pg min/ml vs 11,322.3±1,486.6 pg min/ml; $p<0.05$; Fig. 3a). In fact, the ACTH concentrations in these R121919-pretreated/flumazenil-withdrawn rats were at or below even the concentrations found in the vehicle-pretreated/nonwithdrawn rats at all time points. Pretreatment with R121919 may have diminished the ACTH response to the i.p. injection stress, as well. Comparison of the nonwithdrawn groups reveals that the area under the curve for the ACTH response of the R121919-pretreated/nonwithdrawn rats was decreased by 50.3% as compared to the vehicle-pretreated/nonwithdrawn rats (15,274.7±4,393.4 pg min/ml vs 7,580.6±1,683.3 pg min/ml; $p$=0.06).

With respect to corticosterone concentrations, results obtained by three-way ANOVA with repeated measures on the factor of time indicated significant effects for the factor of time ($F_{7,238}$=39.22; $p<0.0001$). In addition, there were significant interactions between the factors of flumazenil administration×time ($F_{7,266}$=2.07; $p<0.05$) and R121919 administration×flumazenil administration×time ($F_{7,238}$=2.97; $p\leq0.01$). Total area under the curve for corticosterone concentrations integrated over the duration of the experiment did not reveal statistically significant effects for R121919 or flumazenil administration by two-way ANOVA.

As with ACTH, plasma corticosterone values before injection of R121919 or vehicle were low and statistically indistinguishable amongst all groups (3.7–22.8 ng/ml), indicating a similar, nonstressed basal state. Sixty minutes later, serum corticosterone concentrations remained low and were not statistically different from the previous sample (6.6–20.5 ng/ml), again demonstrating the lack of a significant effect of the $CRF_1$ antagonist on basal HPA axis functioning in these rats before stress. Immediately after i.p. injection of flumazenil or vehicle, plasma corticosterone concentrations rose as a result of the precipitation of lorazepam withdrawal and/or the stress of the i.p. injection (Fig. 2). In most groups, the corticosterone response peaked in the 20–30-min timeframe, whereas corticosterone values continued to rise for 90 min after precipitation of withdrawal in the vehicle-pretreated/flumazenil-withdrawn rats. In contrast to the ACTH results, pretreatment with R121919 did not alter the peak plasma corticosterone concentrations achieved after precipitation of lorazepam withdrawal (219.2±41.6 ng/ml at 90 min vs 218.7±16.8 ng/ml at 30 min), likely because the ACTH released, although attenuated by R121919 pretreatment, was sufficient to generate a near maximal corticosterone response. However, the duration of the corticosterone response to benzodiazepine withdrawal was significantly decreased in the group that received pretreatment with the $CRF_1$ antagonist. In these rats, corticosterone concentrations returned to the levels found in the nonwithdrawn rats by 90 min after flumazenil administration, whereas corticosterone concentrations remained elevated in the vehicle-

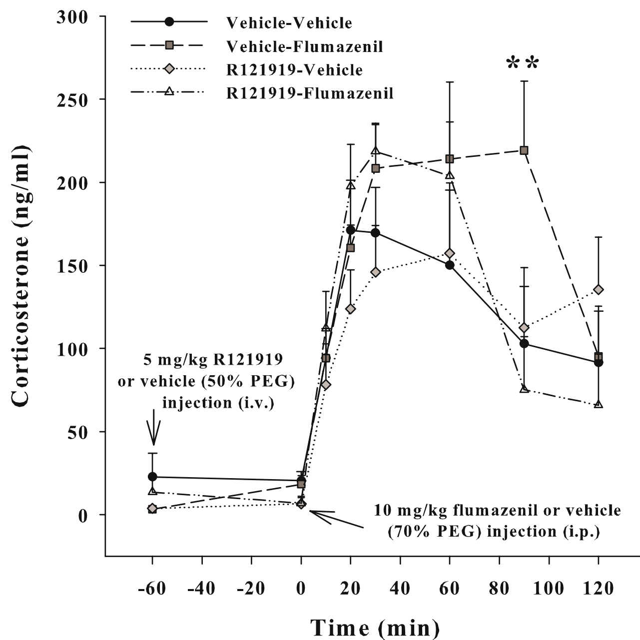


**Fig. 2** Pretreatment with the $CRF_1$ antagonist R121919 decreases the duration of elevated corticosterone concentrations in response to flumazenil-precipitated lorazepam withdrawal. Plasma corticosterone was measured in blood samples obtained from cannulae implanted in the jugular vein of male Sprague–Dawley rats administered lorazepam via subcutaneously implanted osmotic minipumps at a concentration of 10 mg $kg^{-1}$ $day^{-1}$ for 12 days. Rats received pretreatment with either vehicle or 5 mg/kg R121919, followed 60 min later by the administration of vehicle or 10 mg/kg flumazenil to precipitate withdrawal. Each *bar* represents mean±SEM; $n$=8–10 per group at each time point. *Double asterisk* $p$<0.01 vs the R121919-pretreated/flumazenil-withdrawn group at that time point by three-way ANOVA with repeated measures on the factor of time, followed by Student–Newman–Keuls post hoc pairwise analysis

pretreated/flumazenil-withdrawn rats until 120 min after precipitation of withdrawal (Fig. 2). The area under the curve representing the corticosterone response during the 2 h after flumazenil or vehicle administration demonstrated a pattern and direction of change similar to the ACTH results, although the magnitude of the intergroup differences was diminished (Fig. 3b). Pretreatment with R121919 before precipitated lorazepam withdrawal decreased the area under the curve by 21.3% compared to the vehicle-pretreated/flumazenil-withdrawn rats (21,880.2±2,934.2 ng min/ml vs 17,211.1±1,604.0 ng min/ml). This was approximately half of the magnitude of attenuation produced with respect to ACTH. In nonwithdrawn rats, the corticosterone response to the i.p. injection stress alone was decreased by only 9.3% as a result of pretreatment with the $CRF_1$ antagonist (16,412.8±3,174.0 ng min/ml vs 14,888.8±3,189.6 ng min/ml).

### Experiment 2—behavior in the defensive withdrawal paradigm

Results obtained by two-way ANOVA indicated significant effects for the interaction between R121919 administration and flumazenil administration on latency to exit the tube ($F_{1,23}$=9.27, $p$<0.01), total time spent withdrawn in the tube ($F_{1,23}$=18.98, $p$<0.01), and on the number of four paw transitions into the open field ($F_{1,23}$=17.00, $p$<0.01).

During a 15-min testing session in the defensive withdrawal paradigm, latency to exit the tube was lowest in the vehicle-pretreated/nonwithdrawn rats and was markedly increased by the administration of flumazenil to precipitate lorazepam withdrawal (156.0±87.3 vs 785.1±

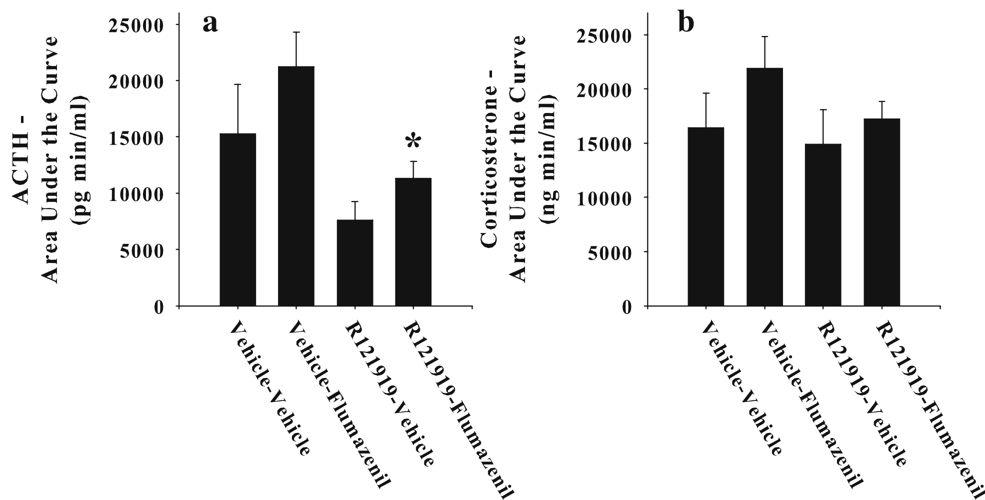


**Fig. 3** The area under the curve for the ACTH response to flumazenil-precipitated lorazepam withdrawal is decreased by pretreatment with the $CRF_1$ antagonist R121919. Each *bar* represents mean±SEM; $n$=8–10 per group. **a** The area under the curve for the ACTH response to flumazenil-precipitated withdrawal (Fig. 1). *Asterisk* $p$<0.05 vs vehicle–flumazenil group by two-way ANOVA followed by Student–Newman–Keuls post hoc pairwise analysis. **b** The area under the curve for the corticosterone response to flumazenil-precipitated lorazepam withdrawal (Fig. 2). The differences between these groups were not statistically significant following analysis by two-way ANOVA

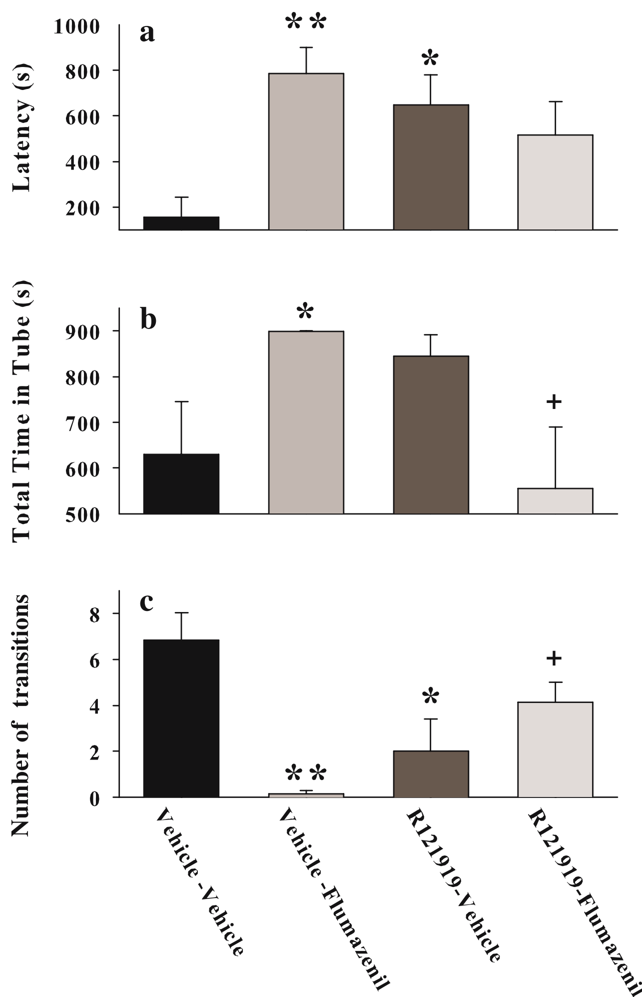


**Fig. 4** Pretreatment with the $CRF_1$ antagonist R121919 decreases the total time spent in defensive withdrawal after flumazenil-precipitation of lorazepam withdrawal. Each *bar* represents mean±SEM; $n=6$ in the vehicle–vehicle group and $n=7$ in all other groups. **a** Five minutes after the administration of vehicle or flumazenil to lorazepam dependent rats, the latency (s) to exit the tube upon placement into the open field was scored by analysis of the videotaped defensive withdrawal session. *Asterisk* $p<0.05$; *double asterisk* $p<0.01$ vs vehicle-pretreated/nonwithdrawn group. **b** Five minutes after the administration of vehicle or flumazenil to lorazepam dependent rats, the total time spent in defensive withdrawal in the tube (s) during the 15 min testing session was scored by analysis of the videotaped defensive withdrawal session. *Asterisk* $p<0.05$ vs vehicle-pretreated/nonwithdrawn group. *Plus sign* $p<0.05$ vs vehicle-pretreated/flumazenil-withdrawn and R121919-pretreated/nonwithdrawn groups. **c** Five minutes after the administration of vehicle or flumazenil to lorazepam dependent rats, the number of four-paw transitions into the open field during the 15 min testing session was scored by analysis of the videotaped defensive withdrawal session. *Asterisk* $p<0.05$, *double asterisk* $p<0.01$ vs vehicle-pretreated/nonwithdrawn group; *plus sign* $p<0.05$ vs vehicle-pretreated/flumazenil-withdrawn group

114.9 s, $p<0.01$; Fig. 4a). Pretreatment with R121919, 60 min before the administration of flumazenil, decreased latency to exit by 34.1% (517.4±145.2 s, n.s.).

On the measure of total time spent in the tube, the vehicle-pretreated/nonwithdrawn rats spent a total of 629.5±115.1 s, out of a possible 900 s, withdrawn in the tube (Fig. 4b). The addition of flumazenil to precipitate lorazepam withdrawal increased anxiety in this paradigm to the extent that only one of the seven rats in this group ever exited the tube, and this rat entered the open field for less than 10 s (899.0±1.0, $p<0.05$ vs vehicle-pretreated/nonwithdrawn rats). Pretreatment with R121919 before the precipitation of lorazepam withdrawal reduced the total time in tube to a level even lower than that observed in the vehicle-pretreated/nonwithdrawn rats (555.6±134.5 s; $p<0.05$ vs vehicle-pretreated/flumazenil-withdrawn rats).

The vehicle-pretreated/nonwithdrawn rats demonstrated an average of 6.8±1.2 four paw transitions into the open field during the 15-min experiment (Fig. 4c). In comparison, the number of four paw transitions into the open field was significantly decreased after flumazenil-precipitated withdrawal (0.143±0.1, $p<0.001$ vs vehicle-pretreated/nonwithdrawn rats). It is difficult to determine if this sharp decrease was noted because the withdrawal-induced anxiety caused these rats to remain in the tube throughout the experiment or because of the decreased motor activity associated with increased muscle tone (Ryan and Boisse 1983), which is an additional symptom of BZ withdrawal. Pretreatment with R121919 before the administration of flumazenil increased the number of transitions to a level similar to that observed in the vehicle-pretreated/nonwithdrawn rats (4.1±1.6; $p<0.05$ vs vehicle-pretreated/flumazenil-withdrawn). Whereas the number of transitions into the open field is generally considered to be a control for locomotor activity, independent of the anxiety level of the rat, an animal that is so anxious as to never fully exit the tube will record no transitions, as occurred in the majority of the vehicle-pretreated/flumazenil-withdrawn rats. Therefore, at least in this study, there appears to be an interaction between these factors with respect to transitions.

### CRF hnRNA expression

CRF hnRNA expression was measured as an early marker of stress-induced CRF gene expression in the PVN. Induction of CRF hnRNA expression was not detectable in the PVN of rats that did not receive flumazenil to precipitate withdrawal. In contrast, 30 min after flumazenil administration, CRF hnRNA expression was apparent in the PVN of each rat examined. Pretreatment with R121919, 60 min before flumazenil injection, decreased CRF hnRNA expression by 32.2% (30.7±2.6 vs 20.8±2.5 nCi/g; $p<0.05$; Fig. 5).

## Discussion

Several studies have provided evidence that increased CRF release contributes to the anxiety and aversive states produced by drug withdrawal and that activation of CRF neuronal

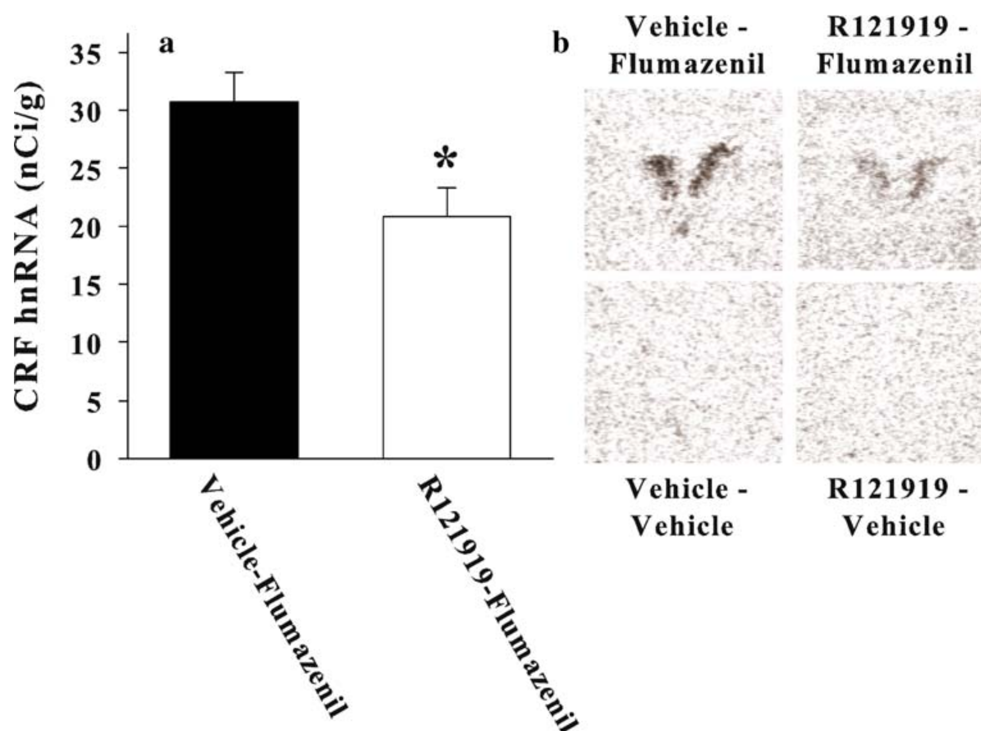


**Fig. 5** Pretreatment with the $CRF_1$ antagonist R121919 decreases CRF hnRNA expression in the PVN after flumazenil-precipitation of lorazepam withdrawal. **a** CRF hnRNA expression was measured via densitometric analysis subsequent to in situ hybridization performed in 20 um brain slices from male Sprague–Dawley rats, 30 min after the administration of vehicle or 10 mg/kg flumazenil to precipitate withdrawal in lorazepam dependent rats. CRF hnRNA expression was not detectable in the PVN of rats that did not receive flumazenil to precipitate withdrawal. Each *bar* represents mean±SEM; $n=7$. One to three slices were quantified per rat. *Asterisk* $p<0.05$ vs vehicle-pretreated/flumazenil-withdrawn group by two-tailed $t$ test. **b** Representative images of CRF hnRNA expression in the PVN after in situ hybridization

systems may be a common neurobiological mechanism of withdrawal from drugs of abuse. In these experiments, we demonstrate that pretreatment of rats with a $CRF_1$ receptor antagonist significantly attenuates the HPA axis activation, anxiogenic behavior, and induction of CRF hnRNA in the PVN that are produced as a consequence of flumazenil-precipitated lorazepam withdrawal. These results suggest that it is the $CRF_1$ receptor subtype that plays a major role in mediating these effects of CRF on neuroendocrine and behavioral responses during BZ withdrawal.

Consistent with our previous studies (Owens et al. 1991; Skelton et al. 2000), elevated concentrations of ACTH and corticosterone provide evidence that the HPA axis was activated as a consequence of BZ withdrawal. Similar results have been reported after cocaine, ethanol, and morphine withdrawal, demonstrating that activation of the endocrine stress response is a common element of withdrawal from drugs that produce physiological dependence (Keith et al. 1983; Roberts et al. 1992; Milanes et al. 1998). Pretreatment of lorazepam-dependent rats with the $CRF_1$ antagonist R121919 before the precipitation of withdrawal by the BZ antagonist, flumazenil, attenuated the HPA axis activation that was produced during BZ withdrawal by decreasing the amplitude of the maximal ACTH response and the duration of corticosterone elevation. This differential time course of the effect of R121919 on ACTH vs corticosterone is consistent with the known activation pattern of the rat HPA axis in response to acute stress, in which ACTH peaks rapidly (typically within 10–15 min) and thereafter declines (typically by 20 min), leading to a sustained elevation in corticosterone concentrations, which frequently persist for 60 min or more (Walberg et al. 2005).

The anxiogenic behavioral response to precipitation of BZ withdrawal, as indicated by an increased latency to exit the tube and the total time spent withdrawn in the tube in the defensive withdrawal test, was diminished by pretreatment with the $CRF_1$ antagonist R121919. A surprising finding was that the latency to exit the tube observed in the BZ-dependent rats treated with R121919, but not precipitated into BZ-withdrawal by flumazenil, was higher than that observed in the vehicle-pretreated/nonwithdrawn rats, which did not receive pretreatment with R121919. $CRF_1$ receptor antagonists have been generally demonstrated in multiple studies to be anxiolytic (Schulz et al. 1996; Heinrichs et al. 1997b; Griebel et al. 1998; Skutella et al. 1998; Smith et al. 1998; Timpl et al. 1998; Liebsch et al. 1999; Okuyama et al. 1999). Furthermore, our laboratory has previously documented that R121919 dose-dependently decreased behavioral evidence of anxiety in this same

paradigm, as evidenced by latency to exit the tube and total time spent in the tube, 60 min after a single subcutaneous administration to rats (Gutman et al. 2003). Additionally, as the order in which the rats were tested was randomized, it is unlikely that a random stressor or variance in the testing environment produced nonrandomly distributed error. It is possible that our current finding is isolated to this data set and not reflective of an underlying psychopharmacologic effect of R121919. Likewise, it is possible that this apparent anxiogenic effect of R121919 in this study may occur in the context of a background of BZ-dependence (which our laboratory has previously reported produces decreases in indices of $CRF_1$ receptor mRNA expression and binding (Skelton et al. 2000)), although we are aware of no direct data to suggest this. An additional potential explanation for this finding is that $CRF_1$ antagonist itself may have a mild sedative effect or produce a decrease in muscle tone, which is overcome in the rats pretreated with this compound and then later precipitated into benzodiazepine withdrawal with flumazenil. The decrease in the number of transitions observed in the nonbenzodiazepine-withdrawn, R121919-pretreated rats is consistent with this explanation. Further interpretation of this finding would be speculative until this data set has been replicated.

In studies of several drugs that produce abuse and dependence, CRF neuronal systems have consistently been demonstrated to be activated as a result of withdrawal. There is evidence of HPA axis activation, increased CRF concentrations in CSF, and increased CRF mRNA expression and/or peptide release in the amygdala temporally correlated with the disappearance of the drug from the blood during withdrawal from cannabis, cocaine, and ethanol (Pich et al. 1995; Rodriguez de Fonseca et al. 1997; Richter and Weiss 1999; McNally and Akil 2002; Maj et al. 2003; Zhou et al. 2003). In fact, CRF concentrations in the amygdala have been reported to remain elevated throughout the time during which the behavioral symptoms of the withdrawal syndromes are prominent.

Because reducing CRFergic neurotransmission with the $CRF_1$ antagonist diminishes the severity of the BZ withdrawal syndrome, this heightened CRF activity appears to be a causal element in the generation of these adverse symptoms. Furthermore, because similar patterns of increased CRF activity are observed after withdrawal from a number of drugs that produce physiological dependence (Pich et al. 1995; Sarnyai et al. 1995; Rodriguez de Fonseca et al. 1997; Milanes et al. 1998; Richter and Weiss 1999; McNally and Akil 2002; Maj et al. 2003; Zhou et al. 2003) and in humans withdrawn from ethanol (Adinoff et al. 1996), the effectiveness of the $CRF_1$ antagonist may generalize to withdrawal from other drugs of abuse and be of therapeutic utility in the treatment of drug withdrawal syndromes in human populations.

The $CRF_1$ antagonist R121919 also attenuated the induction of CRF hnRNA expression in the PVN as a result of lorazepam withdrawal. CRF, acting presumably through a $CRF_1$ receptor-dependent mechanism within the PVN, increases the transcription of its own mRNA in this region (Imaki et al. 1996). By interrupting this positive feedback loop, the $CRF_1$ antagonist can not only prevent existing CRF from activating the $CRF_1$ receptor but also prevent the increased production of newly synthesized CRF that is induced during lorazepam withdrawal, which may be necessary to sustain a heightened stress-response and/or drug-withdrawal reaction over a longer duration.

Because CRF neuronal systems are reliably activated by stress and serve to coordinate the global mammalian stress response, it is not readily apparent whether CRF neurons are simply activated as a consequence of the physiological and psychological stress associated with drug withdrawal or whether they have a more specific role in the causation of at least a subset of the specific symptoms of drug withdrawal. Several previous studies provide support for the latter assertion. Pretreatment with a systemically administered $CRF_1$ antagonist or with a nonselective peptide CRF antagonist administered centrally or locally into the central nucleus of the amygdala (CeA) decreases several of the autonomic and behavioral signs evoked during precipitated morphine withdrawal (Brugger et al. 1998; Iredale et al. 2000; McNally and Akil 2002). Furthermore, reducing the effectiveness of CRFergic neurotransmission in the CeA by either local injection of a nonselective CRF antagonist or by immunotoxin-mediated CRF depletion diminishes the aversive stimulus properties of the negative emotional state produced by morphine withdrawal as utilized in conditioning paradigms (Heinrichs et al. 1995). The effectiveness of CRF antagonists for reducing certain of the symptoms manifested during withdrawal generalizes to other classes of drugs, as well. The anxiety that is produced as a consequence of ethanol withdrawal is attenuated by injection of a CRF antagonist directly into the CeA at a 100-fold lower dose than that required for effectiveness when delivered via the intracerebroventricular route (Baldwin et al. 1991; Rassnick et al. 1993). Mice that are lacking a functional $CRF_1$ receptor also show decreased anxiety as a result of ethanol withdrawal in a light–dark transition test (Timpl et al. 1998). In addition, blocking CRF neuronal activity by central pretreatment with anti-CRF antiserum prevents the induction of anxiety in the elevated plus maze during cocaine withdrawal (Sarnyai et al. 1995). Herein, we demonstrate that anxiety, as measured in the defensive withdrawal behavioral paradigm, which is a core component of drug withdrawal responses in both humans (Gawin and Kleber 1986) and animal models (Baldwin et al. 1989, 1991; Rassnick et al. 1993; Sarnyai et al. 1995), is significantly attenuated by pretreatment with the selective

$CRF_1$ antagonist R121919, likely due to a reduction in $CRF_1$ receptor-mediated neurotransmission within the amygdala before the precipitation of lorazepam withdrawal. These findings provide more evidence that CRF neuronal systems are not only activated as a consequence of the stress of drug withdrawal but also are causally involved in the mediation of certain of the behavioral symptoms of the withdrawal.

In summary, our results demonstrate that pretreatment with the $CRF_1$ antagonist R121919 before precipitation of withdrawal in lorazepam dependent rats reduces withdrawal-induced HPA axis activation, CRF hnRNA expression in the PVN, and behavioral evidence of anxiety in the defensive withdrawal paradigm. These findings implicate activation of CRF neuronal systems, acting via the $CRF_1$ receptor, as an etiological factor in the endocrine and behavioral manifestations of BZ withdrawal in the rat. Given the symptomatic similarity to the BZ withdrawal syndrome in humans, as well as evidence of increased CRF release, $CRF_1$ antagonists are likely to be of future clinical utility in treating BZ withdrawal.

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