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The CRF_1 receptor antagonist, R121919, attenuates the severity of precipitated morphine withdrawal

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Abstract

Corticotropin-releasing factor (CRF) regulates the hypothalamic–pituitary–adrenal axis, coordinates the mammalian stress response, and acting primarily *via* the CRF_1 receptor, has been strongly implicated in the pathophysiology of depression and anxiety. Furthermore, the behavioral and autonomic activation that occurs following withdrawal in drug dependent animals resembles the mammalian stress response. Concordant with this view is evidence of enhanced CRF transcription, release and activity following withdrawal from several drugs of abuse. Conversely, CRF receptor antagonists have been demonstrated to reduce the severity of many drug withdrawal symptoms, implicating a specific role for activation of CRF neurons in mediating the anxiogenic and stress-like reactions observed during withdrawal. To extend these findings, we investigated whether pretreatment with a selective CRF_1 receptor antagonist, R121919, is capable of similarly decreasing the autonomic, behavioral and neuroendocrine activation observed following precipitation of morphine withdrawal in dependent rats. The results indicate that pretreatment with R121919 attenuates the global severity of the precipitated morphine withdrawal syndrome as measured by the Gellert–Holtzman scale. In addition, rats pretreated with R121919 prior to precipitation of morphine withdrawal demonstrated decreased hypothalamic–pituitary–adrenal axis activation, as measured by plasma ACTH concentrations, and decreased early expression of the CRF gene in the paraventricular nucleus of the hypothalamus, as measured by CRF heteronuclear RNA. These findings suggest that activation of CRF neuronal systems *via* the CRF_1 receptor may be one element of the neurobiological mechanisms activated during drug withdrawal and that CRF_1 receptor antagonists may have a potential therapeutic role in the treatment of human drug withdrawal syndromes.

Keywords: Anxiety; Corticotropin-releasing factor (CRF); Drug withdrawal; Opiate; Stress

1. Introduction

The neuropeptide, corticotrophin-releasing factor (CRF), mediates the endocrine response to stress *via* its actions as the major physiological regulator of the hypothalamic–pituitary–adrenal axis. Substantial evidence has accumulated to support the hypothesis that CRF additionally functions as a neurotransmitter in extrahypothalamic limbic structures and brainstem nuclei, and serves to mediate the behavioral and autonomic arms of the stress response in coordination with the endocrine response. In addition to this key role, there is a considerable evidence implicating CRF in the pathophysiology of mood and anxiety disorders (Risbrough and Stein, 2006).

Significant data has also accrued to indicate that activation of CRF neuronal systems is involved in the withdrawal syndromes from a variety of drugs that produce physiological dependence, as this well-studied phenomenon is characterized by anxiety and autonomic activation that in many ways resembles a mammalian stress response. After the chronic administration of benzodiazepines, cocaine, ethanol, or morphine is discontinued there is evidence of heightened hypothalamic–pituitary–

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adrenal axis activity, as measured by plasma adrenocorticotropic hormone (ACTH) and corticosterone concentrations, CRF mRNA expression in the paraventricular nucleus of the hypothalamus, or c-*fos* mRNA induction in CRF-containing neurons in the paraventricular nucleus of the hypothalamus (Keith et al., 1983; Owens et al., 1991; Roberts et al., 1992; Milanes et al., 1998; Hamlin et al., 2004). In addition to stress-induced activation of the neuroendocrine axis, findings of increased CRF mRNA transcription and/or peptide release within the amygdala (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), and/or increased CRF concentrations in the cerebrospinal fluid (CSF; Adinoff et al., 1996) following withdrawal from cannabis, cocaine, ethanol, or morphine indicate increased central CRF neuronal activity.

These results suggest that activation of CRF neuronal systems may be common to withdrawal from drugs of abuse. If this elevated CRF activity is a causal element in the affective and autonomic symptoms of drug withdrawal, then blocking CRF neurotransmission with a CRF receptor antagonist should diminish the withdrawal syndrome. This hypothesis is supported by reports that withdrawal-induced anxiety, following chronic cocaine or ethanol administration, is diminished by pretreatment with a CRF receptor antagonist or CRF antiserum (Baldwin et al., 1991; Rassnick et al., 1993; Sarnyai et al., 1995; Basso et al., 1999). Recent data from our laboratory has indicated that pretreatment with the CRF_1-selective receptor antagonist, 2,5-dimethyl-3-(6-dimethyl-4-methylpyridin-3-yl)-7-dipropylamino-pyrazolo[1,5-*a*]pyrimidine (R121919), attenuates hypothalamic–pituitary–adrenal axis activation, induction of CRF heteronuclear RNA expression in the paraventricular nucleus of the hypothalamus, and behavioral evidence of anxiety following withdrawal from the benzodiazepine, lorazepam (Skelton et al., 2007).

Following morphine withdrawal, in particular, there is significant evidence that pretreatment with CRF receptor antagonists (both non-selective and CRF_1-selective) reduces 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). Further, Contarino and Papaleo (2005) demonstrated that mice bred to lack the CRF_1 receptor did not develop conditioned place aversion associated with the negative affective state of spontaneous morphine withdrawal. Conversely, pretreatment with a peptidergic CRF_2-selective receptor antagonist was found to be largely ineffective (Lu et al., 2000). These findings imply a specific role for activation of CRF, acting *via* the CRF_1 receptor, in mediating the aversive behavioral and autonomic states produced during withdrawal from drugs of dependency, such as benzodiazepines, cocaine, ethanol and morphine. Based on this hypothesis, we sought to expand our previous findings on benzodiazepine withdrawal, by examining the efficacy of the selective CRF_1 receptor antagonist, R121919, in attenuating the morphine withdrawal syndrome as analyzed by a standardized rating system that incorporates behavioral and autonomic measures. In addition, we investigated the utility of R121919 in decreasing activation of the hypothalamic–pituitary–adrenal axis induced by morphine withdrawal, as measured by ACTH and corticosterone concentrations, as well as CRF heteronuclear RNA expression in the paraventricular nucleus of the hypothalamus, never before studied after morphine withdrawal.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats (225–250 g on arrival; Charles River Laboratories, Raleigh NC) were individually housed in an environmentally controlled animal facility on a 12 h light/dark cycle (lights on at 0730 h) with food and water available *ad libitum*. All animals were 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 Press, 1996) was followed.

2.2. Drug treatment

Morphine sulfate was fully dissolved in ddH_2O and placed into Alzet 2ML2 osmotic minipumps (Alza Corp., Palo Alta CA) at a concentration necessary to deliver 20 mg/kg/day of the free base, a dose commonly utilized in rat studies to induce physiologic dependence. In order to produce morphine dependence, the minipumps were subcutaneously implanted in the mid-scapular region under light methoxyfluorane anesthesia, as described in detail in Stout et al. (2002). After 7 days of morphine administration, each rat ($n=10$ per group) was assigned to one of four experimental groups as noted following. At the beginning of the testing, half of the rats were pretreated with the CRF_1 receptor antagonist, R121919 (5 mg/kg), while the other half received vehicle injections (50% polyethylene glycol 400 in ddH_2O). Sixty min later, morphine withdrawal was precipitated in half of each of these groups by administration of the opiate antagonist, naltrexone (0.1 mg/kg), while the other half received a vehicle injection (0.9% NaCl in ddH_2O). Injections of R121919, naltrexone, or their respective vehicles were made subcutaneously into the flank in an injection volume of 1 ml/kg. All injections were made between 0800 and 1200.

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 subcutaneous 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). The dose of naltrexone (0.1 mg/kg) was chosen as it was demonstrated to produce a robust hypothalamic–pituitary–adrenal axis response, indicative of morphine withdrawal.

2.3. Withdrawal rating scale

Immediately after the injection of naltrexone or vehicle, the rats were taken in their home cages into a quiet, isolated room under moderate illumination and observed for 15 min by a rater

Table 1
Modified Gellert–Holtzman scale

Sign	Weighting factor
Graded signs:	
Weight loss (each 1.0% above the weight lost by control rats)	1.33
Number of abdominal constrictions	2 each
Number of wet dog shakes:	
°1–2	2
°3+	4
Checked signs:	
Diarrhea	2
Facial fasciculations or teeth chattering	2
Ptosis	2
Abnormal posture	3
Erection or ejaculation	3
Irritability	3

This scale provides a rating of the overall severity of the morphine withdrawal response and consists of a number of autonomic and behavioral signs which are either graded (counted for each occurrence) or checked (counted as positive if this sign occurs at any point during the observation period).

blinded to the rats' experimental treatment. Withdrawal signs were recorded and scored according to a modified version of the Gellert–Holtzman scale (Gellert and Holtzman, 1978; Table 1) in order to estimate the global severity of the morphine withdrawal response.

The graded signs were counted and scored for each occurrence during the observation period, while the checked signs were scored if present at any point during the observation period. Modifications were made to the original Gellert–Holtzman scale as follows. First, the original weighting factor for weight loss in this scale was 1.0 for weight loss occurring in 2.5 h after the precipitation of withdrawal. In this study, the rats were first weighed at $t=0$, just prior to the injection of naltrexone or vehicle, and again at $t=30$ min, just prior to decapitation. Because weight loss was evaluated during only the first 30 min after the precipitation of withdrawal in this study, the weighting factor was increased to compensate for the diminished weight loss recorded. The new value of 1.33 was assigned after preliminary studies demonstrated that approximately 75% of the maximum weight loss recorded at 2.5 h occurs within the first 30 min. Second, as certain signs (escape attempts, swallowing movements, profuse salivation, and chromodacryorrhea) were not observed by the raters in preliminary testing of naltrexone-precipitated withdrawal in morphine dependent rats, these were removed from the modified rating scale and not recorded during the observation period.

After the 15 min observation period, the rats were returned to the animal housing room for an additional 15 min. Precisely 30 min after the injection of naltrexone or vehicle, the rats were killed by decapitation, and trunk blood was collected on ice in EDTA-containing glass tubes (for plasma) and polycarbonate tubes (for serum). The blood was centrifuged at 5000 ×*g* for 10 min, and the plasma or serum supernatant stored at −80 °C until assay for ACTH or corticosterone levels by radioimmunoassay in plasma and serum, respectively. In addition, the brains were removed, collected on dry ice, and stored at −80 °C until cryostat sectioning.

2.4. ACTH and corticosterone radioimmunoassay

ACTH was measured in duplicate samples of rat plasma by a two-site immunoradiometric assay using the Allegro™ HS-ACTH kit (Nichols Institute, San Juan Capistrano, CA) with a coefficient of variation of 5% and sensitivity (blank±2 S.D.) of 1 pg/ml. Corticosterone was assayed in duplicate samples of rat serum using the ImmunoChem™ Double Antibody kit (ICN Biomedicals, Costa Mesa, CA) with a coefficient of variation of 6% and sensitivity (blank±2 S.D.) of 1.2 ng/ml.

2.5. 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.

2.6. Image analysis

Images from the *in situ* hybridization and receptor autoradiography films were digitized with a Dage-MTI CCD-72

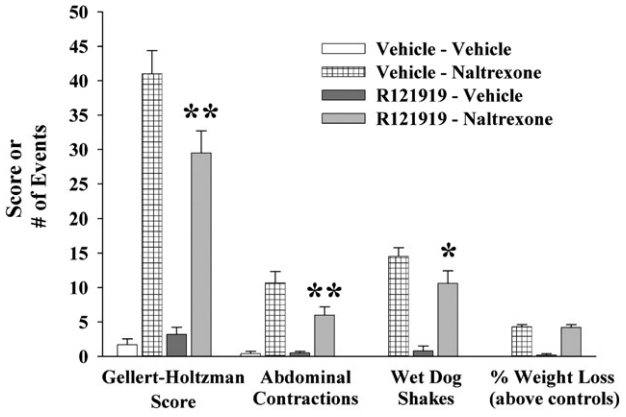

Fig. 1. Pretreatment with the CRF_1 receptor antagonist, R121919, attenuates many of the signs of naltrexone-precipitated morphine withdrawal. Each bar represents mean±S.E.M. $n=10$ per group. Percentage of weight loss (above controls) and the number of abdominal contractions and wet dog shakes were counted during a 15 min observation session that began immediately after the injection of saline or naltrexone (0.1 mg/kg) to precipitate withdrawal. After completion of the observation session, the Gellert–Holtzman score of the overall withdrawal severity was calculated according to Table 1. Naltrexone-precipitation of withdrawal caused a significant increase in all of these parameters — $P<0.001$ by two-way ANOVA followed by Student–Newman–Keuls *post-hoc* pairwise analysis. Abdominal contractions, wet dog shakes, and overall Gellert–Holtzman score were significantly decreased by pretreatment with R121919. $^{**}P<0.01$; $^{*}P<0.05$ *vs.* vehicle-naltrexone group by two-way ANOVA followed by Student–Newman–Keuls *post-hoc* pairwise analysis.

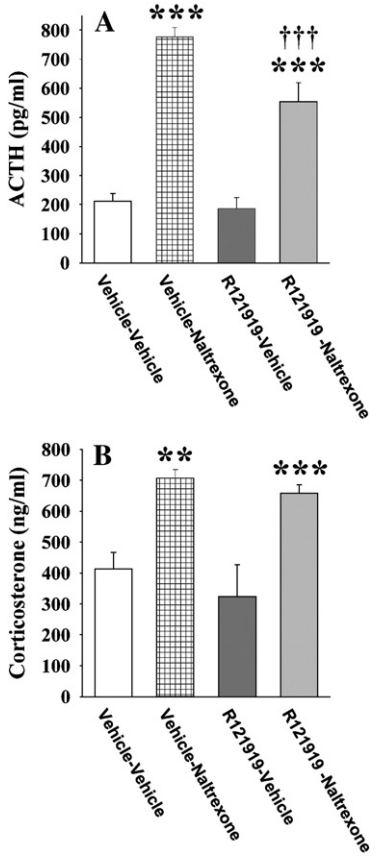

Fig. 2. Hypothalamic–pituitary–adrenal axis activity is increased 30 min after naltrexone-precipitation of morphine withdrawal. Each bar represents mean±S.E.M. $n=10$ per group. (A) Pretreatment with the CRF_1 receptor antagonist, R121919, decreases plasma ACTH following naltrexone-precipitation of morphine withdrawal. ACTH was measured in plasma by radioimmunoassay, 30 min after the administration of vehicle or 0.1 mg/kg naltrexone to precipitate withdrawal in morphine dependent rats. ***$P<0.001$ *vs.* the appropriate vehicle control (vehicle–vehicle or R121919–vehicle); †††$P<0.001$ *vs.* vehicle–naltrexone group by two-way ANOVA followed by Student–Newman–Keuls *post-hoc* pairwise analysis. (B) Serum corticosterone was measured by radioimmunoassay, 30 min after the administration of vehicle or 0.1 mg/kg naltrexone to precipitate withdrawal in morphine dependent rats. ***$P<0.001$ *vs.* R121919–vehicle group; **$P<0.01$ *vs.* vehicle–vehicle group by two-way ANOVA followed by Student–Newman–Keuls *post-hoc* pairwise analysis.

(Michigan City IN) image analysis system equipped with a Nikon camera. Semiquantitative analysis was performed as described in detail in Skelton et al., 2000.

2.7. Drugs

R121919 was a gift from Neurocrine Biosciences (San Diego, CA) and Janssen Pharmaceuticals (Titusville, NJ). Morphine sulfate was a gift from NIDA.

2.8. Statistics

With respect to ACTH and corticosterone concentrations, Gellert–Holtzman score, and graded signs of withdrawal, these results were analyzed by 2-way ANOVA (factors — pretreatment condition (±CRF_1 antagonist) and treatment condition (±opiate antagonist)) followed by Student–Newman–Keuls pairwise testing for *post-hoc* analysis. Between groups comparisons of the checked signs of morphine withdrawal were made *via* exact logistic regression (Mehta and Patel, 1995). Because only minimal CRF heteronuclear RNA expression was observed in the paraventricular nucleus of the hypothalamus of rats which did not received naltrexone (with a single exception), comparisons of this parameter were made between the naltrexone-treated rats only by means of a two-tailed *t*-test. All data are expressed as means±S.E.M.

3. Results

3.1. Gellert–Holtzman scale for precipitated withdrawal signs

As expected, behaviors rated as withdrawal signs were observed primarily in the rats treated with naltrexone to precipitate withdrawal. The Gellert–Holtzman score increased from 2.5±0.7 in the total vehicle-treated rats to 35.3±2.6 ($P<0.001$) in the total naltrexone-treated rats. The low, but measurable, score in the non-withdrawn rats was due to the occasional observation of an abdominal contraction or wet dog shake, as well as the sporadic appearance of facial fasciculations, ptosis, genital grooming, or irritability. These latter checked signs were only noted in the R121919-pretreated rats. However, in the non-withdrawn rats, there was no significant difference in the Gellert–Holtzman score between the R121919-pretreated *vs.* vehicle-pretreated rats (3.2±1.0 *vs.* 1.7±0.8; $P=0.664$; Fig. 1), suggesting that injection of morphine dependent rats with R121919 in the absence of withdrawal did not alter the spontaneous appearance of these scored behavioral signs.

Pretreatment with R121919 significantly decreased by 28.0% (41.0±3.4 *vs.* 29.5±3.2; $P<0.01$; Fig. 1), but did not eliminate, the overall severity of the morphine withdrawal

Table 2
Checked signs of morphine withdrawal

Withdrawal sign	Vehicle–vehicle	Vehicle–naltrexone	R121919–vehicle	R121919–naltrexone
Diarrhea	0%	100%[c]	0%	90%[c]
Facial fasciculations	0%	80%[c]	20%	90%[b]
Ptosis	0%	90%[c]	20%	60%
Abnormal posture	0%	50%[a]	0%	70%[b]
Erection, ejaculation, or genital grooming	0%	50%[a]	10%	20%
Irritability	0%	100%[c]	10%	80%[b]

Each of the listed signs was checked if present in a given rat at any point during the 15 min observation session. The data was then converted to the percentage of rats in a particular experimental group which displayed that sign during the observation period. As expected, the administration of naltrexone to precipitate morphine withdrawal produced significant increases in the proportion of rats demonstrating the checked signs of morphine withdrawal ([a]$P<0.05$, [b]$P<0.01$, [c]$P<0.001$ *vs.* the appropriate control group — *i.e.* R121919–vehicle *vs.* R121919–naltrexone and vehicle–vehicle *vs.* vehicle–naltrexone). Within the naltrexone-treated rats, there were no statistically significant differences in the proportion of rats which demonstrated the checked signs of morphine withdrawal produced by pretreatment with R121919 *vs.* vehicle-pretreatment at the $P<0.05$ level using an exact logistic regression model.

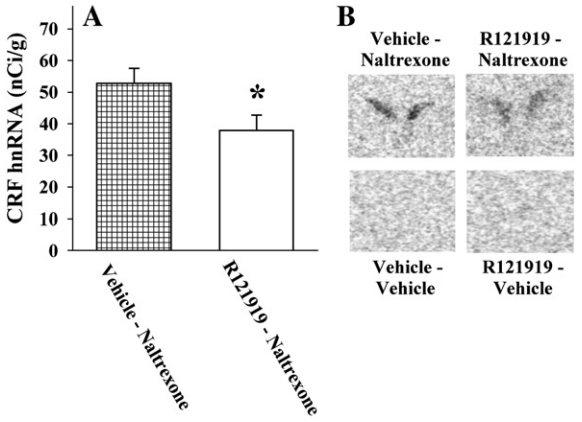

Fig. 3. Pretreatment with the CRF_1 receptor antagonist, R121919, decreases CRF heteronuclear RNA expression in the paraventricular nucleus of the hypothalamus following naltrexone-precipitation of morphine withdrawal. (A) CRF heteronuclear RNA expression was measured *via* densitometric analysis subsequent to *in situ* hybridization performed in 20 μm brain slices from male Sprague–Dawley rats, 30 min after the administration of vehicle or 0.1 mg/kg naltrexone to precipitate withdrawal in morphine dependent rats. CRF heteronuclear RNA expression was not detectable in the paraventricular nucleus of the hypothalamus of rats which did not receive naltrexone to precipitate withdrawal (with the exception of one single rat in the 'R121919–vehicle' group). Each bar represents mean±S.E.M.; $n=10$ per group. 1–3 slices were quantified per rat. ***$P<0.001$ *vs.* vehicle–naltrexone group by two-way ANOVA followed by Student–Newman–Keuls *post-hoc* pairwise analysis. (B) Representative images of CRF heteronuclear RNA expression in the paraventricular nucleus of the hypothalamus following *in situ* hybridization.

syndrome. With respect to the graded signs, the mean number of abdominal contractions was decreased by 43.9% (10.7±1.6 *vs.* 6.0±1.2; $P<0.01$) and wet dog shakes decreased by 26.9% (14.5±1.3 *vs.* 10.6±1.8; $P<0.05$). There was no statistically significant change in weight loss (4.3±0.3% *vs.* 4.2±0.4% above control). With respect to the checked signs, the number of rats per group with diarrhea was decreased by 10.0%, ptosis was decreased by 33.3%, erection and genital grooming were decreased by 60%, and irritability was decreased by 20% (Table 2). However these differences did not reach statistical significance at the $P<0.05$ level.

3.2. Hypothalamic–pituitary–adrenal axis activation and CRF heteronuclear RNA expression

Thirty min after the precipitation of morphine withdrawal, the hypothalamic–pituitary–adrenal axis was activated, as evidenced by increased concentrations of plasma ACTH (199.1±22.4 pg/ml in total non-withdrawn rats *vs.* 665.1±44.1 pg/ml in total morphine-withdrawn rats; $P<0.001$) and serum corticosterone (330.6±45.4 ng/ml *vs.* 682.2±19.6 ng/ml; $P<0.001$) in the withdrawn rats. These relatively elevated plasma concentrations for both ACTH and corticosterone in the non-withdrawn rats (as compared to expected basal values of <20 pg/ml or ng/ml, respectively) indicate hypothalamic–pituitary–adrenal axis stimulation in these rats as well, likely secondary to both the acute stress of the two subcutaneous injections received in this experimental design and to the expected increase in basal ACTH and corticosterone concentrations associated with chronic administration of morphine (Houshyar et al., 2001). Pretreatment with R121919 significantly attenuated the plasma ACTH response to precipitated morphine withdrawal (552.9±66.5 pg/ml *vs.* 777.3±31.4 pg/ml; $P<0.001$; Fig. 2A), whereas, at the 30 min timepoint, the decrease in corticosterone concentrations was not statistically significant (707.4±27.1 ng/ml *vs.* 657.1±27.5 ng/ml; $P=0.565$; Fig. 2B).

CRF heteronuclear RNA expression in the paraventricular nucleus of the hypothalamus was quantified as an additional measure of hypothalamic–pituitary–adrenal axis activity, as well as an index of the recruitment/activation of CRF neuronal systems during the acute phase of precipitated morphine withdrawal. Unlike CRF mRNA, which does not begin to rise until 60–120 min after the onset of a stressor, CRF heteronuclear RNA begins to rise within 5 min after stress and peaks within 15–30 min, making this a more useful measure of activation of CRF gene expression for the current experimental design. CRF heteronuclear RNA expression in the paraventricular nucleus of the hypothalamus was not apparent by *in situ* hybridization in any of the non-withdrawn rats, with the exception of one single R121919-pretreated, non-withdrawn rat. In contrast, CRF heteronuclear RNA expression was noted in the paraventricular nucleus of the hypothalamus of every rat that received a naltrexone injection to precipitate withdrawal. Pretreatment with R121919 significantly attenuated this withdrawal-precipitated induction of CRF heteronuclear RNA expression by 28.3% (52.7±4.7 nCi/g *vs.* 37.8±4.8 nCi/g; $P<0.01$; Fig. 3A).

4. Discussion

The results of this experiment indicate that pretreatment with the CRF_1 receptor antagonist, R121919, significantly attenuates many of the behavioral signs of naltrexone-precipitated morphine withdrawal in Sprague–Dawley rats. In addition, withdrawal-induced activation of the hypothalamic–pituitary–adrenal axis was diminished by R121919, as evidenced by reductions in ACTH release and CRF heteronuclear RNA expression in the paraventricular nucleus of the hypothalamus. Taken together with the results of our previous study (Skelton et al., 2007) which demonstrated the efficacy of R121919 in decreasing the severity of the benzodiazepine withdrawal syndrome, these findings suggest that activation of CRF-mediated neurotransmission *via* the CRF_1 receptor may play a significant role in the complex interplay of biological mechanisms that produce the adverse symptoms of benzodiazepine or morphine withdrawal. Therefore, CRF_1 receptor antagonists may represent a novel class of compounds with generalized therapeutic utility in treating the withdrawal syndromes that develop upon abstinence from a number of drugs that produce physiological dependence.

Because plasma ACTH and corticosterone concentrations, as well as CRF heteronuclear RNA expression, all peak at or near 30 min after the onset of stress, it was at this timepoint after the administration of naltrexone that hypothalamic–pituitary–adrenal axis activation was examined in order to gauge the neuroendocrine response to withdrawal and to reflect the degree

of stress experienced by the rats as a result of withdrawal. As in our prior study of lorazepam withdrawal, ACTH, but not corticosterone concentrations, was found to be decreased at this time. One possible reason for this discrepancy between the levels of the two hormones is that ACTH release occurs more proximally in the hypothalamic–pituitary–adrenal axis to the blockade of CRF_1 receptors. Thus, ACTH concentrations are likely to be more sensitive than corticosterone to CRF_1 receptor antagonist treatment. Furthermore, because each pulse of ACTH leads to the release of considerably greater quantities of corticosterone from the adrenal cortex, serum corticosterone concentrations will likely be attenuated to a smaller degree than plasma ACTH concentrations. Also, if the corticosterone response to morphine withdrawal follows the same course as it did in our prior study of lorazepam withdrawal, then R121919 does not diminish peak levels of corticosterone, but instead causes a more rapid return to baseline levels. At 30 min, corticosterone levels remained elevated in both groups, but a significant difference became apparent at a later time point ($t=90$ min) when the R121919 pretreated rats returned to baseline corticosterone levels before the vehicle-pretreated rats (Skelton et al., 2007). An additional possibility is that non-CRF-dependent, peripheral mechanisms may be activated during morphine withdrawal to increase adrenal secretion of corticosterone.

Similar to results obtained in our prior study of lorazepam withdrawal, R121919 attenuated the withdrawal-induced increase in CRF heteronuclear RNA expression in the paraventricular nucleus of the hypothalamus. Presumably, this effect is a result of reducing the demonstrated ability of CRF to increase the transcription of its own mRNA in the paraventricular nucleus of the hypothalamus through a CRF_1 receptor-dependent mechanism (Imaki et al., 1996). By interrupting this positive feedback loop, R121919 not only prevents existing CRF from activating the CRF_1 receptor, but also inhibits the increased production of newly synthesized CRF during morphine withdrawal, which may be necessary to sustain a heightened stress response and/or drug withdrawal reaction over a longer duration.

In order to mediate its effects on the multiple morphine withdrawal signs observed in this experiment, it is probable that R121919 acts not only in the paraventricular nucleus of the hypothalamus, but at other central nervous system sites, as well. R121919 is readily absorbed across the blood–brain barrier after systemic administration and can reach CRF_1 receptor populations located in many different sites in the brain. One site likely involved in mediating the effects of CRF on opiate withdrawal is the amygdala. Microdialysis studies have revealed that CRF release is increased in the amygdala by up to 400% during the peak symptomatic period of withdrawal from ethanol, cannabis, or cocaine (Pich et al., 1995; Rodriguez de Fonseca et al., 1997; Richter and Weiss, 1999). During morphine withdrawal, CRF mRNA expression has also been demonstrated to be increased in the central nucleus of the amygdala (McNally and Akil, 2002). Furthermore, reducing CRF activity in the amygdala *via* immunotargeted CRF depletion attenuates conditioned place aversion associated with morphine withdrawal (Heinrichs et al., 1995). Similar anxiolytic effects of CRF receptor antagonists have been noted following bilateral CRF receptor antagonist injection into the central nucleus of the amygdala prior to ethanol withdrawal (Rassnick et al., 1993). These findings suggest that antagonism of CRF_1 receptors in the amygdala may be important for decreasing the aversive emotional state and anxiety, as well as certain other of the stress-like symptoms that accompany morphine withdrawal.

Another neuroanatomical site in which the antagonism of CRF_1 receptors is likely to mediate attenuation of morphine withdrawal is the locus coeruleus. This major noradrenergic nucleus has been implicated by biochemical and pharmacological evidence to play the primary causal role in the expression of the autonomic and somatic manifestations of opiate withdrawal (Maldonado, 1997). Activation of the locus coeruleus during opiate withdrawal leads to the release of norepinephrine in forebrain areas on a time course that parallels the manifestation of the behavioral symptoms of withdrawal (Rasmussen et al., 1990). Increases in brain concentrations of the norepinephrine metabolite, 3-methoxy-4-hydroxyphenylglycol (MHPG), as well as the MHPG/norepinephrine ratio, which is reflective of norepinephrine turnover, are evident, as well (Roth et al., 1982; Swann et al., 1982). CRF is released from terminals in the vicinity of the locus coeruleus in response to certain physical and psychological stressors and acts as an excitatory neurotransmitter, leading to increase in locus coeruleus firing and forebrain norepinephrine turnover (Valentino et al., 1993). Furthermore, local application of CRF receptor antagonists can block these effects, indicating that in response to certain stressors, CRF can serve as the primary mediator of locus coeruleus activation (Conti and Foote, 1996; Smagin et al., 1997). In the case of opiate withdrawal, locus coeruleus activation is mediated principally by excitatory amino acid-containing afferent projections from the nucleus paragigantocellularis, as well as intrinsic upregulation of components of the intracellular cAMP signaling pathway (Duman et al., 1988; Ennis and Aston-Jones, 1988; Rasmussen and Aghajanian, 1989; Zhang et al., 1994; Maldonado, 1997). Although CRF is, therefore, not the principal mediator of locus coeruleus activation during morphine withdrawal, it is still probable that CRF is released into the locus coeruleus in response to the stress of withdrawal and contributes to the locus coeruleus activation and the consequent expression of the morphine withdrawal syndrome. By blocking the contribution of CRF to locus coeruleus activation, the resultant attenuation of locus coeruleus activity is reflected in an attenuation of the autonomic and behavioral signs of morphine withdrawal.

In summary, the ability of the CRF_1 receptor antagonist, R121919, to attenuate the behavioral and neuroendocrine manifestations of both morphine and lorazepam withdrawal indicates that this compound may have therapeutic potential in the treatment of drug withdrawal syndromes. This is in agreement with the results of several prior studies demonstrating the utility of CRF receptor antagonists in treating opiate withdrawal as measured by a variety of behavioral, autonomic, and hormonal indices (Milanes et al., 1998; Brugger et al., 1998; Iredale et al., 2000; Lu et al., 2000; Funada et al., 2001; McNally and Akil, 2002; Contarino and Papaleo, 2005). Taken together, these results suggest a potential utility of CRF_1 receptor antagonists in the treatment of human drug withdrawal syndromes and indicate a need for further scientific investigation. Future studies will be

necessary to more rigorously investigate the dose response curve of the CRF_1 receptor antagonist on morphine withdrawal, as well as the differential effects of acute *versus* chronic dosing of R121919. In addition, it would be of interest to examine the effect of R121919 on the overall kinetics of spontaneous morphine withdrawal, the more relevant comparator to the human condition.

5. Conflict of interest statement

Kelly Skelton, M.D. Ph.D., serves on the Speakers' Bureau of AstraZeneca, Bristol-Myers-Squibb, and Pfizer.

Michael J. Owens, Ph.D., has received speakers' honoraria from GlaxoSmithKline, has research grants from Pfizer, GlaxoSmithKline, Merck, Lundbeck, Cyberonics, and Johnson & Johnson, has served (or continues to serve) as consultant for Bristol-Myers-Squibb, Pfizer, Lundbeck, Sepracor, Johnson & Johnson, Sanofi-Avent, and Forest Labs, and has the following patent — "A method to estimate transporter occupancy" (provisional filing).

Charles B. Nemeroff, M.D. Ph.D., in the past 3 years, has consulted to, served on the Speakers' Bureau and/or Board of Directors, has been a grant recipient, and/or owned equity in one or more of the following: Abbott Laboratories, Acadia Pharmaceuticals, American Foundation for Suicide Prevention(AFSP), American Psychiatric Institute for Research and Educations (APIRE), AstraZeneca, BMC-JR LLC, Bristol-Myers-Squibb, CeNeRx, Corcept, Cypress Biosciences, Cyberonics, Eli Lilly, Entrepreneur's Fund, Forest Laboratories, George West Mental Health Foundation, GlaxoSmithKline, i3 DLN, Janssen Pharmaceutica, Lundbeck, National Alliance for Research on Schizophrenia and Depression (NARSAD), Neuronetics, NIMH, NFMH, NovaDel Pharma, Otsuka, Pfizer Pharmaceuticals, Quintiles, Reevax, UCB Pharma, Wyeth-Ayerst.

Currently, Dr. Nemeroff serves on the Scientific Advisory Board for AstraZeneca, Johnson & Johnson, Pharma Neuroboost, Forest Laboratories, Quintiles and NARSAD. He is a grant recipient from NIH, NARSAD and AFSP. He serves on the Board of Directors of AFSP, APIRE, NovaDel Pharmaceuticals and the George West Mental Health Foundation. He owns equity in CeNeRx and Reevax. He owns stock or stock options in Corcept, Cypress Biosciences and NovaDel.

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References

Adinoff, B., Anton, R., Linnoila, M., Guidotti, A., Nemeroff, C.B., Bissette, G., 1996. Cerebrospinal fluid concentrations of corticotropin-releasing hormone (CRH) and diazepam-binding inhibitor (DBI) during alcohol withdrawal and abstinence. Neruopsychopharmacology 15, 288–295.

Baldwin, H.A., Rassnick, S., Rivier, J., Koob, G.F., Britton, K.T., 1991. CRF antagonist reverses the "anxiogenic" response to ethanol withdrawal in the rat. Psychopharmacology 103, 227–232.

Basso, A.M., Spina, M., Rivier, J., Vale, W., Koob, G.F., 1999. Corticotropin-releasing factor antagonist attenuates the "anxiogenic-like" effect in the defensive burying paradigm but not in the elevated plus-maze following chronic cocaine in rats. Psychopharmacology 145, 21–30.

Brugger, S., Sanchez, R., Brugger, A.J., Martinez, J.A., 1998. Icv administration of CRF blocker (CRF_{9-41} α helical) reduces morphine withdrawal in rats. Prog. Neuropsychopharmacol. Biol. Psych. 22, 775–785.

Contarino, A., Papaleo, F., 2005. The corticotrophin-releasing factor receptor-1 pathway mediates the negative affective states of opiate withdrawal. Proc. Natl. Acad. Sci. U. S. A. 102, 18649–18654.

Conti, L.H., Foote, S.L., 1996. Reciprocal cross-desensitization of locus coeruleus electrophysiological responsivity to corticotropin-releasing factor and stress. Brain Res. 722, 19–29.

Duman, R.S., Tallman, J.F., Nestler, E.J., 1988. Acute and chronic opiate-regulation of adenylate cyclase in brain: specific effects in locus coeruleus. J. Pharmacol. Exp. Ther. 246, 1033–1039.

Ennis, M., Aston-Jones, G., 1988. Activation of locus coeruleus from nucleus paragigantocellularis: a new excitatory amino acid pathway in brain. J. Neurosci. 8, 3644–3657.

Funada, M., Hara, C., Wada, K., 2001. Involvement of corticotropin-releasing factor receptor subtype 1 in morphine withdrawal regulation of the brain noradrenergic system. Eur. J. Pharmacol. 430, 277–281.

Gellert, V.F., Holtzman, S.G., 1978. Development and maintenance of morphine tolerance and dependence in the rat by scheduled access to morphine drinking solutions. J. Pharmacol. Exp. Ther. 205, 536–546.

Gutman, D.A., Owens, M.J., Skelton, K.H., Thrivikraman, K.V., Nemeroff, C.B., 2003. The corticotropin-releasing $factor_1$ receptor antagonist R121919 attenuates the behavioral and endocrine responses to stress. J. Pharmacol. Exp. Ther. 304, 874–880.

Hamlin, A.S., Buller, K.M., Day, T.A., Osborne, P.B., 2004. Effect of naloxone-precipitated morphine withdrawal on c-*fos* expression in rat corticotropin-releasing hormone neurons in the paraventricular hypothalamus and extended amygdala. Neurosci. Lett. 362, 39–43.

Heinrichs, S.C., Menzaghi, F., Schulteis, G., Koob, G.F., Stinus, L., 1995. Suppression of corticotropin-releasing factor in the amygdala attenuates aversive consequences of morphine withdrawal. Behav. Pharmacol. 6, 74–80.

Houshyar, H., Galigniana, M.D., Pratt, W.B., Woods, J.H., 2001. Differential responsivity of the hypothalamic–pituitary–adrenal axis to glucocorticoid negative-feedback and corticotropin releasing hormone in rats undergoing morphine withdrawal: possible mechanisms involved in facilitated and attenuated stress responses. J. Neuroendocrinol. 13, 875–886.

Imaki, T., Naruse, M., Harada, S., Chikada, N., Imaki, J., Onodera, H., Demura, H., Vale, W., 1996. Corticotropin-releasing factor up-regulates its own receptor mRNA in the paraventricular nucleus of the hypothalamus. Brain Res. Mol. Brain Res. 38, 166–170.

Iredale, P.A., Alvaro, J.D., Lee, Y., Terwilliger, R., Chen, Y.L., Duman, R.S., 2000. Role of corticotropin-releasing factor receptor-1 in opiate withdrawal. J. Neurochem. 74, 199–208.

Keith, L.D., Crabbe, J.C., Robertson, L.M., Young, E.R., 1983. Ethanol dependence and the pituitary adrenal axis in mice. II. Temporal analysis of dependence and withdrawal. Life Sci. 33, 1889–1897.

Lu, L., Liu, D., Ceng, X., Ma, L., 2000. Differential roles of corticotropin-releasing factor receptor subtypes 1 and 2 in opiate withdrawal and in relapse to opiate dependence. Eur. J. Neurosci. 12, 4398–4404.

Maj, M., Turchan, J., Smialowska, M., Przewlocka, B., 2003. Morphine and cocaine influence on CRF biosynthesis in the rat central nucleus of amygdala. Neuropeptides 37, 105–110.

Maldonado, R., 1997. Participation of noradrenergic pathways in the expression of opiate withdrawal: biochemical and pharmacological evidence. Neurosci. Biobehav. Rev. 21, 91–104.

McNally, G.P., Akil, H., 2002. Role of corticotropin-releasing hormone in the amygdala and bed nucleus of the stria terminalis in the behavioral, pain modulatory, and endocrine consequences of opiate withdrawal. Neuroscience 112, 605–617.

Mehta, C.R., Patel, N.R., 1995. Exact logistic regression: theory and examples. Stat. Med. 14, 2143–2160.

Milanes, M.V., Laorden, M.L., Chapleur-Chateau, M., Burlet, A., 1998. Alterations in corticotropin-releasing factor and vasopressin content in rat brain during morphine withdrawal: correlation with hypothalamic noradrenergic activity and pituitary–adrenal response. J. Pharmacol. Exp. Ther. 285, 700–706.

Owens, M.J., Vargas, M.A., Knight, D.L., Nemeroff, C.B., 1991. The effects of alprazolam on corticotropin-releasing factor neurons in the rat brain: acute time course, chronic treatment and abrupt withdrawal. J. Pharmacol. Exp. Ther. 258, 349–356.

Pich, E.M., Lorang, M., Yeganeh, M., Rodriguez de Fonseca, F., Raber, J., Koob, G.F., Weiss, F., 1995. Increase of extracellular corticotropin-releasing factor-like immunoreactivity levels in the amygdala of awake rats during restraint stress and ethanol withdrawal as measured by microdialysis. J. Neurosci. 15, 5439–5447.

Rasmussen, K., Aghajanian, G.K., 1989. Withdrawal-induced activation of locus coeruleus neurons in opiate-dependent rats: attenuation by lesions of the nucleus paragigantocellularis. Brain Res. 505, 346–350.

Rasmussen, K., Beitner-Johnson, D.B., Krystal, J.H., Aghajanian, G.K., Nestler, E.J., 1990. Opiate withdrawal and the rat locus coeruleus: behavioral, electrophysiological, and biochemical correlates. J. Neurosci. 10, 2308–2317.

Rassnick, S., Heinrichs, S.C., Britton, K.T., Koob, G.F., 1993. Microinjection of a corticotropin-releasing factor antagonist into the central nucleus of the amygdala reverses anxiogenic-like effects of ethanol withdrawal. Brain Res. 605, 25–32.

Richter, R.M., Weiss, F., 1999. In vivo CRF release in rat amygdala is increased during cocaine withdrawal in self-administering rats. Synapse 32, 254–261.

Risbrough, V.B., Stein, M.B., 2006. Role of corticotropin releasing factor in anxiety disorders: a translational research perspective. Horm. Behav. 50, 550–561.

Roberts, A.J., Crabbe, J.C., Keith, L.D., 1992. Genetic differences in hypothalamic–pituitary–adrenal axis responsiveness to acute ethanol and acute ethanol withdrawal. Brain Res. 579, 296–302.

Rodriguez de Fonseca, F., Carrera, M.R.A., Navarro, M., Koob, G.F., Weiss, F., 1997. Activation of corticotropin-releasing factor in the limbic system during cannabinoid withdrawal. Science 276, 2050–2054.

Roth, R.H., Elsworth, J.D., Redmond, D.E., 1982. Clonidine suppression of noradrenergic hyperactivity during morphine withdrawal: biochemical studies in rodents and primates. J. Clin. Psychiatry 43, 42–46.

Sarnyai, Z., Biro, E., Gardi, J., Vecsernyes, M., Julesz, J., Telegdy, G., 1995. Brain corticotropin-releasing factor mediates 'anxiety-like' behavior induced by cocaine withdrawal in rats. Brain Res. 675, 89–97.

Skelton, K.H., Nemeroff, C.B., Knight, D.L., Owens, M.J., 2000. Chronic administration of the triazolobenzodiazepine alprazolam produces opposite effects on corticotropin-releasing factor and urocortin neuronal systems. J. Neurosci. 20, 1240–1248.

Skelton, K.H., Gutman, D.A., Thrivikraman, K.V., Nemeroff, C.B., Owens, M.J., 2007. The CRF_1 receptor antagonist, R121919, attenuates the neuroendocrine and behavioral effects of precipitated lorazepam withdrawal. Psychopharmacology 192, 385–396.

Smagin, G.N., Zhou, J., Harris, R.B., Ryan, D.H., 1997. CRF receptor antagonist attenuates immobilization stress-induced norepinephrine release in the prefrontal cortex in rats. Brain Res. Bull. 42, 431–434.

Stout, S.C., Owens, M.J., Nemeroff, C.B., 2002. Regulation of corticotropin-releasing factor neuronal systems and hypothalamic–pituitary–adrenal axis activity by stress and chronic antidepressant treatment. J. Pharmacol. Exp. Ther. 300, 1085–1092.

Swann, A.C., Elsworth, J.D., Charney, D.S., Jablons, D.M., Roth, R.H., Redmond Jr., D.E., Maas, J.W., 1982. Brain catecholamine metabolites and behavior in morphine withdrawal. Eur. J. Pharmacol. 86, 167–175.

Valentino, R.J., Foote, S.L., Page, M.E., 1993. The locus coeruleus as a site for integrating corticotropin-releasing factor and noradrenergic mediation of stress responses. Ann. N. Y. Acad. Sci. 697, 173–188.

Zhang, T., Feng, Y., Rockhold, R.W., Ho, I.K., 1994. Naloxone-precipitated morphine withdrawal increases pontine glutamate levels in the rat. Life Sci. 55, PL25–PL31.

Zhou, Y., Spangler, R., Ho, A., Kreek, M.J., 2003. Increased CRH mRNA levels in the rat amygdala during short-term withdrawal from chronic 'binge' cocaine. Brain Res. Mol. Brain Res. 114, 73–79.