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CRF_2 receptor-deficiency eliminates opiate withdrawal distress without impairing stress-coping

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Running title: CRF_2 receptor mediates opiate withdrawal distress

Abstract

The opiate withdrawal syndrome is a severe stressor that powerfully triggers addictive drug intake. However, no treatment yet exists that effectively relieve opiate withdrawal distress and spares stress-coping abilities. The corticotropin-releasing factor (CRF) system mediates the stress response but its role in opiate withdrawal distress and bodily strategies aimed to cope with is unknown. CRF-like signaling is transmitted by two receptor pathways, termed CRF_1 and CRF_2. Here, we report that CRF_2 receptor-deficient (CRF_2-/-) mice lack the dysphoria-like and the anhedonia-like states of opiate withdrawal. Moreover, in CRF_2-/- mice opiate withdrawal does not increase the activity of brain dynorphin (DYN), CRF and periaqueductal gray (PAG) circuitry, which are major substrates of opiate withdrawal distress. Nevertheless, CRF_2 receptor-deficiency does not impair brain, neuroendocrine and autonomic stress-coping responses to opiate withdrawal. The present findings point to the CRF_2 receptor pathway as a unique target to relieve opiate withdrawal distress without impairing stress-coping abilities.

Keywords: opiate withdrawal distress; negative affect; stress-coping; corticotropin-releasing factor (CRF) system; CRF_2 receptor pathway.

Introduction

Opiate addiction is a chronic, relapsing disease with a major impact on public health (www.who.int/substance_abuse). Studies show an alarming rise in the recreational use of opiate drugs among adolescents, indicating that the incidence rate of opiate addiction may dramatically increase in the next years[1]. In opiate addicts, heroin "highs" are inexorably followed by a severe opiate withdrawal syndrome composed of influenza-like somatic signs and negative affective symptoms, such as dysphoria, depressed mood and anhedonia[2]. Relief of opiate withdrawal signs and symptoms powerfully motivates compulsive drug-seeking and drug-taking behavior[3, 4]. Moreover, opiate withdrawal serves as a severe stressor that strongly challenges stress-responsive systems[5], thus further reducing the ability to abstain from addictive drug intake. Opiate addiction and withdrawal are currently treated mainly by substitutive opioid receptor agonist drugs, such as methadone and buprenorphine[6]. However, the latter drugs are very addictive and their discontinuation is often followed by relapse to "street" opiates[6]. Novel treatments for opiate addiction and withdrawal are thus urgently needed.

The corticotropin-releasing factor (CRF) system is a major coordinator of behavioral, neuroendocrine and autonomic responses to stressors[7-10]. The CRF system might also mediate the behavioral effects of ethanol- or drug–withdrawal. Indeed, functional antagonism of brain CRF neurotransmission attenuates stress-induced reinstatement of ethanol- or cocaine-seeking behavior[11, 12], decreases ethanol self-administration[13] and reverses anxiety-like behavior[14] induced by ethanol-withdrawal in rats. Accordingly, cessation of ethanol or cocaine intake elevates CRF activity in the amygdala and in the bed nucleus of the stria terminalis[15-18], brain regions implicated in the behavioral effects of substance withdrawal. In mammals, CRF-like signaling is

transmitted by two receptor pathways, termed CRF_1 and CRF_2[19]. Genetically engineered mouse models have allowed the discovery of distinct, and often opposite, functions for the two known CRF receptor pathways. In particular, genetic disruption of the CRF_1 or the CRF_2 receptor pathway decreases or increases, respectively, anxiety-like and hypothalamic-pituitary-adrenal (HPA) axis responses to stressors[20-24]. CRF_1 and CRF_2 receptor pathways might also differentially contribute to the myriad of somatic opiate withdrawal signs. Indeed, CRF_1 or CRF_2 receptor-deficiency exacerbates or attenuates, respectively, the somatic signs of opiate withdrawal[25, 26]. Moreover, CRF_1 receptor-deficiency eliminates the dysphoria-like states of opiate withdrawal[27] but profoundly impairs HPA axis[26, 27] and brain[26] stress-coping responses, suggesting that the CRF_1 receptor pathway might not be a suitable target for relieving opiate withdrawal distress. CRF_2 receptor-deficiency increases HPA axis and negative affective-like responses to stressors other than opiate withdrawal[20-22]. However, the role for the CRF_2 receptor pathway in the negative affective symptoms and the stress response elicited by opiate withdrawal is unknown.

Thus, the present study aims to elucidate the role for the CRF_2 receptor pathway in critical components of opiate withdrawal distress, such as dysphoria and anhedonia, and the underlying brain mechanisms. Furthermore, the contribution of the CRF_2 receptor pathway to brain, neuroendocrine and autonomic responses aimed to cope with the stressful condition of opiate withdrawal is investigated. Noteworthy, in keeping with the clinical setting where opiate withdrawal signs and symptoms "spontaneously" and gradually rise along with the drug removal from the body, behavioral, molecular and neuroendocrine studies are conducted in mice undergoing spontaneous opiate withdrawal. Furthermore, to compare our findings to the literature studies, the role for

the CRF_2 receptor pathway in dysphoria-like behavior is also examined using a more classical opioid-receptor antagonist-precipitated opiate withdrawal paradigm.

Materials and methods

Subjects

Group-housed, littermate female mice on a mixed C57BL/6Jx129 background that are wild-type or CRF_2 receptor null mutant (CRF_2-/-) are used throughout[20]. Mice are 4–9 months old at the time of the experiments and derived from mating CRF_2+/- mice. Wild-type and CRF_2-/- offspring of CRF_2+/- breeders are identified by PCR analysis of tail DNA. The mice are housed in a colony room maintained at 22±2°C on a 12-h light/dark cycle (lights on from 08.00 hours until 20.00 hours). Food and water are available *ad libitum*. All studies are conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and the European Communities Council Directive of 24 November 1986 (86/609/EEC), and are approved by the local Animal Care and Use Committee.

Opiate withdrawal-induced conditioned place aversion (CPA)

The CPA apparatus consists of a rectangular Plexiglas box (length 42 cm, width 21 cm, height 21 cm) divided by a central partition into two chambers of equal size (21x21x21 cm). One compartment has black walls and a smooth Plexiglas floor whereas the other compartment has vertical black and white striped (2 cm) walls and a slightly rough floor. During the test sessions, an aperture (4x4 cm) in the central partition allows the mice to enter both sides of the apparatus whereas during the conditioning trials the individual compartments are closed off from each other. Transparent Plexiglas lids allows observation of the animal's behavior on a video monitor situated in an adjacent room and connected to a camera placed above the apparatus[27]. Also, time spent in each

of the two compartments of the CPA apparatus is quantified by an automated video-tracking system (ViewPoint, Champagne au mont d'or, France). Both the spontaneous and the naloxone-precipitated opiate withdrawal CPA experiments last 14 days and consist of a pre-conditioning test, a conditioning and a post-conditioning phase. On day 1, each mouse is allowed to explore freely the entire CPA apparatus for 20 min and time spent in each of the two compartments is measured (pre-conditioning test). Within each genotype, mice are then divided in two groups with similar pre-conditioning time values in the preferred and the non-preferred compartment of the CPA apparatus. One group is assigned to receive saline and the other increasing morphine doses (20-100 mg/kg). In particular, starting on day 3, every 12 hours (8 a.m.; 8 p.m.) the mice are treated with saline or morphine according to the following protocol: day 3: 20 mg/kg, day 4: 40 mg/kg, day 5: 60 mg/kg, day 6: 80 mg/kg, day 7: 100 mg/kg, day 8: 100 mg/kg, only one injection in the morning. Morphine is administered twice a day since, unlike constant drug delivery devices, it allows partial opiate withdrawal between drug injections, a condition encountered in the clinical setting. In the spontaneous opiate withdrawal (SPO OW) experiment, conditioning trials take place on days 5 to 8, while morphine-treated mice are in an opiate withdrawal state. For this purpose, 8 hours after the morning injection mice are confined for 30 minutes a day into their preferred compartment of the CPA apparatus, as determined on the pre-conditioning test[27]. In the naloxone-precipitated opiate withdrawal (NAL OW) experiment, the mice are treated with saline or morphine, as described above. On days 5 to 8, all mice receive naloxone (0.23 mg/kg, s.c.) 2 hours after the morning saline or morphine injection and are immediately confined for 30 minutes a day into their preferred compartment of the CPA apparatus, as determined on the pre-conditioning test. During the conditioning trials, we

quantify the number of jumps and wet dog shakes, and check for the presence of diarrhea. For each mouse, a global somatic opiate withdrawal score is also calculated as follows: number of jumps + number of wet dog shakes + 3 points for the diarrhea sign. Post-conditioning tests take place 6 days after the last conditioning trial (day 14), once somatic opiate withdrawal signs have largely dissipated in both genotypes[25].

U-50,488H-induced CPA

We use the same CPA apparatus and an experimental procedure similar to that described above. The only difference is that the mice are not exposed to the twice daily treatment with saline or morphine. Also, during the four conditioning trials (days 5 to 8), immediately before being confined to their preferred compartment of the CPA apparatus the mice are treated with saline or the kappa opioid-receptor (KOR) agonist compound U-50,488H (5 mg/kg, s.c.). To evaluate the role for the CRF_1 receptor pathway in the U-50,488H-induced CPA, three additional groups of CRF_2-/- mice are treated with saline or U-50,488H (5 mg/kg, s.c.) immediately prior to the conditioning trials and with antalarmin (20 mg/kg, *per os*) or the appropriate vehicle one hour prior to the conditioning trials. In the latter study, the mice undergo three, instead of four, conditioning trials (days 5 to 7).

Highly palatable food (HPF) preference paradigm

The mice are individually housed 10 days before the beginning of the experiments. Standard chow food (SCF; 3.3 kcal/g, 72.5% carbohydrates, 8.2% fat, 19.3% protein, rodent diet A04, Augy, France) and water are available *ad libitum*. Then, during three consecutive days several baseline measures are collected. In particular, body weight

(BW) and home-cage SCF intake are measured every 12 hours, at the beginning and at the end of the light phase of the 12-h light/dark housing cycle. Moreover, at 16.00 hours 1-h HPF preference tests are performed by offering a choice between a pre-weighed (approximately 4 grams) SCF pellet and three 1-gram HPF pellets. For this purpose, we use a sucrose-enriched HPF (5-TUL, 3.44 kcal/g, 66.7% carbohydrate, 12.7% fat, 20.6% protein; TestDiet). Then, 1 hour later SCF and HPF pellets are removed from the home-cage and intake of both diets calculated. Care is taken to look for spillage in order to measure as accurately as possible food intake. Within genotype, the mice are then assigned to two groups having similar home-cage SCF intake, percentage of HPF and total (SCF+HPF) intake, as measured during the third baseline day. One group is assigned to receive saline and the other increasing morphine doses, as described above. Saline and morphine treatments start 24 hours after the last baseline measure. The mice are weighed immediately before each injection and BW changes calculated as percentage of BW recorded just prior to the first saline or morphine injection. Starting 8 hours after the last saline or morphine injection, 1-h HPF preference tests are performed once daily up to 104 hours following the last injection. Throughout the experiment, BW and home-cage SCF intake are measured, as described above. Moreover, caloric efficiency (CE), calculated as grams of BW gained/kilocalories ingested, is assessed during the three baseline days, the intermittent morphine injections (from the first to 24 hours after the last injection) and 24 to 96 hours after the last injection. For this purpose, the amount of SCF and HPF ingested during the 1-hour HPF preference tests is also taken into account.

In situ hybridization experiments and plasma corticosterone assay

The mice are treated with saline or morphine and exposed to the CPA paradigm, as described above. The only difference is that at the end of the last 30-min conditioning trial, the mice are taken into another room, their brains rapidly removed, frozen in isopentane (-40°C), and stored at -80°C. Blood samples are collected from the trunk, centrifuged (4000 rpm, 15 min) and plasma samples stored at -20°C until corticosterone assay. Plasma corticosterone levels are quantified by radioimmunoassay using a specific corticosterone antibody (ICN Pharmaceuticals). The intra- and inter-assay coefficients of variation are approximately 3.5 and 8%, respectively. For *in situ* hybridization experiments, brains are cut in coronal sections (12 µm) using a cryostat and thaw mounted onto gelatin-coated slides as previously described[28]. The Paxinos and Franklin mouse brain atlas is used to identify the different brain regions examined[29]. *In situ* hybridization is performed with antisense ^{35}S-labelled cRNA probes designed to recognize CRF[30], DYN[31], tyrosine hydroxylase (TH)[28] and *c-fos*[28] mRNAs. All probes are prepared by *in vitro* transcription from 100 ng of linearized plasmid using [^{35}S]UTP (>1,000Ci/mmol; Perkin-Elmer Life Sciences, Courtaboeuf, France) and appropriate RNA polymerases. After alkaline hydrolysis to obtain 0.25 kb cRNA fragments, the ^{35}S-labelled probes are purified on G50-Sephadex and precipitated in sodium acetate 3 M, pH 5.0 (0.1 vol)-absolute ethanol (2.5 vol). Sections are post-fixed in 4% paraformaldehyde for 5 min at room temperature, rinsed twice in 4× sodium chloride–sodium citrate buffer (SSC) and placed into 0.1 M triethanolamine/4× SSC, pH 8.0 for 10 min at room temperature; 0.25% acetic anhydride is added for the last 5 min. After dehydration in graded alcohols, sections are hybridized overnight at 55°C with 10^6 cpm of antisense ^{35}S-labelled probes, in 50 µL hybridization buffer (20 mM Tris–HCl, 1 mM EDTA, 300 mM NaCl, 50% formamide, 10% dextran sulfate, 1% Denhardt). The slides

are rinsed in 4× SSC twice, treated with RNAse A for 15 min at 37°C, washed in decreasing SSC concentrations, at room temperature and then at 65°C with 0.1× SSC for 30 min twice. Slides are dehydrated and then exposed at room temperature to Biomax-MR X-ray film (Kodak) for 2-8 weeks. Quantification of mRNA expression is performed by densitometric analysis on X-ray film and data are expressed as mean of optical density (O.D.) x 1000 ± SEM.

Drugs

Morphine HCl (Francopia, Gentilly, France; 20-100 mg/kg, i.p.), U-50,488H (National Institute on Drug Abuse, Bethesda, MD, U.S.A.; 5 mg/kg, s.c.) and naloxone HCl (0.23 mg/kg, s.c.; Sigma-Aldrich, Lyon, France) are dissolved in physiological saline and injected in a volume of 10 ml/kg. Antalarmin HCl (20 mg/kg, *per os*; Sigma-Aldrich, Lyon, France) is dissolved in acidified saline (pH ≈ 2.5) and injected in a volume of 10 ml/kg. Control mice are injected with the same volume of the appropriate vehicle.

Statistical analysis

Two-way analysis of variance (ANOVA) with genotype (wild-type or CRF_2-/-) and treatment (control, opiate withdrawal or U-50,488H) as independent variables is used to examine the CPA scores, the *in situ* hybridization data (DYN, CRF, *c-fos* and TH mRNAs levels) and the plasma corticosterone levels. A one-way ANOVA is used to examine the CPA scores of the antalarmin/U-50,488H experiment. Jumps, wet dog shakes and global somatic opiate withdrawal scores are examined using the non-parametric Mann-Whitney U test. The 2x2 table chi-square test is used to analyze the presence or absence of the diarrhea sign during the CPA conditioning trials. A three-

way ANOVA with genotype and treatment (control or opiate withdrawal) as between subjects factors and repeated measures as a within-subject factor is used to analyze the percentage of HPF and the total amount of kilocalories ingested during the HPF preference tests. A three-way ANOVA with genotype and treatment (control or morphine) as between subjects factors and repeated measures as a within-subject factor is used to analyze the 12-hour home-cage SCF intake and the percentage of BW changes induced by the stress of intermittent morphine injections and by its cessation. A two-way ANOVA with genotype and treatment (control or morphine) as independent variables is used to examine CE induced by the stress of intermittent morphine injections and by its cessation. The Student-Newman-Keuls post-hoc test is used for individual group comparisons. The accepted value for significance is $P<0.05$.

Results

Absence of dysphoria-like states of opiate withdrawal in CRF_2 receptor-deficient mice

In humans, the opiate withdrawal syndrome is composed of severe negative affective-like states, such as dysphoria and anhedonia, and influenza-like somatic signs[2]. To investigate the role for the CRF_2 receptor pathway in dysphoria-like states induced by opiate withdrawal, we use the CRF_2-/- mouse model[20], a clinically relevant SPO OW CPA paradigm previously validated in our laboratory[27] and a more classical NAL OW CPA paradigm. To closely parallel drug intake patterns of opiate addicts, we use an intermittent morphine injections procedure wherein drug doses are progressively increased. Moreover, in the SPO OW experiment 30-min place conditioning trials are started 8 hours after morphine injection, i.e., following body clearance of the opiate drug and during the maximal expression of somatic opiate withdrawal signs[25, 32]. During the 20-min pre-conditioning test, time spent in the preferred compartment of the CPA apparatus is similar for wild-type and CRF_2-/- mice assigned to the control or the opiate-withdrawn group (Supplementary Table 1). Post-conditioning tests reveal that the wild-type mice consistently avoid the environmental cues of the CPA apparatus previously paired with the SPO OW or the NAL OW (Figures 1a and 1b), indicating dysphoria-like states during the place conditioning trials. In contrast, in both the CPA experiments opiate-withdrawn CRF_2-/- mice do not differ from control wild-type and CRF_2-/- mice (Figures 1a and 1b). Noteworthy, despite the higher CPA scores observed in the wild-type mice during the NAL OW, as compared to the SPO OW experiment, CRF_2-/- mice do not show any sign of dysphoria-like behavior (compare Figures 1a and 1b). Thus, in contrast to the wild-type mice, CRF_2-/- mice do not show any aversion for the environmental cues paired with opiate withdrawal, indicating the absence of

dysphoria-like states. During both the SPO OW and the NAL OW experiments, the impact of CRF_2 receptor-deficiency upon major somatic signs of opiate withdrawal is also examined. Along with increasing morphine doses, during the place conditioning trials of the SPO OW experiment the wild-type mice show a progressive increase in the severity of somatic opiate withdrawal signs, as assessed by the global score parameter (Supplementary Figure 1a). In contrast, opiate-withdrawn CRF_2-/- never differ from control wild-type and CRF_2-/- mice (Supplementary Figure 1a). In particular, opiate-withdrawn wild-type mice make more jumps and wet dog shakes than opiate-withdrawn CRF_2-/-, control wild-type and CRF_2-/- mice (Supplementary Figures 1b and 1c). Noteworthy, none of the opiate-withdrawn CRF_2-/- mice displays the jump sign (Supplementary Figure 1b). Moreover, during the fourth place conditioning trial, 5 out of 10 opiate-withdrawn wild-type mice show the diarrhea sign whereas none of the opiate-withdrawn CRF_2-/- mice show this sign (Supplementary Table 2). On the other hand, no reliable somatic signs of opiate withdrawal are observed in the NAL OW experiment neither in the wild-type nor in the CRF_2-/- mice (data not shown), indicating a selective effect of the naloxone dose used (0.23 mg/kg, s.c.) upon affective-like states. We previously reported that CRF_2 receptor-deficiency attenuates the somatic expression of opiate withdrawal, as assessed 8 to 152 hours after the last morphine injection[25]. Unlike our prior study, the present results provide novel evidence in favor of a role for the CRF_2 receptor pathway also in the progressive increase of somatic opiate withdrawal signs induced by repeated intermittent injections of morphine (Supplementary Figure 1). Thus, CRF_2 receptor-deficiency completely eliminates the dysphoria-like states and the somatic signs of opiate withdrawal occurring between successive injections of increasing morphine doses. These findings clearly indicate an

essential role for the CRF_2 receptor pathway in critical negative affective-like and somatic components of opiate withdrawal distress.

Unaltered CPA abilities in CRF_2 receptor-deficient mice

CRF_2-/- mice show no conditioned aversions to the environmental cues associated with opiate withdrawal. Thus, it can be argued that in CRF_2-/- mice absence of opiate withdrawal-induced CPA might be due to developmental deficits in associative learning processes required for the acquisition and expression of conditioned behavior. To address this issue, an additional cohort of wild-type and CRF_2-/- mice is tested for CPA to the KOR agonist compound U-50,488H[33]. During the 20-min pre-conditioning test, time spent in the preferred compartment of the CPA apparatus is similar for wild-type and CRF_2-/- mice assigned to be treated with saline or U-50,488H (Supplementary Table 1). Post-conditioning tests reveal that wild-type and CRF_2-/- mice display similar levels of CPA for the environmental cues previously paired with the KOR agonist U-50,488H (Supplementary Figure 2a). The latter results clearly indicate that CRF_2 receptor-deficiency does not alter associative learning processes underlying the acquisition and the expression of CPA. These findings also indicate functional levels of the KOR pathway, the preferential target of endogenous DYN peptides[34], in the CRF_2-/- mice. To assess the role for the CRF_1 receptor pathway in the U-50,488H-induced CPA, another cohort of CRF_2-/- mice are pre-treated with the CRF_1 receptor-preferring antagonist antalarmin one hour prior to be conditioned with U-50,488H. Analysis of time spent in the preferred compartment of the CPA apparatus during the pre-conditioning test reveals no group differences ($F_{2,13}$=1.3, P=n.s., data not shown). Similarly to above, the KOR agonist U-50,488H induces reliable CPA in the CRF_2-/-

mice (Supplementary Figure 2b). However, pre-treatment with antalarmin completely abolishes the U-50,488H-induced CPA in the CRF_2-/- mice, indicating that dysphoria-like states induced by stimulation of the KOR pathway are mediated by the CRF_1 receptor pathway (Supplementary Figure 2b). Moreover, the latter results add further support to the notion of preserved CRF_1 receptor activity in the CRF_2-/- mice[20, 21].

Absence of anhedonia-like states of opiate withdrawal in CRF_2 receptor-deficient mice

A prominent negative affective-like state of opiate withdrawal is anhedonia, i.e., an impaired capability to experience reward from natural pleasant activities, such as eating or sex[2]. Stress-induced decrease in the preference for HPF is thought to reflect anhedonia-like states[35]. Thus, we use a HPF preference test to assess the role for the CRF_2 receptor pathway in the anhedonia-like states of opiate withdrawal. In particular, the mice are injected with saline or morphine as in the opiate withdrawal CPA experiment mentioned above, and daily 1-hour HPF preference tests carried out 8 to 104 hours following the last injection. Prior to the beginning of saline or morphine injections, wild-type and CRF_2-/- mice display similar levels of HPF preference (point B, Figure 2a). However, 8 hours after the last morphine injection the opiate-withdrawn wild-type mice display a decrease in the percentage of HPF ingested, indicating the presence of an anhedonia-like state (Figure 2a). In contrast, opiate-withdrawn CRF_2-/- mice do not show any decrease in the percentage of HPF ingested and do not differ from control wild-type and CRF_2-/- mice (Figure 2a). The reduced HPF preference observed in opiate-withdrawn wild-type mice does not depend on energy intake. Indeed, 8 hours after the last saline or morphine injection there is neither genotype nor opiate withdrawal effect in total kilocalories ingested (Figure 2b). However, we find a

genotype-independent increase in total kilocalories ingested 32 and 56 hours after the last morphine injection (Figure 2b), when no more reduction in HPF preference is observed in the opiate-withdrawn wild-type mice. Noteworthy, the amount of kilocalories ingested during the 1-hour HPF preference tests is relatively high, as compared to the kilocalories ingested daily (data not shown), a phenomenon due to the appetitive properties of the HPF used. Thus, our studies demonstrate a fundamental role for the CRF_2 receptor pathway in the anhedonia-like states of opiate withdrawal. Moreover, our results show that CRF_2 receptor-deficiency does not influence the increased HPF-driven energy intake.

CRF_2 receptor-deficiency abolishes the brain substrates of opiate withdrawal distress

Morphine withdrawal increases the expression of the opioid peptide DYN in the brain region of the nucleus accumbens shell (NaccSH)[27]. Increased DYN/KOR activity in the nucleus accumbens may underlie the negative affective-like states of opiate withdrawal[27], and also decrease the extracellular levels of dopamine[36], a neurotransmitter mediating the hedonic properties of drugs of abuse[4] and palatable food[37]. Thus, to investigate the brain substrates underlying the CRF_2 receptor-mediated dysphoria-like and anhedonia-like states of opiate withdrawal, wild-type and CRF_2-/- mice are injected with saline or morphine as above, and NaccSH-DYN expression examined in brains collected 8.5 hours after the last injection. Our results indicate that opiate withdrawal consistently increases NaccSH-DYN expression in the wild-type mice (Figures 3a and 3b). In contrast, opiate withdrawal does not affect NaccSH-DYN expression in the CRF_2-/- mice, which show DYN mRNA levels similar to control wild-type and CRF_2-/- mice (Figures 3a and 3b). We also examine the expression of the

immediate-early gene *c-fos*, a marker of neuronal reactivity[38], in the PAG, a brainstem region implicated in opiate withdrawal distress[39]. Our analyses reveal that opiate withdrawal produces a 2.4-fold increase in PAG-*c-fos* expression in the wild-type mice (Figure 3c). However, opiate withdrawal does not produce any increase in PAG-*c-fos* expression in the CRF_2-/- mice, which display *c-fos* mRNA levels similar to control wild-type and CRF_2-/- mice (Figures 3c and 3d). Increased CRF activity in the central nucleus of the amygdala (CeA) might contribute to the behavioral effects of drug or ethanol withdrawal[5]. However, no prior studies have assessed the role for the CRF_2 receptor pathway in opiate-withdrawal-induced CeA-CRF expression. Here, we demonstrate that opiate withdrawal reliably increases CeA-CRF expression in the wild-type mice (Figures 4a and 4b). However, opiate-withdrawn CRF_2-/- mice do not show any increase in CeA-CRF expression (Figures 4a and 4b), indicating a fundamental role for the CRF_2 receptor pathway in CeA-CRF responses to opiate withdrawal. Thus, our results clearly demonstrate that the activity of brain circuitry underlying negative affective-like and somatic components of opiate withdrawal distress totally depends on functional levels of the CRF_2 receptor pathway.

Preserved stress responses in opiate-withdrawn CRF_2 receptor-deficient mice

The opiate withdrawal syndrome is an extremely severe and challenging stressor. CRF and norepinephrine (NE) pathways originating from the paraventricular nucleus of the hypothalamus (PVN) and the locus caeruleus (LC), respectively, orchestrate brain, neuroendocrine and autonomic stress responses[7, 8, 10]. Moreover, PVN-CRF pathways projecting to the median eminence of the hypothalamus-pituitary system coordinate stress-induced HPA axis activity[9]. Thus, we assess the impact of CRF_2 receptor-

deficiency upon the opiate withdrawal-induced activity of PVN-CRF, HPA axis and LC-NE pathways. Noteworthy, analyses are carried out on the same brains used for the DYN, CRF and *c-fos* expression studies mentioned above. Our results demonstrate that, unlike the CeA-CRF expression, opiate withdrawal similarly increases PVN-CRF expression in wild-type and CRF_2-/- mice (Figures 4b and 4c). Accordingly, CRF_2 receptor-deficiency does not affect plasma corticosterone levels (Supplementary Figure 3), indicating preserved HPA axis and CRF_1 receptor activity[23, 24] in opiate-withdrawn CRF_2-/- mice. However, despite the very elevated plasma corticosterone levels (47-65 μg/100 ml), opiate-withdrawn mice do not differ from control mice, most probably due to a ceiling effect produced by the stressful 30-min confinement to the CPA apparatus. LC-NE activity is examined by assessing the expression of the TH enzyme, the rate-limiting NE synthesis enzyme[40]. Like the PVN-CRF expression, we find that opiate withdrawal similarly increases LC-TH expression in wild-type and CRF_2-/- mice (Figures 5a and 5b). Thus, here we show increased PVN-CRF and LC-TH activity in mice (CRF_2-/-) lacking the somatic signs and the negative affective-like states of opiate withdrawal. These results strongly suggest that activation of PVN-CRF and LC-NE circuitry does not contribute to the malaise of opiate withdrawal. Adaptive stress responses also include time-limited metabolic changes, such as decreased food intake and increased catabolism, which aim to maximize stress-coping[7, 8]. Thus, we investigate the impact of CRF_2 receptor-deficiency upon SCF intake, BW and CE, an index of energy balance status, induced by the stress of intermittent morphine injections and the associated repeated cycles of 12-hour opiate withdrawal. Furthermore, to examine the role for the CRF_2 receptor pathway in the post-stress recovery of metabolic functions, SCF intake, BW and CE are monitored up to 96 hours after the last saline or morphine

injection. Prior to the beginning of saline or morphine injections, wild-type and CRF_2-/- mice display similar daily SCF intake and BW (Supplementary Table 3). The intermittent morphine injections and their cessation induce a genotype-independent decrease and a rebound increase in SCF intake, respectively (Supplementary Figure 4a). Accordingly, CRF_2 receptor-deficiency does not affect BW loss and recovery induced by intermittent morphine injections and by their cessation, respectively (Supplementary Figure 4b). Finally, CRF_2 receptor-deficiency does not affect CE decrease and increase induced by intermittent morphine injections and by their cessation, respectively (Supplementary Figures 5b and 5c). Overall, our findings indicate that CRF_2 receptor-deficiency does not affect either the intensity or the duration of brain, neuroendocrine and autonomic stress-coping responses to opiate withdrawal.

Discussion

The main finding of the present study is that genetic disruption of the CRF_2 receptor pathway completely eliminates the negative affective-like and the somatic components of opiate withdrawal distress. Moreover, unlike wild-type mice, opiate-withdrawn CRF_2 receptor-deficient mice do not show any increase in DYN, CRF and *c-fos* expression in the NaccSH, the CeA and the PAG, respectively, i.e., brain circuitry thought to underlie drug withdrawal distress. Nevertheless, CRF_2 receptor-deficiency does not influence PVN-CRF/HPA axis, LC-TH and autonomic responses essential to cope with the stressful condition of opiate withdrawal.

Despite extensive research, the neural mechanisms underlying opiate withdrawal signs and symptoms remain largely unknown. Using clinically-relevant mouse models of spontaneous drug withdrawal, here we report an essential role for the CRF_2 receptor pathway in the dysphoria-like and the anhedonia-like states of opiate withdrawal. To measure the dysphoric consequences of opiate withdrawal we use the CPA paradigm. In the latter behavioral test, avoidance of environmental cues paired with opiate withdrawal or other stressors is taken as a reliable measure of dysphoria-like states[27, 41]. Our results demonstrate that, unlike the wild-type mice, CRF_2-/- mice do not avoid the environmental cues paired with opiate withdrawal, indicating the absence of dysphoria-like states. Furthermore, using a food preference test designed to assess stress-induced hedonic changes[35], we report that opiate-withdrawn CRF_2-/- mice do not show any reduction in HPF preference, indicating the absence of anhedonia-like states. The present results contrast with the increased anxiety-like responses to stressors other than opiate withdrawal previously observed in CRF_2 receptor-deficient mice[20, 22]. Thus, our findings put forward the original notion of a stressor-dependent role for the CRF_2

receptor pathway in negatively valenced affective-like states. In line with the behavioral findings, here we also show that CRF_2 receptor-deficiency eliminates the brain substrates of opiate withdrawal distress. In particular, we demonstrate that opiate withdrawal increases NaccSH-DYN expression in the wild-type mice. Increased DYN neurotransmission is suggested to underlie negative affective-like responses to stressors. For instance, conditioned aversions for places paired with foot-shock, i.c.v. injection of CRF or i.p. injection of U-50,488H are abolished by the systemic or nucleus accumbens administration of the KOR antagonist nor-binaltorphimine and by prodynorphin-deficiency[41, 42]. Up-regulated DYN/KOR systems activity also decreases dopamine release within the nucleus accumbens[36], a brain mechanism implicated in dysphoria-like states. Nucleus accumbens dopamine circuitry also mediate the hedonic properties of drugs of abuse[4] and natural rewards, such as palatable food[37]. Thus, the increased NaccSH-DYN expression may underlie the dysphoria-like and the anhedonia-like states observed herein in opiate-withdrawn wild-type mice. In contrast with the wild-type mice, opiate withdrawal does not influence the NaccSH-DYN expression in the CRF_2-/- mice, indicating a fundamental role for the CRF_2 receptor pathway in the brain mechanisms underlying the dysphoria-like and the anhedonia-like states of opiate withdrawal.

We also report that CRF_2 receptor-deficiency eliminates the opiate withdrawal-induced neuronal activity, as measured by *c-fos* expression, in the PAG. Systemic administration of naloxone elevates *c-fos* mRNA levels in the PAG in rats implanted with morphine pellets[28]. Moreover, injection of the hydrophilic poorly diffusing opioid receptor antagonist methylnaloxonium into the PAG precipitates somatic signs of opiate withdrawal and produces CPA in rats implanted with morphine pellets[39]. However, to

our knowledge no studies have yet assessed the role for the CRF system in PAG neuronal activity induced by opiate withdrawal. Here, we show that opiate withdrawal reliably increases PAG *c-fos* expression in wild-type mice. Nevertheless, opiate withdrawal does not produce any increase in PAG *c-fos* expression in CRF_2-/- mice, indicating that opiate withdrawal-induced activity of PAG is under the control of the CRF_2 receptor pathway. We also demonstrate that CRF_2 receptor-deficiency abolishes the opiate withdrawal-induced increase in CeA-CRF expression. CeA-CRF circuitry might mediate anxiety-like and substance-seeking behavior induced by cocaine or ethanol withdrawal. For instance, the administration of nonspecific CRF receptor antagonists into the CeA reverses the negative affective-like states and the enhanced ethanol self-administration induced by morphine or ethanol withdrawal in rats[13, 14, 43]. Accordingly, ethanol withdrawal enhances CRF release in the amygdala[16]. However, no prior studies have addressed the relative role for each of the two known CRF receptor pathways in opiate withdrawal-induced CeA-CRF expression. Here, we demonstrate that spontaneous opiate withdrawal increases CeA-CRF expression in wild-type mice. In contrast, CRF_2 receptor-deficiency completely eliminates the increased CRF mRNA levels induced by opiate withdrawal in the CeA, suggesting a presynaptic positive regulation of CRF synthesis and release by the CRF_2 receptor pathway. Another study demonstrates an essential role for CeA-CRF receptor pathways in gamma-aminobutyric acid (GABA) release induced by ethanol dependence[44]. Thus, although further work is needed, the latter evidence points out to a presynaptic control of synthesis and release of neurotransmitters relevant to substance dependence and withdrawal by CRF receptor pathways. Thus, the lack of up-regulated CeA-CRF expression observed herein in opiate-withdrawn CRF_2-/- mice may contribute to the absence of negative affective-like

states, despite preserved CRF_1 receptor activity. Overall, the present results indicate a cardinal role for the CRF_2 receptor pathway in the brain molecular changes thought to mediate the negative affective-like and the somatic components of opiate withdrawal distress. However, further work using more advanced genetic and/or pharmacological tools might be needed to confirm a direct link between the behavioral and the brain gene expression profiles displayed by opiate-withdrawn CRF_2 receptor-deficient mice in the present study. Moreover, since we use a genetic mouse model bearing a whole-body CRF_2 receptor-deficiency, the present study does not allow discerning the relative contribution of peripheral, versus central, CRF_2 receptor pathways to opiate withdrawal distress. Nevertheless, the findings of a recent rat study showing that activation of peripheral CRF_2 receptor pathways by urocortin 2 does not produce any sign of malaise, as assessed by the conditioned taste aversion paradigm[45], suggest that negatively valenced affective-like states are mainly mediated by central CRF_2 receptor pathways.

The opiate withdrawal syndrome is an extremely stressful clinical condition. Effective coping with stressors requires functional stress-responsive systems. CRF and NE pathways originating from the PVN and the LC, respectively, widely project to the neuraxis and coordinate brain, neuroendocrine and autonomic responses essential to cope with stressors[7-10]. For instance, activation of PVN-CRF, HPA axis and LC-NE pathways facilitates arousal, vigilance and attention, stimulates the sympathetic nervous system and inhibits parasympathetic outflow, eating behavior and sleeping, thus favoring adaptation to intrinsic (i.e., hypoglycaemia, decreased blood pressure) or extrinsic (i.e., environmental threat) stressors[7, 8, 10]. In the present study, we report that CRF_2 receptor-deficiency does not impair the increased PVN-CRF expression induced by the stress of opiate withdrawal. The unaltered plasma corticosterone levels observed

in the CRF_2-/- mice might contribute, at least in part, to the opiate withdrawal-induced PVN-CRF expression. Indeed, corticosterone-deficiency produces an aberrant decrease in PVN-CRF responses to opiate withdrawal, which are "normalized" by restoring nadir plasma corticosterone levels[26]. Furthermore, here we show that CRF_2 receptor-deficiency does not influence the opiate withdrawal-induced expression of LC-TH, a key coordinator of sympathetic nervous system responses to stressors[10]. We also report on the role for the CRF_2 receptor pathway in metabolic responses to intermittent injections of increasing morphine doses and to their cessation. Noteworthy, the intermittent morphine injections procedure used herein serves as a severe stressor since it produces repeated cycles of 12-hour opiate withdrawal. Our results demonstrate that, like wild-type mice, CRF_2-/- mice show decreased food intake and BW during the 12-hour opiate withdrawal periods occurring between successive morphine injections and display a negative energy balance status, as revealed by decreased CE. The increased PVN-CRF and LC-TH expression may underlie the decreased food intake, the BW loss and the reduced CE displayed by opiate-withdrawn wild-type and CRF_2-/- mice. Indeed, injection of CRF into the PVN powerfully decreases food intake in rats[46]. Furthermore, activation of PVN-CRF, HPA axis and LC-NE systems leads to primarily catabolic effects and mobilization of energy resources towards the brain, heart and skeletal muscles in order to cope with stressors. Most importantly, in the context of an adequate stress response, such a stress-related metabolic shift toward a generalized catabolic state promptly reverses upon retraction of the stressor. With regard to the latter point, here we show that cessation of the morphine injections is followed by a rapid BW recovery, increased food intake and a positive energy balance status, indicating self-restrained adequate stress-coping responses to opiate withdrawal. Noteworthy, the temporal

dynamics of the latter responses is similar in wild-type and CRF_2-/- mice, demonstrating that CRF_2 receptor-deficiency does not influence either the intensity or the duration of metabolic responses to the stress of opiate withdrawal. Thus, the present results of increased PVN-CRF and LC-TH expression in opiate-withdrawn CRF_2-/- mice, together with a time-limited stressor-dependent catabolic state, strongly indicate that disruption of the CRF_2 receptor pathway spares the ability to cope with the extremely stressful condition of opiate withdrawal.

Studies suggest brain CRF-NE feed-forward loops that might be relevant to adaptive or maladaptive stress responses. For instance, both the CeA and the PVN are major sources of CRF neurons projecting to the LC[10]. Moreover, CRF mediates stress-induced LC-TH immunoreactivity[47], increases the discharge rate of LC-NE neurons[48] and stimulates NE release in LC terminal regions[48, 49]. On the other hand, stimulation of NE receptors increases CRF release in the amygdala and in the hypothalamus[50]. Here, we demonstrate that CRF_2 receptor-deficiency abolishes CeA-CRF but does not affect PVN-CRF and LC-TH responses to the stress of opiate withdrawal. These findings provide initial evidence of a CRF_2 receptor-dependent clear-cut dissociation in the activity of brain systems underlying key features of drug withdrawal and stress-coping abilities.

In conclusion, the present study demonstrates that CRF_2 receptor-deficiency completely abolishes major negative affective-like and somatic components of opiate withdrawal distress yet sparing brain, neuroendocrine and autonomic responses essential for stress-coping. Escape from opiate withdrawal distress is a powerful drive to addictive behavior. Thus, our findings bear important clinical implications for the treatment of opiate addiction and withdrawal: peripherally administered blood-brain

barrier penetrating CRF_2 receptor antagonists might alleviate opiate withdrawal distress without altering stress-coping abilities, thus facilitating opiate abstinence in addicted individuals.

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Conflict of interest

The authors declare no conflict of interest.

Supplementary information is available at *Molecular Psychiatry*'s website.

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Figure legends

Figure 1 CRF_2 receptor-deficiency eliminates the dysphoria-like states of opiate withdrawal. Mean (± s.e.m.) conditioned place aversion (CPA) scores (s) displayed by control (CTL) or opiate-withdrawn (OW) wild-type and CRF_2-/- mice during the **(a)** spontaneous (SPO) or the **(b)** naloxone (NAL)-precipitated opiate withdrawal (OW) experiment (SPO OW, genotype x opiate withdrawal interaction effect: $F_{1,35}$= 5.6, P<0.05; NAL OW, genotype x opiate withdrawal interaction effect: $F_{1,32}$= 4.2, P<0.05). *P<0.01 versus OW CRF_2-/-, CTL wild-type and CTL CRF_2-/- mice, Newman-Keuls *post-hoc* test. For each mouse, a CPA score is calculated as the post- minus the pre-conditioning time spent in the conditioning-paired compartment of the CPA apparatus. N= 9-10/group.

Figure 2 CRF_2 receptor-deficiency abolishes the anhedonia-like states of opiate withdrawal. **(a)** Mean (± s.e.m.) percentage of highly palatable food (HPF) ingested by control (CTL) or opiate-withdrawn (OW) wild-type and CRF_2-/- mice during the daily 1-hour HPF preference tests carried out prior to (B, baseline) and 8 to 104 hours after intermittent saline or morphine injections (genotype x opiate withdrawal x repeated measures interaction effect: $F_{5,150}$= 4.3, P<0.005; ***P<0.0001 versus OW CRF_2-/-, CTL wild-type and CTL CRF_2-/- mice, at the same time point, Newman-Keuls *post-hoc* test). N= 8-9/group. **(b)** Mean (± s.e.m.) total kilocalories (kcal) ingested during the 1-hour HPF preference tests (opiate withdrawal x repeated measures interaction effect: $F_{5,150}$= 9.1, P<0.0001; ##P<0.0005, ###P<0.0001 versus CTL mice at the same time

point, Newman-Keuls *post-hoc* test; genotype x opiate withdrawal x repeated measures interaction effect: $F_{5,150}$= 0.3, P= n.s.). N= 8-9/group.

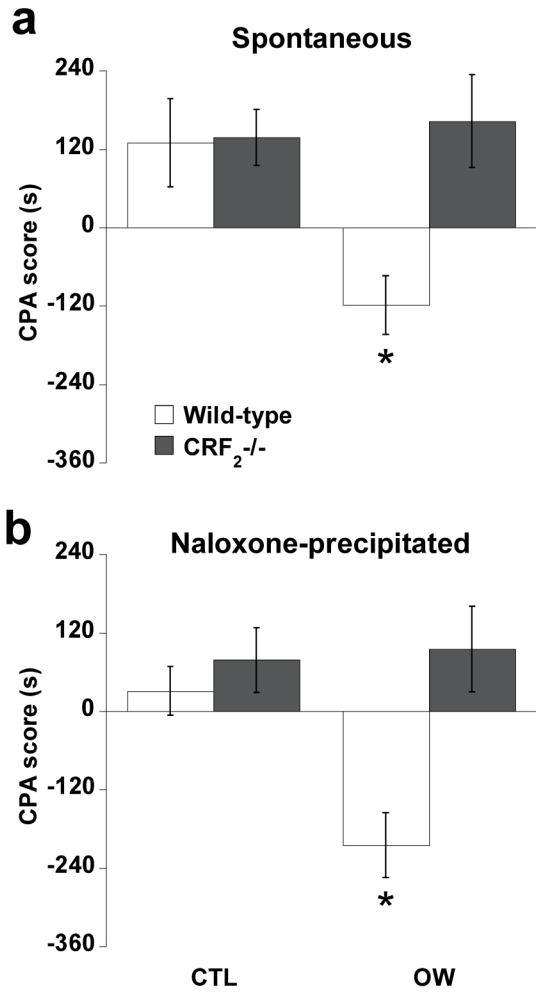
Figure 3 CRF_2 receptor-deficiency eliminates the brain substrates of opiate withdrawal distress. **(a)** Mean (± s.e.m.) dynorphin (DYN) mRNA levels in the nucleus accumbens shell (NaccSH; bregma interval: 1.10/0.86 mm) in control (CTL) or opiate-withdrawn (OW) wild-type and CRF_2-/- mice (genotype x opiate withdrawal interaction effect: $F_{1,28}$= 8.3, P<0.01; *P<0.05 versus OW CRF_2-/-, CTL wild-type and CTL CRF_2-/- mice, Newman-Keuls *post-hoc* test). Results are expressed as optical density (x1000). N= 7-9/group. **(b)** Representative images of brain sections illustrating DYN expression in the NaccSH (S) in CTL or OW wild-type and CRF_2-/- mice. Scale bar= 1 mm. **(c)** Mean (± s.e.m.) *c-fos* mRNA levels in the periaqueductal gray (PAG; bregma interval: -3.16/-3.40 mm) in CTL or OW wild-type and CRF_2-/- mice (genotype x opiate withdrawal interaction effect: $F_{1,36}$= 10.5, P<0.005; *P<0.05 versus OW CRF_2-/-, CTL wild-type and CTL CRF_2-/- mice, Newman-Keuls *post-hoc* test). Results are expressed as optical density (x1000). N= 10/group. **(d)** Representative images of brain sections illustrating *c-fos* expression in the PAG in CTL or OW wild-type and CRF_2-/- mice. Scale bar= 2.5 mm.

Figure 4 Dissociation of brain CRF responses to opiate withdrawal in CRF_2 receptor-deficient mice. **(a)** Mean (± s.e.m.) CRF mRNA levels in the central nucleus of the amygdala (CeA; bregma interval: -0.82/-1.06 mm) in control (CTL) or opiate-withdrawn (OW) wild-type and CRF_2-/- mice (genotype x opiate withdrawal interaction effect: $F_{1,31}$= 4.7, P<0.05; *P<0.01 versus OW CRF_2-/-, CTL wild-type and CTL CRF_2-

/- mice, Newman-Keuls *post-hoc* test). Results are expressed as optical density (x1000). N= 8-10/group. **(b)** Representative images of brain sections illustrating CRF expression in the CeA and in the paraventricular nucleus of the hypothalamus (PVN) in CTL or OW wild-type and CRF_2-/- mice. Scale bar= 2 mm. **(c)** Mean (± s.e.m.) CRF mRNA levels in the PVN (bregma interval: -0.82/-1.06 mm) in CTL or OW wild-type and CRF_2-/- mice (opiate withdrawal main effect: $F_{1,33}$= 60.0, P<0.0001; $^{\#\#}$P<0.0005 versus CTL mice, Newman-Keuls *post-hoc* test; genotype x opiate withdrawal interaction effect: $F_{1,33}$= 0.4, P= n.s.). Results are expressed as optical density (x1000). N= 8-10/group. Noteworthy, in CRF_2-/- mice opiate withdrawal increases the expression of CRF in the PVN but not in the CeA.

Figure 5 Preserved brain stress-coping responses in opiate-withdrawn CRF_2 receptor-deficient mice. **(a)** Mean (±s.e.m.) tyrosine hydroxylase (TH) mRNA levels in the locus caeruleus (LC; bregma interval: -5.40/-5.52 mm) in control (CTL) or opiate-withdrawn (OW) wild-type and CRF_2-/- mice (opiate withdrawal main effect: $F_{1,30}$= 8.5, P<0.01; **P<0.01 versus CTL mice, Newman-Keuls *post-hoc* test; genotype x opiate withdrawal interaction effect: $F_{1,30}$= 0.3, P= n.s.). Results are expressed as optical density (x1000). N= 8-9/group. **(b)** Representative images of brain sections illustrating TH expression in the LC in CTL or OW wild-type and CRF_2-/- mice. Scale bar= 1 mm.

Figure 1


a
Spontaneous
240
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-120
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CPA score (s)
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Wild-type
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-240
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CPA score (s)
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CTL
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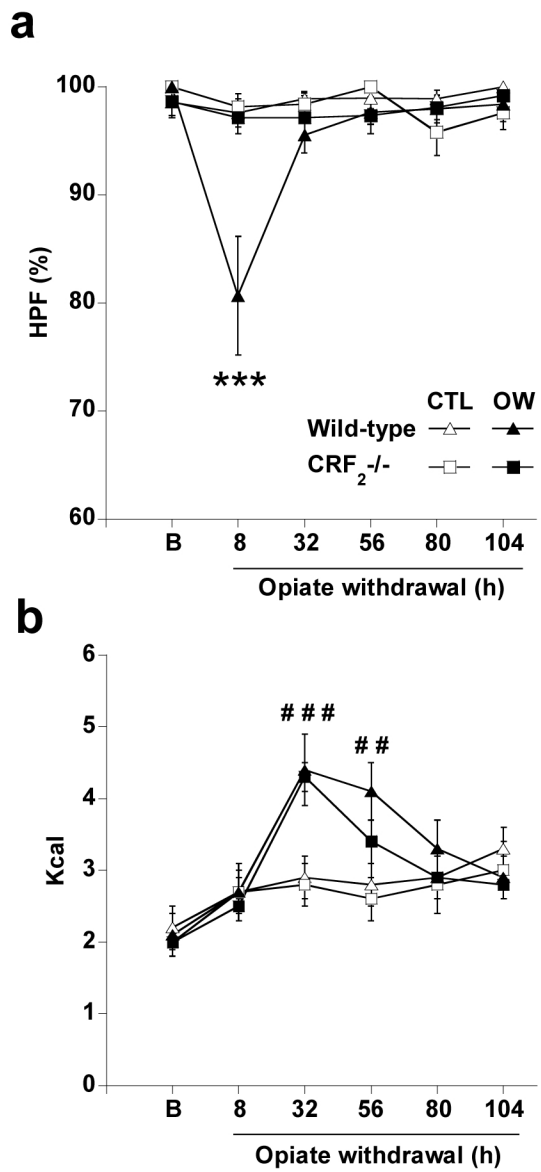
Figure 2


a
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CTL
OW
Wild-type
CRF2-/-
B
8
32
56
80
104
Opiate withdrawal (h)
b
6
5
4
3
2
1
0
Kcal
###
##
B
8
32
56
80
104
Opiate withdrawal (h)

Figure 3

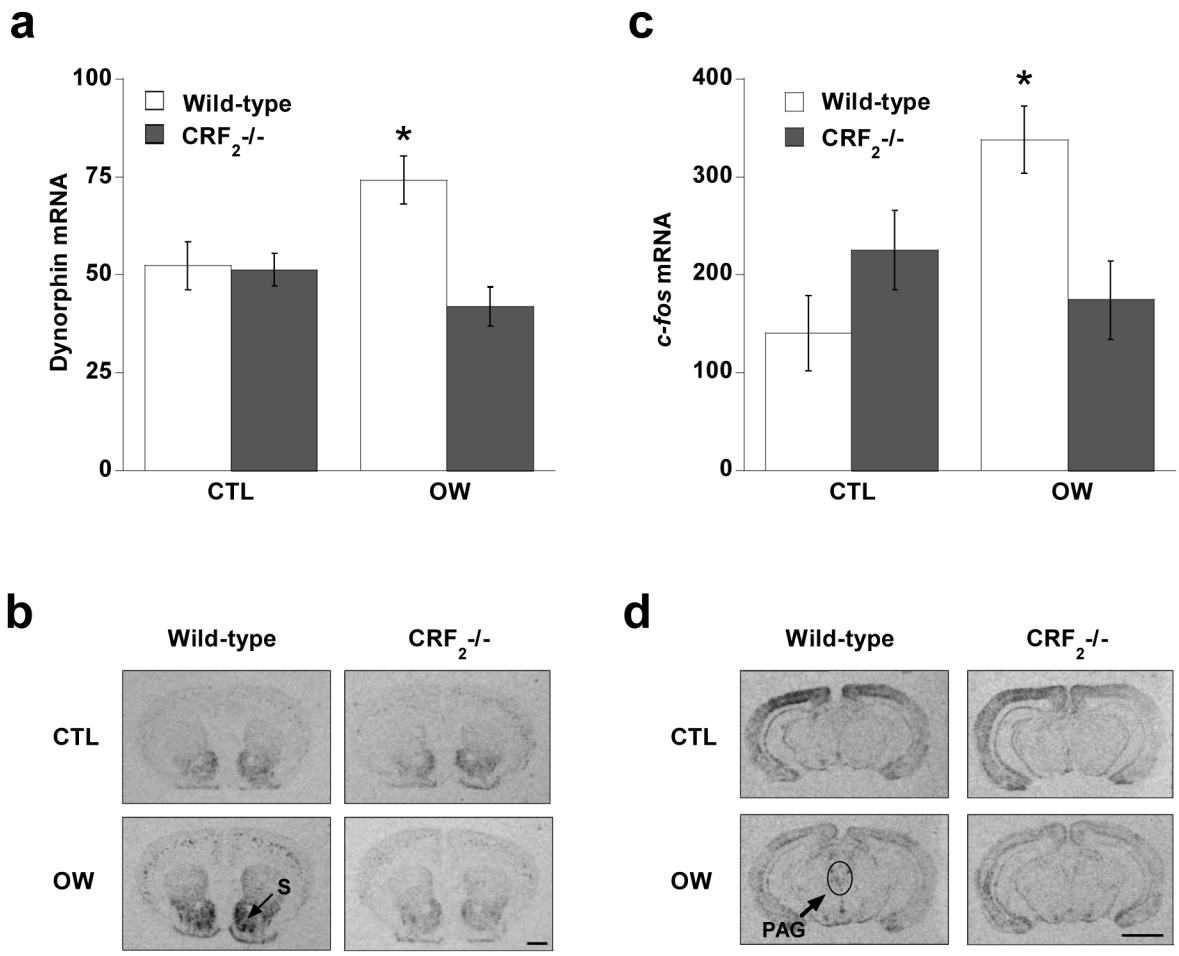
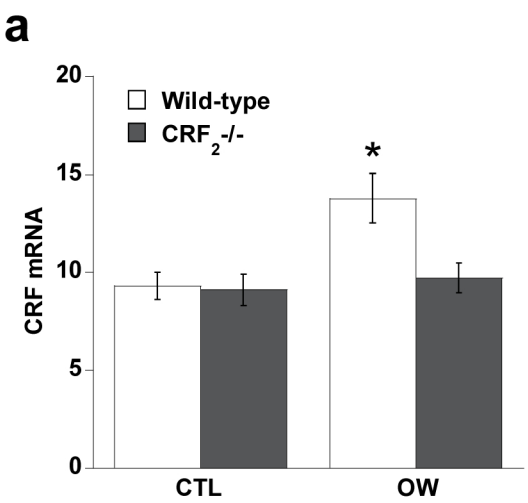
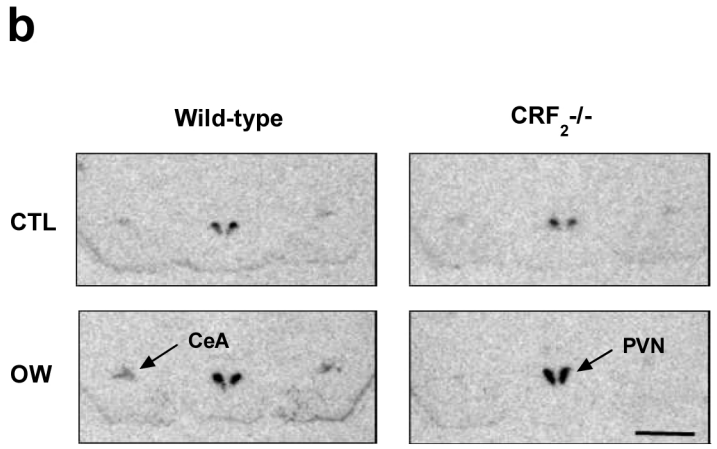

a
100
75
50
25
0
Dynorphin mRNA
Wild-type
CRF2-/-
*
CTL
OW
c
400
300
200
100
0
c-fos mRNA
Wild-type
CRF2-/-
*
CTL
OW
b
Wild-type
CRF2-/-
CTL
OW
S
d
Wild-type
CRF2-/-
CTL
OW
PAG

Figure 4


a
Wild-type
CRF2-/-
CRF mRNA
20
15
10
5
0
*
CTL
OW


b
Wild-type
CRF2-/-
CTL
OW
CeA
PVN

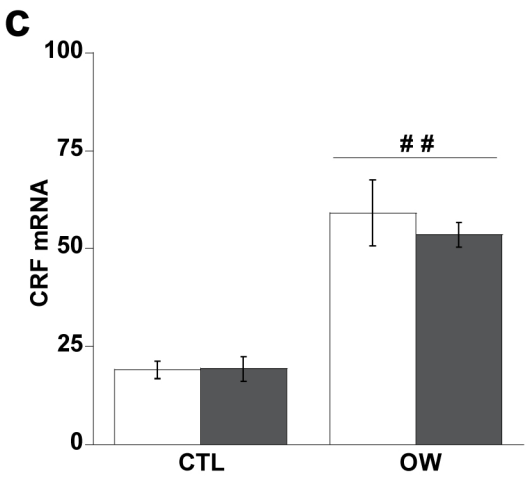
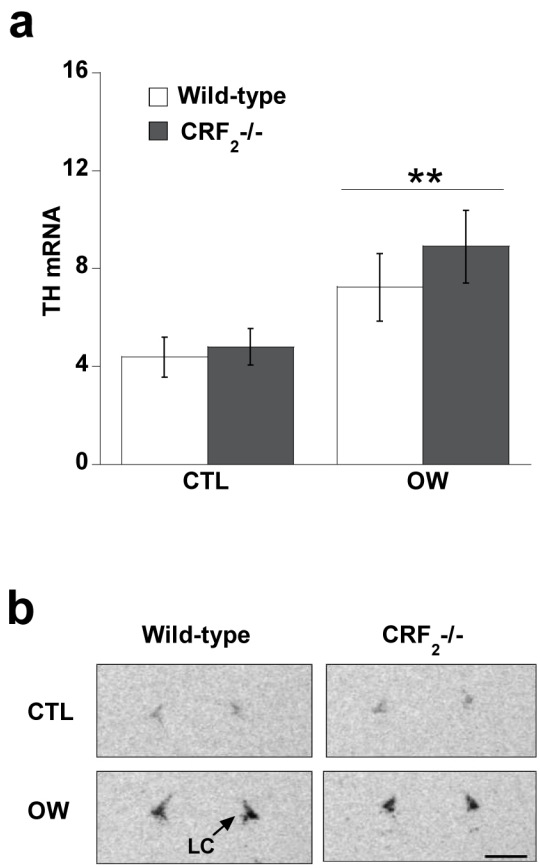

c
CRF mRNA
100
75
50
25
0
# #
CTL
OW

Figure 5


a
16
12
8
4
0
TH mRNA
Wild-type
CRF2-/-
**
CTL
OW
b
Wild-type
CRF2-/-
CTL
OW
LC