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CRF_2 Receptor Deficiency Eliminates the Long-Lasting Vulnerability of Motivational States Induced by Opiate Withdrawal

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Vulnerability to stressful life events is a hallmark of drug dependence that may persist long after cessation of drug intake and dramatically fuel key clinical features, such as deregulated up-shifted motivational states and craving. However, to date, no effective therapy is available for reducing vulnerability to stressful events in former drug users and drug-dependent patients, mostly because of poor knowledge of the mechanisms underlying it. In this study, we report that genetic inactivation of the stress-responsive corticotropin-releasing factor receptor-2 ($CRF_2 -/-$) completely eliminates the reemergence of increased nonrewarded nose-pokes, reflecting up-shifted motivational states, triggered by ethological environmental stressors long after cessation of morphine administration in mice. Accordingly, CRF_2 receptor deficiency completely abolishes the increase in biomarkers of synthesis of major brain motivational substrates, such as ventral tegmental area (VTA) dopamine (DA) and amygdala γ-aminobutyric acid (GABA) systems, associated with the stress-induced reemergence of up-shifted motivational states long after opiate withdrawal. Nevertheless, neither CRF_2 receptor deficiency nor long-term opiate withdrawal affects amygdala CRF or hypothalamus CRF expression, indicating preserved brain stress-coping systems. Moreover, CRF_2 receptor deficiency does not influence the locomotor or the anxiety-like effect of long-term opiate withdrawal. Thus, the present results reveal an essential and specific role for the CRF_2 receptor in the stress-induced reemergence of up-shifted motivational states and related alterations in brain motivational systems long after opiate withdrawal. These findings suggest new strategies for the treatment of the severe and long-lasting vulnerability that inexorably follows drug withdrawal and hinder drug abstinence.
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INTRODUCTION

Vulnerability to stressful life events is a major clinical feature of drug dependence and the associated drug withdrawal syndromes. Notably, despite the apparent recovery, environmental stressors might trigger the reemergence of up-shifted motivational states that may dramatically fuel drug-seeking and drug-taking behavior long after drug discontinuation (Preston and Epstein, 2011; Sinha, 2001). However, the neural mechanisms underlying the long-lasting vulnerability following drug withdrawal remain largely unknown.

The corticotropin-releasing factor (CRF) system coordinates behavioral, neuroendocrine, and autonomic responses to stressors (Koob, 1999; Rivier *et al*, 1982). The CRF system might also be implicated in drug dependence. For instance, CRF receptor antagonists reduce ethanol self-administration, the negative affective-like states of ethanol or cocaine withdrawal, and the stress-induced reinstatement of substance-seeking behavior in rats (Basso *et al*, 1999; Erb *et al*, 1998; Funk *et al*, 2006; Le *et al*, 2000; Shaham *et al*, 1997; Valdez *et al*, 2003). In mammals, CRF-like signaling is transmitted by two types of receptors, termed CRF_1 and CRF_2 (Hauger *et al*, 2003), that may differentially contribute to the multiple signs and symptoms of drug dependence and withdrawal. Indeed, CRF_1 or CRF_2 receptor deficiency completely eliminates the negative affective-like states of opiate withdrawal in mice (Contarino and Papaleo, 2005; Ingallinesi *et al*, 2012). However, CRF_1 receptor deficiency exacerbates whereas CRF_2 receptor deficiency reduces the somatic signs of opiate withdrawal (Papaleo *et al*, 2007, 2008). Drug-withdrawn animals also show altered motivation for drugs or food, especially during initial drug withdrawal phases. For instance, opiate-withdrawn rats and monkeys display increased self-administration of the potent μ-opioid receptor agonists heroin or remifentanil (Cooper *et al*, 2008; Gerak *et al*, 2009). Moreover, opiate-withdrawn rats and mice display increased operant behavior for food, suggesting profound and generalized alterations in motivational processes that may dramatically reduce the ability to overcome drug dependence (Cooper *et al*, 2010; Rouibi and

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Contarino, 2012; Steinfels and Young, 1981). More recently, CRF_2 receptor deficiency is shown to attenuate the motivational effect of initial opiate withdrawal phases (Rouibi and Contarino, 2013). However, the role for the CRF system in the long-lasting vulnerability to stressful life experiences following opiate withdrawal is poorly understood.

Thus, this study investigates the role for the CRF_2 receptor in the motivational states triggered by environmental stressors long after cessation of opiate administration. For this purpose, female and male wild-type and CRF_2 receptor-deficient ($CRF_2-/-$) mice are used (Bale *et al*, 2000). Indeed, although putative CRF_2 receptor-preferring antagonists show higher CRF_2 (*versus* CRF_1) *in vitro* receptor binding affinity (Grace *et al*, 2007; Ruhmann *et al*, 1998), such compounds may interact with both CRF receptors, at least at the behaviorally active doses usually employed (Zorrilla *et al*, 2013), making it difficult to discern CRF_2 from CRF_1 receptor function. In contrast, $CRF_2-/-$ mice show preserved CRF_1 receptor function during opiate withdrawal and might thus be used to understand the specific role for the CRF_2 receptor in drug dependence (Ingallinesi *et al*, 2012; Papaleo *et al*, 2008). Thus, herein wild-type and $CRF_2-/-$ mice are injected with escalating doses of morphine and vulnerability of motivational states to environmental stressors assessed long after drug discontinuation by *ad hoc* designed operant behavior paradigms. To further investigate the neural substrates underlying the CRF_2 receptor-mediated vulnerability, *in situ* hybridization studies are also carried out. In particular, expression of the key catecholamine and γ-aminobutyric acid (GABA) synthesis enzymes tyrosine hydroxylase (TH) and glutamic acid decarboxylase (GAD_{67}) is assessed in stress- and drug dependence-relevant brain regions, such as the ventral tegmental area (VTA), the locus coeruleus (LC), the central (CeA), and the basolateral (BLA) nucleus of the amygdala (Esclapez *et al*, 1994; Ingallinesi *et al*, 2012; Koob, 2008; Papaleo *et al*, 2007; Phelps and LeDoux, 2005; Vrana *et al*, 1993).

MATERIALS AND METHODS

See also the Supplementary Information online.

Subjects

Group-housed littermate wild-type (23 males and 22 females) or $CRF_2-/-$ (22 males and 21 females) mice with a mixed C57BL/6J × 129 strain background are used (Bale *et al*, 2000). Studies are conducted in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and are approved by the local animal care and use committee. All efforts are made to minimize animal suffering and to reduce the number of animals used.

Operant Behavior Studies

The whole experimental design is depicted in Figure 1. To allow acquisition of operant behavior, before drug exposure the mice are daily trained from 1600 to 1800 h using fixed ratio (FR)-1, FR-3, and FR-6 reinforcement schedules, as previously described (Rouibi and Contarino, 2013). Nose-poke into the 'active' hole results in the delivery of a palatable food pellet, whereas nose-poke into the 'inactive' hole has no consequences. Then, the mice are switched to a progressive ratio (PR)-2 reinforcement schedule for 30 days (3 baseline, 6 morphine, and 21 opiate withdrawal days), during which the number of active nose-pokes required to obtain each successive food pellet is increased by 2. During the 6 morphine days, at 0800 and 2000 h the mice are injected i.p. with physiological saline (10 ml/kg) or morphine HCl (Francopia, Gentilly, France), as follows: day 1: 20 mg/kg, day 2: 40 mg/kg, day 3: 60 mg/kg, day 4: 80 mg/kg, day 5: 100 mg/kg, and day 6: 100 mg/kg, only one injection at 0800 h. On opiate withdrawal days 11–21, a reversal operant schedule is applied, ie, the active nose-poke hole becomes inactive, and vice versa. Then, starting on opiate withdrawal day 22, a food withdrawal stressor (FWS) is applied, ie, nose-poke is no more rewarded by the palatable food diet. Moreover, on opiate withdrawal day 32 or 34, within genotype, sex, and drug treatment (saline or morphine), approximately half of the mice are exposed to an elevated platform stressor (EPS) using a counterbalanced design. For this purpose, the mouse is placed on a platform (10 × 10 cm) elevated 40 cm above the ground for 10 min just before confinement to the operant apparatus. A discrimination index is calculated as the percentage of active, or previously active during the extinction sessions, nose-pokes over the total number of nose-pokes (active+inactive) made within a 2-h test session. Finally, on opiate withdrawal day 39, all of the animals are exposed again to the EPS, confined to the operant apparatus for 2 h, and brains rapidly removed thereafter, frozen in isopentane (−40 °C), and stored at − 80 °C.

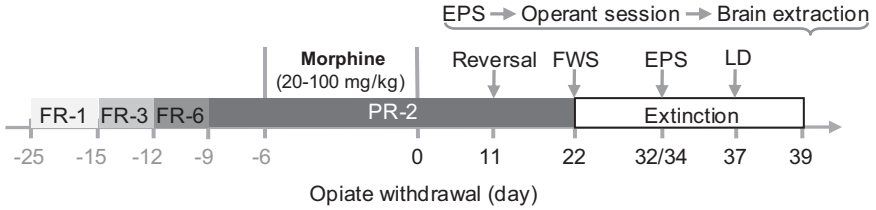

Figure 1 Experimental design. Wild-type or $CRF_2-/-$ mice are first trained in a food-driven operant paradigm under a fixed ratio (FR)-1, -3, and -6 and a progressive ratio (PR)-2 schedule of reinforcement. Morphine is administered i.p. twice a day (0800 and 2000 h) at escalating doses (20–100 mg/kg) and 2-h operant sessions started 8 h after the morning drug injection. Starting on opiate withdrawal day 11, reversal of reward (food) contingency is applied. Starting on opiate withdrawal day 22, the mice undergo a food withdrawal stressor (FWS), the operant sessions being carried out under extinction conditions. Then, the mice are exposed to an elevated platform stressor (EPS) on opiate withdrawal day 32 or 34, according to a within-subject design and tested in the light/dark (LD) exploration test of anxiety on opiate withdrawal day 37. Finally, on opiate withdrawal day 39, all of the mice are again exposed to the EPS, tested for operant behavior (2 h), and brains collected immediately thereafter.

Anxiety-Like Behavior Studies

On opiate withdrawal day 37, the mice are tested for anxiety-like behavior using the light/dark (LD) exploration test (Crawley, 1985). Behaviors scored are: (1) time taken to the first entry into the light chamber, ie, dark/light (DL) latency, (2) time spent in the light chamber, and (3) number of transitions between the two chambers.

In Situ Hybridization Studies

Brain regions are identified using a mouse brain atlas (Paxinos and Franklin, 2001). *In situ* hybridization is performed as previously described (Ingallinesi *et al*, 2012) with antisense ^{35}S-labeled complementary RNA probes designed to recognize GAD_{67} (Frenois *et al*, 2005), TH, and CRF (Ingallinesi *et al*, 2012) mRNAs. GAD_{67} gene expression is assessed in the BLA, the CeA, the nucleus accumbens shell (NaccSh) and the cingulate cortex (CinCtx). TH expression is assessed in the VTA and the LC and CRF expression in the CeA and the paraventricular nucleus of the hypothalamus (PVN).

Statistical Analysis

The analysis of variance (ANOVA) with genotype (wild-type *vs* $CRF_2-/-$), sex (male *vs* female), and drug treatment (saline *vs* morphine) as between-subjects factors and repeated tests as a within-subject factor is used to analyze nose-poke, discrimination index, horizontal activity, anxiety-like behavior, and mRNA expression. The Newman–Keuls *post hoc* test is used for individual group comparisons. The accepted value for significance is $P<0.05$.

RESULTS

See also the Supplementary Information online.

CRF_2 Receptor Deficiency Eliminates the Reemergence of Motivational Up-Shifts Induced by a FWS Following Long-Term Opiate Withdrawal

Control or opiate-withdrawn mice of either genotype rapidly acquire reversal operant behavior during the opiate withdrawal days 11–20 (Supplementary Information; Supplementary Figure S1A and B). To assess the role for the CRF_2 receptor in the reemergence of stress-related motivational states following long-term opiate withdrawal, an ethological FWS is applied starting on opiate withdrawal day 22. Removal of the palatable food reinforcer differentially affects nose-poke in the previously active hole in opiate-withdrawn wild-type or $CRF_2-/-$ mice (genotype × drug treatment interaction effect: $F_{1,79}=7.12$, $P<0.01$), independently of the sex. Indeed, opiate-withdrawn wild-type mice make more nose-pokes than opiate-withdrawn $CRF_2-/-$ and control wild-type or $CRF_2-/-$ mice ($P<0.001$). In contrast, opiate-withdrawn $CRF_2-/-$ do not differ from control mice ($P=0.95$), indicating an essential role for the CRF_2 receptor in the reemergence of up-shifted motivational states induced by the FWS (Figure 2a, and see insert). However, no genotype × sex × drug treatment interaction effect is observed. A repeated test effect ($F_{10,790}=78.81$, $P<0.0001$) indicates that, starting from opiate withdrawal day 25, overall the mice make less nose-pokes than on the last reversal day (opiate withdrawal day 21, $P<0.0001$; Figure 2a), indicating a relatively rapid extinction of the previously food-reinforced behavior. Although discrimination indices remain relatively elevated, a genotype × drug treatment interaction effect ($F_{1,79}=5.94$, $P<0.05$) reveals higher discrimination indices in opiate-withdrawn wild-type than in opiate-withdrawn $CRF_2-/-$ or control mice ($P<0.05$, Figure 2b, and see insert), possibly linked to their increased motivational state. However, no genotype × sex × drug treatment interaction effect is observed. Moreover, a sex × drug treatment interaction effect ($F_{1,79}=4.25$, $P<0.05$) reveals lower horizontal activity in male, *but not* female, opiate-withdrawn mice, as compared with same-sex control mice ($P<0.05$, Supplementary Figure S2A). However, no genotype × sex × drug treatment interaction effect is observed. Thus, following long-term opiate withdrawal, CRF_2 receptor deficiency totally eliminates the motivational effect of the ethological FWS without affecting ambulation.

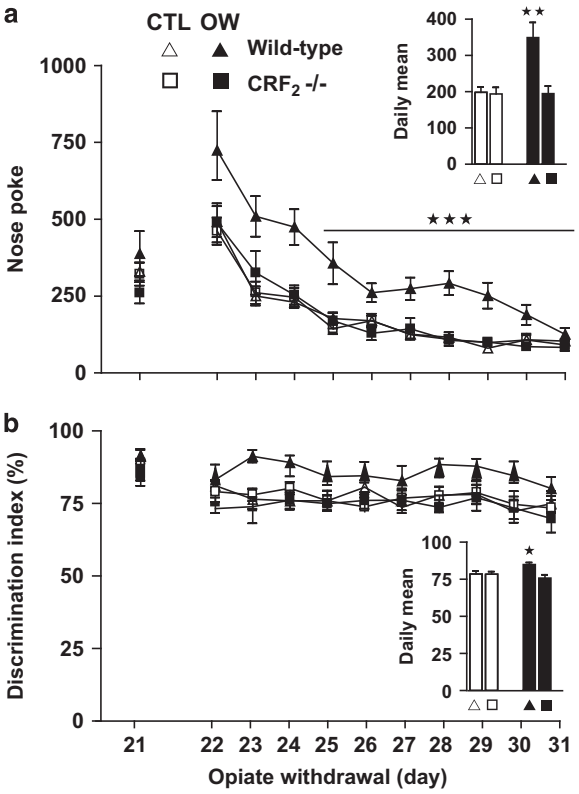

Figure 2 CRF_2 receptor deficiency eliminates the reemergence of up-shifted motivational states induced by a food withdrawal stressor (FWS) following long-term opiate withdrawal. Nose-poke in the previously active hole (a) and discrimination index (b) displayed by control (CTL) or opiate-withdrawn (OW) wild-type or $CRF_2-/-$ mice on opiate withdrawal days 21–31. The FWS is applied starting on opiate withdrawal day 22. Values represent mean ± SEM. $N=21$–24/group; each group includes male and female mice. Inserts illustrate the daily mean of nose-poke or discrimination index. $^*P<0.05$, $^{**}P<0.005$ *vs* OW $CRF_2-/-$ and CTL wild-type and $CRF_2-/-$ mice. $^{***}P<0.0001$ *vs* opiate withdrawal day 21, repeated measure effect. See also Supplementary Figure S2A for horizontal activity (beam-breaks) during the 2-h operant behavior tests carried out on opiate withdrawal days 21–31.

CRF_2 Receptor Deficiency Eliminates the Reemergence of Motivational Up-Shifts Induced by an EPS Following Long-Term Opiate Withdrawal

To investigate whether the effect of CRF_2 receptor deficiency upon the reemergence of up-shifted motivational states generalizes to other ethological stressful experiences, an EPS is applied on opiate withdrawal day 32 or 34. Exposure to the EPS affects nose-poke in a genotype- and drug treatment-dependent manner (stress × genotype × drug treatment interaction effect: $F_{1,79} = 5.76$, $P < 0.05$). Indeed, following exposure to the EPS, opiate-withdrawn wild-type mice make more nose-pokes than opiate-withdrawn $CRF_2 -/-$ and control mice ($P < 0.0005$, Figure 3a). In contrast, opiate-withdrawn $CRF_2 -/-$ mice do not differ from control mice and show similar nose-pokes during the stress and the no-stress day ($P = 0.27$, Figure 3a). Notably, the lack of effect in control drug-naive mice indicates the relatively mild nature of the EPS used. However, no genotype × sex × drug treatment × stress interaction effect is observed. The EPS does not affect discrimination index in opiate-withdrawn mice of either genotype (stress × genotype × drug treatment interaction effect: $F_{1,79} = 0.34$, $P = 0.56$) or sex (stress × sex × drug treatment interaction effect: $F_{1,79} = 0.17$, $P = 0.68$; Figure 3b). However, following EPS exposure, opiate-withdrawn female, *but not* male, mice display higher horizontal activity than same-sex control mice (sex × drug treatment interaction effect: $F_{1,79} = 9.33$, $P < 0.005$; Supplementary Figure S2B). However, no genotype × sex × drug treatment interaction effect is observed. These results demonstrate that CRF_2 receptor deficiency completely eliminates the EPS-induced reemergence of motivational up-shifts long after opiate withdrawal and further support dissociation between motivation and ambulation.

CRF_2 Receptor-Independent Anxiety-Like Effects of Long-Term Opiate Withdrawal

To assess emotional-like behavior that might contribute to stress-induced motivational states, anxiety-like behavior is evaluated by the LD test on opiate withdrawal day 37. Opiate-withdrawn mice show increased DL latency ($F_{1,79} = 12.92$, $P < 0.001$), decreased transitions ($F_{1,79} = 15.07$, $P < 0.0005$), and decreased time spent in the light chamber of the LD apparatus ($F_{1,79} = 20.40$, $P < 0.0001$) as compared with control mice (Supplementary Figure S3A–C). A marginally significant genotype × drug treatment interaction effect ($F_{1,79} = 3.87$, $P = 0.052$; Supplementary Figure S3B) is also found for the transitions but not for DL latency or time spent in the light chamber. However, no genotype × sex × drug treatment interaction effect is observed for any of the three anxiety-like parameters. Thus, CRF_2 receptor deficiency does not affect anxiety-like behavior associated with long-term opiate withdrawal.

CRF_2 Receptor-Linked VTA-TH, BLA-, and CeA-GAD_{67} Expression Following Long-Term Opiate Withdrawal

On opiate withdrawal day 38, opiate-withdrawn wild-type mice of either sex show nose-poke levels similar to those observed on the day preceding the first EPS exposure (data not shown). Thus, to further search for the neural substrates implicated in the CRF_2 receptor-mediated vulnerability to environmental stressors linked to long-term opiate withdrawal, on opiate withdrawal day 39, the mice are once again exposed to the EPS, tested for nonrewarded operant behavior, and brains extracted immediately thereafter for gene expression studies. Reexposure to the EPS affects wild-type and $CRF_2 -/-$ mice in a sex-linked manner (genotype × sex interaction effect: $F_{1,79} = 4.73$, $P < 0.05$). Similar to the first EPS exposure, a genotype × drug treatment interaction effect ($F_{1,40} = 4.67$, $P < 0.05$) reveals that opiate-withdrawn male wild-type mice make more nose-pokes than opiate-withdrawn male $CRF_2 -/-$ and control mice ($P < 0.005$); in contrast, opiate-withdrawn male $CRF_2 -/-$ mice do not differ from control drug-naive mice ($P = 0.36$, Supplementary Figure S4A). However, a drug treatment

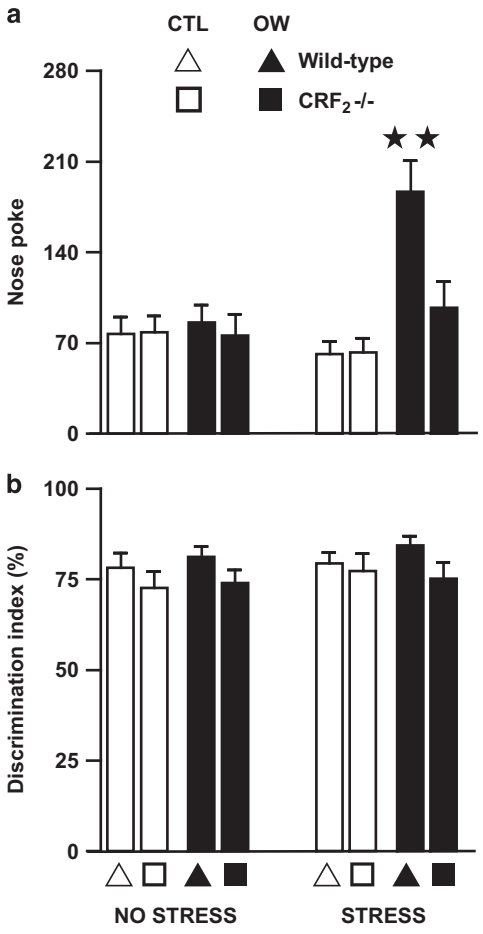

Figure 3 CRF_2 receptor deficiency eliminates the reemergence of up-shifted motivational states induced by an elevated platform stressor (EPS) following long-term opiate withdrawal. Nose-poke in the previously active hole (a) and discrimination index (b) displayed by unstressed (NO STRESS) or stressed (STRESS) control (CTL) or opiate-withdrawn (OW) wild-type or $CRF_2 -/-$ mice. No-stress and stress tests are carried out on opiate withdrawal days 32 or 34 using a counterbalanced design, and nose-poke is not rewarded. On the stress day, the mice are exposed to the EPS for 10 min just before being tested. Values represent mean ± SEM. $N = 21$–24/group; each group includes male and female mice. $^{**}P < 0.0005$ *vs* all other groups, including the NO-STRESS condition. See also Supplementary Figure S2B for horizontal activity (beam-breaks) during the 2-h operant behavior test carried out following the first EPS exposure (opiate withdrawal day 32 or 34).

effect ($F_{1,39} = 16.25$, $P < 0.0005$) reveals that either wild-type or $CRF_2 -/-$ opiate-withdrawn female mice make more nose-pokes than control mice ($P < 0.0005$, Supplementary Figure S4B). Thus, the role for the CRF_2 receptor in brain gene expression associated with stress-induced motivational up-shifts is assessed in male mice.

Long-term opiate withdrawal differentially affects VTA-TH expression in wild-type or $CRF_2 -/-$ mice (genotype × drug treatment interaction effect: $F_{1,37} = 7.97$, $P < 0.01$; Figure 4a). The *post hoc* individual group comparisons reveal no significant differences. However, percentage of same genotype control VTA-TH expression is increased in opiate-withdrawn wild-type mice but decreased in opiate-withdrawn $CRF_2 -/-$ mice ($P < 0.001$, Figure 4b and c; Student's *t*-test). In contrast, neither genotype ($F_{1,37} = 0.64$, $P = 0.43$) nor drug treatment ($F_{1,37} = 0.01$, $P = 0.94$) affects LC-TH expression (Figure 4d–f). Similar to the VTA, long-term opiate withdrawal differentially affects GAD_{67} expression in wild-type or $CRF_2 -/-$ mice in both the BLA (genotype × drug treatment interaction effect: $F_{1,38} = 5.19$, $P < 0.05$; Figure 5a) and CeA (genotype × drug treatment interaction effect: $F_{1,37} = 4.26$, $P < 0.05$; Figure 5c). The *post hoc* individual group comparisons reveal no significant differences. However, percentage of same genotype control GAD_{67} expression is increased in opiate-withdrawn wild-type mice but decreased in opiate-withdrawn $CRF_2 -/-$ mice in both the BLA ($P < 0.05$, Figure 5b and e; Student's *t*-test) and CeA ($P < 0.05$, Figure 5d and e; Student's *t*-test). These results indicate a CRF_2 receptor-mediated biosynthesis of VTA dopamine (DA) and amygdala GABA in long-term opiate-withdrawn mice showing a stress-induced reemergence of up-shifted motivational states. However, long-term opiate withdrawal does not affect CRF expression in wild-type or $CRF_2 -/-$ mice in either the PVN (genotype × drug treatment interaction effect: $F_{1,20} = 0.42$, $P = 0.53$; Supplementary Figure S5A and C) or the CeA (genotype × drug treatment interaction effect: $F_{1,37} = 0.05$, $P = 0.83$; Supplementary Figure S5B and C), indicating no change in other major brain stress-responsive systems. Moreover, neither genotype nor drug treatment nor genotype × drug treatment interaction effects are observed in GAD_{67} mRNA expression in the NaccSh or the CinCtx (Supplementary Table S1).

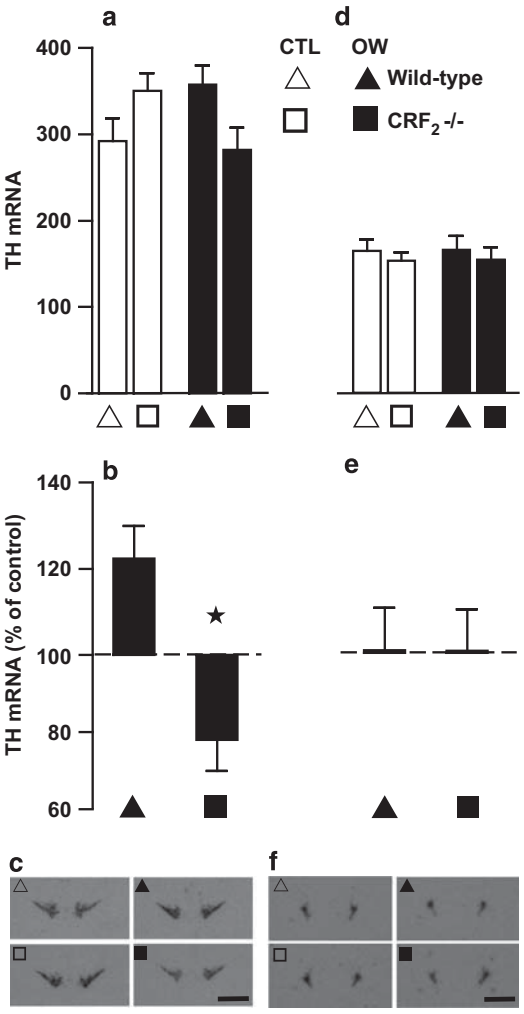

Figure 4 CRF_2 receptor-dependent increase in brain dopamine activity following long-term opiate withdrawal. Optical density (a, d), percentage of same genotype control optical density (b, e), and representative images of brain sections (c, f) illustrating tyrosine hydroxylase (TH) expression in the ventral tegmental area (VTA) (a–c) or in the locus coeruleus (LC) (d–f) in male control (CTL) or opiate-withdrawn (OW) wild-type or $CRF_2 -/-$ mice following reexposure to an elevated platform stressor (EPS) on opiate withdrawal day 39. The mice are exposed to EPS for 10 min, and then immediately confined to the operant behavior apparatus for 2 h and brains rapidly collected thereafter. Bregma intervals are −2.93/−3.04 for the VTA and −5.40/−5.70 for the LC. Scale bar = 1 mm. $N = 9$–11/group. Results represent mean ± SEM of optical density (×1000). *$P < 0.0001$ *vs* OW wild-type mice, Student's *t*-test. See also Supplementary Figures S4 and S5 for nose-pokes during the 2-h operant behavior test or CRF expression in the paraventricular nucleus of the hypothalamus (PVN) or the central nucleus of the amygdala (CeA), respectively, following reexposure to the EPS.

DISCUSSION

Summary of the Results

This study demonstrates a crucial role for the CRF_2 receptor in vulnerability to environmental stressors of motivational states following long-term opiate withdrawal. Indeed, CRF_2 receptor deficiency abolishes the reemergence of motivational up-shifts induced by a FWS in drug-withdrawn mice. Furthermore, exposure to a relatively mild EPS ~2 weeks later once again triggers the reemergence of up-shifted motivational states in opiate-withdrawn wild-type, but *not* $CRF_2 -/-$, mice. Accordingly, the stress-induced reemergence of motivational states is associated with an increase in biosynthesis markers relevant to motivational processes, such as VTA-TH, BLA-, and CeA-GAD_{67}, that is abolished by CRF_2 receptor deficiency.

The CRF_2 Receptor Mediates the Stress-Induced Reemergence of Up-Shifted Motivational States Following Long-Term Opiate Withdrawal

Upon application of a reversal learning reinforcement schedule, control and opiate-withdrawn wild-type and $CRF_2 -/-$ mice similarly acquire a food-driven operant behavior, indicating that neither motivation nor cognitive function is affected by CRF_2 receptor deficiency or opiate withdrawal. Nevertheless, removal of the positive food

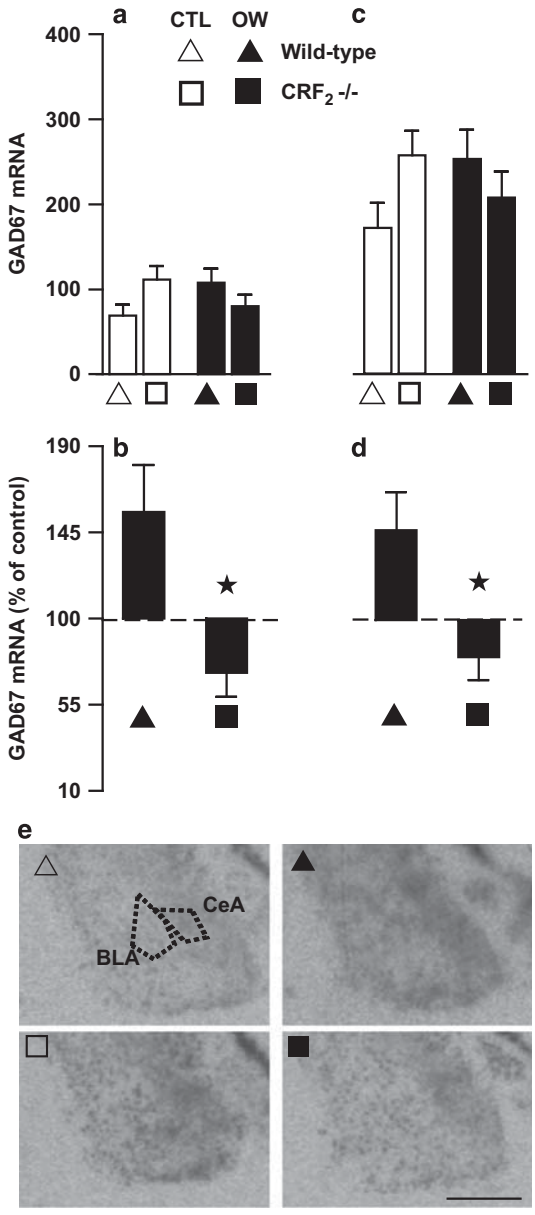

Figure 5 CRF_2 receptor-dependent increase in brain GABA activity following long-term opiate withdrawal. Optical density (a, c), percentage of same genotype control optical density (b, d), and representative images of brain sections (e) illustrating glutamic acid decarboxylase (GAD_{67}) expression in the basolateral amygdala (BLA) (a, b, e) or the central amygdala (CeA) (c–e) in male control (CTL) or opiate-withdrawn (OW) wild-type or $CRF_2 -/-$ mice following reexposure to an elevated platform stressor (EPS) on opiate withdrawal day 39. The mice are exposed to EPS for 10 min, and then immediately confined to the operant behavior apparatus for 2 h and brains rapidly collected thereafter. Bregma interval is −0.79/−1.22. Scale bar = 1 mm. $N = 9–12$/group. Results represent mean ± SEM of optical density (×1000). *$P < 0.01$ *vs* OW wild-type mice, Student's *t*-test. See also Supplementary Figures S4 and S5 for nose-pokes during the 2-h operant behavior test or CRF expression in the paraventricular nucleus of the hypothalamus (PVN) or the CeA, respectively, following reexposure to the EPS.

reinforcer (ie, the FWS) on opiate withdrawal day 22 increases nonrewarded operant behavior in opiate-withdrawn wild-type mice, unmasking a vulnerable state. In contrast, opiate-withdrawn $CRF_2 -/-$ mice do not differ from drug-naive mice, revealing an essential role for the CRF_2 receptor in the reemergence of motivational states triggered by environmental stressors long after drug withdrawal. Throughout this study, overt somatic signs of opiate withdrawal are not observed in either wild-type or $CRF_2 -/-$ mice. CRF_2 receptor deficiency effectively reduces the somatic signs of opiate withdrawal (Papaleo *et al*, 2008). Moreover, by 6 days after the last morphine injection, somatic opiate withdrawal also largely dissipates in the wild-type mice (Papaleo *et al*, 2008). Thus, it is unlikely that somatic opiate withdrawal signs account for the long-lasting genotype differences in motivational states observed herein. The neural mechanisms underlying drug-induced vulnerability remain poorly understood. The FWS used herein might exacerbate the stressful experience of opiate withdrawal in wild-type mice, thus increasing behavior aimed at obtaining palatable food in the attempt to alleviate a severe stress-like state. Accordingly, rats or mice withdrawn from palatable food diets display a stress-like state, including hypophagia, anhedonia, increased anxiety-like, and hypothalamus–pituitary–adrenal (HPA) axis responses to stressors, that can be attenuated by the CRF_1 receptor-preferring antagonist R121919 (Cottone *et al*, 2009; Sharma *et al*, 2013). Although other interpretations could be provided, several factors suggest that the stress-induced nonrewarded operant behavior observed herein in the long-term opiate-withdrawn wild-type mice reflects up-shifted motivational states. For instance, prior studies fail to support lowered motivational thresholds during opiate withdrawal. Indeed, opiate withdrawal elevates the reward threshold for intracranial self-stimulation (ICSS), instead of lowering it, indicating decreased sensitivity of the brain reward system (Bruijnzeel *et al*, 2006). Moreover, herein the impact of stressors upon nose-poking is examined in the absence of the rewarding stimulus, ie, under extinction conditions, throughout a relatively long period (~18 days). In this regard, studies suggest that under extinction conditions behavior is mainly motivated by the incentive salience (ie, how much the reward is wanted) rather than by the hedonic value (ie, how much the reward is liked) of the rewarding stimulus (Berridge *et al*, 2009). Thus, the stress-induced increase in nose-poking observed herein in long-term opiate-withdrawn wild-type mice likely reflects elevated (up-shifted) motivational states rather than lowered motivational thresholds.

Following recovery of prestress levels of nonrewarded operant behavior, exposure to another relatively mild environmental stressor (ie, the EPS) once again induces motivational up-shifts in long-term opiate-withdrawn wild-type mice. Notably, the EPS used herein may be considered a relatively mild stressor as it does not influence operant behavior or cognitive performance of drug-naive mice (Morisot *et al*, 2014). The present results thus provide new and solid evidence of a long-lasting, but 'hidden', vulnerability in drug-withdrawn subjects that is unmasked by stressful life events and may reemerge as deregulated up-shifted motivational states. Such motivational up-shifts are likely because of long-term opiate withdrawal rather than morphine itself. Indeed, using the same morphine regimen as that employed herein, no motivational changes are observed when the operant test session is carried out 8 h after the drug injection (Rouibi and Contarino, 2012, 2013). However, motivational up-shifts are observed starting 32 h

after the last morphine injection and may last at least up to 12 days after cessation of morphine administration (Rouibi and Contarino, 2012, 2013). Thus, the stress-induced motivational states observed herein in long-term opiate withdrawn mice may not occur if morphine dosing is continued up to the stress exposure.

Unlike the wild-type mice, the EPS-induced reemergence of motivational alterations is totally abolished in opiate-withdrawn $CRF_2-/-$ mice, revealing a crucial role for the CRF_2 receptor in vulnerability associated with long-term drug withdrawal. CRF receptor antagonists might attenuate the stress-induced reinstatement of ethanol- or cocaine-seeking behavior in substance-withdrawn rats (Erb *et al*, 1998; Le *et al*, 2000; Shaham *et al*, 1997). However, the relative role for each of the two known CRF receptor types is not clear. For instance, prior studies using putative CRF_1 or CRF_2 receptor-preferring antagonists provide opposite findings on the role for each CRF receptor type in the stress-induced reinstatement of drug-seeking behavior in cocaine-withdrawn rats (Blacktop *et al*, 2011; Wang *et al*, 2007). However, it is possible that the currently available CRF receptor antagonists, especially the putative CRF_2 receptor-preferring antagonists, are poorly selective and might thus interact with both CRF receptor types, at least at the behaviorally active doses usually employed (Zorrilla *et al*, 2013). In contrast, opiate-withdrawn CRF_2 receptor-deficient mice display unaltered CRF_1 receptor-dependent neuroendocrine and behavioral responses (Ingallinesi *et al*, 2012; Papaleo *et al*, 2008), suggesting that resilience to the stress-induced reemergence of motivational alterations is specifically because of the lack of functional CRF_2 receptors. The CRF_2 receptor has been largely implicated in the stress response (Bale *et al*, 2000; Coste *et al*, 2000; Kishimoto *et al*, 2000). However, herein drug-naive mice of either genotype do not differ following exposure to the FWS or the EPS. Furthermore, long-term opiate-withdrawn wild-type and $CRF_2-/-$ mice display similar locomotor activity and anxiety-like behavior, indicating that CRF_2 receptor-mediated motivational states are independent of ambulatory or negative affective-like effects of drug withdrawal. Accordingly, studies indicate dissociation of motivational, ambulatory, somatic, and affective-like effects of opiate withdrawal (Contarino and Papaleo, 2005; Delfs *et al*, 2000; Papaleo *et al*, 2007; Rouibi and Contarino, 2012). Nevertheless, the FWS and the EPS used herein reliably increase nonrewarded nose-poking in long-term opiate-withdrawn wild-type, *but not* $CRF_2-/-$, mice. Thus, although CRF_2 receptor-dependent changes in stress susceptibility cannot be totally ruled out, the present results mainly suggest a role for the CRF_2 receptor in the long-lasting vulnerability of motivational states induced by opiate withdrawal.

This study also reveals sex-linked stress responses in opiate-withdrawn $CRF_2-/-$ mice. Indeed, unlike opiate-withdrawn *male* $CRF_2-/-$ mice, upon the second exposure to the EPS, opiate-withdrawn *female* $CRF_2-/-$ mice show the stress-induced reemergence of increased operant behavior. Prior studies report sex-linked differences in stress responses. For instance, female rats ingest less food than male rats in response to an emotional stressor, a sex effect abolished by ovariectomy and by the CRF_1 receptor-preferring antagonist CRA1000 (Kuriyama and Shibasaki, 2004). Moreover, a repeated restraint stress procedure increases c-Fos immunoreactivity in the amygdala to a larger extent in female than in male rats, an effect that may underlie behavioral responses to stressors and that is also abolished by ovariectomy (Khurana and Devaud, 2007; Phelps and LeDoux, 2005). Sex-linked differences in various effects of drugs of abuse are also reported (for a review see Hudson and Stamp, 2011). For instance, the pharmacological stressor yohimbine elicits higher levels of cue-induced reinstatement of cocaine-seeking behavior in female than in male rats. Moreover, the latter effect is more pronounced during the proestrus phase of the estrous cycle, suggesting a facilitating action of the ovarian hormones estrogens and progestins (Feltenstein *et al*, 2011). Evidence also indicates CRF_2 receptor mRNA expression in brain regions relatively rich in gonadotropin-releasing hormone (GnRH) neurons, such as the medial septum, the preoptic area, and the anterior hypothalamus, and in luteinizing hormone (LH) and follicle-stimulating hormone (FSH) gonadotropic cells of the anterior pituitary (Kageyama *et al*, 2003; Van Pett *et al*, 2000), further suggesting that in female animals behavioral and neuroendocrine responses to stressors may result from complex CRF–sex hormones interactions. Relatively few behavioral studies implicate the CRF system in sex-linked differences in stress responses. In particular female, *but not* male, urocortin 2-deficient mice show decreased immobility levels in the forced swim and in the tail suspension tests as well as altered HPA axis activity as compared with wild-type mice (Chen *et al*, 2006). Moreover, male, *but not* females, mice born to $CRF_2+/-$ or $CRF_2-/-$ mothers display higher anxiety-like behavior than mice born to wild-type mothers, independently of their CRF_2 genotype (Bale *et al*, 2002). However, a direct comparison between the latter and the present study is difficult to make. Nevertheless, all of the male and the female mice used herein derive from $CRF_2+/-$ mothers (ie, heterozygote X heterozygote nonsibling matings), ruling out a role for the mother's genotype and/or nurturing effects linked to the mother's genotype in sex- and/or genotype-linked differences. Recent studies provide more insight into the role for the CRF system in sex-linked differences in stress responses. Notably, a swim stress induces association of CRF receptors to β-arrestin2 and receptor internalization in male rats; in contrast, the same stressor decreases the ratio of cytoplasmic-to-total CRF receptor levels in female rats, suggesting CRF receptor recruitment to the plasma membrane and increased stress susceptibility (Bangasser *et al*, 2010). Thus, ovarian hormones and/or sex-linked differences in plasma membrane CRF receptor availability might underlie the loss of resilience to the final stress exposure observed herein in the opiate-withdrawn female, *but not* male, $CRF_2-/-$ mice. However, further studies are warranted to test the latter hypotheses.

CRF_2 Receptor Deficiency Abolishes the Increased Biosynthesis of VTA DA and Amygdala GABA Associated with Long-Term Opiate Withdrawal

To further investigate the neural substrates underlying CRF_2 receptor-mediated stress-induced motivational up-shifts following long-term opiate withdrawal, neuronal markers of brain DA and norepinephrine biosynthesis are also assessed. For this purpose, expression of TH, the rate-limiting enzyme of catecholamine synthesis, is assessed in

the VTA and the LC, major substrates of motivational states and stress responses (Salamone, 1994; Valentino and Van Bockstaele, 2008). Long-term opiate withdrawal elevates VTA-TH expression in the vulnerable wild-type mice, providing initial evidence of increased brain DA biosynthesis long after drug discontinuation. However, CRF_2 receptor deficiency totally abolishes the opiate withdrawal-linked VTA-TH expression, revealing an essential role for the CRF_2 receptor in brain DA biosynthesis relevant to motivational states. Prior studies suggest a role for the CRF system in the activity of VTA DA systems. For instance, CRF antagonists attenuate NMDA receptor-mediated excitatory postsynaptic currents of VTA DA neurons (Ungless *et al*, 2003). Moreover, intra-VTA infusion of the nonselective CRF antagonist α-helical CRF attenuates footshock stress-induced reinstatement of cocaine-seeking behavior (Wang *et al*, 2005). CRF_1 and CRF_2 receptors are reported to be expressed in 15% or 65% of VTA-TH-positive neurons, respectively (Korotkova *et al*, 2006). However, the specific role for the CRF_2 receptor in VTA DA biosynthesis associated with long-term drug withdrawal is poorly understood. Herein, long-term opiate-withdrawn $CRF_2-/-$ mice do not show any increase in VTA-TH expression following exposure to an environmental stressor as compared with drug-naive mice. Thus, the present results provide initial evidence of an essential role for the CRF_2 receptor in the long-lasting vulnerability of brain motivational systems following drug withdrawal.

This study also suggests elevated amygdala GABA neurotransmission in long-term opiate-withdrawn mice showing a stress-induced reemergence of up-shifted motivational states. Indeed, opiate-withdrawn wild-type mice display elevated expression of the key GABA synthesis enzyme GAD_{67} in either the BLA or the CeA. Stressful life events might increase GABA activity in the amygdala. For instance, a predator (dog) stress elevates extracellular GABA content in the sheep amygdala (Cook, 2004). Moreover, naloxone-precipitated opiate withdrawal increases the number of *c-fos*-positive GABA neurons in the CeA of rats or mice (Bagley *et al*, 2011; Frenois *et al*, 2005). However, to our knowledge, studies assessing amygdala GABA biosynthesis in long-term drug-withdrawn animals are lacking. The present data provide initial evidence of increased GAD_{67} expression in the BLA and the CeA of stress-vulnerable opiate-withdrawn mice, indicating elevated amygdala GABA activity long after drug withdrawal. Furthermore, they reveal a major role for the CRF system in amygdala GABA biosynthesis associated with long-term drug withdrawal. Indeed, unlike the wild-type mice, opiate-withdrawn $CRF_2-/-$ mice do not show any increase in GAD_{67} expression as compared with drug-naive mice, indicating that amygdala GABA biosynthesis linked to drug withdrawal requires functional CRF_2 receptors. Exposure to the EPS procedure might affect gene expression. However, as all of the mice (control or opiate-withdrawn wild-type and $CRF_2-/-$ mice) are stressed before the final operant behavior session and the subsequent brain extraction, the present gene expression differences are most likely because of genotype and/or drug withdrawal and cannot be simply ascribed to the EPS procedure. Moreover, the increased TH and GAD_{67} expression reported herein might or might not be associated with elevated DA or GABA neurotransmission. However, rat and mouse studies indicate simultaneous elevations in TH or GAD_{67} mRNA expression and dialysate (extracellular) DA or GABA levels in the VTA or the amygdala, respectively, suggesting concurrent increases in DA or GABA neurotransmission and related mRNA expression (Ding *et al*, 1998; Ferrari *et al*, 2002; Tasan *et al*, 2011).

Overall, the present results suggest the implication of VTA DA and amygdala GABA circuitry in the long-lasting motivational alterations following drug withdrawal. Amygdalar nuclei receive massive DA inputs from the VTA (Asan, 1998; Freedman and Cassell, 1994) and bath application of DA enhances the excitability of BLA GABA neurons (Kroner *et al*, 2005). The BLA is the primary 'input' amygdalar nucleus and is composed of GABA interneurons and glutamate neurons projecting to the CeA, the primary 'output' amygdalar nucleus (Pitkanen *et al*, 1997; Woodruff *et al*, 2006). The CeA is mostly composed of GABA interneurons and neurons projecting to brain regions implicated in the stress response, such as the bed nucleus of the stria terminalis, the periaqueductal gray matter, and the hypothalamus (Pitkanen *et al*, 2000). Besides, CeA neurons projecting to the VTA might provide a negative feedback mechanism to attenuate VTA DA activity elicited by stressful experiences (Watabe-Uchida *et al*, 2012). Herein, initial evidence is provided in favor of increased VTA-TH and BLA- and CeA-GAD_{67} expression in long-term opiate-withdrawn wild-type mice showing a stress-induced reemergence of up-shifted motivational states. In contrast, opiate-withdrawn $CRF_2-/-$ mice do not show any motivational change or increased VTA-TH and BLA- and CeA-GAD_{67} expression as compared with drug-naive mice. Thus, taken together, the present behavioral and gene expression findings suggest a link between VTA DA and amygdala GABA activity and stress-induced motivational alterations in long-term drug-withdrawn mice. Besides, neither CRF_2 receptor deficiency nor long-term opiate withdrawal affects LC-TH, CeA-, or PVN-CRF expression. Similarly, the latter brain stress systems are not affected by long-term (42 days) cocaine withdrawal (Morisot *et al*, 2014). In contrast, wild-type mice as those used herein show increased LC-TH, CeA-, and PVN-CRF expression 8 h after morphine administration (Ingallinesi *et al*, 2012; Papaleo *et al*, 2007). Thus, unlike early drug withdrawal, the behavioral effects of long-term drug withdrawal phases may be independent of LC-TH, CeA-, or PVN-CRF expression.

The CRF_2 Receptor Null Mutation Does Not Induce Overt Developmental Deficits

As available CRF receptor antagonists do not yet allow addressing the specific role for the CRF_2 (*versus* the CRF_1) receptor in behavior, this study is carried out using a genetically engineered mouse model bearing a life-long disruption of the CRF_2 receptor. Thus, some issues need to be taken into consideration and might limit the interpretation of the present results. In particular, although not yet demonstrated, developmental deficits in motivational and/or cognitive processes might be associated to the life-long CRF_2 receptor deficiency. However, the present and past studies (Rouibi and Contarino, 2013) show that drug-naive $CRF_2-/-$ mice do not differ from wild-type mice in food-motivated operant behavior, as assessed by several FR and PR

reinforcement schedules. Moreover, CRF_2 receptor deficiency does not impair the ability to learn operant tasks and to discriminate reward-reinforced behavior (Rouibi and Contarino, 2013), suggesting preserved cognitive ability. Accordingly, $CRF_2-/-$ mice show unaltered associative learning or recognition memory (Ingallinesi *et al*, 2012; Morisot *et al*, 2014), further ruling out *general* developmental deficits in the life-long CRF_2 receptor-deficient mice.

CONCLUSION AND PERSPECTIVES

In vulnerable former substance users, environmental stressors might trigger deregulated motivational up-shifts that powerfully drive drug-seeking and drug-taking behavior, even after very long periods of drug abstinence; yet the underlying brain mechanisms remain largely unknown. This study unravels an essential role for the CRF_2 receptor in the reemergence of up-shifted motivational states triggered by ethological environmental stressors long after opiate discontinuation. Furthermore, CRF_2 receptor deficiency completely abolishes the elevated expression of biomarkers of brain motivational systems associated with long-term opiate withdrawal. This suggests that CRF_2 receptor antagonism might attenuate the severe and long-lasting vulnerability to stressful events following drug withdrawal, thus enhancing the ability to abstain from drug intake.

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The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

NM, KR, and AC designed the study and analyzed the data; KR conducted the behavioral experiments and helped with the preparation of the manuscript; NM conducted the *in situ* hybridization experiments; NM and AC wrote the manuscript.

REFERENCES

Asan E (1998). The catecholaminergic innervation of the rat amygdala. *Adv Anat Embryol Cell Biol* **142**: 1–118.

Bagley EE, Hacker J, Chefer VI, Mallet C, McNally GP, Chieng BC *et al* (2011). Drug-induced GABA transporter currents enhance GABA release to induce opioid withdrawal behaviors. *Nat Neurosci* **14**: 1548–1554.

Bale TL, Contarino A, Smith GW, Chan R, Gold LH, Sawchenko PE *et al* (2000). Mice deficient for corticotropin-releasing hormone receptor-2 display anxiety-like behaviour and are hypersensitive to stress. *Nat Genet* **24**: 410–414.

Bale TL, Picetti R, Contarino A, Koob GF, Vale WW, Lee KF (2002). Mice deficient for both corticotropin-releasing factor receptor 1 (CRFR1) and CRFR2 have an impaired stress response and display sexually dichotomous anxiety-like behavior. *J Neurosci* **22**: 193–199.

Bangasser DA, Curtis A, Reyes BA, Bethea TT, Parastatidis I, Ischiropoulos H *et al* (2010). Sex differences in corticotropin-releasing factor receptor signaling and trafficking: potential role in female vulnerability to stress-related psychopathology. *Mol Psychiatry* **15**: 877, 896–904.

Basso AM, Spina M, Rivier J, Vale W, Koob GF (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 (Berl)* **145**: 21–30.

Berridge KC, Robinson TE, Aldridge JW (2009). Dissecting components of reward: 'liking', 'wanting', and learning. *Curr Opin Pharmacol* **9**: 65–73.

Blacktop JM, Seubert C, Baker DA, Ferda N, Lee G, Graf EN *et al* (2011). Augmented cocaine seeking in response to stress or CRF delivered into the ventral tegmental area following long-access self-administration is mediated by CRF receptor type 1 but not CRF receptor type 2. *J Neurosci* **31**: 11396–11403.

Bruijnzeel AW, Lewis B, Bajpai LK, Morey TE, Dennis DM, Gold M (2006). Severe deficit in brain reward function associated with fentanyl withdrawal in rats. *Biol Psychiatry* **59**: 477–480.

Chen A, Zorrilla E, Smith S, Rousso D, Levy C, Vaughan J *et al* (2006). Urocortin 2-deficient mice exhibit gender-specific alterations in circadian hypothalamus-pituitary-adrenal axis and depressive-like behavior. *J Neurosci* **26**: 5500–5510.

Contarino A, Papaleo F (2005). The corticotropin-releasing factor receptor-1 pathway mediates the negative affective states of opiate withdrawal. *Proc Natl Acad Sci USA* **102**: 18649–18654.

Cook CJ (2004). Stress induces CRF release in the paraventricular nucleus, and both CRF and GABA release in the amygdala. *Physiol Behav* **82**: 751–762.

Cooper ZD, Shi YG, Woods JH (2010). Reinforcer-dependent enhancement of operant responding in opioid-withdrawn rats. *Psychopharmacology* **212**: 369–278.

Cooper ZD, Truong YN, Shi YG, Woods JH (2008). Morphine deprivation increases self-administration of the fast- and short-acting mu-opioid receptor agonist remifentanil in the rat. *J Pharmacol Exp Ther* **326**: 920–929.

Coste SC, Kesterson RA, Heldwein KA, Stevens SL, Heard AD, Hollis JH *et al* (2000). Abnormal adaptations to stress and impaired cardiovascular function in mice lacking corticotropin-releasing hormone receptor-2. *Nat Genet* **24**: 403–409.

Cottone P, Sabino V, Roberto M, Bajo M, Pockros L, Frihauf JB *et al* (2009). CRF system recruitment mediates dark side of compulsive eating. *Proc Natl Acad Sci USA* **106**: 20016–20020.

Crawley JN (1985). Exploratory behavior models of anxiety in mice. *Neurosci Biobehav Rev* **9**: 37–44.

Delfs JM, Zhu Y, Druhan JP, Aston-Jones G (2000). Noradrenaline in the ventral forebrain is critical for opiate withdrawal-induced aversion. *Nature* **403**: 430–434.

Ding R, Asada H, Obata K (1998). Changes in extracellular glutamate and GABA levels in the hippocampal CA3 and CA1 areas and the induction of glutamic acid decarboxylase-67 in dentate granule cells of rats treated with kainic acid. *Brain Res* **800**: 105–113.

Erb S, Shaham Y, Stewart J (1998). The role of corticotropin-releasing factor and corticosterone in stress- and cocaine-induced relapse to cocaine seeking in rats. *J Neurosci* **18**: 5529–5536.

Esclapez M, Tillakaratne NJ, Kaufman DL, Tobin AJ, Houser CR (1994). Comparative localization of two forms of glutamic acid decarboxylase and their mRNAs in rat brain supports the concept of functional differences between the forms. *J Neurosci* **14**: 1834–1855.

Feltenstein MW, Henderson AR, See RE (2011). Enhancement of cue-induced reinstatement of cocaine-seeking in rats by yohimbine: sex differences and the role of the estrous cycle. *Psychopharmacology (Berl)* **216**: 53–62.

Ferrari R, Le Novere N, Picciotto MR, Changeux JP, Zoli M (2002). Acute and long-term changes in the mesolimbic dopamine pathway after systemic or local single nicotine injections. *Eur J Neurosci* **15**: 1810–1818.

Freedman LJ, Cassell MD (1994). Distribution of dopaminergic fibers in the central division of the extended amygdala of the rat. *Brain Res* **633**: 243–252.

Frenois F, Stinus L, Di Blasi F, Cador M, Le Moine C (2005). A specific limbic circuit underlies opiate withdrawal memories. *J Neurosci* **25**: 1366–1374.

Funk CK, O'Dell LE, Crawford EF, Koob GF (2006). Corticotropin-releasing factor within the central nucleus of the amygdala mediates enhanced ethanol self-administration in withdrawn, ethanol-dependent rats. *J Neurosci* **26**: 11324–11332.

Gerak LR, Galici R, France CP (2009). Self administration of heroin and cocaine in morphine-dependent and morphine-withdrawn rhesus monkeys. *Psychopharmacology (Berl)* **204**: 403–411.

Grace CR, Perrin MH, Cantle JP, Vale WW, Rivier JE, Riek R (2007). Common and divergent structural features of a series of corticotropin releasing factor-related peptides. *J Am Chem Soc* **129**: 16102–16114.

Hauger RL, Grigoriadis DE, Dallman MF, Plotsky PM, Vale WW, Dautzenberg FM (2003). International Union of Pharmacology. XXXVI. Current status of the nomenclature for receptors for corticotropin-releasing factor and their ligands. *Pharmacol Rev* **55**: 21–26.

Hudson A, Stamp JA (2011). Ovarian hormones and propensity to drug relapse: a review. *Neurosci Biobehav Rev* **35**: 427–436.

Ingallinesi M, Rouibi K, Le Moine C, Papaleo F, Contarino A (2012). CRF2 receptor-deficiency eliminates opiate withdrawal distress without impairing stress coping. *Mol Psychiatry* **17**: 1283–1294.

Kageyama K, Li C, Vale WW (2003). Corticotropin-releasing factor receptor type 2 messenger ribonucleic acid in rat pituitary: localization and regulation by immune challenge, restraint stress, and glucocorticoids. *Endocrinology* **144**: 1524–1532.

Khurana RC, Devaud LL (2007). Sex differences in neurotransmission parameters in response to repeated mild restraint stress exposures in intact male, female and ovariectomised female rats. *J Neuroendocrinol* **19**: 511–520.

Kishimoto T, Radulovic J, Radulovic M, Lin CR, Schrick C, Hooshmand F *et al* (2000). Deletion of crhr2 reveals an anxiolytic role for corticotropin-releasing hormone receptor-2. *Nat Genet* **24**: 415–419.

Koob GF (1999). Corticotropin-releasing factor, norepinephrine, and stress. *Biol Psychiatry* **46**: 1167–1180.

Koob GF (2008). A role for brain stress systems in addiction. *Neuron* **59**: 11–34.

Korotkova TM, Brown RE, Sergeeva OA, Ponomarenko AA, Haas HL (2006). Effects of arousal- and feeding-related neuropeptides on dopaminergic and GABAergic neurons in the ventral tegmental area of the rat. *Eur J Neurosci* **23**: 2677–2685.

Kroner S, Rosenkranz JA, Grace AA, Barrionuevo G (2005). Dopamine modulates excitability of basolateral amygdala neurons in vitro. *J Neurophysiol* **93**: 1598–1610.

Kuriyama H, Shibasaki T (2004). Sexual differentiation of the effects of emotional stress on food intake in rats. *Neuroscience* **124**: 459–465.

Le AD, Harding S, Juzytsch W, Watchus J, Shalev U, Shaham Y (2000). The role of corticotrophin-releasing factor in stress-induced relapse to alcohol-seeking behavior in rats. *Psychopharmacology (Berl)* **150**: 317–324.

Morisot N, Le Moine C, Millan MJ, Contarino A (2014). CRF2 receptor-deficiency reduces recognition memory deficits and vulnerability to stress induced by cocaine withdrawal. *Int J Neuropsychopharmacol* **17**: 1969–1979.

Papaleo F, Ghozland S, Ingallinesi M, Roberts AJ, Koob GF, Contarino A (2008). Disruption of the CRF(2) receptor pathway decreases the somatic expression of opiate withdrawal. *Neuropsychopharmacology* **33**: 2878–2887.

Papaleo F, Kitchener P, Contarino A (2007). Disruption of the CRF/CRF1 receptor stress system exacerbates the somatic signs of opiate withdrawal. *Neuron* **53**: 577–589.

Paxinos G, Franklin KBJ (2001). *The Mouse Brain in Stereotaxic Coordinates*. Academic Press: San Diego.

Phelps EA, LeDoux JE (2005). Contributions of the amygdala to emotion processing: from animal models to human behavior. *Neuron* **48**: 175–187.

Pitkanen A, Pikkarainen M, Nurminen N, Ylinen A (2000). Reciprocal connections between the amygdala and the hippocampal formation, perirhinal cortex, and postrhinal cortex in rat. A review. *Ann NY Acad Sci* **911**: 369–391.

Pitkanen A, Savander V, LeDoux JE (1997). Organization of intra-amygdaloid circuitries in the rat: an emerging framework for understanding functions of the amygdala. *Trends Neurosci* **20**: 517–523.

Preston KL, Epstein DH (2011). Stress in the daily lives of cocaine and heroin users: relationship to mood, craving, relapse triggers, and cocaine use. *Psychopharmacology (Berl)* **218**: 29–37.

Rivier C, Brownstein M, Spiess J, Rivier J, Vale W (1982). In vivo corticotropin-releasing factor-induced secretion of adrenocorticotropin, beta-endorphin, and corticosterone. *Endocrinology* **110**: 272–278.

Rouibi K, Contarino A (2012). Increased motivation to eat in opiate-withdrawn mice. *Psychopharmacology* **221**: 675–684.

Rouibi K, Contarino A (2013). The corticotropin-releasing factor receptor-2 mediates the motivational effect of opiate withdrawal. *Neuropharmacology* **73**: 41–47.

Ruhmann A, Bonk I, Lin CR, Rosenfeld MG, Spiess J (1998). Structural requirements for peptidic antagonists of the corticotropin-releasing factor receptor (CRFR): development of CRFR2beta-selective antisauvagine-30. *Proc Natl Acad Sci USA* **95**: 15264–15269.

Salamone JD (1994). The involvement of nucleus accumbens dopamine in appetitive and aversive motivation. *Behav Brain Res* **61**: 117–133.

Shaham Y, Funk D, Erb S, Brown TJ, Walker CD, Stewart J (1997). Corticotropin-releasing factor, but not corticosterone, is involved in stress-induced relapse to heroin-seeking in rats. *J Neurosci* **17**: 2605–2614.

Sharma S, Fernandes MF, Fulton S (2013). Adaptations in brain reward circuitry underlie palatable food cravings and anxiety induced by high-fat diet withdrawal. *Int J Obes (Lond)* **37**: 1183–1191.

Sinha R (2001). How does stress increase risk of drug abuse and relapse? *Psychopharmacology (Berl)* **158**: 343–359.

Steinfels GF, Young GA (1981). Effects of narcotic abstinence on schedule-controlled behavior in dependent rats. *Pharmacol Biochem Behav* **14**: 393–395.

Tasan RO, Bukovac A, Peterschmitt YN, Sartori SB, Landgraf R, Singewald N *et al* (2011). Altered GABA transmission in a mouse model of increased trait anxiety. *Neuroscience* **183**: 71–80.

Ungless MA, Singh V, Crowder TL, Yaka R, Ron D, Bonci A (2003). Corticotropin-releasing factor requires CRF binding protein to potentiate NMDA receptors via CRF receptor 2 in dopamine neurons. *Neuron* **39**: 401–407.

Valdez GR, Zorrilla EP, Roberts AJ, Koob GF (2003). Antagonism of corticotropin-releasing factor attenuates the enhanced responsiveness to stress observed during protracted ethanol abstinence. *Alcohol* **29**: 55–60.

Valentino RJ, Van Bockstaele E (2008). Convergent regulation of locus coeruleus activity as an adaptive response to stress. *Eur J Pharmacol* **583**: 194–203.

Van Pett K, Viau V, Bittencourt JC, Chan RK, Li HY, Arias C *et al* (2000). Distribution of mRNAs encoding CRF receptors in brain and pituitary of rat and mouse. *J Comp Neurol* **428**: 191–212.

Vrana SL, Vrana KE, Koves TR, Smith JE, Dworkin SI (1993). Chronic cocaine administration increases CNS tyrosine hydroxylase enzyme activity and mRNA levels and tryptophan hydroxylase enzyme activity levels. *J Neurochem* **61**: 2262–2268.

Wang B, Shaham Y, Zitzman D, Azari S, Wise RA, You ZB (2005). Cocaine experience establishes control of midbrain glutamate and dopamine by corticotropin-releasing factor: a role in stress-induced relapse to drug seeking. *J Neurosci* **25**: 5389–5396.

Wang B, You ZB, Rice KC, Wise RA (2007). Stress-induced relapse to cocaine seeking: roles for the CRF(2) receptor and CRF-binding protein in the ventral tegmental area of the rat. *Psychopharmacology (Berl)* **193**: 283–294.

Watabe-Uchida M, Zhu L, Ogawa SK, Vamanrao A, Uchida N (2012). Whole-brain mapping of direct inputs to midbrain dopamine neurons. *Neuron* **74**: 858–873.

Woodruff AR, Monyer H, Sah P (2006). GABAergic excitation in the basolateral amygdala. *J Neurosci* **26**: 11881–11887.

Zorrilla EP, Roberts AJ, Rivier JE, Koob GF (2013). Anxiolytic-like effects of antisauvagine-30 in mice are not mediated by CRF2 receptors. *PLoS One* **8**: e63942.

Supplementary Information accompanies the paper on the Neuropsychopharmacology website (http://www.nature.com/npp)