

CRF₂ 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 (CRF₂−/−) 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, CRF₂ 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 CRF₂ receptor deficiency nor long-term opiate withdrawal affects amygdala CRF or hypothalamus CRF expression, indicating preserved brain stress-coping systems. Moreover, CRF₂ 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 CRF₂ 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.

Neuropsychopharmacology (2015) **40**, 1990–2000; doi:10.1038/npp.2015.49; published online 11 March 2015

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 CRF₁ and CRF₂ (Hauger *et al*, 2003), that may differentially contribute to the multiple signs and symptoms of drug dependence and withdrawal. Indeed, CRF₁ or CRF₂ receptor deficiency completely eliminates the negative affective-like states of opiate withdrawal in mice (Contarino and Papaleo, 2005; Ingallinesi *et al*, 2012). However, CRF₁ receptor deficiency exacerbates whereas CRF₂ 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 remifentanyl (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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Received 10 September 2014; revised 6 February 2015; accepted 8 February 2015; accepted article preview online 12 February 2015

Contarino, 2012; Steinfelds and Young, 1981). More recently, CRF₂ 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 CRF₂ receptor in the motivational states triggered by environmental stressors long after cessation of opiate administration. For this purpose, female and male wild-type and CRF₂ receptor-deficient (CRF₂ -/-) mice are used (Bale *et al*, 2000). Indeed, although putative CRF₂ receptor-preferring antagonists show higher CRF₂ (*versus* CRF₁) *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 CRF₂ from CRF₁ receptor function. In contrast, CRF₂ -/- mice show preserved CRF₁ receptor function during opiate withdrawal and might thus be used to understand the specific role for the CRF₂ receptor in drug dependence (Ingallinesi *et al*, 2012; Papaleo *et al*, 2008). Thus, herein wild-type and CRF₂ -/- 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 CRF₂ 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 (GAD₆₇) 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 CRF₂ -/- (22 males and 21 females) mice with

a mixed C57BL/6J \times 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 \times 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 .

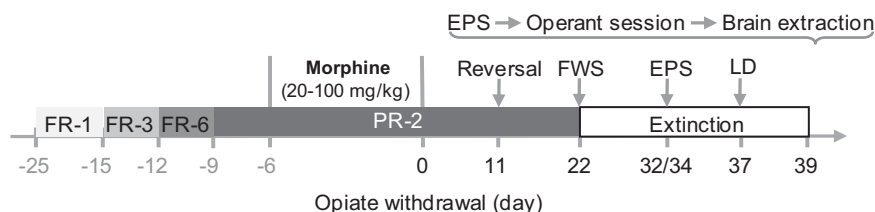


Figure 1 Experimental design. Wild-type or CRF₂ -/- mice are first trained in a food-driven operant paradigm under a fixed ratio (FR)-1, -3, and -6 and a progressive ratio (PR)-2 schedule of reinforcement. Morphine is administered *i.p.* twice a day (0800 and 2000 h) at escalating doses (20–100 mg/kg) 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 ³⁵S-labeled complementary RNA probes designed to recognize GAD₆₇ (Frenois et al, 2005), TH, and CRF (Ingallinesi et al, 2012) mRNAs. GAD₆₇ 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 CRF₂^{-/-}), 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.

CRF₂ 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 CRF₂ 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 CRF₂^{-/-} 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 CRF₂^{-/-} and control wild-type or CRF₂^{-/-} mice ($P < 0.001$). In contrast, opiate-withdrawn CRF₂^{-/-} do not differ from control mice ($P = 0.95$), indicating an essential role for the CRF₂ 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 ($F_{10,790} = 78.81$,

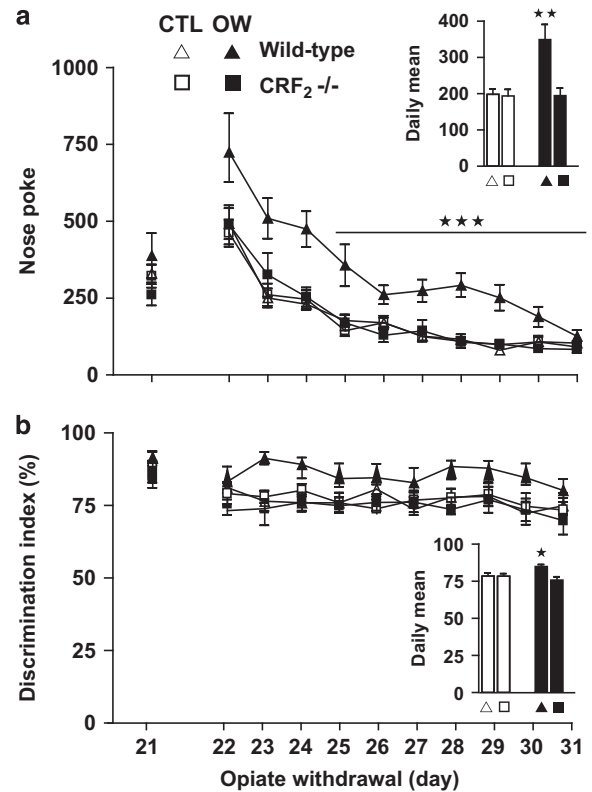


Figure 2 CRF₂ receptor deficiency eliminates the reemergence of up-shifted motivational states induced by a food withdrawal stressor (FWS) following long-term opiate withdrawal. Nose-poke in the previously active hole (a) and discrimination index (b) displayed by control (CTL) or opiate-withdrawn (OW) wild-type or CRF₂^{-/-} 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 CRF₂^{-/-} and CTL wild-type and CRF₂^{-/-} 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.

$P < 0.0001$) 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 CRF₂^{-/-} 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, CRF₂ receptor deficiency totally eliminates the motivational effect of the ethological FWS without affecting ambulation.

CRF₂ Receptor Deficiency Eliminates the Reemergence of Motivational Up-Shifts Induced by an EPS Following Long-Term Opiate Withdrawal

To investigate whether the effect of CRF₂ 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 \times genotype \times 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 CRF₂^{-/-} and control mice ($P < 0.0005$, Figure 3a). In contrast, opiate-withdrawn CRF₂^{-/-} 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-naïve mice indicates the relatively mild nature of the EPS used. However, no genotype \times sex \times drug treatment \times stress interaction effect is observed. The EPS does not affect discrimination index in opiate-withdrawn mice of either genotype (stress \times genotype \times drug treatment interaction effect: $F_{1,79} = 0.34$, $P = 0.56$) or sex (stress \times sex \times 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 \times drug treatment interaction effect: $F_{1,79} = 9.33$, $P < 0.005$; Supplementary Figure S2B). However, no genotype \times sex \times drug treatment interaction effect is observed. These results demonstrate that CRF₂ receptor deficiency completely eliminates the EPS-induced reemergence of motivational up-shifts long after opiate withdrawal and further support dissociation between motivation and ambulation.

CRF₂ 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 \times 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 \times sex \times drug treatment interaction effect is observed for any of the three anxiety-like parameters. Thus, CRF₂ receptor deficiency does not affect anxiety-like behavior associated with long-term opiate withdrawal.

CRF₂ Receptor-Linked VTA-TH, BLA-, and CeA-GAD₆₇ 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

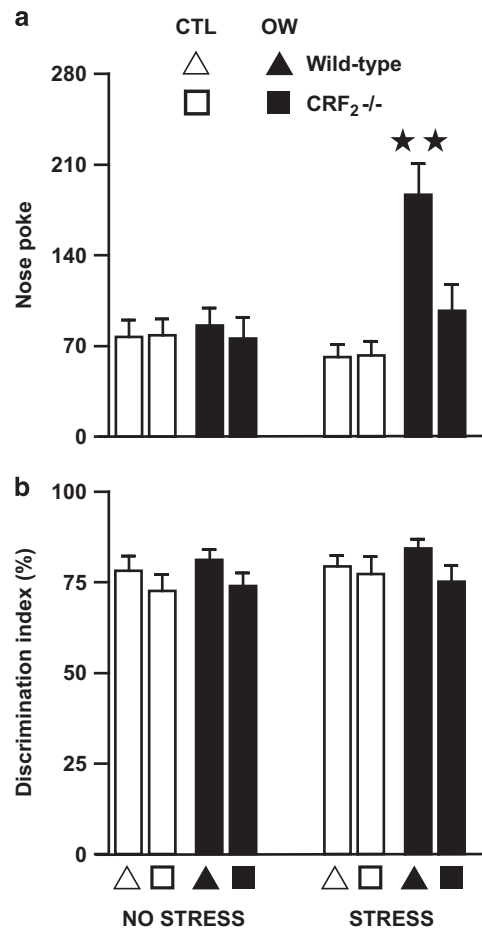


Figure 3 CRF₂ receptor deficiency eliminates the reemergence of up-shifted motivational states induced by an elevated platform stressor (EPS) following long-term opiate withdrawal. Nose-poke in the previously active hole (a) and discrimination index (b) displayed by unstressed (NO STRESS) or stressed (STRESS) control (CTL) or opiate-withdrawn (OW) wild-type or CRF₂^{-/-} 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 \pm 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).

implicated in the CRF₂ 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 CRF₂^{-/-} mice in a sex-linked manner (genotype \times sex interaction effect: $F_{1,79} = 4.73$, $P < 0.05$). Similar to the first EPS exposure, a genotype \times 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 CRF₂^{-/-} and control mice ($P < 0.005$); in contrast, opiate-withdrawn male CRF₂^{-/-} mice do not differ from control drug-naïve mice ($P = 0.36$, Supplementary Figure S4A). However, a drug treatment

effect ($F_{1,39} = 16.25$, $P < 0.0005$) reveals that either wild-type or CRF₂^{-/-} opiate-withdrawn female mice make more nose-pokes than control mice ($P < 0.0005$, Supplementary Figure S4B). Thus, the role for the CRF₂ 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 CRF₂^{-/-} mice (genotype \times 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 CRF₂^{-/-} 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 GAD₆₇ expression in wild-type or CRF₂^{-/-} mice in both the BLA (genotype \times drug treatment interaction effect: $F_{1,38} = 5.19$, $P < 0.05$; Figure 5a) and CeA (genotype \times 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 GAD₆₇ expression is increased in opiate-withdrawn wild-type mice but decreased in opiate-withdrawn CRF₂^{-/-} 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 CRF₂ 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 CRF₂^{-/-} mice in either the PVN (genotype \times drug treatment interaction effect: $F_{1,20} = 0.42$, $P = 0.53$; Supplementary Figure S5A and C) or the CeA (genotype \times 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 \times drug treatment interaction effects are observed in GAD₆₇ mRNA expression in the NaccSh or the CinCtx (Supplementary Table S1).

DISCUSSION

Summary of the Results

This study demonstrates a crucial role for the CRF₂ receptor in vulnerability to environmental stressors of motivational states following long-term opiate withdrawal. Indeed, CRF₂ 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* CRF₂^{-/-}, 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-GAD₆₇, that is abolished by CRF₂ receptor deficiency.

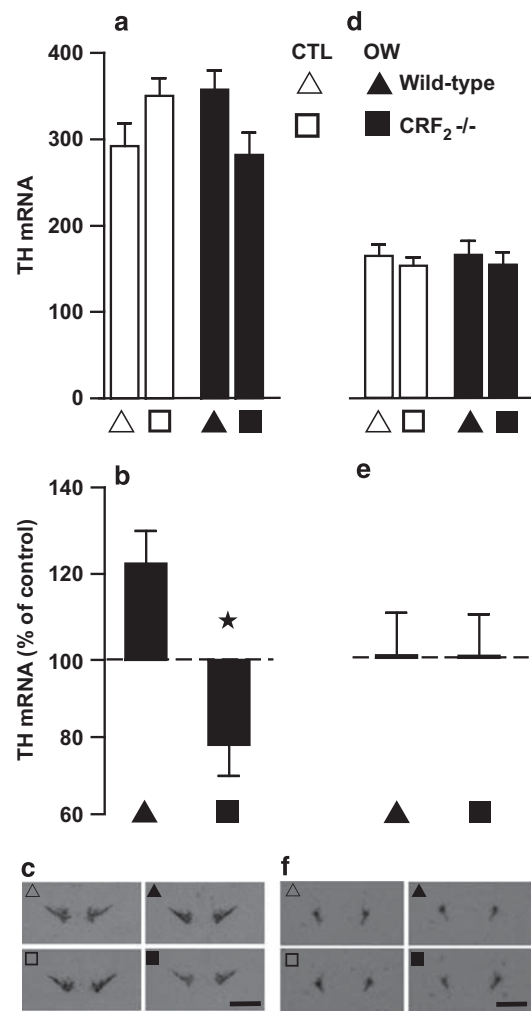


Figure 4 CRF₂ receptor-dependent increase in brain dopamine activity following long-term opiate withdrawal. Optical density (a, d), percentage of same genotype control optical density (b, e), and representative images of brain sections (c, f) illustrating tyrosine hydroxylase (TH) expression in the ventral tegmental area (VTA) (a–c) or in the locus coeruleus (LC) (d–f) in male control (CTL) or opiate-withdrawn (OW) wild-type or CRF₂^{-/-} 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 \pm SEM of optical density ($\times 1000$). * $P < 0.0001$ 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.

The CRF₂ 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 CRF₂^{-/-} mice similarly acquire a food-driven operant behavior, indicating that neither motivation nor cognitive function is affected by CRF₂ receptor deficiency or opiate withdrawal. Nevertheless, removal of the positive food

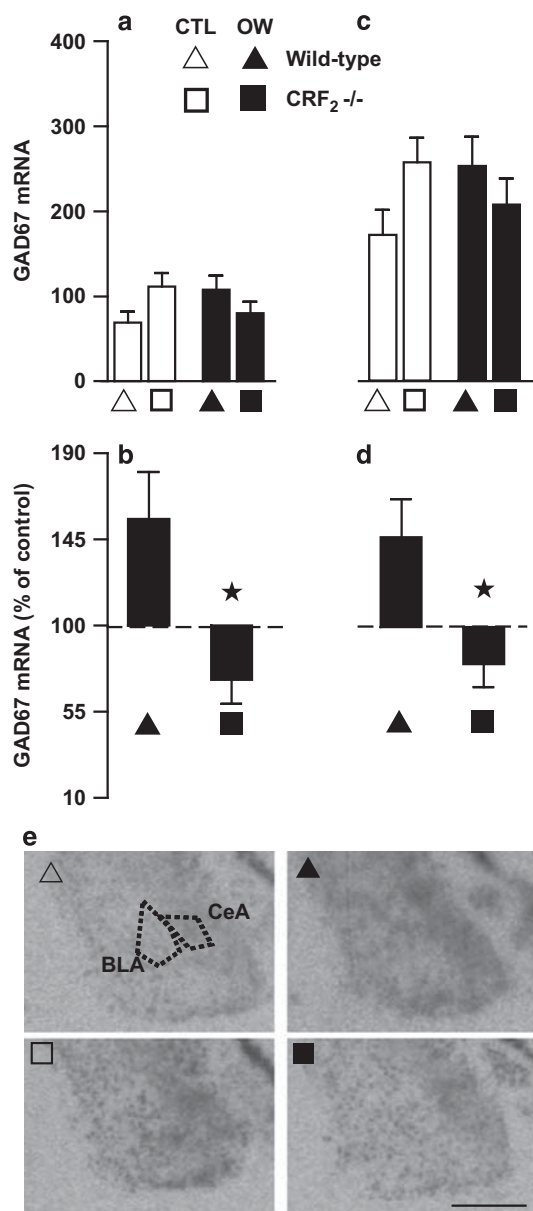


Figure 5 CRF₂ receptor-dependent increase in brain GABA activity following long-term opiate withdrawal. Optical density (a, c), percentage of same genotype control optical density (b, d), and representative images of brain sections (e) illustrating glutamic acid decarboxylase (GAD₆₇) expression in the basolateral amygdala (BLA) (a, b, e) or the central amygdala (CeA) (c–e) in male control (CTL) or opiate-withdrawn (OW) wild-type or CRF₂^{-/-} 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 \pm SEM of optical density ($\times 1000$). $*P < 0.01$ 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 CRF₂^{-/-} mice do not differ

from drug-naïve mice, revealing an essential role for the CRF₂ 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 CRF₂^{-/-} mice. CRF₂ 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 CRF₁ 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-naïve 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 CRF₂ -/- mice, revealing a crucial role for the CRF₂ 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 CRF₁ or CRF₂ 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 CRF₂ 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 CRF₂ receptor-deficient mice display unaltered CRF₁ 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 CRF₂ receptors. The CRF₂ receptor has been largely implicated in the stress response (Bale *et al*, 2000; Coste *et al*, 2000; Kishimoto *et al*, 2000). However, herein drug-naïve mice of either genotype do not differ following exposure to the FWS or the EPS. Furthermore, long-term opiate-withdrawn wild-type and CRF₂ -/- mice display similar locomotor activity and anxiety-like behavior, indicating that CRF₂ 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* CRF₂ -/-, mice. Thus, although CRF₂ receptor-dependent changes in stress susceptibility cannot be totally ruled out, the present results mainly suggest a role for the CRF₂ receptor in the long-lasting vulnerability of motivational states induced by opiate withdrawal.

This study also reveals sex-linked stress responses in opiate-withdrawn CRF₂ -/- mice. Indeed, unlike opiate-withdrawn *male* CRF₂ -/- mice, upon the second exposure to the EPS, opiate-withdrawn *female* CRF₂ -/- 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 CRF₁ 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 progesterins (Feltenstein *et al*, 2011). Evidence also indicates CRF₂ 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 CRF₂ +/- or CRF₂ -/- mothers display higher anxiety-like behavior than mice born to wild-type mothers, independently of their CRF₂ 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 CRF₂ +/- 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, CRF₂ -/- mice. However, further studies are warranted to test the latter hypotheses.

CRF₂ 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 CRF₂ 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, CRF₂ receptor deficiency totally abolishes the opiate withdrawal-linked VTA-TH expression, revealing an essential role for the CRF₂ 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). CRF₁ and CRF₂ 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 CRF₂ receptor in VTA DA biosynthesis associated with long-term drug withdrawal is poorly understood. Herein, long-term opiate-withdrawn CRF₂^{-/-} 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 CRF₂ 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 GAD₆₇ 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 GAD₆₇ 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 CRF₂^{-/-} mice do not show any increase in GAD₆₇ expression as compared with drug-naive mice, indicating that amygdala GABA biosynthesis linked to drug withdrawal requires functional CRF₂ receptors. Exposure to the EPS procedure might affect gene expression. However, as all of the mice (control or opiate-withdrawn wild-type and CRF₂^{-/-} 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 GAD₆₇ 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 GAD₆₇ 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-GAD₆₇ expression in long-term opiate-withdrawn wild-type mice showing a stress-induced reemergence of up-shifted motivational states. In contrast, opiate-withdrawn CRF₂^{-/-} mice do not show any motivational change or increased VTA-TH and BLA- and CeA-GAD₆₇ 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 CRF₂ 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 CRF₂ Receptor Null Mutation Does Not Induce Overt Developmental Deficits

As available CRF receptor antagonists do not yet allow addressing the specific role for the CRF₂ (*versus* the CRF₁) receptor in behavior, this study is carried out using a genetically engineered mouse model bearing a life-long disruption of the CRF₂ 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 CRF₂ receptor deficiency. However, the present and past studies (Rouibi and Contarino, 2013) show that drug-naive CRF₂^{-/-} mice do not differ from wild-type mice in food-motivated operant behavior, as assessed by several FR and PR

reinforcement schedules. Moreover, CRF₂ 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, CRF₂^{-/-} 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 CRF₂ 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 CRF₂ receptor in the reemergence of up-shifted motivational states triggered by ethological environmental stressors long after opiate discontinuation. Furthermore, CRF₂ receptor deficiency completely abolishes the elevated expression of biomarkers of brain motivational systems associated with long-term opiate withdrawal. This suggests that CRF₂ 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.

FUNDING AND DISCLOSURE

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

We thank Dr Catherine Le Moine, Marie-Line Fournier, and Ousama Dayoub for technical assistance with the *in situ* hybridization studies. This study is supported by the Université de Bordeaux, the Conseil Régional d'Aquitaine, and the Centre National de la Recherche Scientifique (CNRS), France. Funding sources have no involvement in the conduct of the research and/or the preparation of the manuscript.

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.

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