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Disruption of the CRF_2 Receptor Pathway Decreases the Somatic Expression of Opiate Withdrawal

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Escape from the extremely aversive opiate withdrawal symptoms powerfully motivates compulsive drug-seeking and drug-taking behaviors. The corticotropin-releasing factor (CRF) system is hypothesized to mediate the motivational properties of drug dependence. CRF signaling is transmitted by two receptor pathways, termed CRF_1 and CRF_2. To investigate the role for the CRF_2 receptor pathway in somatic opiate withdrawal, in the present study we used genetically engineered mice deficient in the CRF_2 receptor ($CRF_2-/-$). We employed a novel, clinically relevant mouse model of 'spontaneous' opiate withdrawal as well as a classical opioid receptor antagonist (naloxone)-precipitated opiate withdrawal paradigm. To induce opiate dependence, mice were treated with intermittent escalating morphine doses (20–100 mg/kg, i.p.). We found that 8–128 h after the last opiate injection, $CRF_2-/-$ mice showed decreased levels of major somatic signs of spontaneous opiate withdrawal, such as paw tremor and wet dog shake, as compared to wild-type mice. Similarly, challenge with naloxone 2 h after the last morphine injection induced lower levels of paw tremor and wet dog shake in $CRF_2-/-$ mice as compared to wild-type mice. Despite the differences in somatic signs, wild-type and $CRF_2-/-$ mice displayed similar plasma corticosterone responses to opiate dosing and withdrawal, indicating a marginal role for the hypothalamus–pituitary–adrenal axis in the CRF_2 receptor mediation of opiate withdrawal. Our results unravel a novel role for the CRF_2 receptor pathway in opiate withdrawal. The CRF_2 receptor pathway might be a critical target of therapies aimed at alleviating opiate withdrawal symptoms and reducing relapse to drug intake.

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INTRODUCTION

Opiate dependence is a chronic, relapsing disorder with a major impact on public health. Recent reports show an alarming rise in the recreational use of opiate drugs among adolescents, indicating that the incidence rate of opiate dependence may dramatically increase in the next years (NIDA, 2005; OEDT, 2006). In opiate-dependent individuals, heroin 'highs' are followed by a severe opiate withdrawal syndrome composed of somatic signs and symptoms, and negative affective states (O'Brien, 1996). Besides a positive reinforcement theory of drug addiction (Shaham *et al*, 2003), studies indicate that avoidance of, and/or escape from, the extremely aversive opiate withdrawal symptoms powerfully motivates compulsive opiate-seeking and opiate-taking behavior (Carrera *et al*, 1999; Kenny *et al*, 2006; Lu *et al*, 2005; Schulteis and Koob, 1996). The opiate withdrawal syndrome is currently treated mainly by drugs mimicking opiate action, such as methadone (Gonzalez *et al*, 2004; Kreek *et al*, 2002). Opiate-like drugs have greatly ameliorated the management of opiate withdrawal and dependence. However, the abrupt discontinuation of such medications is often followed by relapse into another cycle of opiate abuse and dependence (Kreek *et al*, 2002). The development of novel treatments thus remains a major goal and relies heavily on the understanding of the neural mechanisms underlying the opiate withdrawal syndrome.

The corticotropin-releasing factor (CRF) system is a major coordinator of endocrine and behavioral responses to stress (Heinrichs *et al*, 1994; Rivier and Vale, 1983). The CRF system might also mediate the motivational properties of drug dependence. CRF receptor antagonists reduce stress-induced reinstatement of drug-seeking behavior and ameliorate negative affective-like states associated with alcohol, cocaine, or opiate withdrawal (Erb *et al*, 1998;

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Sarnyai *et al*, 1995; Stinus *et al*, 2005; Valdez *et al*, 2002b). The latter studies point to the CRF system as a promising target of novel and effective therapies for drug dependence. In mammals, CRF signaling is mediated by two receptor pathways, termed CRF_1 and CRF_2 (Hauger *et al*, 2003). Genetically engineered mouse models have allowed the discovery of distinct and often opposite functions for the two known CRF receptor pathways (Bale and Vale, 2004; Contarino and Gold, 2002). In particular, CRF_1 receptor-deficient mice display decreased hypothalamus–pituitary–adrenal (HPA) axis as well as behavioral reactions to stressors (Contarino *et al*, 1999; Muller *et al*, 2003; Smith *et al*, 1998; Timpl *et al*, 1998). In net contrast, CRF_2 receptor-deficient mice show increased HPA axis and affective-like responses to stressors (Bale *et al*, 2000; Bale and Vale, 2003; Coste *et al*, 2000, 2006; Kishimoto *et al*, 2000). In line with a major role for the CRF_1 receptor pathway in stress-responsive circuitry, we found that genetic disruption of the CRF_1 receptor pathway eliminated the negative affective-like states of opiate withdrawal (Contarino and Papaleo, 2005). However, in net contrast to affective-like indices, disruption of the CRF_1 receptor pathway exacerbated the somatic expression of opiate withdrawal (Papaleo *et al*, 2007). Thus, our previous studies indicate a complex physiopathological role for the CRF_1 receptor pathway in opiate dependence. However, the role for the CRF_2 receptor pathway in opiate dependence remains largely unexplored.

To examine the specific contribution of the CRF_2 receptor pathway to somatic opiate withdrawal signs, in the present study we used CRF_2 receptor-deficient mice (Bale *et al*, 2000). For this purpose, we employed a clinically relevant mouse model of opiate withdrawal recently developed in our laboratory (Papaleo and Contarino, 2006) as well as a classical opioid receptor antagonist-precipitated opiate withdrawal paradigm (Matthes *et al*, 1996).

MATERIALS AND METHODS

Subjects

Group-housed, littermate female mice on a mixed C57BL/6Jx129 background that were wild-type or CRF_2 receptor-null mutant ($CRF_2-/-$) were used throughout (Bale *et al*, 2000). Mice were 4–5 months old at the time of the experiments and derived from mating $CRF_2+/-$ mice. Wild-type and $CRF_2-/-$ offspring of $CRF_2+/-$ breeders were identified by PCR analysis of tail DNA. The mice were housed in a colony room maintained at $22\pm2^{\circ}C$ on a 12-h light/dark cycle (lights on from 0800 hours until 2000 hours). Mice used in the naloxone-precipitated opiate withdrawal study were group-housed in a reversed cycle room, with lights on from 2000 hours until 0800 hours, and opiate withdrawal tests were conducted during the dark phase of the 12-h light/dark cycle. Food and water were available *ad libitum*. All studies were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and the European Communities Council Directive of 24 November 1986 (86/609/EEC), and were approved by the local Animal Care and Use Committee.

Experimental Procedures

The spontaneous opiate withdrawal study was carried out in the Laboratoire Homéostasie–Allostasie-Pathologie (EA 3666), University of Bordeaux, France. During the 5 days preceding the beginning of drug injections, the mice were handled every other day for 1 min for a total of three times. During the following 6 days, wild-type and $CRF_2-/-$ mice were treated every 12 h (0800 and 2000 hours) with increasing doses of morphine according to the following protocol: 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; day 6: 100 mg/kg; only one injection in the morning. Then, the mice were tested daily 8, 32, 56, 80, 104, 128, and 152 h after the last morphine injection. For this purpose, mice were individually placed into transparent Plexiglas cylinders (diameter: 23 cm, height: 50 cm) and observed continuously for 30 min for the occurrence of somatic signs of opiate withdrawal. The opioid receptor antagonist-precipitated opiate withdrawal study was carried out at the Molecular & Integrative Neurosciences Department, The Scripps Research Institute (La Jolla, CA, USA). The mice were injected with vehicle or morphine as in the spontaneous opiate withdrawal study, and 2 h after the last injection, they were treated with naloxone (1 mg/kg). The mice were individually placed into transparent plastic cylinders (diameter: 30 cm, height: 60 cm) placed on blotting paper 15 min before the naloxone injection and observed continuously for the occurrence of somatic signs of opiate withdrawal up to 30 min after the naloxone injection.

Jumps, paw tremors, wet dog shakes, body stretches, and sniffing were counted as the number of events occurring during the total test time (graded signs); chewing, teeth chattering, diarrhea, body tremor, piloerection, and palpebral ptosis were instead recorded as the number of 5-min intervals in which it occurred (checked signs, maximal score = 6). Body weights (BWs) were recorded each time the mice were injected as well as immediately before and after the 30-min opiate withdrawal tests, and the percentage of BW changes was calculated. To obtain a comprehensive index of the severity of somatic opiate withdrawal including all of the signs examined, for each mouse a global opiate withdrawal score was calculated by summing the raw values of somatic signs (Gellert and Holtzman, 1978).

Evaluation of the HPA Axis Activity during Opiate Treatment and Withdrawal

Another cohort of wild-type and $CRF_2-/-$ mice was treated with increasing doses of morphine (20–100 mg/kg; i.p.), as described above. Just prior to (12-h opiate withdrawal) and 30 and 90 min after the last opiate injection, three tail blood samples were collected from each mouse. Furthermore, 8 h after the last morphine injection, wild-type and $CRF_2-/-$ mice were tested for somatic opiate withdrawal signs, as described above, and blood was collected from the trunk immediately thereafter. Plasma samples were stored at $-20^{\circ}C$ until corticosterone assay. Plasma corticosterone levels were quantified by radioimmunoassay using a specific corticosterone antibody (ICN Pharmaceuticals). The intra- and interassay coefficients of variation were approximately 3.5 and 8%, respectively.

Drugs

Morphine HCl (20–100 mg/kg, i.p.; Francopia, Gentilly, France) and naloxone HCl (1 mg/kg, s.c.) were dissolved in physiological saline and injected in a volume of 10 ml/kg of BW. Control mice were injected with the same volume of saline.

Statistical Analysis

A two-way analysis of variance (ANOVA) with genotype (wild-type or $CRF_2-/-$) as a between-subject factor and time as a within-subject repeated measure factor was used to examine percentage of BW loss during the intermittent morphine injections and the 30-min somatic opiate withdrawal tests carried out 8–152 h after the last morphine injection, the percentage of BW recovery following discontinuation of opiate treatment and plasma corticosterone levels being measured just prior to and 30 and 90 min after the last morphine injection. Graded somatic signs of opiate withdrawal were subjected to the Lilliefors test for normality using the Statistica software; data that did not follow a normal distribution were analyzed by the non-parametric Mann–Whitney *U*-test, whereas data that showed normal distributions were examined by parametric tests (eg, ANOVA). Thus, paw tremor, wet dog shake, body stretch, and global opiate withdrawal scores observed during each 30-min test of the spontaneous opiate withdrawal study as well as jump and sniffing of the naloxone-precipitated opiate withdrawal test were analyzed by the Mann–Whitney *U*-test. Paw tremor, wet dog shake, global opiate withdrawal scores, and percentage of BW loss observed during the naloxone-precipitated opiate withdrawal test were analyzed by a two-way ANOVA with genotype (wild-type, $CRF_2-/-$) and treatment (vehicle, morphine) as independent variables. The Student–Newman–Keuls *post hoc* test was used for individual group comparisons. Checked somatic signs of opiate withdrawal were analyzed by the Mann–Whitney *U*-test. The accepted value for significance was $P<0.05$. For illustration purposes, data analyzed by non-parametric statistics were represented as median (lower, upper quartiles), whereas data analyzed by parametric statistics were represented as mean ± SEM.

RESULTS

Disruption of the CRF_2 Receptor Pathway Decreases the Somatic Expression of Spontaneous Opiate Withdrawal

In mice, the abrupt discontinuation of chronic opiate administration is followed by a constellation of somatic signs, such as jumping (escape attempt), paw tremor, wet dog shake, body stretch, diarrhea, chewing, teeth chattering, and palpebral ptosis (Papaleo and Contarino, 2006).

Prior to the beginning of morphine dosing, BWs were 29.6 ± 2.0 or 28.4 ± 0.6 g (mean ± SEM) for wild-type or $CRF_2-/-$ mice, respectively ($P=$ NS, Student's *t*-test). Twice-daily treatment with increasing morphine doses caused a progressive BW loss. Analysis of percent BW loss revealed no genotype effect ($F_{1,16}=1.09$, $P=$ NS), a time effect ($F_{5,80}=114.81$, $P<0.0001$), but no genotype × time interaction effect ($F_{5,80}=0.99$, $P=$ NS). Intermittent morphine administration produced comparable BW loss in wild-type and $CRF_2-/-$ mice (Supplementary Figure 1a). Also, following morphine discontinuation, wild-type and $CRF_2-/-$ mice displayed similar BW recovery ($F_{1,16}=0.00$, $P=$ NS; Supplementary Figure 1b).

Starting 8 h after the last morphine injection, the mice were tested every 24 h for the occurrence of somatic opiate withdrawal signs. Analysis of somatic signs of opiate withdrawal revealed that $CRF_2-/-$ mice displayed less paw tremors than wild-type mice 8 h ($U=2.0$, $P<0.001$), 32 h ($U=12.5$, $P<0.05$), 56 h ($U=12.0$, $P<0.05$), 80 h ($U=12.5$, $P<0.05$), and 104 h ($U=15.5$, $P<0.05$) after the last morphine injection (Figure 1a). Furthermore, $CRF_2-/-$ mice made less wet dog shakes than wild-type mice 32 h ($U=17.5$, $P<0.05$), 56 h ($U=16.0$, $P<0.05$), 104 h ($U=9.5$, $P<0.005$), and 128 h ($U=18.0$, $P<0.05$) after the last opiate injection (Figure 1b). Throughout the repeated 30-min tests carried out 8–152 h after the last morphine injection, genetic inactivation of the CRF_2 receptor pathway did not affect the expression of opiate withdrawal-induced body stretch ($U=27.5$–38.5, $P=$ NS; data not shown), palpebral ptosis ($U=29.0$–38.5, $P=$ NS; data not shown), chewing ($U=24.0$–39.0, $P=$ NS; Figure 2a), and diarrhea ($U=22.0$–40.0, $P=$ NS; Figure 2b). Also, during the repeated tests, wild-type and $CRF_2-/-$ mice displayed similar BW losses ($F_{1,16}=0.31$, $P=$ NS; data not shown).

Analysis of global opiate withdrawal scores revealed that 8 h ($U=7.0$, $P<0.005$), 32 h ($U=11.0$, $P<0.01$), 56 h ($U=9.0$, $P<0.01$), 80 h ($U=9.0$, $P<0.01$), 104 h ($U=10.0$, $P<0.01$), and 128 h ($U=11.0$, $P<0.01$) after the last morphine injection, $CRF_2-/-$ mice displayed lower levels of global opiate withdrawal than opiate-withdrawn wild-type mice (Figure 3). Genotypes did not differ 152 h after the

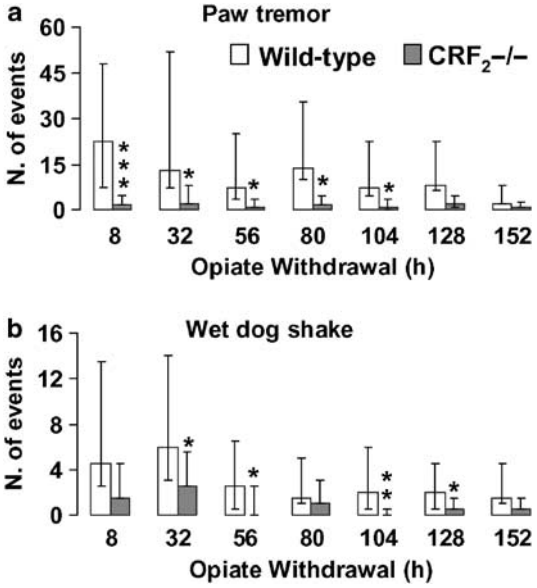

Figure 1 Discrete graded somatic signs of spontaneous opiate withdrawal. (a) Paw tremors and (b) wet dog shakes displayed by opiate-withdrawn wild-type or $CRF_2-/-$ mice during repeated daily 30-min tests carried out 8–152 h after the last morphine injection. Values represent the median (lower, upper quartile). $N=8$–10 per genotype. $^{*}P<0.05$, $^{**}P<0.005$, $^{***}P<0.001$ *vs* opiate-withdrawn wild-type mice at the same time point.

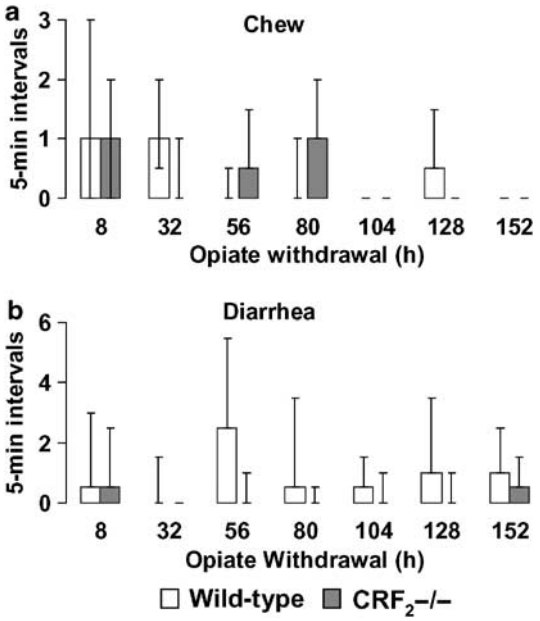

Figure 2 Discrete checked somatic signs of spontaneous opiate withdrawal. (a) Chews and (b) diarrhea displayed by opiate-withdrawn wild-type or $CRF_2-/-$ mice during repeated daily 30-min tests carried out 8–152 h after the last morphine injection. Values represent the median (lower, upper quartile). $N = 8–10$ per genotype.

last opiate administration ($U = 28.0$, $P =$ NS; Figure 3). Thus, at least up to 128 h after the last morphine injection, $CRF_2-/-$ mice displayed reduced levels of spontaneous somatic opiate withdrawal as compared to wild-type mice.

Unaltered Plasma Corticosterone Responses to Opiate Dosing and Withdrawal in CRF_2 Receptor-Deficient Mice

Analysis of plasma corticosterone levels measured just prior to (12-h opiate withdrawal) and 30 and 90 min after the last morphine injection revealed no genotype effect ($F_{1,10} = 0.88$, $P =$ NS), a time effect ($F_{2,20} = 61.28$, $P < 0.0001$), but no genotype × time interaction effect ($F_{2,20} = 1.18$, $P =$ NS), indicating that CRF_2 receptor deficiency did not affect HPA axis activity related to either opiate dosing or withdrawal (Figure 4a). Notably, plasma corticosterone levels were higher 30 and 90 min after than prior to morphine injection (eg, during opiate withdrawal, $P < 0.0005$; Figure 4a). Plasma corticosterone levels were also measured immediately after a 30-min spontaneous opiate withdrawal test carried out 8 h after the last morphine injection. Similar to the above, opiate-withdrawn $CRF_2-/-$ mice displayed less paw tremors ($U = 3.0$, $P < 0.05$), wet dog shakes ($U = 6.0$, $P = 0.05$), and lower global opiate withdrawal scores ($U = 3.0$, $P < 0.05$) than opiate-withdrawn wild-type mice (data not shown). However, at the end of the behavioral test, opiate-withdrawn $CRF_2-/-$ and wild-type mice showed similar plasma corticosterone levels ($t_{10} = 1.54$, $P =$ NS; Student's t-test), ruling out a role for the HPA axis in the CRF_2 receptor-regulated expression of somatic opiate withdrawal (Figure 4b). Notably, plasma corticosterone levels detected immediately after the 30-min somatic opiate withdrawal test were higher than those observed just prior to the last morphine injection (12-h opiate withdrawal), most likely due to an opiate withdrawal-linked HPA axis response to behavioral testing (for comparisons, see Figure 4a and b).

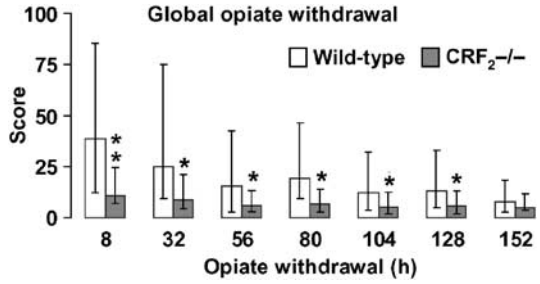

Figure 3 Global score of spontaneous somatic opiate withdrawal. Comprehensive scores (sum of the somatic sign values) displayed by opiate-withdrawn wild-type or $CRF_2-/-$ mice during repeated daily 30-min tests carried out 8–152 h after the last morphine injection. Values represent the median (lower, upper quartile). $N = 8–10$ per genotype. $^*P < 0.01$, $^{**}P < 0.005$ *vs* opiate-withdrawn wild-type mice at the same time point.

Disruption of the CRF_2 Receptor Pathway Decreases the Somatic Expression of Naloxone-Precipitated Opiate Withdrawal

Two hours after the last vehicle or morphine injection, wild-type and $CRF_2-/-$ mice were challenged with naloxone (1 mg/kg, s.c.) and immediately tested for the occurrence of somatic signs of opiate withdrawal. Examination of graded signs revealed a genotype × opiate treatment interaction effect for paw tremor ($F_{1,23} = 4.99$, $P < 0.05$). Opiate-treated wild-type mice made more paw tremors than vehicle-treated wild-type or $CRF_2-/-$ mice ($P < 0.0005$; Figure 5a). In contrast, opiate-treated $CRF_2-/-$ mice did not differ from vehicle-treated wild-type or $CRF_2-/-$ mice. Moreover, opiate-treated $CRF_2-/-$ mice made less paw tremors than opiate-treated wild-type mice ($P < 0.005$; Figure 5a). We also found a genotype × opiate treatment interaction effect for the wet dog shake sign ($F_{1,23} = 7.68$, $P < 0.05$). Both wild-type and $CRF_2-/-$ mice treated with morphine made more wet dog shakes than vehicle-treated wild-type or $CRF_2-/-$ mice ($P < 0.001$; Figure 5b). However, opiate-treated $CRF_2-/-$ mice made less wet dog shakes than opiate-treated wild-type mice ($P < 0.0005$; Figure 5b). CRF_2 receptor deficiency did not affect the expression of jumping and sniffing. Neither wild-type nor $CRF_2-/-$ mice treated with vehicle and challenged with naloxone displayed jumping or sniffing. In contrast, naloxone produced reliable levels of jumping in opiate-treated wild-type ($U = 0.0$, $P < 0.005$ *vs* vehicle-treated wild-type mice; Figure 5c) or $CRF_2-/-$ mice ($U = 10.5$, $P < 0.05$ *vs* vehicle-treated $CRF_2-/-$ mice; Figure 5c). Naloxone also produced reliable levels of sniffing in opiate-treated wild-type ($U = 0.0$, $P < 0.005$ *vs* vehicle-treated wild-type mice; Figure 5d) or $CRF_2-/-$ mice ($U = 3.5$, $P < 0.005$ *vs* vehicle-treated $CRF_2-/-$ mice; Figure 5d). Opiate-treated $CRF_2-/-$ mice did not differ from opiate-treated wild-type mice in the expression of jumping ($U = 13.5$, $P =$ NS; Figure 5c) or sniffing ($U = 10.0$, $P =$ NS; Figure 5d).

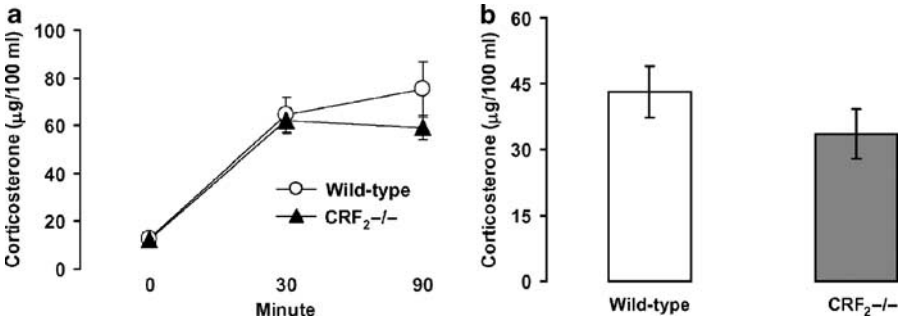

Figure 4 HPA axis responses to opiate dosing and withdrawal. Plasma corticosterone levels displayed by opiate-withdrawn wild-type or $CRF_2-/-$ mice (a) just prior to (0: 12-h opiate withdrawal) and 30 and 90 min after the last morphine injection and (b) immediately after a 30-min somatic opiate withdrawal test carried out 8 h after the last morphine injection. During 6 consecutive days, wild-type and $CRF_2-/-$ mice were treated every 12 h with increasing morphine doses (20–100 mg/kg; i.p.). Values represent mean ± SEM. $N = 6$ per genotype.

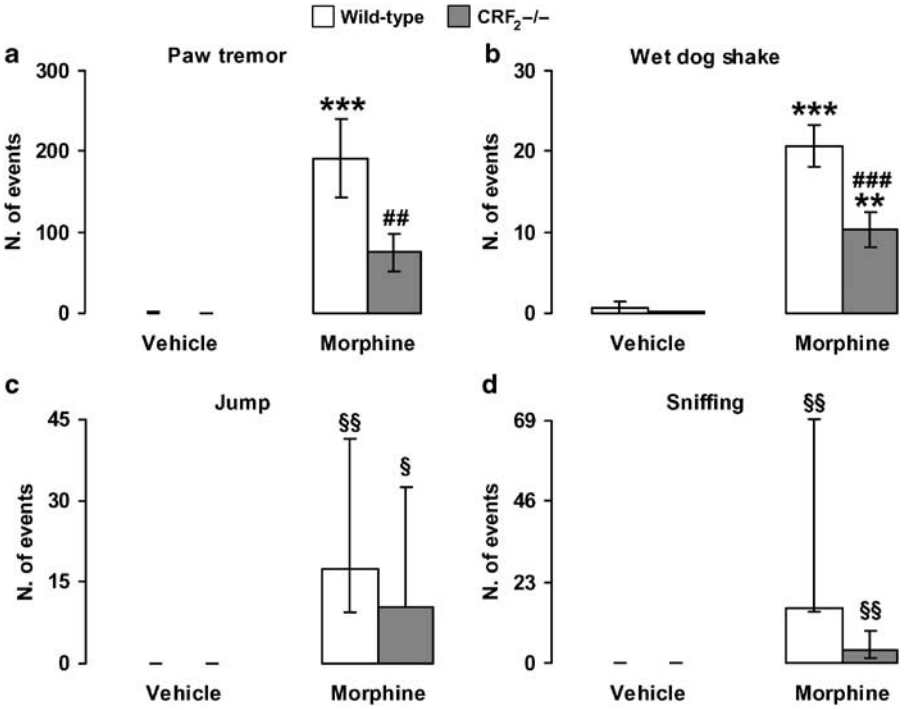

Figure 5 Discrete graded somatic signs of naloxone-precipitated opiate withdrawal. (a) Paw tremors, (b) wet dog shakes, (c) jumps, and (d) sniffing displayed by vehicle- or morphine-treated wild-type or $CRF_2-/-$ mice during a 30-min test carried out immediately after naloxone (1 mg/kg, s.c.) dosing. Naloxone was administered 2 h after the last vehicle or morphine injection. Values represent mean ± SEM for paw tremor and wet dog shake, and median (lower, upper quartile) for jump and sniffing. $N = 6$–8 per group. $^{**}P < 0.001$, $^{***}P < 0.0005$ *vs* vehicle-treated mice; $^{\#\#}P < 0.005$, $^{\#\#\#}P < 0.0005$ *vs* morphine-treated wild-type mice; $^{\S}P < 0.05$, $^{\S\S}P < 0.005$ *vs* same genotype vehicle-treated mice.

Evaluation of checked somatic signs of naloxone-precipitated opiate withdrawal revealed an opiate treatment effect for chewing, body tremor, palpebral ptosis, and piloerection. Opiate-treated wild-type mice displayed more chews ($U = 0.0$, $P < 0.005$; Figure 6a), body tremors ($U = 0.0$, $P < 0.005$; Figure 6b), palpebral ptoses ($U = 3.0$, $P < 0.01$; Figure 6c), and piloerection ($U = 0.0$, $P < 0.005$; Figure 6d) than vehicle-treated wild-type mice. Similarly, opiate-treated $CRF_2-/-$ mice displayed more chews ($U = 0.0$, $P < 0.001$; Figure 6a), body tremors ($U = 3.5$, $P < 0.005$; Figure 6b), palpebral ptoses ($U = 7.0$, $P < 0.01$; Figure 6c), and piloerection ($U = 7.0$, $P < 0.01$; Figure 6d) than vehicle-treated $CRF_2-/-$ mice. Opiate-treated $CRF_2-/-$ mice did not differ from opiate-treated wild-type mice in the expression of chewing ($U = 13.5$, $P =$ NS), body tremor ($U = 19.0$, $P =$ NS), palpebral ptosis ($U = 23.5$, $P =$ NS), and piloerection ($U = 20.5$, $P =$ NS). In the naloxone-precipitated opiate withdrawal study, neither the genotype nor the opiate treatment affected the expression of the diarrhea sign. Diarrhea events were as follows: for vehicle-treated mice: wild-type = 0.0 (0.0, 0.0), $CRF_2-/-$ = 0.0 (0.0, 0.0), and for opiate-treated mice: wild-type = 0.0 (0.0, 1.0), $CRF_2-/-$ = 0.0 (0.0, 1.0); median (lower, upper quartile). Also, during the 30-min naloxone-precipitated opiate withdrawal test, the percentage of BW loss was as follows: for vehicle-treated mice: wild-type = 0.95 ± 0.4, $CRF_2-/-$ = 0.45 ± 0.1, and for opiate-treated mice: wild-type = 1.08 ± 0.4, $CRF_2-/-$ = 0.42 ± 0.3 (mean ± SEM); analysis of

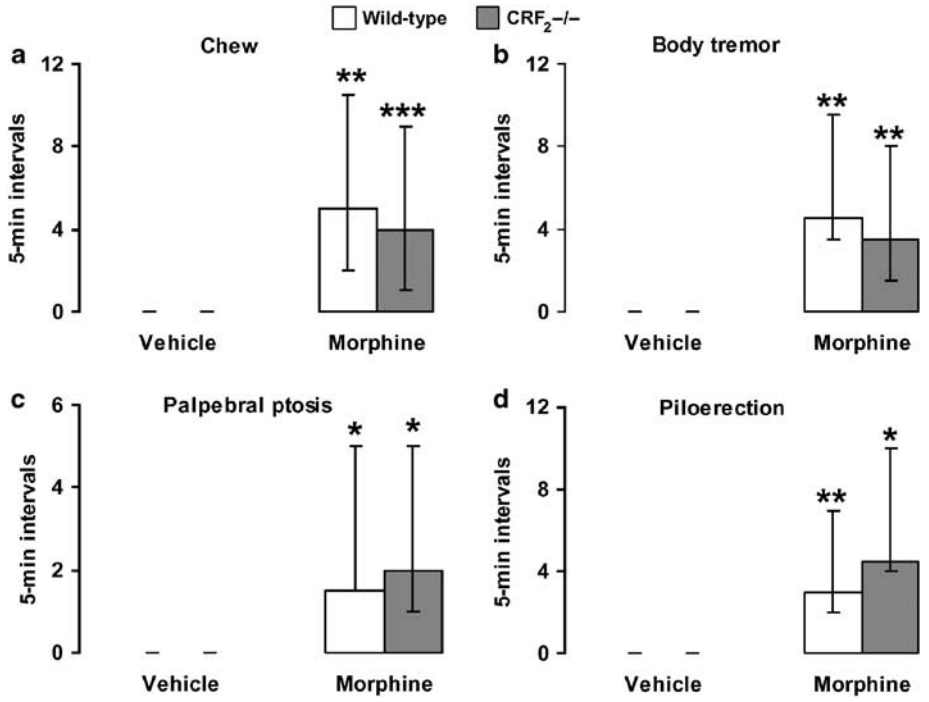

Figure 6 Discrete checked somatic signs of naloxone-precipitated opiate withdrawal. (a) Chews, (b) body tremors, (c) palpebral ptoses, and (d) piloerection displayed by vehicle- or morphine-treated wild-type or CRF_2−/− mice during a 30-min test carried out immediately after naloxone (1 mg/kg, s.c.) dosing. Naloxone was administered 2 h after the last vehicle or morphine injection. Values represent the median (lower, upper quartile). $N = 6$–8 per group. $^{*}P < 0.01$, $^{**}P < 0.005$, $^{***}P < 0.001$ *vs* same genotype vehicle-treated mice.

the latter parameter revealed no genotype effect ($F_{1,23} = 2.67$, P = NS), no opiate treatment effect ($F_{1,23} = 0.06$, P = NS), and no genotype × opiate treatment interaction effect ($F_{1,23} = 0.04$, P = NS). Thus, similar to the spontaneous opiate withdrawal study described above, CRF_2 receptor deficiency sharply decreased the expression of major somatic signs of opiate withdrawal, such as paw tremor and wet dog shake, also in chronically opiate-treated mice challenged with the opioid receptor antagonist naloxone.

An analysis of global scores revealed a genotype effect ($F_{1,23} = 7.33$, $P < 0.05$), an opiate treatment effect ($F_{1,23} = 28.78$, $P < 0.0001$), and a genotype × opiate treatment interaction effect ($F_{1,23} = 7.06$, $P < 0.05$). Opiate-treated wild-type mice displayed higher global scores than vehicle-treated wild-type or CRF_2−/− mice ($P < 0.0005$; Figure 7). In net contrast, global scores displayed by opiate-treated CRF_2−/− mice did not differ from those of vehicle-treated wild-type or CRF_2−/− mice (P = NS; Figure 7). Moreover, opiate-treated CRF_2−/− mice displayed lower global opiate withdrawal scores than opiate-treated wild-type mice ($P < 0.0005$; Figure 7). Thus, the lack of functional CRF_2 receptor levels overall decreased the expression of naloxone-precipitated somatic opiate withdrawal signs, reducing global scores of opiate-treated CRF_2−/− mice to drug-naive mice levels.

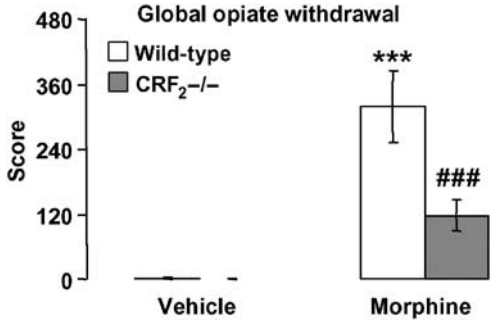

Figure 7 Global score of naloxone-precipitated somatic opiate withdrawal. Comprehensive scores (sum of the somatic sign values) displayed by vehicle- or morphine-treated wild-type or CRF_2−/− mice during a 30-min test carried out immediately after naloxone (1 mg/kg, s.c.) dosing. Naloxone was administered 2 h after the last vehicle or morphine injection. Values represent mean ± SEM. $N = 6$–8 per group. $^{***}P < 0.0005$ *vs* vehicle-treated mice. $^{###}P < 0.0005$ *vs* morphine-treated wild-type mice.

DISCUSSION

This report demonstrates, by both a spontaneous opiate withdrawal paradigm recently developed in our laboratory (Papaleo and Contarino, 2006) and a classical opioid receptor antagonist-precipitated opiate withdrawal procedure (Matthes *et al*, 1996), a crucial role for the CRF_2 receptor pathway in the somatic expression of opiate withdrawal. In particular, mice lacking functional levels of the CRF_2 receptor pathway displayed decreased somatic reactions to the stressful condition of opiate withdrawal, as compared to wild-type mice.

To mimic the clinical setting, in the present study mice were treated with intermittent escalating morphine doses and opiate withdrawal-related somatic signs assessed following 'spontaneous' clearance of the opiate drug from the body. Using such a clinically oriented experimental

paradigm, we found that CRF_2−/− mice showed lower levels of major somatic signs of opiate withdrawal, such as paw tremor and wet dog shake, than wild-type mice. Genotype differences were observed at several time points, that is, 8–128 h after the last opiate administration, ruling out a genotype-dependent time-shift in the expression of somatic opiate withdrawal. CRF_2 receptor-deficient mice also showed lower global opiate withdrawal scores than wild-type mice, which were evident as soon as 8 h after the last opiate injection, and lasted at least up to 128 h following drug discontinuation. We also investigated the impact of CRF_2 receptor deficiency upon the somatic expression of opioid receptor antagonist (naloxone)-precipitated opiate withdrawal. Prior to naloxone challenge, mice were repeatedly treated with increasing morphine doses as in the spontaneous opiate withdrawal study. We found that CRF_2 receptor deficiency dampened the expression of paw tremor and wet dog shake and decreased global opiate withdrawal scores, a behavioral result very similar to that observed in the spontaneous opiate withdrawal study. Notably, the spontaneous and the opioid receptor antagonist-precipitated opiate withdrawal studies were carried out in two different laboratories (see the Materials and Methods section), strengthening the reliability and significance of our results. Thus, both the spontaneous and the naloxone-precipitated opiate withdrawal studies reported here clearly indicate that genetic disruption of the CRF_2 receptor pathway sharply reduces the severity of major somatic signs of opiate withdrawal, suggesting that activation of the CRF_2 receptor pathway may positively modulate the somatic expression of opiate withdrawal.

The present study also provides novel evidence in favor of major differences between spontaneous and opioid receptor antagonist-precipitated opiate withdrawal in mice. In particular, in either wild-type or CRF_2 receptor-deficient mice, naloxone increased the expression of almost all of the somatic opiate withdrawal signs examined, as compared to the spontaneous opiate withdrawal condition. Moreover, the opioid receptor antagonist triggered somatic signs, such as piloerection, body tremor, sniffing, and jumping, which were not observed during the spontaneous opiate withdrawal study. Accordingly, previous studies showed that challenging opiate-dependent rats with opioid receptor antagonists induced behavioral signs that greatly differed, in both intensity and type, from those observed in animals undergoing spontaneous opiate withdrawal (Cicero *et al*, 2002; Houshyar *et al*, 2004; Linseman, 1977; Mucha *et al*, 1979; Papaleo and Contarino, 2006; Papaleo *et al*, 2007; Ruiz *et al*, 1996).

Genetically engineered mouse models have allowed the identification of distinct and often opposite functions for the two known CRF receptor pathways in HPA axis and affective-like behavioral responses to stress (Bale and Vale, 2004; Contarino and Gold, 2002). In particular, either CRF_1 receptor deficiency or functional antagonism of the CRF_1 receptor pathway decreased anxiety-like and depression-like behavior (Contarino *et al*, 1999; Griebel *et al*, 2002; Hodgson *et al*, 2007; Nielsen *et al*, 2004; Okuyama *et al*, 1999; Smith *et al*, 1998; Timpl *et al*, 1998; Zorrilla *et al*, 2002). Also, CRF_1−/− mice displayed deficient HPA axis responses to stressful events (Contarino and Papaleo, 2005; Papaleo *et al*, 2007; Smith *et al*, 1998; Timpl *et al*, 1998). In net contrast, genetic disruption of the CRF_2 receptor pathway increased the expression of anxiety-like and depression-like behavior, and exacerbated HPA axis responses to an immobilization stress (Bale *et al*, 2000; Bale and Vale, 2003; Coste *et al*, 2000, 2006; Kishimoto *et al*, 2000). Finally, CRF_1 and CRF_2 receptor pathways were shown to have opposite roles on information processing mechanisms that regulate responses to stressors. In particular, either genetic inactivation or pharmacological antagonism of the CRF_1 receptor pathway increased prepulse inhibition (PPI) of defensive startle responses by sensory stimuli, whereas pharmacological antagonism of the CRF_2 receptor pathway decreased PPI (Risbrough *et al*, 2004). Recently, studies also suggested distinct roles for the CRF_1 (*vs* the CRF_2) receptor pathway in ethanol dependence. In particular, during acute ethanol withdrawal, CRF_1 receptor activity was shown to mediate excessive ethanol self-administration in dependent animals, whereas activation of the CRF_2 receptor pathway reduced ethanol intake as well as anxiety-like behavior induced by ethanol withdrawal (Funk *et al*, 2007; Valdez *et al*, 2004). We have recently reported that genetic inactivation as well as pharmacological antagonism of the CRF_1 receptor pathway increased and prolonged the somatic expression of opiate withdrawal (Papaleo *et al*, 2007). In contrast to CRF_1 receptor deficiency, in the present study we demonstrate that CRF_2 receptor-deficient mice displayed reduced somatic signs of opiate withdrawal as compared to wild-type mice. Notably, both in our previous and present studies, we used exactly the same experimental paradigm to investigate the role for the CRF_1 or the CRF_2 receptor pathway in opiate withdrawal. Thus, together with our recent study in CRF_1 receptor-null mutant mice (Papaleo *et al*, 2007), the present results provide initial and compelling evidence of opposite roles for the two known CRF receptor pathways in somatic opiate withdrawal. In particular, following abrupt discontinuation of opiate administration, CRF_1 receptor signaling may dampen, whereas the CRF_2 receptor pathway may function to facilitate the somatic expression of opiate withdrawal.

The present findings of decreased somatic opiate withdrawal signs in CRF_2−/− mice are in apparent contrast with studies showing that CRF_2−/− mice displayed increased behavioral responses to stressful events (Bale *et al*, 2000; Bale and Vale, 2003; Coste *et al*, 2006; Kishimoto *et al*, 2000). However, in contrast to CRF_1 receptors, the role for the CRF_2 receptor pathway in stress-responsive circuitry is somewhat less clear. Studies have shown anxiolytic-like effects of CRF_2 receptor activation (Valdez *et al*, 2002a, 2003; Venihaki *et al*, 2004). Moreover, genetic disruption of the CRF_2 receptor pathway resulted in increased anxiety-like and depressive-like behaviors (Bale *et al*, 2000; Bale and Vale, 2003; Coste *et al*, 2006; Kishimoto *et al*, 2000). In contrast, other reports suggested a stress-like activity for the CRF_2 receptor pathway (Henry *et al*, 2006; Ho *et al*, 2001; Radulovic *et al*, 1999; Risbrough *et al*, 2003, 2004). It should be pointed out that the *in vivo* receptor selectivity of the CRF-related peptides and/or peptide doses used in the studies cited above remains uncertain. In the present study, we used a genetic model of CRF_2 receptor inactivation, which might provide a level of molecular specificity that is rarely achieved by pharmacological studies. Prior to being

tested for somatic expression of opiate withdrawal, every 12 h wild-type and CRF_2−/− mice were repeatedly treated with increasing doses of morphine. The twice-daily partial opiate withdrawal that accompanied our drug regimen may have served as a severe chronic stressor. Accordingly, 12 h after morphine dosing, rats treated with a morphine regimen similar to that used in the present study displayed stress-like alterations in the brain, HPA axis, and metabolic functions (Houshyar *et al*, 2004). We also found elevated CRF expression in the paraventricular nucleus of the hypothalamus, a main brain region coordinating HPA axis responses to stress, of opiate-withdrawn wild-type mice treated with the same morphine regimen as that used in the present study (Papaleo *et al*, 2007). Thus, the present results clearly indicate decreased somatic reactions to the stressful event of opiate withdrawal in CRF_2 receptor-deficient mice. However, further studies are needed to better characterize the role for the CRF_2 receptor pathway in drug withdrawal-related stressful conditions.

We recently demonstrated a major role for the HPA axis in behavioral, molecular, and neuroendocrine alterations relevant to the somatic expression of opiate withdrawal (Papaleo *et al*, 2007). In particular, during the stressful condition of opiate withdrawal, CRF_1 receptor-deficient mice showed increased somatic signs as well as profound impairments in the HPA axis and extra-hypothalamic brain stress-responsive circuitry. Interestingly, we found that aberrant behavioral and molecular responses to the stress of opiate withdrawal observed in CRF_1 receptor-deficient mice were largely dependent on the HPA axis impairments associated with the CRF_1 receptor-null mutation. Accordingly, treatment of the CRF_1−/− mice with non-stressful amounts of corticosterone during repeated intermittent dosing with morphine effectively reduced the somatic signs of opiate withdrawal and restored 'wild-type-like' brain responses to the stressful condition of opiate withdrawal (Papaleo *et al*, 2007). Most likely, the 'beneficial' effects of exogenous corticosterone observed in the CRF_1 receptor-deficient mice were due to enduring changes in the HPA system that were taking place throughout the intermittent morphine treatment (Papaleo *et al*, 2007). Thus, 'normalizing' HPA axis activity during intermittent opiate exposure may effectively reduce the severe somatic consequences of opiate withdrawal. In the present study, we provide initial evidence showing that CRF_2−/− mice displayed an intact HPA axis response to drug-related stressful conditions (opiate dosing and opiate withdrawal). In particular, our results clearly show that CRF_2−/− mice were able to mount relatively high plasma corticosterone responses to either opiate dosing (48–114 μg/100 ml) or opiate withdrawal (23–71 μg/100 ml). However, since wild-type and CRF_2−/− mice showed similar plasma corticosterone responses to either opiate dosing or withdrawal, the present findings do not support a role for the HPA axis in the decreased somatic expression of opiate withdrawal displayed by CRF_2 receptor-deficient mice.

The results of the present study bear important implications for research aimed at developing novel medications for the opiate withdrawal syndrome. Human and animal studies indicate that avoidance of, and/or escape from, the extremely stressful condition of opiate withdrawal powerfully motivates compulsive heroin-seeking and heroin-taking behavior (Baker *et al*, 2004; Carrera *et al*, 1999; Kenny *et al*, 2006; Lu *et al*, 2005; Schulteis and Koob, 1996). Here, we report that genetic disruption of the CRF_2 receptor pathway sharply reduced the expression of major somatic signs of opiate withdrawal. Notably, under both a clinically relevant spontaneous opiate withdrawal and a classical opioid receptor antagonist-precipitated opiate withdrawal paradigm, mice lacking functional levels of the CRF_2 receptor pathway displayed decreased somatic reactions to the stress of opiate withdrawal. These findings unravel a novel role for the CRF_2 receptor pathway in opiate withdrawal and suggest new pharmacological strategies for the management of the opiate withdrawal syndrome.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declared that they have no financial conflict of interest.

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