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RESEARCH PAPER

# Corticotropin-releasing factor receptor 2-deficiency eliminates social behaviour deficits and vulnerability induced by cocaine

**Correspondence** Angelo Contarino, INCIA, UMR 5287, Univ. Bordeaux, CNRS, 146 rue Léo Saignat, 33000 Bordeaux, France. E-mail: angelo.contarino@u-bordeaux.fr

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Nadège Morisot[1,2], Romain Monier[1,2], Catherine Le Moine[1,2], Mark J Millan[3] and Angelo Contarino[1,2]

*[1]INCIA, UMR 5287, Univ. Bordeaux, Bordeaux, France, [2]INCIA, UMR 5287, CNRS, Bordeaux, France, and [3]Centre for Therapeutic Innovation in Neuropsychiatry, Institut de Recherches Servier, Croissy-sur-Seine, France*

## BACKGROUND AND PURPOSE

Poor social behaviour and vulnerability to stress are major clinical features of stimulant use disorders. The corticotropin-releasing factor (CRF) system mediates stress responses and might underlie substance use disorders; however, its involvement in social impairment induced by stimulant substances remains unknown. CRF signalling is mediated by two receptor types, $CRF_1$ and $CRF_2$. In the present study we investigated the role of the $CRF_2$ receptor in social behaviour deficits, vulnerability to stress and related brain alterations induced by cocaine administration and withdrawal.

## EXPERIMENTAL APPROACH

$CRF_2$ receptor-deficient ($CRF_2-/-$) and littermate wild-type mice were repeatedly tested in the three-chamber task for sociability (i.e. preference for an unfamiliar conspecific vs. an object) and social novelty preference (SNP; i.e. preference for a novel vs. a familiar conspecific) before and after chronic cocaine administration. An *in situ* hybridization assay was used to assess gene expression of the stress-responsive arginine vasopressin (AVP) and oxytocin (OT) neuropeptides in the hypothalamus.

## KEY RESULTS

$CRF_2$ receptor deficiency eliminated the sociability deficit induced by cocaine withdrawal. Moreover, $CRF_2-/-$ mice did not show either the stress-induced sociability deficit or the increased AVP and OT expression associated with long-term cocaine withdrawal, indicating resilience to stress. Throughout, wild-type and $CRF_2-/-$ mice displayed SNP, suggesting that cocaine withdrawal-induced sociability deficits were not due to impaired detection of social stimuli.

## CONCLUSIONS AND IMPLICATIONS

These findings demonstrate a central role for the $CRF_2$ receptor in social behaviour deficits and biomarkers of vulnerability induced by cocaine withdrawal, suggesting new therapeutic strategies for stimulant use disorders.

## Abbreviations

AVP, arginine vasopressin; CRF, corticotropin-releasing factor; EPS, elevated platform stressor; ITI, intertrial time interval; OT, oxytocin; PVN, paraventricular nucleus of the hypothalamus; SNP, social novelty preference; SON, supraoptic nucleus of the hypothalamus; SSC, saline–sodium citrate

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## Introduction

Stimulant use disorders are among the most prevalent substance use disorders, carrying a heavy burden of disability and death worldwide (http://www.unodc.org/wdr2016). These conditions are characterized by enhanced processing of substance-induced reward and reduced valuation of non-substance rewards, including social stimuli (Volkow *et al.*, 2011). As a result, stimulant users often display dysfunctional social behaviour (APA, 2013; Preller *et al.*, 2014). Furthermore, stressful life events and social 'breakdown' are hypothesized to contribute to the high rate of relapse to substance-seeking and -taking behaviour (Sinha, 2001; Preston and Epstein, 2011; Volkow *et al.*, 2011). However, the brain alterations underlying social dysfunction and vulnerability induced by stimulant substances remain largely unknown.

The **corticotropin-releasing factor** (CRF) system might be involved in substance use disorders. Indeed, **cocaine** withdrawal increases CRF activity in the amygdala and the frontal cortex, brain regions relevant to the behavioural effects of substances of abuse (Richter and Weiss, 1999; Zorrilla *et al.*, 2001; Noel *et al.*, 2013). Accordingly, CRF antagonism attenuates negative affective-like states and stress-induced substance-seeking behaviour in cocaine-withdrawn rats (Sarnyai *et al.*, 1995; Basso *et al.*, 1999; Shaham *et al.*, 2003; Koob, 2008). CRF signalling is mediated by two receptor types, named **$CRF_1$** and **$CRF_2$** (Hauger *et al.*, 2003). There is compelling evidence indicating that they fulfil distinct roles in the effects of substances of abuse. For instance, $CRF_1$ or $CRF_2$ receptor deficiency increases or decreases, respectively, the somatic signs and the recognition memory deficits induced by morphine withdrawal in mice (Papaleo *et al.*, 2007; 2008; Morisot and Contarino, 2016). Moreover, $CRF_2$ receptor deficiency reduces the negative affective-like states of early morphine withdrawal phases and eliminates the stress-induced re-emergence of recognition memory deficits and motivational states in long-term morphine- or cocaine-withdrawn mice (Ingallinesi *et al.*, 2012; Rouibi and Contarino, 2013; Morisot *et al.*, 2014; 2015; Morisot and Contarino, 2016). Studies also suggest a complex role for the CRF system in social behaviour. For instance, i.c.v. administration of CRF facilitates partner preference in male prairie voles (DeVries *et al.*, 2002). Moreover, transgenic CRF overexpression, urocortin 3 or $CRF_2$ receptor deficiency enhance social investigation or social memory, as assessed in social discrimination tasks (Deussing *et al.*, 2010; Kasahara *et al.*, 2011). However, CRF responses to stressors are differently modulated by the two major 'social' hormones **oxytocin** (OT) and **arginine vasopressin** (AVP) (Stoop, 2012). Indeed, AVP is co-released with CRF from the paraventricular nucleus of the hypothalamus (PVN) to potentiate stress responses, whereas OT reduces the stress-induced PVN-CRF transcription (Bulbul *et al.*, 2011; Jurek *et al.*, 2015; Herman and Tasker, 2016). Interestingly, $CRF_2$ receptors are expressed both in the PVN and the supraoptic nucleus of the hypothalamus (SON), main sources of OT and AVP production in the brain (Van Pett *et al.*, 2000; Stoop, 2012). Moreover, other studies have suggested the OT and AVP systems have a role in the actions of cocaine. For instance, cocaine administration decreased OT and vasopressin peptide levels in the hypothalamus and hippocampus, reduced vasopressin peptide levels in the amygdala and increased OT receptor binding in the lateral septum and the amygdala (Sarnyai *et al.*, 1992; Georgiou *et al.*, 2016b). Moreover, cocaine withdrawal increased the expression of PVN-AVP, OT receptor binding in the medial and lateral septum and the amygdala, decreased novel object recognition memory and increased anxiety-like behaviour in mice and rats (Zhou *et al.*, 2011; Georgiou *et al.*, 2016b). Interestingly, studies have also shown that administration of vasopressin or OT decreases cocaine self-administration or cue-induced reinstatement of cocaine seeking in rats respectively (van Ree *et al.*, 1988; Leong *et al.*, 2017). Nevertheless, the role of the CRF/$CRF_2$ receptor system in social behaviour deficits and brain AVP/OT changes induced by stimulant substances remains poorly understood.

Thus, in the present study we investigated the involvement of the $CRF_2$ receptor in social dysfunction and stress vulnerability induced by repeated administration of cocaine and withdrawal from cocaine. For this purpose, we used the widely employed and validated three-chamber task for social behaviour in mice, which is thought to reliably measure sociability and social novelty preference (SNP), independently of emotional-like states (Silverman *et al.*, 2010; Moy *et al.*, 2013). Social behaviour was first assessed in substance-naïve wild-type and $CRF_2$ receptor-deficient ($CRF_2-/-$) mice. Then, mice were treated with escalating doses of cocaine, and social behaviour examined immediately and throughout a relatively long period after the last substance administration. We also assessed the role of the $CRF_2$ receptor in social behaviour vulnerability to an environmental ethological stressor long time after cocaine discontinuation. To further search for brain alterations associated with the long-lasting $CRF_2$ receptor-dependent vulnerability induced by cocaine withdrawal, OT and AVP gene expression were measured in the PVN and the SON, brain circuits implicated in social behaviour and stress responses (McCall and Singer, 2012; Neumann and Landgraf, 2012).

## Methods

### *Subjects*

Knockout mouse models are essential to evaluate the contribution of specific signalling pathways in key behavioural phenotypes relevant to addiction research. Thus, herein, $CRF_2$ receptor null mutant ($CRF_2-/-$) and wild-type mice were used to assess the role of the $CRF_2$ receptor in cocaine-induced social behaviour deficits. Littermate wild-type ($n$ = 32), $CRF_2$ receptor heterozygous ($CRF_2+/-$; $n$ = 53) and $CRF_2-/-$ ($n$ = 34) male mice were obtained from mating of $CRF_2+/-$ mice using a trio breeding programme (one male was mated with two female mice). The specific pathogen-free mouse colony was on a C57BL/6J × 129 background and originated from Bale *et al.* (2000). The colony room (22 ± 2°C, relative humidity: 50–60%) was maintained on a 12 h light/dark cycle (lights on at 0800 h). The genotype of the mice was determined by PCR analysis of tail DNA. Mice were housed in groups of 2–4 in polycarbonate cages (29.5 × 11.5 × 13 cm; $L$ × $W$ × $H$) containing bedding and

a cotton nestlet (SAFE, Augy, France). They had *ad libitum* access to standard laboratory food (3.3 kcal·g$^{-1}$; SAFE) and water. The mice were 3–7 months old at the beginning of the experiment; such a relatively large age range did not influence social behaviour in cocaine-naïve or -treated mice (data not shown). At the beginning of the experiment, body weight of the mice was 24–44 g (32.5 ± 0.5 g; mean ± SEM). Animals were monitored daily for the presence of adverse effects of the experimental treatment, and veterinary advice was sought if animals displayed signs of distress. All studies were conducted in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were approved by the local Animal Care and Use Committee. Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath and Lilley, 2015).

## *Three-chamber apparatus*

The three-chamber apparatus was a rectangular box (60 × 40 × 20 cm, *L* × *W* × *H*) divided in three equal chambers and made of transparent Plexiglas. Dividing walls had small square doors (8 cm) that could be manually opened and closed. The central chamber was empty, and each side chamber contained a round wire cage (12 cm diameter and 14 cm high with bars spaced 1 cm apart) in which an object or a mouse could be placed.

## *Three-chamber testing in cocaine-naïve mice*

Prior to the beginning of the experiment, during three consecutive days, each animal was handled for 1 min·day$^{-1}$. The experiments were conducted during the light phase of the 12 h light/dark cycle in a quiet room dimly illuminated (10 lx). The three-chamber task allowed the study of (i) sociability (i.e. preference for an unfamiliar conspecific vs. an object) and (ii) SNP (i.e. preference for a novel vs. a familiar conspecific) (Silverman *et al.*, 2010), as depicted in Figure 1A. To reduce the number of animals used, mice were repeatedly tested in the three-chamber task, as depicted in Figure 1B. Due to the limited number of animals that could be tested daily in the three-chamber paradigm and to the breeding capacity of the mouse colony, behavioural experiments were identically carried out using three independent cohorts of mice. No statistical difference was found between the independent experiments, and therefore, the results were pooled. Within each animal cohort and across test days, all of the experimental conditions (i.e. genotype, treatment and stress) were pseudorandomized. During the habituation phase, the subject mouse was confined to the central chamber for 5 min; then, the doors were opened, so the mouse could freely explore the three chambers and the empty wire cages for 10 min. During the subsequent 10 min sociability phase, the subject mouse was allowed to explore the entire apparatus with one wire cage containing an unfamiliar mouse and the other an object. The unfamiliar mice were substance-naïve $CRF_2$+/− male mice age matched with the subject mice. They were handled (1 min·day$^{-1}$) and habituated to the wire cages (10 min·day$^{-1}$) over the 3 days preceding the first three-chamber test. The position (left- or right-side of chamber) of the unfamiliar mouse was counterbalanced within each experimental group. Then, a 10 min SNP test was carried out, during which a novel mouse was placed in the wire cage that previously contained the object, and the subject mouse could choose between the already investigated familiar and the novel mouse. To assess social recognition memory, the mice were tested once a week using an intertrial time interval (ITI) between the sociability and the SNP tests (Silverman *et al.*, 2010; Millan and Bales, 2013). In particular, on a given test day, within each experimental group, one-third of the animals were tested using the 0 h ITI, one-third using the 2 h ITI and one-third using the 4 h ITI. Between each test, the apparatus was cleaned with water and the wire cages with 50% ethanol. The experiments were recorded on a video system. The time spent exploring each wire cage and the number of entries made into each side chamber by the subject mouse were scored by a trained observer. Exploration was defined

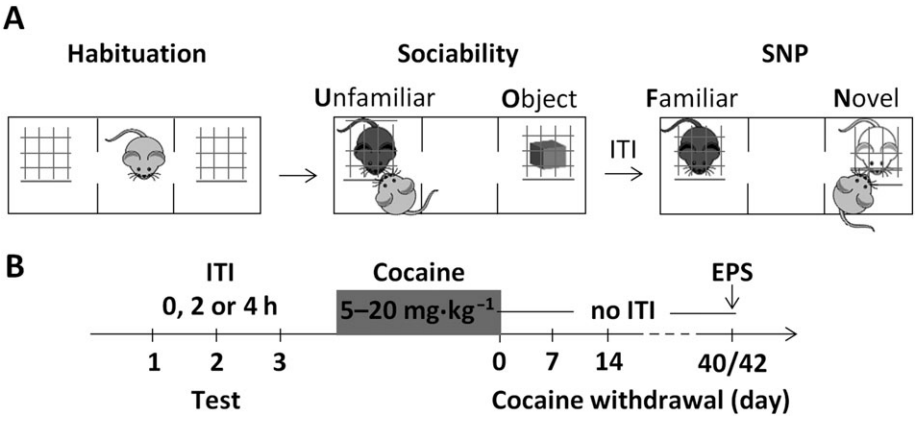



**Figure 1**

Experimental procedure. (A) Drawing illustrating the habituation, sociability and the SNP phases of the three-chamber test. (B) Wild-type and $CRF_2$−/− mice are tested (ticks) once a week in the three-chamber task using a 0, 2 or 4 h ITI between the sociability and the SNP test. Then, over an 11 day period, cocaine (5–20 mg·kg$^{-1}$, i.p.) is injected twice a day (0800–2000 h), except for the last day when only one injection is given in the morning, immediately prior to testing (cocaine withdrawal day 0). Following cessation of cocaine administration, the mice are tested weekly in the three-chamber task throughout a relatively long period using a 0 h ITI between the sociability and the SNP test. Then, using a within-subject design, on cocaine withdrawal day 40 or 42, the mice are exposed or not to an EPS 1 h prior to the three-chamber test.

as the animal directing the nose within 0.5 cm of the wire cage. An entry was defined as all four paws in one side of the chamber.

## Cocaine administration paradigm

Following the initial three-chamber tests, within genotype mice were pseudorandomly assigned to two treatment groups (saline or cocaine) having similar age and three-chamber performance, as assessed with the 2 h ITI (data not shown). For the biochemical experiment, within genotype mice were pseudorandomly assigned to two groups (saline or cocaine) having a similar age. Mice received i.p. injections (10 mL·kg$^{-1}$) of physiological saline or cocaine hydrochloride (Coopération Pharmaceutique Française, Melun, France) every 12 h (0800–2000 h) for 11 consecutive days, as follows: day 1: 5 mg·kg$^{-1}$; days 2 and 3: 10 mg·kg$^{-1}$; days 4–6: 15 mg·kg$^{-1}$; days 7–10: 20 mg·kg$^{-1}$; and day 11: 20 mg·kg$^{-1}$, only one injection in the morning. The latter treatment was chosen because it produced cognitive deficits and vulnerability to stress (Morisot *et al.*, 2014). The mice were weighed immediately before each injection and body weight changes calculated as a percentage of the body weight recorded just prior to the first injection.

## Three-chamber testing after cocaine administration and withdrawal

The three-chamber tests were carried out immediately and once a week after the last injection, up to cocaine withdrawal day 42. To control for the ability to detect social stimuli without loading on cognitive function, no ITI was applied between the sociability and the SNP test (Silverman *et al.*, 2010; Millan and Bales, 2013). To assess vulnerability to stress, on cocaine withdrawal day 40 within each group, approximately half of the mice were exposed to an elevated platform stressor (EPS) for 10 min and tested in the three-chamber task 1 h later; the other half remained undisturbed in the home cage (no stress) prior to being tested in the three-chamber task. Two days later (cocaine withdrawal day 42), the mice previously stressed were tested under no-stress conditions and *vice versa*. The elevated platform was a square (10 × 10 cm) made of dark grey polypropylene, elevated 40 cm above the floor.

## AVP and OT in situ hybridization histochemistry

To avoid the any effects the SNP testing may have on brain changes, the biochemistry studies were carried out using a fourth independent cohort of mice. In particular, wild-type and $CRF_2$−/− mice were treated with saline or cocaine, as described above. Then, 40 or 42 days after the last injection, they were exposed to the EPS for 10 min and killed by rapid cervical dislocation 1 h and 25 min later. Brains were rapidly removed, frozen in isopentane (−40°C) and stored at −80°C. The 1 h and 25 min interval was chosen to mimic the time elapsing between the EPS exposure and the end of the sociability phase during the three-chamber tests. Brains were cut in coronal sections (12 μm) using a cryostat and thaw mounted onto gelatin-coated slides to be processed for *in situ* hybridization. This was performed with oligonucleotide probes designed to recognize AVP (modified from Vacher *et al.*, 2002) or OT (modified from Patisaul *et al.*, 2003) mRNA, as previously described (Papaleo *et al.*, 2007). Oligonucleotide probes were labelled by tailing with [$^{35}$S]-dATP (PerkinElmer SAS, Courtaboeuf, France) using terminal deoxynucleotide transferase (Promega, Madison, WI, USA). The specific activity of the oligonucleotide probes was 58 × 10$^7$ and 78 × 10$^7$-cpm·μg$^{-1}$ for AVP or OT respectively. After being labelled, the probes were precipitated in absolute ethanol and 5 M sodium chloride, dried and resuspended at a concentration of 2-pg·μL$^{-1}$ in the hybridization buffer (50% deionized formamide, 10% dextran sulphate, 20 mM Tris–HCl, 1 mM EDTA, 300 mM NaCl, 200 μg·mL$^{-1}$ denatured salmon sperm DNA, 1% Denhardt, 0.1% SDS and 240 μg·mL$^{-1}$ tRNA). The sections were fixed with 4% paraformaldehyde in phosphate buffer 0.1 M (pH 7.2) for 5 min at room temperature, rinsed twice for 30 min with 4× saline–sodium citrate (SSC; 1% Denhardt), acetylated into 4× SSC (0.25% acetic anhydride, 1.33% triethanolamine; pH 8) for 10 min at room temperature and then dehydrated in graded alcohol. The slides were then incubated horizontally overnight at 40°C with the hybridization solution containing the $^{35}$S-labelled probe (45 μL per slide). At the end of the incubation, the slides were washed in decreasing concentrations of SSC (1× SSC at room temperature, 1× SSC at 40°C and 0.1× SSC at 40°C) and dehydrated in ethanol. The slides were then exposed at room temperature to Amersham Hyperfilms MP (Dominique Dutscher SAS, Brumath, France) over 2–4 days. Quantification of mRNA was performed by densitometry on X-ray films. Optical density (OD) was measured within regions defining the PVN or the SON (bregma interval: −0.70/−0.94 mm), as identified using the Paxinos and Franklin mouse brain atlas (Paxinos and Franklin, 2001). For each mouse, two measurements were performed for each brain region, one per hemisphere. These measurements were then averaged, and the outcome was considered as a representative value. For statistical analysis, the data were expressed as OD × 10$^3$.

## Statistical analysis

Each mouse was assigned a unique identification number that was used to conduct blind testing and data analysis. To prevent initial side preference from biasing the results, only animals exploring each side compartment of the three-chamber apparatus for no more than 70% of the 10 min habituation phase were included in the study (inclusion criterion). To obtain sociability or SNP ratios, the percentage of time spent investigating the wire cage containing the unfamiliar or the novel mouse over the total time spent investigating the two wire cages was calculated respectively. Since under drug-naïve conditions all of the animals met the inclusion criteria, a two-way ANOVA with genotype (wild-type, $CRF_2$−/−) as a between-subjects factor and repeated sociability tests or ITI as a within-subject factor was used. However, after drug administration, repeated measure analyses could not be used because of missing values during one or more of the three-chamber tests (i.e. animals not meeting the inclusion criterion). Therefore, within each test day, sociability, SNP ratios or entries were analysed using a two-way ANOVA with genotype and treatment (saline and cocaine) as between-subjects factors. Moreover, since three-chamber performance is considered a yes-or-no result, within each experimental group, time spent by the subject mouse exploring the unfamiliar mouse or the object, the novel or the familiar

mouse, during the sociability or the SNP tests, respectively, was compared using Student's paired *t*-test, as previously suggested (Silverman *et al.*, 2015; Kazdoba *et al.*, 2016). Entries into the two side chambers of the apparatus were also measured to control for sedation or hyperactivity (Moy *et al.*, 2013). A three-way ANOVA was used to analyse body weight changes, with genotype and treatment as between-subjects factors and days as a within-subject factor. A two-way ANOVA was used to analyse AVP and OT mRNA levels with genotype and treatment as between-subjects factors. The accepted value for significance was $P < 0.05$. If main or interaction effects were significant, Fisher's least significant difference *post hoc* test was used for individual group comparisons. Statistical analyses were performed using the Statistica software (version 10, TIBCO Software Inc, Palo Alto, CA, USA). Data graphs were created using GraphPad Prism (GraphPad Software, La Jolla, CA, USA). The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2015).

### Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding *et al.*, 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander *et al.*, 2017).

## Results

### Unaltered social behaviour in substance-naïve $CRF_2$ receptor-deficient mice

The impact of $CRF_2$ receptor deficiency upon social behaviour was first evaluated prior to cocaine administration. Although $CRF_2-/-$ mice showed a higher sociability ratio than wild-type mice ($P < 0.05$; Table 1 and Figure 2A), either genotype spent more time exploring the unfamiliar mouse than the object ($P < 0.05$; Supporting Information Table S1 and Figure S1A). Repeated testing reduced entry into the two side chambers of the apparatus ($P < 0.05$; Table 1). Indeed, mice made more entries during the first than the second or the third three-chamber test ($P < 0.05$; Figure 2B). During the SNP tests, social memory followed a genotype-independent ITI-linked decay ($P < 0.05$; Table 1). Indeed, when a 2 h ITI was applied between the sociability and the SNP test, mice showed lower or higher SNP ratios, as compared with no or a 4 h ITI respectively ($P < 0.05$; Figure 2C). Moreover, either genotype spent more time exploring the novel than the familiar mouse when no or a 2 h ITI was applied between the sociability and the SNP test ($P < 0.05$; Supporting Information Table S1 and Figure S1B). However, when a 4 h ITI was used, wild-type and $CRF_2-/-$ mice spent similar time exploring the novel and the familiar mouse, indicating a genotype-independent ITI-induced SNP deficit (Supporting Information Table S1 and Figure S1B). During the SNP tests, neither genotype nor ITI influenced entry into the two side chambers of the apparatus (Table 1 and Figure 2D). Thus, under substance-naïve conditions, $CRF_2$ receptor deficiency does not alter either sociability or SNP.

### $CRF_2$ receptor deficiency eliminates the sociability deficits associated with early cocaine withdrawal phases

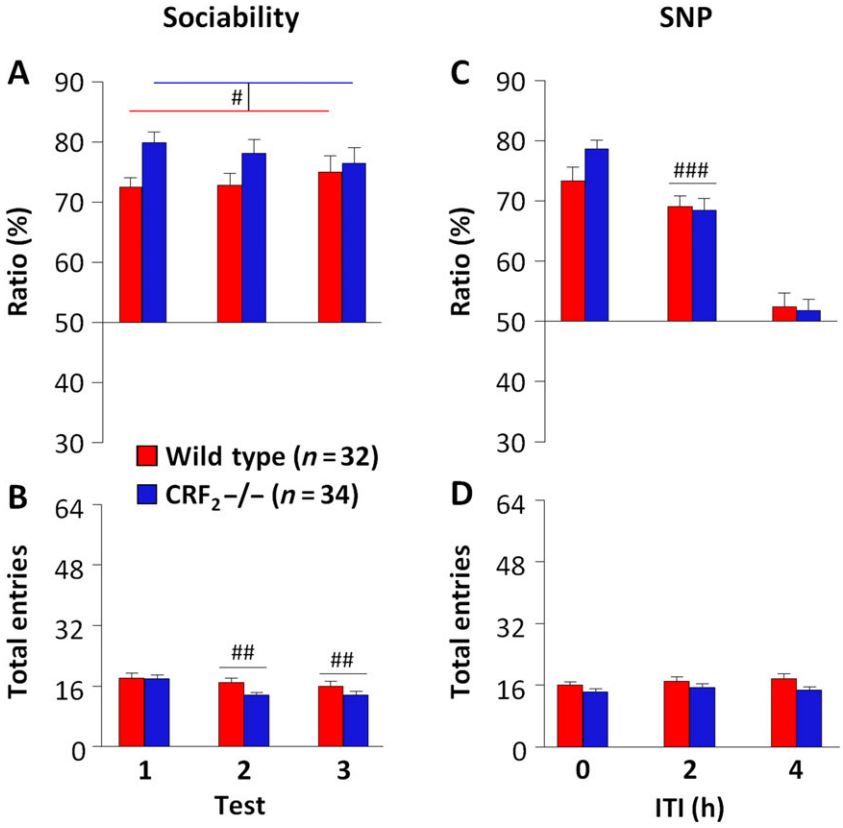
The intermittent administration of escalating cocaine doses induced a genotype-independent body weight loss (genotype effect: $F_{1, 60} = 1.79$, $P = 0.19$; treatment effect: $F_{1, 60} = 24.77$, $P < 0.05$; day effect: $F_{9, 540} = 25.91$, $P < 0.05$; and treatment × day effect: $F_{9, 540} = 11.64$, $P < 0.05$) that was evident starting 12 h after the 10th injection ($P < 0.05$, Supporting Information Figure S2). Analysis of sociability ratios immediately after the last cocaine injection revealed a genotype-independent treatment effect ($P < 0.05$; Table 2). Indeed, cocaine-treated mice showed a lower sociability ratio than saline-treated mice ($P < 0.05$; Figure 3A). Notably, cocaine-treated wild-type and $CRF_2-/-$ mice spent less time investigating the unfamiliar mouse than the object, suggesting social avoidance ($P < 0.05$; Supporting Information Table S2 and Figure S3A). Cocaine-treated mice also showed more entries than saline-treated mice ($P < 0.05$; Table 2 and Figure 3B), suggesting an influence of hyperactivity on the lack of sociability. Seven days after the last administration, cocaine withdrawal affected sociability ratios in a genotype-dependent manner ($P < 0.05$; Table 2). Notably, cocaine-withdrawn wild-type mice showed lower sociability

**Table 1**

Statistical analysis of sociability ratio, SNP ratio and entries into the two side compartments of the three-chamber apparatus displayed by drug-naïve wild-type and $CRF_2-/-$ mice

| | Sociability | | SNP | |
|---|---|---|---|---|
| | Ratio | Entries | Ratio | Entries |
| Geno | $F_{1, 64} = 4.33$<br>$P < 0.05$ | $F_{1, 64} = 3.51$<br>$P = 0.07$ | $F_{1, 64} = 0.89$<br>$P = 0.34$ | $F_{1, 64} = 3.43$<br>$P = 0.07$ |
| RM | $F_{2, 128} = 0.07$<br>$P = 0.93$ | $F_{2, 128} = 6.50$<br>$P < 0.005$ | $F_{2, 128} = 79.19$<br>$P < 0.05$ | $F_{2, 128} = 1.27$<br>$P = 0.28$ |
| Geno × RM | $F_{2, 128} = 1.24$<br>$P = 0.29$ | $F_{2, 128} = 1.41$<br>$P = 0.25$ | $F_{2, 128} = 1.42$<br>$P = 0.25$ | $F_{2, 128} = 0.45$<br>$P = 0.64$ |

Geno, genotype; RM, repeated measure (sociability: tests 1, 2 and 3; SNP: 0, 2 and 4 h ITI between the sociability and the SNP test).




## Figure 2

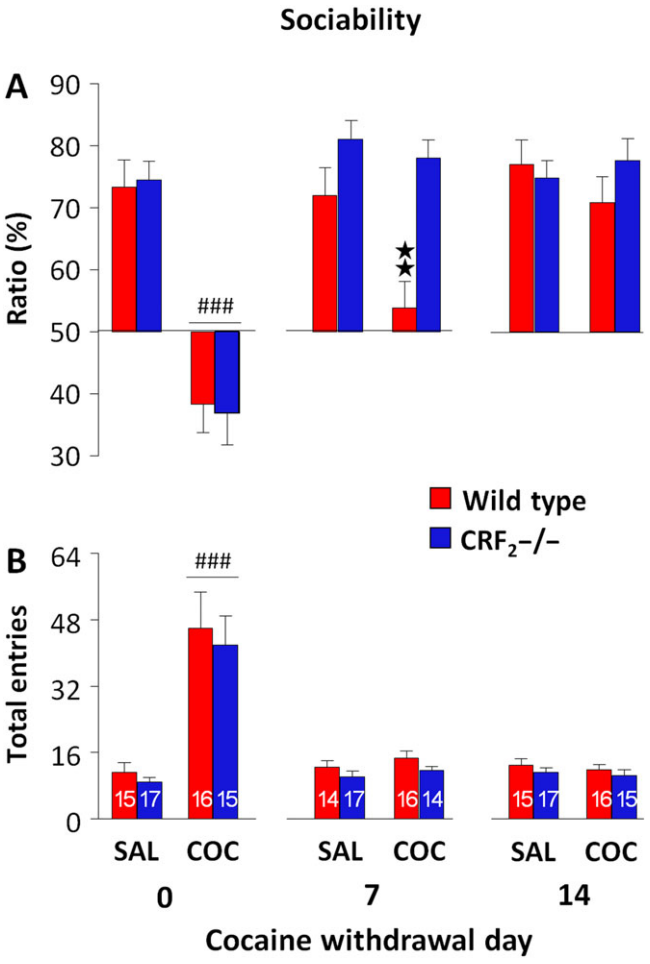
$CRF_2$ receptor deficiency does not influence sociability or social recognition memory under basal drug-naïve conditions. (A) Sociability and (C) SNP ratio (%) displayed by drug-naïve wild-type and $CRF_2-/-$ mice repeatedly tested (tests 1 to 3) once a week in the three-chamber task. A 0, 2 or 4 h ITI was pseudorandomly applied between the sociability and the SNP test. The ratio was calculated as the percentage of time spent investigating the wire cage containing the unfamiliar or the novel mouse over the total time spent investigating the two wire cages during the sociability or the SNP test respectively. (B, D) Total entries made into the two side compartments of the three-chamber apparatus during the sociability or the SNP test. Values represent mean ± SEM. $^{\#}P < 0.05$ versus wild-type mice, genotype effect. $^{\#\#}P < 0.05$ versus test 1, repeated measure effect. $^{\#\#\#}P < 0.05$ versus 0 and 4 h ITI, repeated measure effect.

## Table 2

Statistical analysis of sociability ratio and entries into the two side compartments of the three-chamber apparatus displayed by wild-type and $CRF_2-/-$ mice immediately (0), 7 or 14 days after the last saline or cocaine administration

| | **Sociability** | | | | | |
|---|---|---|---|---|---|---|
| | **Days after the last administration** | | | | | |
| | **0** | | **7** | | **14** | |
| | **Ratio** | **Entries** | **Ratio** | **Entries** | **Ratio** | **Entries** |
| Geno | $F_{1, 59} = 0.08$<br>$P = 0.78$ | $F_{1, 59} = 0.36$<br>$P = 0.55$ | $F_{1, 60} = 20.35$<br>$P < 0.05$ | $F_{1, 60} = 3.31$<br>$P = 0.07$ | $F_{1, 59} = 0.46$<br>$P = 0.50$ | $F_{1, 59} = 1.74$<br>$P = 0.19$ |
| Treat | $F_{1, 59} = 69.46$<br>$P < 0.05$ | $F_{1, 59} = 34.91$<br>$P < 0.05$ | $F_{1, 60} = 7.99$<br>$P < 0.05$ | $F_{1, 60} = 1.83$<br>$P = 0.18$ | $F_{1, 59} = 0.21$<br>$P = 0.65$ | $F_{1, 59} = 0.45$<br>$P = 0.50$ |
| Geno × Treat | $F_{1, 59} = 0.0003$<br>$P = 0.98$ | $F_{1, 59} = 0.007$<br>$P = 0.93$ | $F_{1, 60} = 4.19$<br>$P < 0.05$ | $F_{1, 60} = 0.03$<br>$P = 0.87$ | $F_{1, 59} = 1.53$<br>$P = 0.22$ | $F_{1, 59} = 0.03$<br>$P = 0.87$ |

Geno, genotype; Treat, treatment (saline or cocaine).




## Figure 3

$CRF_2$ receptor deficiency eliminates sociability deficits induced by cocaine withdrawal. (A) Sociability ratio (%) displayed by saline (SAL)- or cocaine (COC)-treated wild-type and $CRF_2-/-$ mice. The three-chamber tests were carried out immediately (0), 7 and 14 days after the last saline or cocaine administration. (B) Total entries made into the two side compartments of the three-chamber apparatus during the sociability test. The *n* for each experimental group is presented in the columns in panel B. Values represent mean ± SEM. $^{**}P < 0.05$ versus all other groups on cocaine withdrawal day 7. $^{###}P < 0.05$ versus saline groups on day 0, genotype-independent effect.

ratios than cocaine-withdrawn $CRF_2-/-$ and saline-withdrawn mice ($P < 0.05$; Figure 3A). Indeed, unlike the sociability displayed by cocaine-withdrawn $CRF_2-/-$ and saline-withdrawn mice ($P < 0.05$), cocaine-withdrawn wild-type mice spent a similar time investigating the unfamiliar mouse and the object, further indicating sociability deficits (Supporting Information Table S2 and Figure S3A). The genotype-dependent sociability observed on cocaine withdrawal day 7 was not due to locomotor activity since the experimental groups did not differ in the number of entries (Table 2 and Figure 3B). Finally, 14 days after the last injection, neither genotype nor treatment affected sociability ratios (Table 2 and Figure 3A). Indeed, similarly to cocaine-withdrawn $CRF_2-/-$ and saline-withdrawn mice, cocaine-withdrawn wild-type mice spent more time investigating the unfamiliar mouse than the object, indicating recovered sociability ($P < 0.05$; Supporting Information Table S2 and Figure S3A). Moreover, neither genotype nor treatment affected the number of entries (Table 2 and Figure 3B). These results indicate that $CRF_2$ receptor deficiency eliminates the sociability deficits induced by cocaine withdrawal.

### *Cocaine administration and withdrawal do not impair SNP*

Immediately after the last injection, cocaine decreased SNP ratios, independently of genotype ($P < 0.05$; Table 3 and Figure 4A). However, all of the experimental groups investigated the novel more than the familiar mouse, indicating SNP ($P < 0.05$; Supporting Information Table S2 and Figure S3B). Cocaine treatment also genotype-independently increased the number of entries ($P < 0.05$; Table 3 and Figure 4B). Consistently, on cocaine withdrawal day 7 or 14, neither genotype nor treatment affected SNP ratios (Table 3 and Figure 4A). Indeed, saline- or cocaine-withdrawn mice of either genotype spent more time investigating the novel than the familiar mouse ($P < 0.05$; Supporting Information Table S2 and Figure S3B). Moreover, 7 and 14 days after the last administration, neither genotype nor treatment affected the number of entries (Table 3 and Figure 4B). These results indicate that cocaine administration and withdrawal do not affect the ability to detect social novel stimuli.

### *$CRF_2$ receptor deficiency eliminates the long-lasting vulnerability of social behaviour induced by cocaine withdrawal*

Forty or 42 days after the last administration, under no-stress conditions, neither genotype nor treatment affected sociability ratios (Table 4 and Figure 5A). Indeed, all of the experimental groups spent more time investigating the unfamiliar mouse than the object, indicating sociability ($P < 0.05$; Supporting Information Table S3 and Figure S4A). However, following exposure to the EPS, cocaine withdrawal affected sociability ratios in a genotype-dependent manner ($P < 0.05$; Table 4). Indeed, cocaine-withdrawn wild-type mice showed lower sociability ratios than cocaine-withdrawn $CRF_2-/-$ and saline-withdrawn mice ($P < 0.05$; Figure 5A). Accordingly, cocaine-withdrawn wild-type mice spent similar time exploring the unfamiliar mouse and the object (Supporting Information Table S3 and Figure S4A), indicating a stress-induced re-emergence of sociability deficits. In contrast, cocaine-withdrawn $CRF_2-/-$ mice exposed to the EPS spent more time investigating the unfamiliar mouse than the object ($P < 0.05$; Supporting Information Table S3 and Figure S4A), indicating resilience to stress. The EPS did not impair sociability in saline-withdrawn wild-type and $CRF_2-/-$ mice ($P < 0.05$; Supporting Information Table S3 and Figure S4A), suggesting a relatively mild nature of this stressor in substance-naïve mice. Finally, both under no-stress and stress conditions, neither genotype nor treatment affected the number of entries (Table 4 and Figure 5B), ruling out a role for locomotor activity in the stress-induced sociability deficit displayed by cocaine-withdrawn wild-type mice. Thus, $CRF_2$ receptor deficiency abolishes the long-lasting

## Table 3

Statistical analysis of SNP ratio and entries into the two side compartments of the three-chamber apparatus displayed by wild-type and $CRF_2$−/− mice immediately (0), 7 or 14 days after the last saline or cocaine administration

| | SNP | | | | | |
|---|---|---|---|---|---|---|
| | Days after the last administration | | | | | |
| | 0 | | 7 | | 14 | |
| | Ratio | Entries | Ratio | Entries | Ratio | Entries |
| Geno | $F_{1, 59} = 0.60$<br>$P = 0.44$ | $F_{1, 59} = 0.84$<br>$P = 0.36$ | $F_{1, 60} = 0.75$<br>$P = 0.39$ | $F_{1, 60} = 1.15$<br>$P = 0.29$ | $F_{1, 59} = 0.57$<br>$P = 0.45$ | $F_{1, 59} = 0.27$<br>$P = 0.60$ |
| Treat | $F_{1, 59} = 13.87$<br>$P < 0.05$ | $F_{1, 59} = 36.91$<br>$P < 0.05$ | $F_{1, 60} = 0.48$<br>$P = 0.49$ | $F_{1, 60} = 1.95$<br>$P = 0.17$ | $F_{1, 59} = 0.05$<br>$P = 0.82$ | $F_{1, 59} = 0.47$<br>$P = 0.49$ |
| Geno × Treat | $F_{1, 59} = 1.23$<br>$P = 0.27$ | $F_{1, 59} = 0.00$<br>$P = 1.00$ | $F_{1, 60} = 1.98$<br>$P = 0.16$ | $F_{1, 60} = 0.00$<br>$P = 1.00$ | $F_{1, 59} = 0.10$<br>$P = 0.75$ | $F_{1, 59} = 2.88$<br>$P = 0.09$ |

Geno, genotype; Treat, treatment (saline or cocaine).

social behaviour vulnerability to stress induced by cocaine withdrawal.

### *Stress does not impair SNP in long-term cocaine-withdrawn mice*

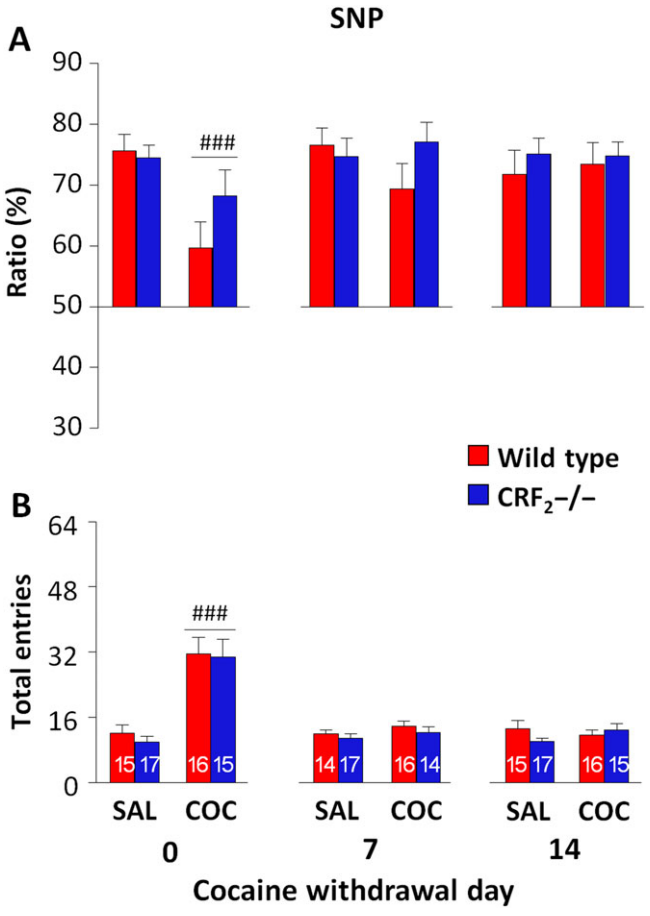
During the SNP tests carried out 40 or 42 days after the last administration, under no-stress conditions, neither genotype nor treatment affected SNP ratios (Table 4 and Figure 5C). However, following exposure to the EPS, cocaine-withdrawn mice showed higher SNP ratios than saline-withdrawn mice, independently of genotype ($P < 0.05$; Table 4 and Figure 5C). Within-group comparisons revealed that saline- and cocaine-withdrawn wild-type and $CRF_2$−/− mice exposed or not to the EPS spent more time investigating the novel than the familiar mouse ($P < 0.05$; Supporting Information Table S3 and Figure S4B). Finally, neither genotype nor treatment affected the number of entries, both under no-stress or stress conditions (Table 4, Figure 5D). These results indicate that stress does not alter the ability to detect social novel stimuli, either in substance-naïve or in long-term cocaine-withdrawn mice.

### *$CRF_2$ receptor deficiency abolishes the increased AVP and OT expression induced by cocaine withdrawal*

Stressors increase AVP and OT activity in the PVN and the SON (Wotjak *et al.*, 1998; Yang *et al.*, 2012; Yan *et al.*, 2014). Interestingly, the $CRF_2$ receptor is expressed both in the PVN and the SON (Van Pett *et al.*, 2000). However, to our knowledge, the role for the CRF system in brain AVP and OT activity induced by cocaine withdrawal remains unexplored. To address the latter issue, 40 or 42 days after the last saline or cocaine administration, wild-type and $CRF_2$−/− mice were exposed to the EPS, and brains were examined for AVP and OT mRNA expression. Cocaine withdrawal affected PVN-AVP expression in a genotype-dependent manner (genotype effect: $F_{1, 26} = 5.13$, $P < 0.05$; treatment effect: $F_{1, 26} = 0.01$, $P = 0.91$; and genotype × treatment interaction effect: $F_{1, 26} = 5.54$, $P < 0.05$). Notably, cocaine-withdrawn wild-type mice had higher PVN-AVP expression than cocaine-withdrawn $CRF_2$−/− mice ($P < 0.05$; Figure 6A, B). In contrast, cocaine-withdrawn $CRF_2$−/− mice did not differ from saline-withdrawn mice (Figure 6A, B). However, neither genotype ($F_{1, 26} = 0.03$, $P = 0.85$) nor treatment ($F_{1, 26} = 0.09$, $P = 0.76$) affected SON-AVP expression (genotype × treatment interaction effect: $F_{1, 26} = 1.11$, $P = 0.74$; Figure 6A, B). Similarly, neither genotype ($F_{1, 26} = 0.85$, $P = 0.36$) nor treatment ($F_{1, 26} = 2.84$, $P = 0.10$) affected PVN-OT expression (genotype × treatment interaction effect: $F_{1, 26} = 1.35$, $P = 0.25$; Figure 6C, D). However, cocaine withdrawal affected SON-OT expression in a genotype-dependent manner (genotype effect: $F_{1, 26} = 0.88$, $P = 0.36$; treatment effect: $F_{1, 26} = 0.39$, $P = 0.54$; and genotype × treatment interaction effect: $F_{1, 26} = 6.54$, $P < 0.05$). Indeed, cocaine-withdrawn wild-type mice showed higher SON-OT expression than cocaine-withdrawn $CRF_2$−/− or saline-withdrawn wild-type mice ($P < 0.05$; Figure 6C, D). In contrast, cocaine-withdrawn $CRF_2$−/− mice did not differ from saline-withdrawn mice (Figure 6C, D). These results indicate a brain region-specific AVP or OT gene expression after a relatively long period of cocaine withdrawal. Moreover, they show that the cocaine withdrawal-induced expression of AVP and OT is dependent on the $CRF_2$ receptor.

## Discussion

The present study shows that cessation of intermittent administration of escalating doses of cocaine disrupts social behaviour in wild-type, but not in $CRF_2$−/− mice, revealing an essential role for the $CRF_2$ receptor in sociability deficits induced by substance use and withdrawal. Furthermore, following a rather long 6 week period of cocaine withdrawal, exposure to a relatively mild stressor triggers the re-emergence of sociability deficits in wild-type, but not in $CRF_2$−/− mice, emphasizing a critical role for the $CRF_2$ receptor also in the long-lasting vulnerability following substance withdrawal. Accordingly, in wild-type, but not in $CRF_2$−/− mice, long-term cocaine withdrawal and stress exposure increase the hypothalamic expression of AVP and OT,




## Figure 4

$CRF_2$ receptor deficiency does not affect SNP. (A) SNP ratio (%) displayed by saline (SAL)- or cocaine (COC)-treated wild-type and $CRF_2$−/− mice. The three-chamber tests were carried out immediately (0), 7 and 14 days after the last saline or cocaine administration. (B) Total entries made into the two side compartments of the three-chamber apparatus during the SNP test. The *n* for each experimental group is presented in panel B. Values represent mean ± SEM. $^{###}P < 0.05$ versus saline groups on day 0, genotype-independent effect.

signalling a vulnerable state. Interestingly, neither cocaine withdrawal nor stress susceptibility is associated with deficits in SNP, indicating preserved ability to detect and discern social stimuli.

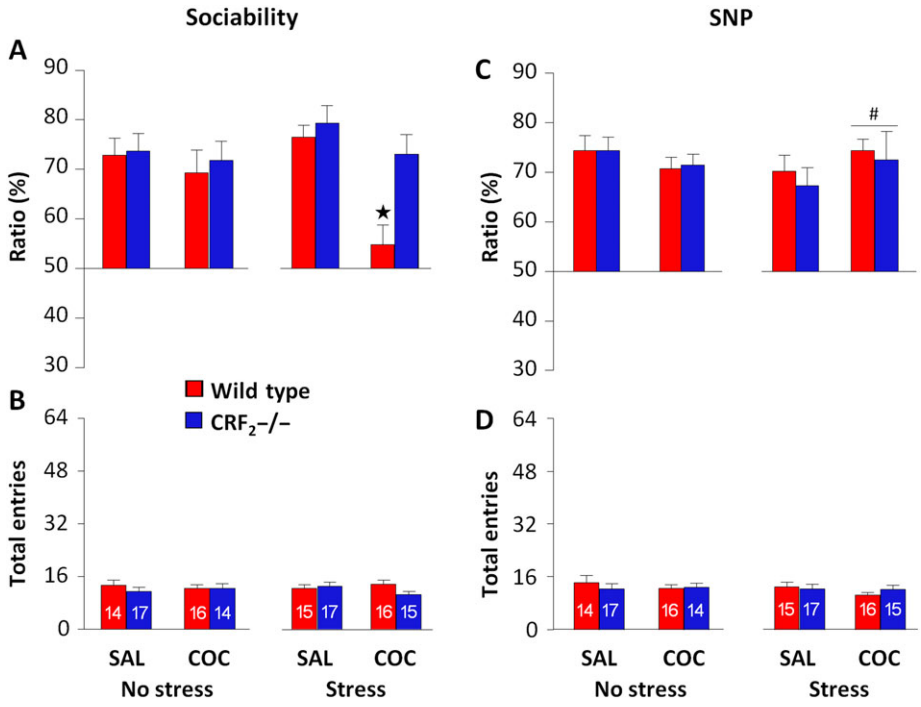
To assess the impact of $CRF_2$ receptor deficiency upon sociability and social memory span, prior to cocaine administration, wild-type and $CRF_2$−/− mice were repeatedly tested using a variable time interval between the sociability and the SNP tests. Wild-type and $CRF_2$−/− mice displayed similar sociability and social memory span. The latter results are in apparent contrast with a study showing that $CRF_2$−/− mice exhibit a preference for familiar (nest mate) mice and avoid novel unknown mice, as compared with wild-type mice (Shemesh *et al.*, 2016). Differences in experimental conditions may underlie the discrepancies between the latter and the present study. Notably, in the latter study, a male 'subject' mouse could remain in a remote (non-social) chamber or interact with a nest-mate familiar sibling or a novel mouse. $CRF_2$ receptor deficiency is known to increase anxiety-like behaviour (Bale *et al.*, 2000; Kishimoto *et al.*, 2000). Thus, it is possible that in the latter study, the anxiogenic-like effect of the $CRF_2$ receptor null mutation contributed to the avoidance of the novel unknown, ethologically more threatening mouse. In contrast, the three-chamber task used herein is thought to reliably measure social approach behaviour in mice, independently of emotional-like or cognitive states (Silverman *et al.*, 2010). Indeed, ethanol withdrawal decreased exploration of a three-chamber apparatus, indicating increased anxiety-like behaviour, without impairing sociability (Moy *et al.*, 2013). Conversely, herein, cocaine withdrawal impaired sociability without affecting exploration, as assessed by entries into the two side compartments of the three-chamber apparatus. Moreover, on cocaine withdrawal day 14, both genotypes displayed sociability and SNP. However, we previously reported that on cocaine withdrawal day 14, both genotypes display recognition memory deficits (Morisot *et al.*, 2014), indicating that substance withdrawal-induced cognitive dysfunction does not affect social behaviour, as assessed by the three-chamber task. Accordingly, cocaine-withdrawn mice did not show social behaviour deficits but displayed impaired novel object recognition memory,

## Table 4

Statistical analysis of sociability ratio, SNP ratio and entries into the two side compartments of the three-chamber apparatus displayed by saline- or cocaine-treated wild-type and $CRF_2$−/− mice 40 or 42 days after the last administration

| | Sociability | | | | SNP | | | |
|---|---|---|---|---|---|---|---|---|
| | No stress | | Stress | | No stress | | Stress | |
| | Ratio | Entries | Ratio | Entries | Ratio | Entries | Ratio | Entries |
| Geno | $F_{1, 57} = 1.22$<br>$P = 0.27$ | $F_{1, 57} = 0.42$<br>$P = 0.52$ | $F_{1, 58} = 5.64$<br>$P < 0.05$ | $F_{1, 58} = 1.20$<br>$P = 0.28$ | $F_{1, 57} = 0.03$<br>$P = 0.84$ | $F_{1, 57} = 0.10$<br>$P = 0.75$ | $F_{1, 58} = 0.78$<br>$P = 0.38$ | $F_{1, 58} = 0.54$<br>$P = 0.47$ |
| Treat | $F_{1, 57} = 1.25$<br>$P = 0.27$ | $F_{1, 57} = 0.00$<br>$P = 0.98$ | $F_{1, 58} = 2.02$<br>$P = 0.16$ | $F_{1, 58} = 0.32$<br>$P = 0.57$ | $F_{1, 57} = 0.55$<br>$P = 0.46$ | $F_{1, 57} = 0.23$<br>$P = 0.63$ | $F_{1, 58} = 4.48$<br>$P < 0.05$ | $F_{1, 58} = 0.71$<br>$P = 0.40$ |
| Geno × Treat | $F_{1, 57} = 0.01$<br>$P = 0.92$ | $F_{1, 57} = 0.41$<br>$P = 0.53$ | $F_{1, 58} = 4.37$<br>$P < 0.05$ | $F_{1, 58} = 2.10$<br>$P = 0.15$ | $F_{1, 57} = 0.86$<br>$P = 0.36$ | $F_{1, 57} = 0.48$<br>$P = 0.49$ | $F_{1, 58} = 0.31$<br>$P = 0.58$ | $F_{1, 58} = 1.75$<br>$P = 0.19$ |

On cocaine withdrawal day 40 or 42, mice were exposed (stress) or not (no stress) to the EPS, using a within-subject experimental design. Geno, genotype; Treat, treatment (saline or cocaine).




## Figure 5

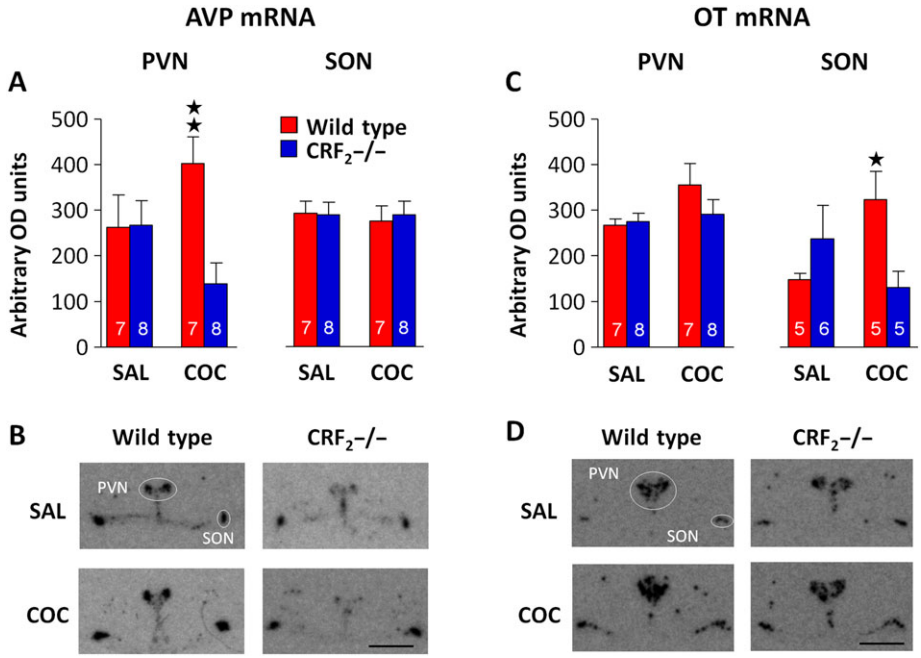
$CRF_2$ receptor deficiency abolishes the long-lasting social behaviour vulnerability to stress induced by cocaine (COC) withdrawal. (A) Sociability and (C) SNP ratio (%) displayed by saline (SAL)- or cocaine-treated wild-type and $CRF_2-/-$ mice exposed (stress) or not (no stress) to the EPS. The three-chamber tests were carried out 40 or 42 days after the last administration using a within-subject (stress vs. no stress) experimental design. (B, D) Total entries made into the two side compartments of the three-chamber apparatus during the sociability or the SNP test. The *n* for each experimental group is presented in panels B and D. Values represent mean ± SEM. $^{*}P < 0.05$ versus all other stressed groups. $^{\#}P < 0.05$ versus stressed saline groups, genotype-independent effect.

as determined, respectively, 12 and 13 days after the last drug injection (Georgiou *et al.*, 2016b).

In wild-type mice, the sociability deficits produced by the intermittent administration of escalating doses of cocaine lasted at least up to 7 days after substance discontinuation. In contrast, cocaine-withdrawn $CRF_2-/-$ mice showed a social behaviour profile similar to substance-naïve mice, indicating an essential role for the $CRF_2$ receptor in cocaine-induced sociability deficits. Noteworthy, despite disrupted sociability, wild-type mice displayed SNP, indicating that the substance withdrawal-induced sociability deficit was not due to alterations in the detection or processing of social stimuli. To our knowledge, no study has yet reported impaired sociability without a deficit in SNP. Indeed, prior studies reported SNP impairment alone or together with sociability deficits in mouse models of autism (Sala *et al.*, 2011; Moy *et al.*, 2013). This suggests that withdrawal from stimulant substances might produce a unique pattern of social dysfunction, namely, impaired sociability in spite of preserved social novelty recognition.

Herein, exposure to a mild environmental stressor that did not affect social behaviour of substance-naïve mice induced the re-emergence of sociability deficits in cocaine-withdrawn wild-type mice, even relatively long time (6 weeks) after the last substance administration. This observation parallels human patients where withdrawal from substances of abuse is followed by a strikingly long-lasting period of vulnerability to stressful life events. Indeed, stress exposure is considered a major risk factor for relapse to substance intake in former substance users (Sinha, 2001). Accordingly, laboratory animal studies showed that physical, psychological or pharmacological stressors reinstate substance-seeking behaviour and induce negative affective-like states, even after relatively long periods of substance withdrawal (Shaham *et al.*, 2003; Blatchford *et al.*, 2005; Breese *et al.*, 2005; Morisot *et al.*, 2015). The present results extend to social behaviour the deleterious consequences of substance withdrawal and the associated long-lasting vulnerability to stress. Most importantly, unlike wild-type mice, following stress exposure, cocaine-withdrawn $CRF_2-/-$ mice did not show sociability deficits, indicating an essential role for the $CRF_2$ receptor in the long-lasting vulnerability following discontinuation of stimulant substances. Together with the prior results showing a key role for the $CRF_2$ receptor in the stress-induced re-emergence of cognitive dysfunction and up-shifted motivational states in opiate-withdrawn or cocaine-withdrawn mice (Morisot *et al.*, 2014; 2015; Morisot and Contarino, 2016), the present findings further point to the $CRF_2$ receptor as a key neural substrate of the long-lasting vulnerability induced by substances of abuse.

Investigations into the molecular mechanisms underlying the $CRF_2$ receptor-dependent sociability deficits in long-term cocaine-withdrawn mice revealed altered brain AVP and OT gene expression. Specifically, following stress exposure, cocaine-withdrawn wild-type mice displayed elevated expression of AVP in the PVN and OT in the SON, as




## Figure 6

$CRF_2$ receptor-dependent expression of brain AVP and OT after long-term cocaine (COC) withdrawal. (A, C) Optical density (OD) and (B, D) representative images of brain sections illustrating (A, B) AVP or (C, D) OT mRNA levels in the paraventricular (PVN) and the supraoptic (SON) nuclei of the hypothalamus in saline (SAL)- or cocaine-withdrawn wild-type and $CRF_2-/-$ mice following exposure to the EPS. Mice received chronic saline or cocaine treatment, as illustrated in Figure 1. On cocaine withdrawal day 40 or 42, the mice were exposed to the EPS for 10 min, and brains were rapidly collected 1 h and 25 min later. Bregma intervals are −0.70/−0.94 mm. Scale bar = 1 mm. The *n* for each experimental group is presented in panels A and C. Values represent mean ± SEM. Panel A: $^{**}P < 0.05$ versus $CRF_2-/-$ cocaine-withdrawn mice. Panel C: $^{*}P < 0.05$ versus $CRF_2-/-$ cocaine-withdrawn and wild-type saline-treated mice.

compared with substance-naïve mice. Repeated cocaine or nicotine administration has been shown to decrease OT and vasopressin peptide levels in the hypothalamus and hippocampus, to diminish vasopressin peptide levels in the amygdala and to increase **OT receptor** binding in the lateral septum or the amygdala (Sarnyai *et al.*, 1992; Zanos *et al.*, 2015; Georgiou *et al.*, 2016b). Decreased OT peptide levels in the hypothalamus, increased OT receptor binding in the lateral septum and amygdala and decreased social behaviour have also been shown in morphine-withdrawn mice (Zanos *et al.*, 2014). Moreover, cocaine or methamphetamine withdrawal increased the expression of PVN-AVP, OT receptor binding or immunoreactivity in the septum or the subthalamic nucleus, decreased novel object recognition memory and sucrose preference and increased anxiety-like behaviour in mice or rats (Zhou *et al.*, 2011; Baracz *et al.*, 2016; Georgiou *et al.*, 2016a,b). In addition, extensive research supports a major involvement of OT and AVP in social memory and social interaction (Neumann and Landgraf, 2012). However, the functional significance of the increased brain AVP and OT gene expression induced by substances of abuse remains largely unknown. One possibility is that withdrawal from substances of abuse induces a compensatory activation of AVP and OT systems in order to cope with this stressful and vulnerable condition. This hypothesis is supported by studies suggesting that AVP and OT are involved in coping with stress. Indeed, substance-naïve rats exposed to the forced swim or the tail suspension stressors showed increased levels of AVP and OT in several hypothalamic nuclei, including the PVN and the SON, the frontal cortex and the amygdala (Yang *et al.*, 2012; Yan *et al.*, 2014). Moreover, rats repeatedly exposed to a forced swim stressor displayed increased AVP or OT release within the PVN or the SON respectively (Wotjak *et al.*, 1998). Notably, functional antagonism of brain AVP and OT signalling by i.c.v. injection of AVP or OT receptor antagonists increased despair behaviour (immobility time) induced by the forced swim or the tail suspension stressors, suggesting that the increased AVP and OT activity served to attenuate the deleterious effects of stressful experiences (Yang *et al.*, 2012; Yan *et al.*, 2014). Within this framework, the elevated hypothalamic AVP and OT gene expressions observed herein in cocaine-withdrawn mice might serve as valuable biomarkers of the long-lasting vulnerability following substance withdrawal. However, since hypothalamic AVP and OT expressions were not investigated in non-stressed animals, the present findings might conceivably have been a consequence of long-term cocaine withdrawal, independently of EPS exposure.

Contrary to cocaine-withdrawn wild-type mice, following stress exposure, cocaine-withdrawn $CRF_2-/-$ mice did not show any increase in the expression of AVP or OT in the PVN or the SON. As mentioned above, studies have suggested that the CRF system is involved in social interaction and social memory (reviewed in Hostetler and Ryabinin, 2013). The $CRF_2$ receptor is expressed both in the PVN and the SON, major sources of brain AVP and OT (Van Pett *et al.*,

2000). Moreover, double-labelling *in situ* hybridization studies have shown co-localization of $CRF_2$ receptor mRNA with OT and AVP mRNA in the SON (Arima and Aguilera, 2000). However, to the best of our knowledge, no studies have yet demonstrated a functional link between the CRF system and the deregulated activity of AVP and OT systems induced by substances of abuse. In the present study, we report that $CRF_2$ receptor deficiency eliminates both the stress-induced re-emergence of sociability deficits and the elevated hypothalamic expression of AVP and OT in long-term cocaine-withdrawn animals. This suggests that a relatively mild environmental stressor might activate brain CRF pathways which, *via* $CRF_2$ receptors, might mediate vulnerability to stress and the hypothalamic expression of AVP and OT in order to counteract the long-lasting cocaine withdrawal-induced deleterious impact of stressors. Conversely, the lack of functional $CRF_2$ receptors may confer stress resilience, which *per se* does not require AVP or OT elevations to cope with stressful events after substance withdrawal. These findings indicate a central role for the $CRF_2$ receptor in stress vulnerability and the closely related induction of AVP and OT gene expression following cocaine discontinuation. Nevertheless, more studies are warranted to further explore the functional interplay between the stress-responsive CRF, AVP and OT systems and their relative contribution to the long-lasting vulnerable state of substance-withdrawn individuals.

In conclusion, the present results demonstrate impaired social behaviour in mice withdrawn from chronic cocaine administration. Notably, $CRF_2$ receptor deficiency eliminated the sociability deficits and the long-lasting vulnerability to stress induced by cocaine discontinuation. Accordingly, $CRF_2$ receptor deficiency also abolished the increased expression of the stress-responsive AVP and OT systems that was provoked by cocaine withdrawal, further demonstrating a major role for the $CRF_2$ receptor in the deleterious consequences of cocaine administration and withdrawal. Finally, the present findings suggest that pharmacological blockade of the $CRF_2$ receptor might attenuate the social breakdown commonly observed in stimulant use disorders and help abstinent patients to refrain from reinitiating substance-seeking and substance-taking behaviour.

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## Author contributions

N.M. and A.C. designed the study; N.M. and R.M. conducted the behavioural and the *in situ* hybridization experiments; N.M., R.M., C.L.M. and A.C. analysed the data; and N.M., M.J.M. and A.C. wrote the manuscript.

## Conflict of interest

The authors declare no conflicts of interest.

## Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies, publishers and other organisations engaged with supporting research.

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## Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article.

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**Table S1** Statistical analysis of sociability and social novelty preference (SNP) displayed by drug-naïve wild-type and $CRF_2$−/− mice. Significant Ps mean higher exploration of the unfamiliar mouse *vs*. the object or the novel *vs*. the familiar mouse during the sociability or the SNP test, respectively. ITI: inter-trial time interval between the sociability and the SNP test. N = 32–34/genotype.

**Table S2** Statistical analysis of sociability and social novelty preference (SNP) displayed by wild-type and $CRF_2$−/− mice immediately (0), 7 or 14 days after the last saline or cocaine administration. Significant Ps mean higher or lower (*) exploration of the unfamiliar mouse *vs*. the object or the novel *vs*. the familiar mouse during the sociability or the SNP test, respectively. N = 15–17/group (see Figure 3 and Figure 4 for detailed group size). SAL: saline. COC: cocaine.

**Table S3** Statistical analysis of sociability and social novelty preference (SNP) displayed by saline- or cocaine-treated wild-type and $CRF_2$−/− mice 40 or 42 days after the last administration. On cocaine withdrawal day 40 or 42, mice were exposed (Stress) or not (No stress) to the elevated platform stressor (EPS), using a within-subject experimental design. Significant Ps mean higher exploration of the unfamiliar mouse *vs*. the object or the novel *vs*. the familiar mouse during the sociability or the SNP test, respectively. N = 14–17/group (see Figure 5 for detailed group size). SAL: saline. COC: cocaine.

**Figure S1** $CRF_2$ receptor-deficiency does not alter sociability or social memory span. Time (s) spent exploring the wire cages containing an object (O) or an unfamiliar mouse (U) during the (A) sociability test or a familiar (F) or a novel (N) mouse during the (B) social novelty preference (SNP) test by wild-type and $CRF_2$−/− mice repeatedly tested (tests 1 to 3) once a week in the 3-chamber task. A 0, 2 or 4 h inter-trial time interval (ITI) was applied between the sociability and the SNP test. Value represent mean ± S.E.M. $^{***}$ $P < 0.0005$ *vs*. O or F, Student paired t-test.

**Figure S2** $CRF_2$ receptor-deficiency does not affect cocaine-induced body weight loss. Body weight change, calculated as percentage of body weight recorded just prior (P) to the first injection, displayed by saline (SAL)- or cocaine (COC)-treated wild-type and $CRF_2$−/− mice. Represented is the body

weight change as measured 12 h after the second injection of the cocaine dose indicated. Values represent mean ± S.E.M $^{###}P < 0.0005$ *vs*. SAL mice, genotype-independent effect.

**Figure S3** $CRF_2$ receptor-deficiency eliminates sociability deficits induced by cocaine withdrawal. Time (s) spent exploring the wire cages containing an object (O) or un unfamiliar mouse (U) during the (A) sociability test or a familiar (F) or a novel (N) mouse during the (B) social novelty preference (SNP) test by saline (SAL)- or cocaine (COC)-treated wild-type and $CRF_2-/-$ mice. The 3-chamber tests were carried out immediately (0), 7 and 14 days after the last saline or cocaine administration. N of each experimental group is reported under the X axis. Values represent mean ± S.E.M. $^{*}P < 0.05$ *vs*. O or F, Student paired t-test.

**Figure S4** $CRF_2$ receptor-deficiency abolishes the long-lasting social behavior vulnerability to stress induced by cocaine withdrawal. Time (s) spent exploring the wire cages containing an object (O) or un unfamiliar mouse (U) during the (A) sociability test or a familiar (F) or a novel (N) mouse during the (B) social novelty preference (SNP) test by wild-type or $CRF_2-/-$ mice exposed (Stress) or not (No Stress) to the elevated platform stressor (EPS). The 3-chamber tests were carried out 40 or 42 days after the last administration of saline (SAL) or cocaine (COC). Moreover, the EPS was applied on cocaine withdrawal day 40 or 42, using a within-subject experimental design. N of each experimental group is reported under the X axis. Values represent mean ± S.E.M. $^{*}P < 0.05$ *vs*. O or F, Student paired t-test.