eLife

Neuroscience

Disruption of the CRF_1 receptor eliminates morphine-induced sociability deficits and firing of oxytocinergic neurons in male mice

Reviewed Preprint
v2 • January 14, 2025
Revised by authors

Reviewed Preprint
v1 • September 25, 2024

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eLife Assessment

The revised report provides **valuable** findings for the field, suggesting a relationship between CRF1 receptors, sociability deficits in morphine-treated male mice yet not females, and a potential mechanism involving oxytocin neurons in the paraventricular nucleus of the hypothalamus. Generally, the strength of evidence is **solid** in terms of the methods, data, and analyses. This work will be of interest to those interested in social behavior and addiction.

https://doi.org/10.7554/eLife.100849.2.sa2

Abstract

Substance-induced social behavior deficits dramatically worsen the clinical outcome of substance use disorders; yet, the underlying mechanisms remain poorly understood. Herein, we investigated the role for the corticotropin-releasing factor receptor 1 (CRF_1) in the acute sociability deficits induced by morphine and the related activity of oxytocin (OXY)-and arginine-vasopressin (AVP)-expressing neurons of the paraventricular nucleus of the hypothalamus (PVN). For this purpose, we used both the CRF_1 receptor-preferring antagonist compound antalarmin and the genetic mouse model of CRF_1 receptor-deficiency. Antalarmin completely abolished sociability deficits induced by morphine in male, but not in female, C57BL/6J mice. Accordingly, genetic CRF_1 receptor-deficiency eliminated morphine-induced sociability deficits in male mice. *Ex vivo* electrophysiology studies showed that antalarmin also eliminated morphine-induced firing of PVN neurons in male, but not in female, C57BL/6J mice. Likewise, genetic CRF_1 receptor-deficiency reduced morphine-induced firing of PVN neurons in a CRF_1 gene expression-dependent manner. The electrophysiology results consistently mirrored the behavioral results, indicating a link between morphine-induced PVN activity and sociability deficits. Interestingly, in male mice antalarmin abolished morphine-induced firing in neurons co-expressing OXY and AVP, but not in neurons expressing only AVP. In contrast, in female mice antalarmin did not affect morphine-induced firing of neurons co-expressing OXY and AVP or only OXY, indicating a selective sex-specific

role for the CRF_1 receptor in opiate-induced PVN OXY activity. The present findings demonstrate a major, sex-linked, role for the CRF_1 receptor in sociability deficits and related brain alterations induced by morphine, suggesting new therapeutic strategy for opiate use disorders.

Introduction

Opiate substances often induce severe social behavior deficits, such as poor sociability, social isolation and elevated aggressiveness (Babor et al., 1976; Gerra et al., 2004). Notably, opiate-induced social behavior deficits dramatically contribute to addictive-like substance consumption, favoring the development and maintenance of opiate use disorders (OUD) (APA, 2013; Pomrenze et al., 2022). Thus, it has been hypothesized that treatments increasing positive peer relationships might considerably reduce substance seeking and taking and ameliorate the clinical outcome of substance-dependent patients (Heilig et al., 2016; Venniro et al., 2018). However, the development of novel, effective, therapy heavily relies on a better understanding of the brain mechanisms underlying the harmful effects of substances of abuse; yet, to date the neurobiological substrates of substance-induced social behavior deficits remain largely unknown.

The corticotropin-releasing factor (CRF) system is a main orchestrator of behavioral and neuroendocrine responses to stress (Dedic et al., 2018; Koob, 2008). The CRF system might also underlie the behavioral and brain effects of substances of abuse (Koob, 2008). CRF signaling is mediated by two types of receptors, named CRF_1 and CRF_2 (Hauger et al., 2003). Relatively recent studies shed some light on the role for each of the two known CRF receptor types in social behavior deficits induced by repeated administration of and withdrawal from substances of abuse. For instance, genetic inactivation of the CRF_2 receptor (CRF_2-/-) reduced sociability deficits and vulnerability to stress associated with long-term cocaine withdrawal in male mice (Morisot et al., 2018). Moreover, genetic inactivation of the CRF_1 receptor (CRF_1-/-) decreased morphine withdrawal-induced sociability deficits in female mice and hostility-driven interest for a same-sex conspecific in male mice (Piccin and Contarino, 2022). The CRF system may also interact with other brain systems implicated in social behavior. In particular, extensive literature points out to the two closely related neuropeptides oxytocin (OXY) and arginine-vasopressin (AVP) as main substrates of social interaction, parenting behavior and intermale aggressiveness (Jurek and Neumann, 2018). For instance, chemogenetic activation or inhibition of OXY-expressing neurons within the paraventricular nucleus of the hypothalamus (PVN) respectively increased or decreased social approach in male mice (Resendez et al., 2020). Accordingly, social stimuli increased the activity of PVN OXY-expressing neurons, as assessed by *in vivo* two-photon calcium imaging (Resendez et al., 2020). Moreover, coordinated responses of PVN parvocellular and magnocellular OXY-expressing neurons to somatosensory stimuli mediated social interaction in female rats (Tang et al., 2020). In contrast, male *Shank3b* knock-out mice, i.e., a mouse model of autistic-like behavior, showed a marked reduction in PVN OXY-expressing neurons and decreased social approach (Peça et al., 2011; Resendez et al., 2020). Furthermore, targeted chemogenetic silencing of PVN OXY neurons in male rats impaired short-and long-term social recognition memory (Thirtamara Rajamani et al., 2024). Likewise OXY and AVP, CRF is largely expressed in the PVN (Jiang et al., 2018; Sawchenko et al., 1993). Interestingly, whole-cell patch-clamp studies showed that CRF-and OXY-expressing neurons are highly intermingled within the PVN, suggesting local cell-to-cell interactions (Jamieson et al., 2017). CRF might also modulate hypothalamic OXY and AVP responses to substances of abuse. For instance, long-term cocaine-withdrawn male CRF_2-/- mice showed neither the stress-induced sociability deficits nor the related increased expression of OXY or AVP in the supraoptic nucleus of the hypothalamus (SON) or the PVN (Morisot et al., 2018). Thus, CRF, OXY and AVP systems may be potential targets of effective therapy for diseases characterized by dysfunctional social behavior, including substance use disorders. However, to date very little is known about their implication in social behavior deficits induced by substances of abuse.

Thus, herein we investigated the role for the CRF/CRF_1 receptor pathway in the acute social behavior deficits following opiate administration. In particular, using the three-chamber task for sociability in mice, the role for the CRF_1 receptor in sociability deficits induced by morphine was assessed by both pharmacological (i.e., the CRF_1 receptor-preferring antagonist antalarmin) and genetic (i.e., the CRF_1 receptor-deficient mouse model) approaches (Moy et al., 2004; Smith et al., 1998; Webster et al., 1996). Moreover, to understand CRF role in brain OXY and AVP responses to morphine, *ex vivo* electrophysiology studies assessed the effect of antalarmin and genetic CRF_1 receptor-deficiency upon morphine-induced firing of PVN OXY-and/or AVP-immunoreactive neurons. Notably, to fully adhere to the Sex as a Biological Variable (SABV) initiative and given the well-established influence of sex upon the addictive-like properties of substances of abuse, herein male and female mice were used throughout (Becker and Koob, 2016; Clayton, 2018).

Results

Pharmacological CRF_1 receptor antagonism eliminates morphine-induced sociability deficits in male, but not in female, mice

The acute effects of morphine upon social behavior were investigated using the three-chamber test for sociability in mice, as previously reported (Piccin et al., 2022). During the habituation phase of the test, male C57BL/6J mice spent similar time in the regions of interests (ROIs, side half-chambers) of the apparatus (**Fig. 1A-B** and Table S2). Analysis of the sociability phase revealed a *pretreatment* X *treatment* X *repeated measures* interaction effect (Table S2). Unlike saline-treated mice, vehicle/morphine-treated mice spent similar time in the ROIs containing the unfamiliar conspecific or the object (P=0.823), indicating sociability deficits (**Fig. 1C**). In contrast, antalarmin/morphine-treated mice spent more time with the unfamiliar conspecific than with the object (P<0.005), indicating unaltered sociability (**Fig. 1C**). Accordingly, analysis of sociability ratio revealed a *pretreatment* effect ($F_{1,32}$=11.598, P<0.005), a *treatment* effect ($F_{1,32}$=4.713, P<0.05) and a *pretreatment* X *treatment* interaction effect ($F_{1,32}$=8.718, P<0.01). Vehicle/morphine-treated mice showed lower sociability ratio than vehicle/saline-treated mice (P<0.005, **Fig. 1D**). In contrast, antalarmin/morphine-treated mice did not differ from saline-treated mice (P=0.661) and showed higher sociability ratio than vehicle/morphine-treated mice (P<0.005, **Fig. 1D**). During the habituation phase, morphine-treated female C57BL/6J mice spent less time in the ROIs of the apparatus than saline-treated mice (P<0.0005), independently of vehicle or antalarmin pretreatment (**Fig. 1E** and Table S2). Moreover, analysis of the sociability phase revealed a *treatment* X *repeated measures* interaction effect but no *pretreatment* X *treatment* X *repeated measures* interaction effect (Table S2). Indeed, unlike saline-treated mice, morphine-treated mice spent similar time in the ROIs containing the unfamiliar conspecific or the object (P=0.259), independently of vehicle or antalarmin pretreatment (**Fig. 1F**). Accordingly, analysis of sociability ratio revealed no *pretreatment* effect ($F_{1,21}$=0.035, P=0.852), a *treatment* effect ($F_{1,21}$=8.698, P<0.01) but no *pretreatment* X *treatment* interaction effect ($F_{1,21}$=0.018, P=0.894). Morphine-treated mice showed lower sociability ratio than saline-treated mice (P<0.05), independently of vehicle or antalarmin pretreatment (**Fig. 1G**). Sociability ratio was also examined by a three-way ANOVA with sex (males *vs.* females), pretreatment (vehicle *vs.* antalarmin) and treatment (saline *vs.* morphine) as between-subjects factors. The latter analysis revealed an almost significant *sex* X *pretreatment* X *treatment* interaction effect ($F_{1,53}$=3.287, P=0.075), which could not allow for post-hoc individual group comparisons. Nevertheless, post-hoc tests revealed that male mice treated with antalarmin/morphine showed higher sociability ratio than female mice treated with antalarmin/morphine (P<0.05). During the three-chamber test, morphine-treated male, but not female, mice travelled more distance than saline-treated mice (P<0.05), independently of vehicle or antalarmin pretreatment (Table S3 and Fig. S1A-B). Also, overall mice travelled more distance during the habituation than during the sociability phase

(P<0.05), indicating familiarization with the test apparatus (Table S3 and Fig. S1A-B). Thus, the present results indicate a sex-linked role for the CRF_1 receptor in social behavior deficits induced by morphine. Moreover, morphine effects upon sociability seemed unrelated to locomotor activity.

Pharmacological CRF_1 receptor antagonism eliminates morphine-induced firing of PVN neurons in male, but not in female, mice

To investigate the neural substrates of CRF_1 receptor-mediated sociability deficits induced by morphine, electrophysiology studies examined firing frequency of PVN neurons (**Fig. 2A**). In male C57BL/6J mice, analysis of firing frequency of all of the recorded cells (n=110) revealed a *pretreatment* effect ($F_{1,106}$=7.894, P<0.01), a *treatment* effect ($F_{1,106}$=13.350, P<0.0005) and a *pretreatment* X *treatment* interaction effect ($F_{1,106}$=4.208, P<0.05). Vehicle/morphine-treated mice showed higher firing frequency than vehicle/saline-treated mice (P<0.0005, **Fig. 2B**). In contrast, antalarmin/morphine-treated mice did not differ from saline-treated mice (P=0.552) and showed lower firing frequency than vehicle/morphine-treated mice (P<0.005, **Fig. 2B**). On the other hand, analysis of firing frequency of all of the recorded cells (n=93) in female C57BL/6J mice revealed no *pretreatment* effect ($F_{1,89}$=0.049, P=0.826), a *treatment* effect ($F_{1,89}$=20.476, P<0.0001) but no *pre-treatment* X *treatment* interaction effect ($F_{1,89}$=1.045, P=0.310). Morphine similarly increased firing frequency in vehicle-or antalarmin-pretreated mice, as compared to saline-treated mice (P<0.0005, **Fig. 2D**). Firing frequency of all of the recorded cells was also examined by a three-way ANOVA with sex (males *vs.* females), pretreatment (vehicle *vs.* antalarmin) and treatment (saline *vs.* morphine) as between-subjects factors. The latter analysis revealed a *sex* X *pretreatment* X *treatment* interaction effect ($F_{1,195}$=4.765, P<0.05). Post-hoc individual group comparisons revealed that male mice treated with vehicle/morphine showed higher firing frequency than all other male and female groups (P<0.0005). Moreover, male mice treated with antalarmin/morphine showed lower firing frequency than male mice treated with vehicle/morphine (P<0.0005). In contrast, female mice treated with antalarmin/morphine did not differ from female mice treated with vehicle/morphine (P=0.914). These results indicate a critical role for the CRF_1 receptor in PVN neuronal activity induced by morphine in male, but not in female, mice. Notably, the sex-dependent effects of antalarmin upon neuronal firing closely mimicked the social behavior results, indicating a link between PVN activity and sociability deficits induced by morphine.

Genetic inactivation of the CRF_1 receptor eliminates morphine-induced sociability deficits

The specific role for the CRF_1 receptor in morphine-induced sociability deficits was further investigated using the genetic mouse model of CRF_1 receptor-deficiency. We first tested n=3 CRF_1+/+ and n=3 CRF_1+/- male mice using the same morphine dose (2.5 mg/kg) employed in the C57BL/6J mice. However, during the whole 10-min habituation phase of the three-chamber test, all of the six morphine-treated animals remained in the central chamber of the apparatus, suggesting that the morphine dose used was relatively high. Thus, we decided to use a substantially lower morphine dose, i.e., 0.625 mg/kg (**Fig. 3A**). During the habituation phase, morphine (0.625 mg/kg) reduced the time spent in both ROIs of the three-chamber apparatus in CRF_1+/- (P<0.05 *vs.* saline-treated CRF_1+/- mice), but not in CRF_1+/+ or CRF_1-/-, male mice (**Fig. 3B** and Table S4). Analysis of the sociability phase revealed a *genotype* X *treatment* X *repeated measures* interaction effect (Table S4). Unlike saline-treated mice, morphine-treated CRF_1+/+ and CRF_1+/- mice spent similar time in the ROIs containing the unfamiliar conspecific or the object (P=0.873), indicating sociability deficits (**Fig. 3C**). In contrast, morphine-treated CRF_1-/- mice spent more time with the conspecific than with the object (P<0.005), indicating unaltered sociability (**Fig. 3C**). Accordingly, analysis of sociability ratio revealed no *genotype* effect ($F_{2,58}$=2.641, P=0.080), a *treatment* effect ($F_{1,58}$=7.478, P<0.01) and a *genotype* X *treatment* interaction effect ($F_{2,58}$=4.994, P<0.01). Morphine-

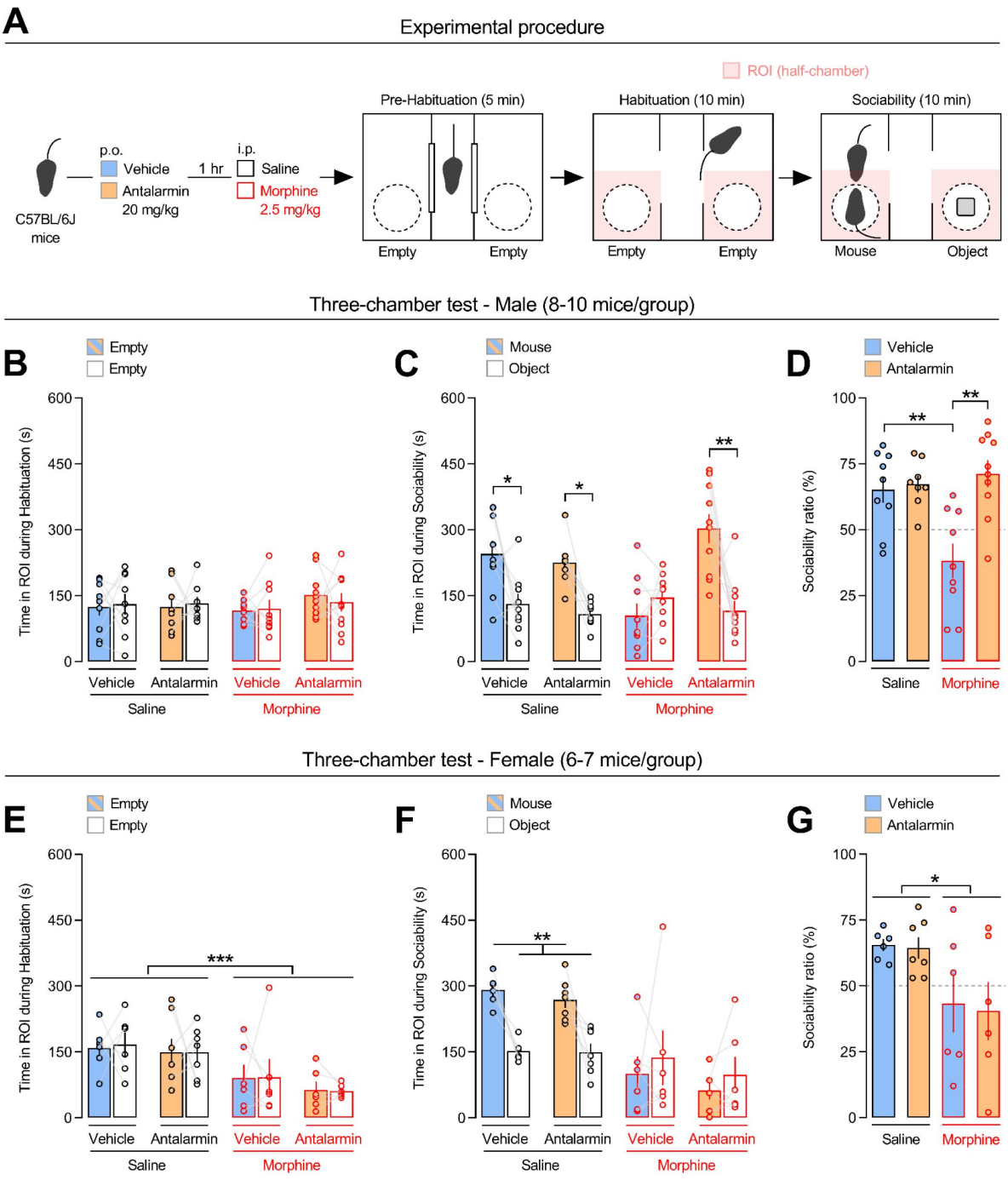

Fig. 1.

Pharmacological antagonism of the CRF_1 receptor eliminates morphine-induced sociability deficits in male, but not in female, mice.

(A) Experimental procedure. Male and female C57BL/6J mice were injected *per os* (p.o.) with either vehicle or the CRF_1 receptor-preferring antagonist antalarmin (20 mg/kg). One hour later, they were injected intraperitoneally (i.p.) with either saline or morphine (2.5 mg/kg) and tested in the three-chamber task for sociability. Time (s) spent in the regions of interest (ROIs, side half-chambers) of the three-chamber apparatus by male (**B** and **C**) and female (**E** and **F**) mice during the (**B** and **E**) habituation or the (**C** and **F**) sociability phase of the test. During the habituation phase, the ROIs contained empty wire cages; during the sociability phase, the wire cages contained an unfamiliar same-sex mouse or an object **(A)**. Sociability ratio (%) displayed by **(D)** male and **(G)** female mice. The number of animals within each experimental group is reported in Table S1A. Values represent mean±SEM. *P<0.05, **P<0.005, ***P<0.0005.

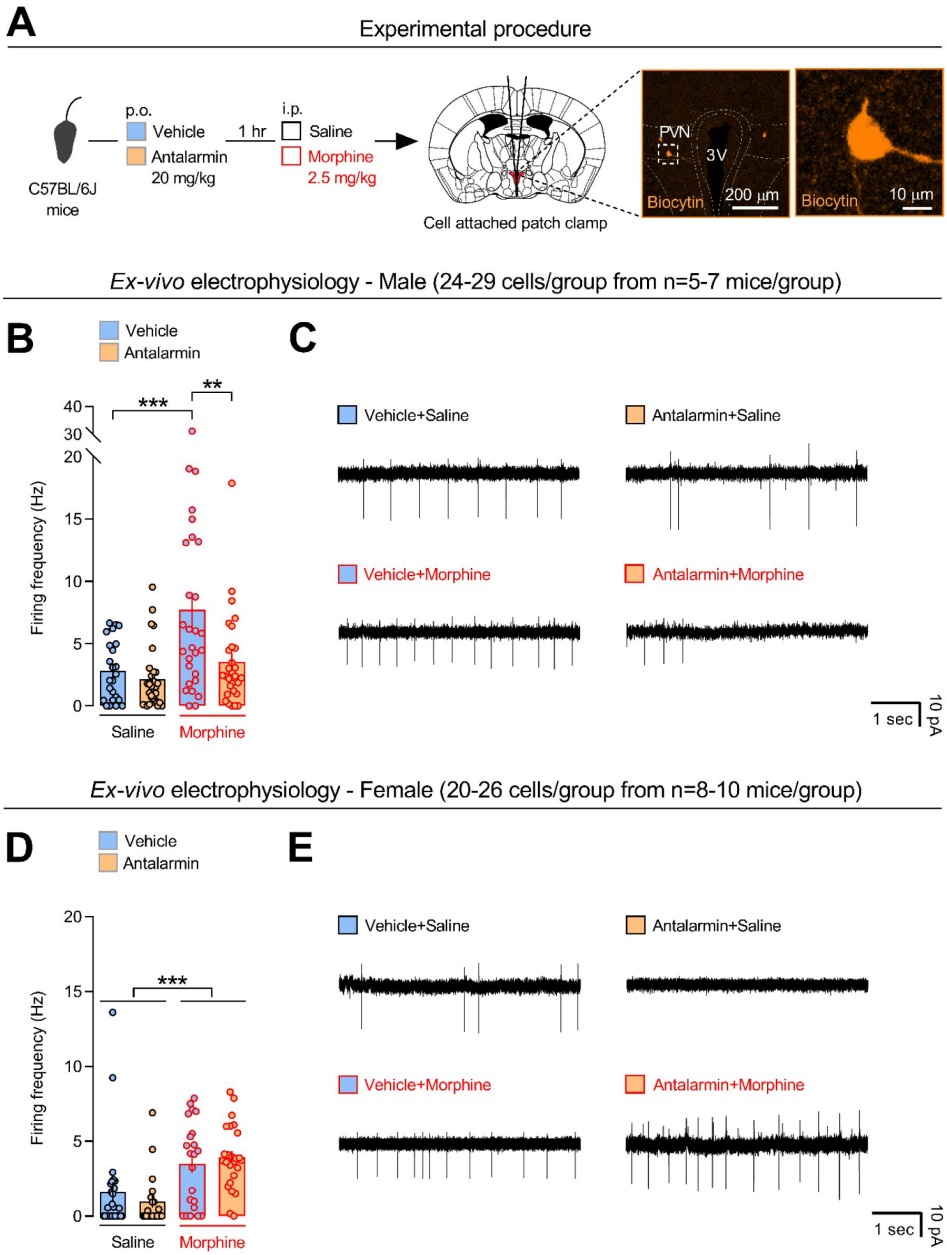

Fig. 2.

Pharmacological antagonism of the CRF_1 receptor eliminates neuronal firing induced by morphine in male, but not in female, mice.

(**A**) Experimental procedure. Male and female C57BL/6J mice were injected *per os* (p.o.) with either vehicle or the CRF_1 receptor-preferring antagonist antalarmin (20 mg/kg). One hour later, they were injected intraperitoneally (i.p.) with either saline or morphine (2.5 mg/kg). Ten minutes after, brains were removed and cell-attached patch-clamp recordings of paraventricular nucleus of the hypothalamus (PVN) neurons performed from brain slices. Scale bars: 200 and 10 µm. Firing frequency (Hz) of PVN neurons displayed by (**B**) male and (**D**) female mice treated with either vehicle or antalarmin followed by either saline or morphine. Images showing electrophysiological recordings from PVN neurons of the four (**C**) male and the four (**E**) female experimental groups. The number of *total* patched and recorded cells within each experimental group is reported in Table S1C. Values represent mean±SEM. **P<0.005, ***P<0.0005.

treated CRF_1+/+ mice showed lower sociability ratio than saline-treated CRF_1+/+ mice (P<0.05, **Fig. 3D**). In contrast, morphine-treated CRF_1-/- mice did not differ from saline-treated mice (P=0.819) and showed higher sociability ratio than morphine-treated CRF_1+/+ and CRF_1+/- mice (P<0.05, **Fig. 3D**). Interestingly, unlike CRF_1+/+ and CRF_1-/- mice, morphine-treated CRF_1+/- mice almost differed from saline-treated CRF_1+/- mice (P=0.065), suggesting a gene expression-dependent effect of CRF_1 receptor-deficiency (**Fig. 3D**). During the three-chamber test, overall morphine did not affect distance travelled (Table S4). Moreover, saline-treated mice and morphine-treated CRF_1+/+, but not CRF_1+/- or CRF_1-/-, mice travelled more distance during the habituation than during the sociability phase (P<0.05, Fig. S2 and Table S4), further indicating dissociation between the locomotor and the sociability effects of morphine. Thus, the similar results obtained with CRF_1-/- and antalarmin-treated C57BL/6J male mice strengthened the notion of a key role for the CRF_1 receptor in sociability deficits induced by morphine.

We then assessed the effect of morphine (0.625 mg/kg) in female CRF_1 receptor-deficient mice. However, as shown in Table S5, during the habituation phase of the three-chamber test only 2/8 CRF_1+/+ mice treated with saline and 2/8 CRF_1+/+ mice treated with morphine visited both side chambers of the apparatus. Also, despite all saline-treated CRF_1+/- mice (n=4) visited both side chambers of the apparatus, this occurred only in 3/8 CRF_1+/- mice treated with morphine. Thus, we could not obtain a reliable amount of data using a reasonable number of female mice, at least under our experimental conditions and with the 0.625 mg/kg morphine dose.

Genetic CRF_1 receptor-deficiency eliminates morphine-induced firing of PVN neurons

Analysis of firing frequency of PVN neurons in male CRF_1 receptor-deficient mice revealed a *genotype* effect ($F_{2,119}$=8.498, P<0.0005), a *treatment* effect ($F_{1,119}$=31.816, P<0.0001) and a *genotype* X *treatment* interaction effect ($F_{2,119}$=7.224, P<0.005). Morphine (0.625 mg/kg) increased firing frequency in CRF_1+/+ (P<0.0005) and in CRF_1+/- (P<0.005), but not in CRF_1-/- (P=0.987), mice, as compared to same-genotype saline-treated mice (**Fig. 3E**). Notably, morphine-treated CRF_1+/- mice showed lower or higher firing frequency than morphine-treated CRF_1+/+ (P<0.05) or CRF_1-/- (P<0.005) mice, respectively, indicating a CRF_1 gene expression-dependent effect (**Fig. 3E**). These results further support an essential role for the CRF_1 receptor in PVN neuronal firing induced by morphine. Moreover, the lack of morphine effects upon neuronal firing and sociability in CRF_1-/- mice indicates once more a link between PVN activity and social behavior.

Pharmacological CRF_1 receptor antagonism eliminates morphine-induced firing of PVN OXY-expressing neurons in male, but not in female, mice

In male C57BL/6J mice, 40 cells expressed both OXY and AVP, 49 cells expressed AVP but not OXY, 6 cells expressed OXY but not AVP and 15 cells expressed neither OXY nor AVP (**Fig. 4B**). Vehicle/morphine-treated mice showed higher firing frequency of OXY/AVP-expressing neurons than vehicle/saline-treated mice (P<0.0005; **Fig. 4C** and Table S6). In contrast, antalarmin/morphine-treated mice did not differ from saline-treated mice (P=0.782) and showed lower firing frequency than vehicle/morphine-treated mice (P<0.0005, **Fig. 4C**). On the other hand, morphine increased firing frequency of neurons expressing AVP, but not OXY, independently of vehicle or antalarmin pretreatment (P<0.05; **Fig. 4D** and Table S6). In female C57BL/6J mice, 31 cells expressed both OXY and AVP, 38 cells expressed OXY but not AVP, 7 cells expressed AVP but not OXY and 17 cells expressed neither OXY nor AVP (**Fig. 4E**). Morphine increased firing frequency of neurons co-expressing OXY and AVP, independently of vehicle or antalarmin pretreatment (P<0.005; **Fig. 4F** and Table S6). Similarly, morphine increased firing frequency of neurons expressing OXY, but not AVP, independently of vehicle or antalarmin

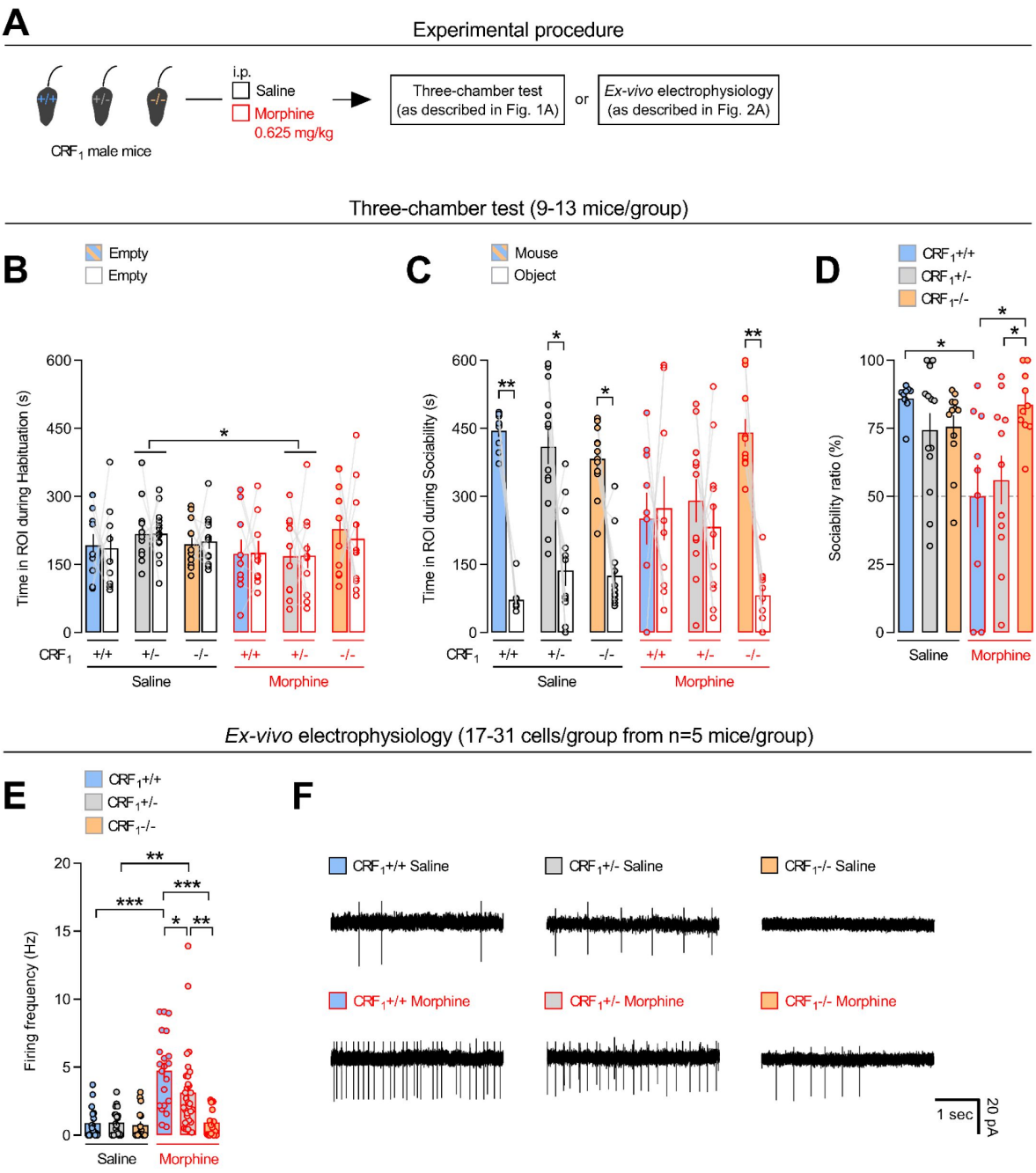

Fig. 3.

Genetic inactivation of the CRF_1 receptor eliminates morphine-induced sociability deficits and neuronal firing.

(**A**) Experimental procedure. Male CRF_1+/+, CRF_1+/- and CRF_1-/- mice were injected intraperitoneally (i.p.) with either saline or morphine (0.625 mg/kg) and tested in the three-chamber task for sociability. Additional groups of male CRF_1+/+, CRF_1+/- and CRF_1-/- mice were injected with either saline or morphine (0.625 mg/kg) and cell-attached patch-clamp recordings of paraventricular nucleus of the hypothalamus (PVN) neurons performed from brain slices. Time (s) spent in the regions of interest (ROIs, side half-chambers) of the three-chamber apparatus (see **Fig. 1A**) during the (**B**) habituation and the (**C**) sociability phase of the test, (**D**) sociability ratio (%) and (**E**) firing frequency (Hz) of PVN neurons by saline- or morphine-treated CRF_1+/+, CRF_1+/- and CRF_1-/- mice. (**F**) Images showing electrophysiological recordings from PVN neurons of the six experimental groups. The number of animals and the number of patched and recorded cells within each experimental group is reported in Table S1B. Values represent mean±SEM. *P<0.05, **P<0.005, ***P<0.0005.

pretreatment (P<0.005; **Fig. 4G** and Table S6). These results indicate a sex-specific role for the CRF_1 receptor in morphine-induced firing of PVN OXY-expressing neurons, suggesting that CRF modulates brain OXY responses to opiate substances.

Discussion

The present study demonstrates a major, sex-linked, role for the CRF_1 receptor in social behavior alterations induced by morphine. Indeed, male, but not female, mice treated with the CRF_1 receptor-preferring antagonist antalarmin did not show the sociability deficits induced by morphine. Accordingly, genetic inactivation of the CRF_1 receptor eliminated morphine-induced sociability deficits in male mice. Antalarmin also abolished morphine-induced firing of PVN neurons in male, but not in female, mice. Consistently, in male mice CRF_1 receptor-deficiency decreased morphine-induced firing of PVN neurons in a CRF_1 gene expression-dependent manner. Thus, the electrophysiology results reliably mirrored the behavioral results, suggesting a link between morphine-induced neuronal activity and sociability deficits. Furthermore, in male, but not in female, mice antalarmin eliminated morphine-induced firing in PVN neurons expressing OXY, suggesting sex-specific CRF-OXY interactions.

In agreement with our previous study (Piccin et al., 2022), morphine consistently and similarly decreased sociability in male and female mice. Prior work reported sex-linked behavioral effects of opiate substances. For instance, female rats displayed greater motivation to take heroin and self-administered greater amounts of heroin or oxycodone than male rats (Cicero et al., 2003; Fulenwider et al., 2020; George et al., 2021). Moreover, female mice showed elevated heroin self-administration and increased sensitivity to the rewarding properties of morphine, as compared to male mice (Piccin et al., 2022; Towers et al., 2019). Thus, unlike other behavioral effects of opiate substances, sex might have a marginal role in opiate-induced impairment of sociability. Nevertheless, herein CRF_1 receptor antagonism by antalarmin prevented morphine-induced sociability deficits in male, but not in female, mice. To date, very few studies have examined CRF role in social behavior effects of substances of abuse. For instance, genetic inactivation of the CRF_2 receptor reduced sociability deficits associated with long-term cocaine withdrawal in male mice (Morisot et al., 2018). Moreover, genetic CRF_1 receptor-deficiency decreased opiate withdrawal-induced sociability deficits in female mice, as assessed one week after cessation of repeated morphine administration (Piccin and Contarino, 2022). The latter findings contrast with the present lack of effect of antalarmin in female mice. However, although it might be difficult to compare pharmacological and genetic studies, the possibility exists for a differential implication of the CRF_1 receptor in social behavior deficits induced by a single opiate administration or by withdrawal from repeated opiate administration. Nonetheless, the present findings might bear importance for opiate-related diseases since a single morphine administration may induce long-lasting behavioral and brain alterations relevant to substance use disorders (Vanderschuren et al., 2001). Thus, identifying the neural substrates underlying initial opiate effects might be critical to advance our quest towards the development of effective treatments for substance use disorders.

Genetically engineered mouse models might provide a level of molecular specificity that is rarely achieved by pharmacological tools. Thus, to specifically assess the role for the CRF_1 receptor in morphine-induced sociability deficits, herein CRF_1 receptor-deficient mice were also used (Smith et al., 1998). However, following preliminary experiments showing that male CRF_1+/+ and CRF_1+/-mice treated with morphine (2.5 mg/kg) did not explore the three-chamber apparatus, a lower morphine dose was employed. Like in C57BL/6J mice, morphine (0.625 mg/kg) reliably impaired sociability in CRF_1+/+ and CRF+/- male mice, indicating that the two morphine doses used herein were suitable to compare the effect of pharmacological and genetic disruption of the CRF_1 receptor. Some differences were though observed between CRF_1 receptor-deficient and C57BL/6J mice treated with saline. In particular, though we did not perform direct statistical comparisons,

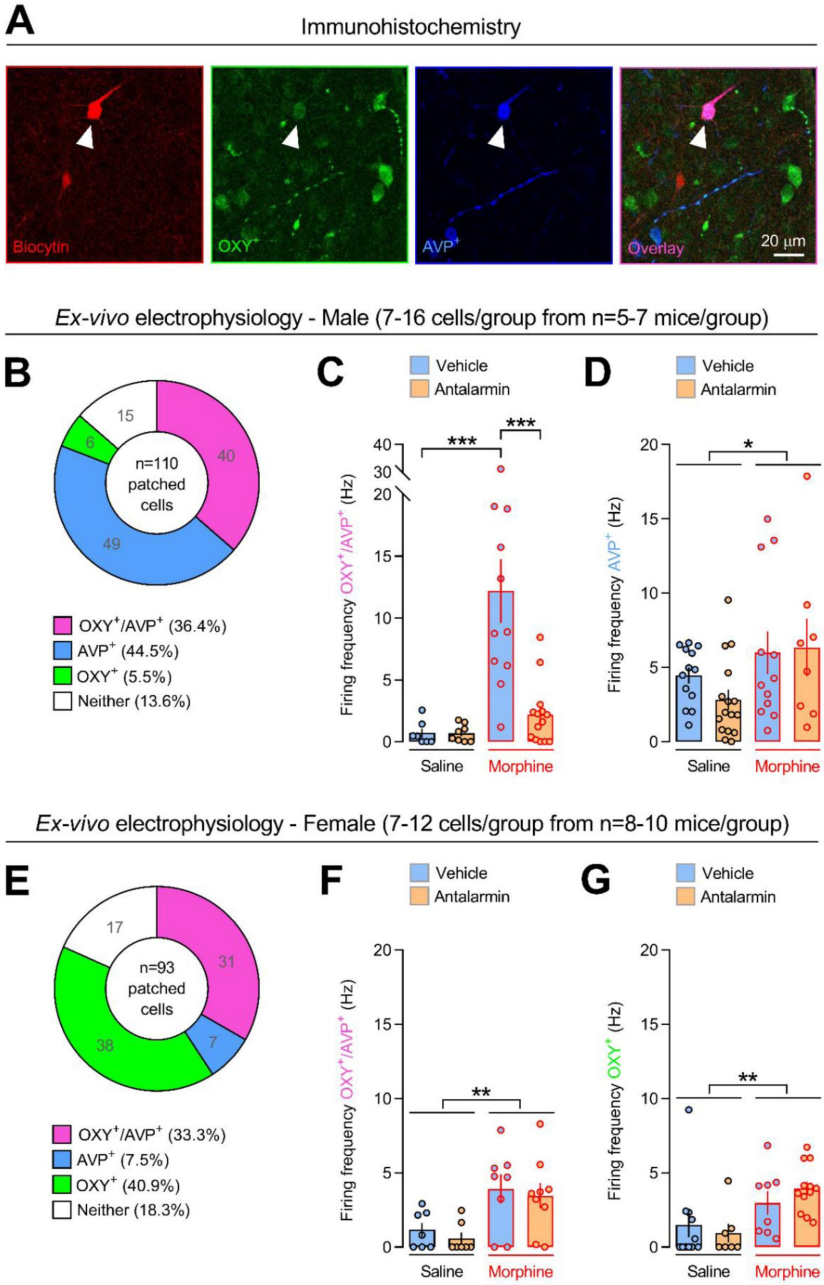

Fig. 4.

Pharmacological antagonism of the CRF_1 receptor eliminates morphine-induced firing of oxytocin-expressing neurons in male, but not in female, mice.

(**A**) Immunohistochemical images of a paraventricular nucleus of the hypothalamus (PVN) neuron co-expressing oxytocin (OXY^+) and arginine-vasopressin (AVP^+). Scale bar: 20 µm. Number of patched and recorded PVN neurons expressing OXY and/or AVP or neither of the two neuropeptides in (**B**) male and (**E**) female C57BL/6J mice. Firing frequency (Hz) of PVN neurons expressing OXY and/or AVP in (**C** and **D**) male and (**F** and **G**) female C57BL/6J mice treated with either vehicle or antalarmin (20 mg/kg) followed by either saline or morphine (2.5 mg/kg), as shown in **Fig. 2A**. The number of patched and recorded cells within each experimental group is reported in Table S1C. Values represent mean±SEM. *P<0.05, **P<0.005, ***P<0.0005.

percentage of time spent with the unfamiliar conspecific seemed higher in saline-treated CRF_1+/+, CRF_1+/- and CRF_1-/- male mice, as compared to saline-treated C57BL/6J male mice. It is difficult to understand the factors underlying the latter results. However, male mice bearing a mixed (B6x129PF2/J) genetic background also showed higher sociability levels than C57BL/6J male mice (Moy et al., 2004). Thus, it is possible that the mixed (C57BL/6Jx129S4/SvJae) genetic background of the CRF_1 receptor-deficient mice used herein contributed, at least in part, to increase social approach, as compared to inbred C57BL/6J mice.

Nevertheless, despite the latter differences, CRF_1 receptor-deficiency (CRF_1-/-) completely eliminated the sociability deficits induced by morphine in male mice, further supporting the notion of an essential role for the CRF_1 receptor in opiate-induced disruption of social behavior. Unlike CRF_1+/+ mice, CRF_1-/- mice showed sex-independent hypothalamus-pituitary-adrenal (HPA) axis deficits under basal and stressful conditions, as revealed by plasma adrenocorticotropic hormone (ACTH) and corticosterone assays (Papaleo et al., 2007; Smith et al., 1998; Timpl et al., 1998). Thus, it could be argued that the lack of morphine effects found in CRF_1-/- mice was due to HPA axis alterations. However, the present antalarmin results might, at least in part, rule out the latter hypothesis. Indeed, antalarmin is a non-peptide CRF_1 receptor-preferring antagonist that, upon systemic administration, readily crosses the blood-brain barrier and is behaviorally active (Zorrilla and Koob, 2010). Notably, antalarmin did affect neither basal nor stress-induced ACTH and corticosterone levels in male rats and mice (Jutkiewicz et al., 2005; Pérez-Tejada et al., 2013). Accordingly, the dose of antalarmin (20 mg/kg) used herein increased the somatic signs of morphine withdrawal without affecting plasma corticosterone (Papaleo et al., 2007). Thus, the present similar results obtained with antalarmin and CRF_1-/- mice argue in favor of a marginal role for the HPA axis in CRF_1 receptor-mediated sociability deficits induced by morphine.

Throughout the present studies, locomotor activity during the three-chamber test did not seem to account for the CRF_1 receptor-mediated sociability deficits induced by morphine. For instance, antalarmin-and vehicle-treated male C57BL/6J mice showed similar locomotor but different sociability responses to morphine (**Fig. 1D** and Fig. S1A). Moreover, morphine-treated CRF_1+/- and CRF_1-/- mice travelled similar distance but showed different social behavior (**Fig. 3D** and Fig. S2). Finally, overall mice travelled more distance during the habituation than during the sociability phase, an effect usually observed in the three-chamber test (Piccin and Contarino, 2020a, 2020b).

The PVN is a main source of brain CRF (Sawchenko et al., 1993). Moreover, CRF released within the PVN may act on intra-PVN CRF_1 receptor-expressing neurons (Jiang et al., 2019, 2018). Thus, to further explore the mechanisms of CRF_1 receptor-mediated sociability deficits, we examined neuronal responses to morphine in the PVN. We found that morphine consistently elevated the firing frequency of PVN neurons in male and female C57BL/6J mice, and in male CRF_1+/+ and CRF_1+/- mice. Acute morphine treatment increases CRF level in the hypothalamus and HPA axis activity (Buckingham, 1982; Ignar and Kuhn, 1990). Accordingly, stimulation of presynaptic mu-opioid receptors located on PVN GABA terminals might reduce GABA release and thus elevate PVN CRF activity (Wamsteeker Cusulin et al., 2013). Thus, although herein we did not examine CRF expression, it is likely that morphine increased the activity of PVN CRF neurons. We also show that CRF_1 receptor antagonism by antalarmin completely eliminated morphine-induced PVN neuronal firing in male, but not in female, mice. Likewise, in male mice genetic inactivation of the CRF_1 receptor decreased morphine-induced PVN neuronal firing in a CRF_1 gene expression-dependent manner. Sex-linked differences in brain distribution and activity of the CRF system might underlie the present findings. For instance, female rats displayed higher CRF expression in the PVN and in the central nucleus of the amygdala (CeA), as compared to male rats (Iwasaki-Sekino et al., 2009). However, using a CRF_1 reporter mouse line maintained on a C57BL/6 background, studies showed higher levels of the CRF_1 receptor in the PVN of adult (2 months) and old (20-24 months) male mice, as compared to adult and old female mice (Rosinger et al., 2019). Interestingly, adult gonadectomy (6 weeks) decreased PVN CRF_1 receptor-

immunoreactive cells in male, but not in female, mice, indicating a sex-linked modulation of CRF_1 receptor expression by gonadal hormones (Rosinger et al., 2019). Moreover, female rats showed higher CRF_1 receptor-GTP-binding protein (G_s) coupling and higher CRF_1 receptor cellular internalization than male rats in cortical and locus coeruleus (LC) tissues, respectively (Bangasser et al., 2010). Notably, swim stress and transgenic CRF overexpression increased or decreased LC CRF_1 receptor cellular internalization in male or female rats and mice, respectively (Bangasser et al., 2013, 2010). Thus, it is possible that sex-linked differences in PVN CRF_1 receptor expression, CRF_1 receptor intracellular signaling or cellular compartmentalization contributed to the sex-linked behavioral and brain effects of antalarmin reported herein. However, more studies are warranted to address the latter hypotheses.

The present immunohistochemistry studies showed that, in male and female C57BL/6J mice, approximately half of the patched PVN cells expressed both OXY and AVP. However, in male mice a relatively large portion of the stained cells expressed AVP, but not OXY. In net contrast, in female mice a large portion of the stained cells expressed OXY, but not AVP. The latter sex differences resonate with previous studies. Indeed, AVP-or OXY-positive neurons were shown to be more numerous in the PVN of male or female animals, respectively, in a variety of species, including humans (Dumais and Veenema, 2016). Interestingly, herein morphine disrupted sociability but increased the firing frequency of PVN neurons expressing OXY and/or AVP. At first glance, the present results might seem at odds with the alleged prosocial role for OXY systems. However, PVN OXY neurons extensively project to several brain areas where they may differentially modulate social behavior in a brain site-specific manner (Jurek and Neumann, 2018). For instance, genetically-driven activation or inhibition of PVN OXY neurons projecting to the ventral tegmental area (VTA) respectively increased or decreased social interaction in male mice (Hung et al., 2017). In contrast, OXY infusion into the bed nucleus of the stria terminalis (BNST) dose-dependently decreased social approach in both male and female California mice (Duque-Wilckens et al., 2020). Moreover, both pharmacological antagonism of OXY receptors and genetic inhibition of OXY synthesis within the BNST attenuated social defeat stress-induced reduction of social interaction in female California mice, further indicating a negative modulation of social behavior by BNST OXY activity (Duque-Wilckens et al., 2020, 2018). CRF_1 receptor mRNA and OXY mRNA were shown to co-localize in PVN neurons in male rats (Arima and Aguilera, 2000). Also, PVN CRF_1 receptor-expressing neurons are thought to make bidirectional connections with PVN OXY-expressing neurons, suggesting intra-PVN CRF-OXY interactions relevant to stress responses and social behavior (Jiang et al., 2019). Thus, activation of PVN CRF_1 receptors by morphine-induced CRF release might modulate the activity of PVN OXY neurons projecting to the VTA or the BNST and related social behavior. On the other hand, since CRF_1 receptors are highly expressed in the VTA, dopamine activity might also be directly modulated by intra-VTA CRF/CRF_1 receptor pathways (Chen et al., 2014). Accordingly, intra-VTA administration of CRF receptor antagonists attenuated social defeat stress-induced dopamine release in the nucleus accumbens shell (Boyson et al., 2014). However, more studies are needed to determine the role for brain site-specific CRF/CRF_1 receptor pathways in OXY and dopamine activity underlying the social behavior effects of substances of abuse.

Stressful events strongly activate brain OXY systems (Jurek and Neumann, 2018). For instance, male rats exposed to the forced swim or the tail suspension stressor showed increased OXY peptide levels in several brain areas, including the PVN and the SON (Yan et al., 2014). Notably, intracerebroventricular injection of an OXY receptor antagonist dose-dependently increased stress-induced immobility, suggesting that OXY activity served to cope with stress (Yan et al., 2014). Acute morphine administration may elicit a stress-like state, as revealed by elevated CRF mRNA in the CeA and HPA axis activity in male rats (Ignar and Kuhn, 1990; Maj et al., 2003). Within this framework, the present results of morphine-induced firing of PVN OXY-positive neurons suggest the presence of a stress-like state, which may disrupt social behavior. Thus, morphine may activate brain CRF systems which, via CRF_1 receptors, may increase the activity of PVN OXY neurons in order to counteract stress effects. In contrast, antalarmin completely

eliminated morphine-induced firing of PVN OXY-expressing neurons and sociability deficits in male mice. This suggests that pharmacological disruption of the stress-responsive CRF_1 receptor confers stress resilience, which *per se* does not require PVN OXY activity to cope with a stress-like state, leaving unaltered the expression of social behavior. On the other hand, antalarmin did not affect the activity of neurons expressing AVP, but not OXY, in male mice. Accordingly, prior work indicated a minor role for AVP in stress resilience and sociability, as compared to OXY (Lukas et al., 2011; Neumann and Landgraf, 2012). However, PVN-targeted genetic or pharmacological studies are needed to determine the role for PVN CRF_1 receptors in opiate-induced sociability deficits and related PVN OXY activity. Heroin self-administration may up-regulate CRF_1 receptor mRNA level in VTA dopamine neurons in male rats (Galaj et al., 2023). Nevertheless, to our knowledge, to date no studies have investigated the effect of acute morphine administration upon PVN CRF_1 receptor expression. Thus, together with PVN-targeted genetic or pharmacological manipulation of CRF_1 receptor activity, assessing CRF_1 receptor expression in the PVN might help to understand the brain substrates of opiate-induced disruption of social behavior. Finally, in female mice antalarmin affected neither morphine-induced sociability deficits nor firing of OXY/AVP-or OXY-expressing neurons, revealing a sex-specific role for the CRF_1 receptor in opiate-induced activity of brain OXY systems and related social behavior.

In summary, herein we provide initial evidence of a major, sex-linked, role for the CRF_1 receptor in social behavior and brain alterations induced by morphine. Indeed, disruption of CRF_1 receptor function consistently eliminated morphine-induced sociability deficits and PVN neuronal firing in male, but not in female, mice. These findings suggest that inhibition of CRF_1 receptor activity may relieve severe social behavior deficits commonly observed in OUD patients. Moreover, the present results point out to sex as a critical biological variable of studies assessing novel treatments for substance use disorders.

Materials and methods

Animals

Male and female C57BL/6J mice were bred in-house and derived from mice originally purchased from Janvier Labs (Le Genest-Saint-Isle, France). Male and female CRF_1+/+, CRF_1+/- and CRF_1-/- mice previously generated on a mixed C57BL/6Jx129 background were bred in-house from mating CRF_1+/- mice and genotype identified by PCR analysis of tail DNA (Smith et al., 1998). The colony room (22±2°C, relative humidity: 50–60%) was maintained on a 12 h light/dark cycle (lights on at 08:00). Mice were housed in groups of 2-4 in transparent polycarbonate cages (29.5 x 11.5 x 13 cm, L x W x H) containing bedding and a cotton nestlet (SAFE, Augy, France) and were 12-28 weeks old at testing. Standard laboratory food (3.3 kcal/g; SAFE, Augy, France) and water were available *ad libitum*. All studies were conducted in accordance with the European Communities Council Directive 2010/63/EU, were approved by the local Animal Care and Use Committee and complied with the ARRIVE Guidelines (Kilkenny et al., 2010).

Three-chamber sociability task

The three-chamber task allowed the study of the preference for an unfamiliar same-sex conspecific *vs*. an object and was carried out as previously reported (Piccin et al., 2022). The three-chamber apparatus was a rectangular box (60 x 40 x 20 cm, L x W x H) divided in three equal chambers and made of dark grey polypropylene. Dividing transparent Plexiglas walls had small squared doors (8 x 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, 14 cm high, with bars spaced 1 cm apart) placed in one half-portion of the chamber. The three-chamber test was conducted during the light phase of the 12 h light/dark cycle and light intensity in the apparatus was ~10 lux. The subject mice were handled 1 min/day during the three days preceding the three-chamber experiment. On the fourth day, C57BL/6J mice were treated with either vehicle or

antalarmin (20 mg/kg) and returned to their home-cage. One hour later, they were treated with either saline or morphine (2.5 mg/kg) and immediately tested in the three-chamber task (**Fig. 1A**). We did not monitor the estrous cycle in female mice. The normal estrous cycle of laboratory mice is 4-5 days in length, and it is divided into four phases (proestrus, estrus, metestrus and diestrus). The three-chamber experiments were generally carried out over a 5-day period, thus spanning across the entire estrous cycle. In particular, on each test day approximately the same number of mice was assigned to each experimental group. Thus, within each group the number of female mice tested on each phase of the estrous cycle was likely similar. Moreover, studies indicated no significant difference over different phases of the estrous cycle in social interaction, anxiety-like and anhedonia-like behavioral tests in C57BL/6J female mice (Zeng et al., 2023; Zhao et al., 2021). CRF_1+/+, CRF_1+/- and CRF_1-/- mice were treated with either saline or morphine (0.625 mg/kg) and immediately tested in the three-chamber task (**Fig. 3A**). The three-chamber test consisted of three phases: pre-habituation, habituation and sociability. During the pre-habituation phase, the subject mouse was confined to the central chamber for 5 min. Then, the doors were opened and the mouse could freely explore the three chambers and the empty wire cages for 10 min (habituation phase). During the subsequent 10 min, the subject mouse could freely explore the entire apparatus with one wire cage containing an unfamiliar same-sex mouse and the other an object, i.e., a plastic bottle cap (sociability phase). The unfamiliar mice were C57BL/6J mice sex-and age-matched with the subject mice. During the three days preceding testing, the unfamiliar mice were handled 1 min/day and habituated to the wire cages for 10 min/day, with the wire cage habituation taking place in the three-chamber apparatus on the second and third day. The position (left or right side chamber) of the unfamiliar mouse was counterbalanced within each experimental group. Between each tested mouse, the apparatus was cleaned with water and the wire cages with 70% ethanol and then water. Videos were acquired and analyzed with a home-made tracking system. In particular, time (s) spent by the tested mouse in the regions of interest (ROIs, side half-chambers) containing the wire cages was taken as a measure of sociability (**Fig. 1A**). Indeed, our prior studies reliably demonstrated that the latter measure positively correlated with the number of nose-to-nose contacts with the unfamiliar mouse (Piccin and Contarino, 2020b). Moreover, ratio of time spent in the ROI containing the unfamiliar mouse positively correlated with the ratio of time spent with the nose in the wire cage containing the unfamiliar mouse (Piccin and Contarino, 2020b). Furthermore, to control for locomotor activity, distance (m) travelled throughout the whole apparatus during the habituation and the sociability phases of the test was examined. Sociability ratio was calculated as percentage of time spent in the ROI containing the unfamiliar mouse over the total time spent in both ROIs containing the wire cages.

Brain slice preparation

C57BL/6J mice were injected with either vehicle or antalarmin (20 mg/kg) and, one hour later, with either saline or morphine (2.5 mg/kg). CRF_1 receptor-deficient mice were just injected with either saline or morphine (0.625 mg/kg). Ten minutes after saline or morphine administration, mice were anesthetized by intraperitoneal (i.p.) injection of ketamine (100 mg/kg) / xylazine (10 mg/kg) until reflexes to tail-or toe-pinching were lost. Before brain removal, animals were intracardially perfused with an ice-cold bubbled (95% O_2 / 5% CO_2) sucrose-based saline solution containing (in mM): NaH_2PO_4 1.25, KCl 2.5, $CaCl_2$ 0.5, $MgSO_4$ 10, D-glucose 10, $NaHCPO_3$ 26. Brains were rapidly removed and 300 μm coronal slices containing the PVN were cut using a vibroslicer (Leica VT100S, Leica Biosystems, Germany). Slices were then allowed to recover for at least 1 h at 30°C in a holding chamber filled with oxygenated (95 % O_2 / 5 % CO_2) artificial cerebrospinal fluid (aCSF) composed of (in mM): NaCl 126, KCl 2.5, $CaCl_2$ 2, $MgSO_4$ 2, NaH_2PO_4 1.25, $NaHCO_3$ 26, glucose 10 (pH 7.3, 290 mOsm).

Electrophysiology studies

Cell-attached patch-clamp recordings from PVN neurons were made at room temperature in voltage-clamp conditions under continuous perfusion of oxygenated aCSF composed of (in mM): NaCl 126, KCl 3, $CaCl_2$ 1.6, $MgSO_4$ 1.5, NaH_2PO_4 1.25, $NaHCO_3$ 26, glucose 10. Throughout recordings, GABAergic and glutamatergic inputs were blocked with gabazine (1 µM) and 10µM of the NMDA and non-NMDA receptor antagonists D(-)-2-amino5-phosphonopentanoic acid (AP5) and 6,7-dinitroquinoxaline-2,3(1H,4H) dione (DNQX). Neurons were visualized with an upright Nikon Eclipse FN1 microscope (Nikon, Japan) with infrared illumination. Recording borosilicate electrodes were filled with an internal solution containing K-Gluconate 120 mM, KCl 20 mM, $MgCl_2$ 1.3 mM, EGTA 1 mM, HEPES 10 mM, $CaCl_2$ 0.1 mM, GTP 0.03 mM, cAMP 0.1 mM, leupeptine 0.01 mM, D-Mannitol 77 mM and Na 2 ATP 3 mM (pH 7.3). Moreover, biocytin 0.1% was added to the internal solution in order to post-visualize recorded neurons. Data were collected online with a Multiclamp 700B amplifier (Molecular Devices, USA) and acquired with Axograph X software (Axograph, Australia). Electrophysiology recordings were analyzed offline using the Axograph X software.

Immunohistochemistry and imaging

The phenotype of the patched and recorded cells was assessed by immunohistochemistry. After electrophysiological recording, slices were fixed with 4% paraformaldehyde overnight at 4°C. Biocytin was then revealed with FITC-Streptavidin (1/300, Vector Laboratories). OXY and AVP immunohistochemical labeling was performed at the same time using as first antibodies mouse anti-OXY monoclonal antibody (1/1000, Millipore MAB5296) and T-5048 Guinea pig anti (Arg^8)-vasopressin antibody (1/1000, BMA biomedicals). Alexa fluor 488 goat anti-mouse IgG (1/500, Life technology) and Alexa fluor 647 donkey anti-guinea pig IgG (1/500, Life technology) were used as secondary antibodies. Immunostainings were acquired using a confocal Zeiss LSM900 microscope. Serial optical sections were obtained at a Z-step of 1.2 µm and imaged using an objective 10X or 20X /1.00 numerical aperture.

Drugs

Antalarmin hydrochloride (20 mg/kg; TOCRIS, Lille, France) was dissolved in acidified saline (pH ~2.5) and injected *per os* (p.o.) by gavage. Morphine hydrochloride (0.625 or 2.5 mg/kg; Francopia, Gentilly, France) was dissolved in physiological saline and injected i.p. Control mice were injected p.o. or i.p. with the appropriate vehicle (acidified or physiological saline) and volume of administration was always 10 ml/kg. The morphine dose was chosen based on our prior studies showing that morphine (2.5 mg/kg, i.p.) impaired sociability in male and female C57BL/6J mice, without affecting locomotor activity (Piccin et al., 2022). Also, the antalarmin dose and route of administration were chosen based on our prior reports of behavioral effects of antalarmin (20 mg/kg) administered p.o. (Contarino et al., 2017; Ingallinesi et al., 2012; Piccin and Contarino, 2020b). Notably, the oral route of administration for antalarmin was chosen for its translational relevance, as it could be easily employed in clinical trials assessing the therapeutic value of pharmacological CRF_1 receptor antagonists.

Statistical analysis

Each mouse was assigned a unique identification number that was used to conduct blind testing and data analysis. To prevent strong initial preferences from biasing the three-chamber sociability results, animals exploring each ROI containing the wire cage for more than 80% (or less than 20%) of the total time spent in both ROIs during the habituation phase (10 min) were excluded from data analysis. The number of animals excluded within each experimental group is reported in Table S1A-B. For simplification and illustration purposes, data obtained in male and female mice were reported on separate figures. Thus, within each sex, the three-way repeated measures analysis of variance (ANOVA) with pretreatment (vehicle *vs.* antalarmin) and treatment (saline *vs.*

morphine) as between-subjects factors and side (mouse *vs.* object) or test phase (habituation *vs.* sociability) as a within-subject factor was used to analyze time spent in the ROIs or distance travelled during the three-chamber test by C57BL/6J mice. A three-way repeated measures ANOVA with genotype (CRF_1+/+ *vs.* CRF_1+/- *vs.* CRF_1-/-) and treatment (saline *vs.* morphine) as between-subjects factors and side (mouse *vs.* object) or test phase (habituation *vs.* sociability) as a within-subject factor was used to analyze time spent in the ROIs or distance travelled during the three-chamber test by CRF_1 receptor-deficient mice. The two-way ANOVA with pretreatment (vehicle *vs.* antalarmin) or genotype (CRF_1+/+ *vs.* CRF_1+/- *vs.* CRF_1-/-) and treatment (saline *vs.* morphine) as between-subjects factors was used to analyze sociability ratio and the firing frequency (Hz) results of the electrophysiology studies. Sociability ratio and firing frequency displayed by C57BL/6J mice were also examined by a three-way ANOVA with sex (males *vs.* females), pretreatment (vehicle *vs.* antalarmin) and treatment (saline *vs.* morphine) as between-subjects factors. The accepted value for significance was $P<0.05$. Following significant interaction effects, the Newman-Keuls post-hoc test was used for individual group comparisons. Statistical analyses were performed using the Statistica software (Version 10). Data graphs were created using GraphPad Prism and Adobe Illustrator.

Acknowledgements

The authors would like to thank Dr Philippe Ciofi (INSERM U1215) for the precious help with the oxytocin and vasopressin studies.

Additional information

Funding sources

This study was supported by the *Fondation pour la Recherche Médicale* (Grant No. DPA20140629794 to AC and JB), the *Agence Nationale de la Recherche* (Grant No. ANR-21-CE37-0019-01 to AC), the University of Bordeaux and the *Centre National de la Recherche Scientifique* (CNRS), France. Funding sources had no further role in study design, in the collection, analysis and interpretation of data, in the writing of the report and in the decision to submit the paper for publication.

Author contributions

AP and AC designed research. AP, AEA and JB performed research. AP, AEA, SSB and AC analyzed data. AC wrote and edited the paper with input from all authors.

Competing interests

The authors declare no competing interest.

Additional files

Supplemental Figures

Supplemental Tables

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Reviewer #1 (Public review):

Summary:

The use of antalarmin, a selective CRF1 receptor antagonist, prevents the deficits in sociability in (acutely) morphine-treated males, but not in females. In addition, cell attached experiments show a rescue to control levels of the morphine-induced increased firing in PVN neurons from morphine-treated males. Similar results are obtained in CRF receptor 1-/- male mice, confirming the involvement of CRF receptor 1-mediated signaling in both sociability deficits and neuronal firing changes in morphine-treated male mice.

Strengths:

In the revised version of the paper the authors respond to some reviewers's points with a new statistical analysis of behavioral data and a new discussion of previous literature.

Weaknesses:

Following reviewers' comments, the authors provided mechanistic insights of their findings with new experiments.

https://doi.org/10.7554/eLife.100849.2.sa1

Author response:

The following is the authors' response to the original reviews.

> ***Public Reviews:***
>
> ***Reviewer #1 (Public review):***
>
> *Summary:*
>
> *The use of antalarmin, a selective CRF1 receptor antagonist, prevents the deficits in sociability in (acutely) morphine-treated males, but not in females. In addition, cell-attached experiments show a rescue to control levels of the morphine-induced increased firing in PVN neurons from morphine-treated males. Similar results are obtained in CRF receptor 1-/- male mice, confirming the involvement of CRF receptor 1-mediated signaling in both sociability deficits and neuronal firing changes in morphine-treated male mice.*
>
> *Strengths:*
>
> *The experiments and analyses appear to be performed to a high standard, and the manuscript is well written and the data clearly presented. The main finding, that CRF-receptor plays a role in sociability deficits occurring after acute morphine administration, is an important contribution to the field.*
>
> *Weaknesses:*
>
> *The link between the effect of pharmacological and genetic modulation of CRF 1 receptor on sociability and on PVN neuronal firing, is less well supported by the data presented. No evidence of causality is provided.*
>
> *Major points:*
>
> *(1) The results of behavioral tests and the neural substrate are purely correlative. To find causality would be important to selectively delete or re-express CRF1 receptor sequence in the VPN. Re-expressing the CRF1 receptor in the VPN of male mice and testing them for social behavior and for neuronal firing would be the easier step in this direction.*

We agree with this comment and have acknowledged that further studies, such as genetic or pharmacological inactivation of CRF_1 receptors selectively in the paraventricular nucleus of the hypothalamus (PVN), are warranted to address this issue (page 17, line 25 to page 18, line 1).

We would also like to mention that our manuscript title intentionally presented our findings separately without implying causality. Our idea was simply to pair the behavioral data to neural activity within a network of interest, i.e., the PVN CRF-oxytocin (OXY)/arginine-vasopressin (AVP) network, which is thought to play a critical role at the interface of substance use disorders and social behavior. Accordingly, we previously reported that genetic CRF_2 receptor deficiency reliably eliminated sociability deficits and hypothalamic OXY and AVP expression induced by cocaine withdrawal (Morisot et al., 2018). Thus, the present manuscript reliably shows that CRF_1 receptor-mediated effects of acute morphine administration upon social behavior are consistently mirrored by neural activity changes within the PVN, and particularly within its OXY^+/AVP^+ neuronal populations. In addition, we demonstrate that the latter effects are sex-linked, which is in line with previous reports of

sex-biased CRF_1 receptor roles in rodents (Rosinger et al., 2019; Valentino et al., 2013) and humans (Roy et al., 2018; Weber et al., 2016).

> *(2) It would be interesting to discuss the relationship between morphine dose and CRF1 receptor expression.*

We are not aware of studies reporting CRF_1 receptor expression following acute morphine administration. However, repeated heroin self-administration was shown to increase CRF_1 receptor expression in the ventral tegmental area (VTA). We have mentioned the latter study in the present revised version of our manuscript at page 18, lines 1-2.

> *(3) It would be important to show the expression levels of CRF1 receptors in PVN neurons in controls and morphine-treated mice, both males and females.*

We agree with this reviewer comment and, in the present version of the manuscript, have mentioned that examination of CRF_1 receptor expression in the PVN might help to understand the brain mechanisms underlying morphine effects upon social behavior (page 18, lines 2-6). Moreover, at page 15, lines 11-19 we have mentioned studies showing higher levels of the CRF_1 receptor in the PVN of adult (2 months) and old (20-24 months) male mice, as compared to adult and old female mice (Rosinger et al., 2019). Thus, differences in PVN CRF_1 receptor expression between male and female mice might underlie the sex-linked effects of CRF_1 receptor antagonism by antalarmin reported in our manuscript.

> *(4) It would be important to discuss the mechanisms by which CRF1 receptor controls the firing frequency of APV+/OXY+ neurons in the VPN of male mice.*

Using the *in situ* hybridization technique, studies reported relatively low expression of the CRF_1 receptor in the PVN (Van Pett et al., 2000). However, more recent studies using genetic approaches identified a substantial population of CRF_1 receptor-expressing neurons within the PVN (Jiang et al., 2019, 2018). These CRF_1 receptor-expressing neurons are believed to respond to local CRF release and likely form bidirectional connections with both CRF and OXY+/AVP+ neurons (Jiang et al., 2019, 2018). Thus, one proposed mechanism of action is that morphine increases intra-PVN release of CRF, which may act on intra-PVN CRF_1 receptor-expressing neurons. The latter neurons might in turn influence the activity of PVN OXY+/AVP+ neurons, which largely project to the VTA and the bed nucleus of the stria terminalis (BNST) to modulate social behavior. Within this framework, pharmacological or genetic inactivation of CRF_1 receptors might deregulate the activity of intra-PVN CRF-OXY/AVP interactions and thus interfere with opiate-induced social behavior deficits. In particular, the latter phenomenon might be more pronounced in male mice since they express more CRF_1 receptor-positive neurons in the PVN, as compared to female mice (Rosinger et al., 2019). The putative mechanisms of action described herein are also mentioned at page 16, lines 12 to page 17, line 7 of the present revised version of the manuscript.

> *Minor points:*
>
> *(1) The phase of the estrous cycles in which females are analyzed for both behavior and electrophysiology should be stated.*

The normal estrous cycle of laboratory mice is 4-5 days in length, and it is divided into four phases (proestrus, estrus, metestrus and diestrus). The three-chamber experiments were generally carried out over a 5-day period, thus spanning across the entire estrous cycle. In particular, on each test day approximately the same number of mice was assigned to each experimental group. Thus, within each group the number of female mice tested on each

phase of the estrous cycle was likely similar. Moreover, except for firing frequency displayed by vehicle/morphine-treated mice, female and male mice showed similar results *variability*, indicating a marginal role for the estrous cycle in the spread of data. We would also like to mention relatively recent studies indicating no significant difference over different phases of the estrous cycle in the social interaction test as well as in anxiety-like and anhedonia-like behavioral tests in C57BL/6J female mice (Zhao et al., 2021). Accordingly, similar findings were also reported by other authors who found no difference across the diestrus and estrus phases of the estrous cycle in C57BL/6J female mice tested in behavioral assays of anxiety-like, depression-like and social interaction (Zeng et al., 2023).

A paragraph has been added to page 20, lines 1-9 of the present version of the manuscript to explain why we did not monitor the estrous cycle in female mice.

> *(2) It would be important to show the statistical analysis between sexes.*

Following this reviewer comment, we examined the sociability ratio results by a three-way ANOVA with sex (males *vs.* females), pretreatment (vehicle *vs.* antalarmin) and treatment (saline *vs.* morphine) as between-subjects factors. The latter analysis revealed an almost significant sex X pretreatment X treatment interaction effect ($F_{1,53}$=3.287, P=0.075), which could not allow for post-hoc individual group comparisons. Nevertheless, Newman-Keuls post-hoc comparisons revealed that male mice treated with antalarmin/morphine showed higher sociability ratio than female mice treated with antalarmin/morphine (P<0.05). The latter statistical results have been added to the present revised version of the manuscript at page 7, lines 2-8.

We also examined neuronal firing frequency by a three-way ANOVA with sex (males *vs.* females), pretreatment (vehicle *vs.* antalarmin) and treatment (saline *vs.* morphine) as between-subjects factors. Analysis of firing frequency of all of the recorded cells in C57BL/6J mice revealed a sex X pretreatment X treatment interaction effect ($F_{1,195}$=4.765, P<0.05). Newman-Keuls post-hoc individual group comparisons revealed that male mice treated with vehicle/morphine showed higher firing frequency than all other male and female groups (P<0.0005). Moreover, male mice treated with antalarmin/morphine showed lower firing frequency than male mice treated with vehicle/morphine (P<0.0005). In net contrast, female mice treated with antalarmin/morphine did not differ from female mice treated with vehicle/morphine (P=0.914). The latter statistical results have been added to the present revised version of the manuscript at page 8, lines 4-12. Finally, similar results were obtained following the three-way ANOVA (sex X pretreatment X treatment) of firing frequency recorded in the subset of neurons co-expressing OXY and AVP (data not shown).

Thus, sex-linked responses to morphine were detected also by three-way ANOVAs including sex as a variable. However, in the revised version of the manuscript we did not include novel figures combining the two sexes because it would have been largely redundant with the figures already reported, especially with Fig. 1D, Fig. 1G, Fig. 2B and Fig. 2D.

> ***Reviewer #2 (Public review):***
>
> *This manuscript reports a series of studies that sought to identify a biological basis for morphine-induced social deficits. This goal has important translational implications and is, at present, incompletely understood in the field. The extant literature points to changes in periventricular CRF and oxytocin neurons as critical substrates for morphine to alter social behavior. The experiments utilize mice, administered morphine prior to a sociability assay. Both male and female mice show reduced sociability in this procedure. Pretreatment with the CRF1 receptor antagonist, antalarmin, clearly abolished the morphine effect in males, and the data are compelling. Consistently, CRF1-/- male mice appeared to be spared of the effect of morphine (while wild-type and het mice had*

> *reduced sociability). The same experiment was reported as non-feasible in females due to the effect of dose on exploratory behavior per se. Seeking a neural correlate of the behavioral pharmacology, acute cell-attached recordings of PVN neurons were made in acute slices from mice pretreated with morphine or anatalarmin. Morphine increased firing frequencies, and both antalarmin and CRF1-/- mice were spared of this effect. Increasing confidence that this is a CRF1 mediated effect, there is a gene deletion dose effect where het's had an intermediate response to morphine. In general, these experiments are well-designed and sufficiently powered to support the authors' inferences. A final experiment repeated the cell-attached recordings with later immunohistochemical verification of the recorded cells as oxytocin or vasopressin positive. Here the data are more nuanced. The majority of sampled cells were positive for both oxytocin and vasopressin, in cells obtained from males, morphine pretreatment increased firing in this population and was CRF1 dependent, however in females the effect of morphine was more modest without sensitivity to CRF1. Given that only ~8 cells were only immunoreactive for oxytocin, it may be premature to attribute the changes in behavior and physiology strictly to oxytocinergic neurons.*
>
> *In sum, the data provide convincing behavioral pharmacological evidence and a regional (and possibly cellular) correlation of these effects suggesting that morphine leads to sociality deficits via CRF interacting with oxytocin in the hypothalamus. While this hypothesis remains plausible, the current data do not go so far as directly testing this mechanism in a site or cell-specific way.*

We agree with this reviewer's comment and acknowledge that further studies are needed to better understand the neural substrates of CRF_1 receptor-mediated sociability deficits induced by morphine. This has been mentioned at page 17, line 25 to page 18, line 6 of the present revised version of the manuscript.

> *With regard to the presentation of these data and their interpretation, the manuscript does not sufficiently draw a clear link between mu-opioid receptors, their action on CRF neurons of the PVN, and the synaptic connectivity to oxytocin neurons. Importantly, sex, cell, and site-specific variations in the CRF are well established (see Valentino & Bangasser) yet these are not reviewed nor are hypotheses regarding sex differences articulated at the outset. The manuscript would have more impact on the field if the implications of the sex-specific effects evident here were incorporated into a larger literature.*

At page 15, line 19 to page 16, line 2 of the present version of the manuscript, we have mentioned prior studies reporting differences in CRF_1 receptor signaling or cellular compartmentalization between male and female rodents (Bangasser et al., 2013, 2010). However, the latter studies were conducted in cortical or locus coeruleus brain tissues. Thus, more studies are needed to examine CRF_1 receptor signaling or cellular compartmentalization in the PVN and their relationship to the sex-linked results reported in our manuscript.

> *With regards to the model proposed in the discussion, it seems that there is an assumption that ip morphine or antalarmin have specific effects on the PVN and that these mediate behavior - but this is impossible to assume and there are many meaningful alternatives (for example, both MOR and CRF modulation of the raphe or accumbens are worth exploration).*

We focused our discussion on PVN OXY/AVP systems because ourelectrophysiology studies examined neurons expressing OXY and/or AVP in this brain area. However, we understand that other brain areas/systems might mediate the effect of systemic administration of the

CRF_1 receptor antagonist antalarmin or whole-body genetic disruption of the CRF_1 receptor upon morphine-induced social behavior deficits. For this reason, at page 16, line 12 to page 17, line 7 of the present version of the manuscript we have mentioned the possible involvement of BNST OXY or VTA dopamine systems in the CRF_1 receptor-mediated social behavior effects of morphine reported herein. Indeed, literature suggests important CRF-OXY and CRF-dopamine interactions in the BNST and the VTA, which might be relevant to the expression of social behavior. Nevertheless, to date the implication of the latter brain systems interactions in social behavior alterations induced by substances of abuse remains to be elucidated.

> *While it is up to the authors to conduct additional studies, a demonstration that the physiology findings are in fact specific to the PVN would greatly increase confidence that the pharmacology is localized here. Similarly, direct infusion of antalarmin to the PVN, or cell-specific manipulation of OT neurons (OT-cre mice with inhibitory dreadds) combined with morphine pre-exposure would really tie the correlative data together for a strong mechanistic interpretation.*

We agree with this reviewer's comment that the suggested experiments would greatly increase the understanding of the brain mechanisms underlying the social behavior deficits induced by opiate substances. We have acknowledged this at page 17, line 25 to page 18, line 6.

> *Because the work is framed as informing a clinical problem, the discussion might have increased impact if the authors describe how the acute effects of CRF1 antagonists and morphine might change as a result of repeated use or withdrawal.*

Prior studies reported behavioral and neuroendocrine (hypothalamus-pituitary-adrenal axis) effects of chronic systemic administration of CRF_1 receptor antagonists, such as R121919 and antalarmin (Ayala et al., 2004; Dong et al., 2018). However, to our knowledge, no studies have directly compared the behavioral effects of acute *vs.* repeated administration of CRF_1 receptor antagonists. We previously reported that *acute* administration of antalarmin increased the expression of somatic opiate withdrawal in mice, indicating that this compound is effective following withdrawal from repeated morphine administration (Papaleo et al., 2007). Nevertheless, further studies are needed to specifically address this reviewer's comment.

> ***Reviewer #3 (Public review):***
>
> *Summary:*
>
> *In the current manuscript, Piccin et al. identify a role for CRF type 1 receptors in morphine-induced social deficits using a 3-chamber social interaction task in mice. They demonstrate that pre-treatment with a CRFR1 antagonist blocks morphine-induced social deficits in male, but not female, mice, and this is associated with the CRF R1 antagonist blocking morphine-induced increases in PVN neuronal excitability in male but not female mice. They followed up by using a transgenic mouse CRFR1 knockout mouse line. CRFR1 genetic deletion also blocked morphine-induced social deficits, similar to the pharmacological approach, in male mice. This was also associated with morphine-induced increases in PVN neuronal excitability being blocked in CRFR1 knockout mice. Interestingly they found that the pharmacological antagonism of the CRFR1 specifically blocked morphine-induced increases in oxytocin/AVP neurons in the PVN in male mice.*
>
> *Strengths:*

> *The authors used both male and female mice where possible and the studies were fairly well controlled. The authors provided sufficient methodological detail and detailed statistical information. They also examined measures of locomotion in all of the behavioral tasks to separate changes in sociability from overall changes in locomotion. The experiments were well thought out and well controlled. The use of both the pharmacological and genetic approaches provides converging lines of evidence for the role of CRFR1 in morphine-induced social deficits. Additionally, they have identified the PVN as a potential site of action for these CRFR1 effects.*
>
> *Weaknesses:*
>
> *While the authors included both sexes they analyzed them independently. This was done for simplicity's sake as they have multiple measures but there are several measures where the number of factors is reduced and the inclusion of sex as a factor would be possible.*

Please, see above our response to the same comment made by Reviewer 1.

> *Additionally, single doses of both the CRFR1 antagonist and morphine are used within an experiment without justification for the doses. In fact, a lower dose of morphine was needed for the genetic CRFR1 mouse line. This would suggest that the dose of morphine being used is likely causing some aversion that may be more present in the females, as they have lower overall time in the ROI areas of both the object and the mouse following morphine exposure.*

The morphine dose was chosen based on our prior study showing that morphine (2.5 mg/kg) impaired sociability in male and female C57BL/6J mice, without affecting locomotor activity (Piccin et al., 2022). Also, the antalarmin dose (20 mg/kg) and the route of administration (*per os*) was chosen based on our prior studies demonstrating behavioral effects of this CRF_1 receptor antagonist administered *per os* (Contarino et al., 2017; Ingallinesi et al., 2012; Piccin and Contarino, 2020). This is now mentioned in the “materials and methods” section of the present revised version of the manuscript at page 23, lines 6-13. We also agree with this reviewer that female mice seemed more sensitive to morphine than male mice. Indeed, during the habituation phase of the three-chamber test female mice treated with morphine (2.5 mg/kg) spent less time in the ROIs containing the empty wire cages, as compared to saline-treated female mice (Fig. 1E). However, morphine did not affect locomotor activity in female mice (Fig. S1B), suggesting independency between social approach and ambulation.

> *As for the discussion, the authors do not sufficiently address why CRFR1 has an effect in males but not females and what might be driving that difference, or why male and female mice have different distribution of PVN cell types during the recordings.*

At page 15, line 11 to page 16, line 2, we have mentioned possible mechanisms that might underlie the sex-linked results reported in our manuscript. Moreover, at page 16, lines 6-9 we have mentioned a seminal review reporting sex-linked expression of PVN OXY and AVP in a variety of animal species that is similar to the present results. Nevertheless, as mentioned in the “discussion” section, further studies are needed to elucidate the neural substrates underlying sex-linked effects of opiate substances upon social behavior.

> *Additionally, the authors attribute their effect to CRF and CRFR1 within the PVN but do not consider the role of extrahypothalamic CRF and CRFR1. While the PVN does contain the largest density of CRF neurons there are other CRF neurons, notably in the central amygdala and BNST, that have been shown to play important roles in the impact of stress on drug-related behavior. This also holds true for the expression of CRFR1 in other*

> *regions of the brain, including the VTA, which is important for drug-related behavior and social behavior. The treatments used in the current manuscript were systemic or brain-wide deletion of CRFR1. Therefore, the authors should consider that the effects could be outside the PVN.*

Even if they suggest a role for PVN CRF_1-OXY circuits, we are aware that the present data do not support a direct link between behavior and PVN CRF_1 receptors. Thus, at page 16, line 12 to page 17, line 7 of the present version of the manuscript we have mentioned some studies showing a role for PVN OXY, BNST OXY or VTA dopamine systems in social behavior. Interestingly, the latter brain systems are thought to interact with the CRF system. However, more studies are warranted to understand the implication of CRF-OXY or CRF-dopamine interactions in social behavior deficits induced by substances of abuse.

> ***Recommendations for the authors:***
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> ***Reviewer #2 (Recommendations for the authors):***
>
> *I commend the authors on crafting a well-written and clear manuscript with excellent figures. Furthermore, the data analysis and rigor are quite high. I have a few suggestions in the order they appear in the manuscript:*
>
> *The introduction has a number of abrupt transitions. For example, the sentence beginning with "Besides," in paragraph 2 jumps from CRF to oxytocin and vasopressin without a transition or justification. In all, vasopressin may be better removed from the introduction. There is sufficient evidence in the literature to support the CRF-OT circuit that might mediate behavioral pharmacology and this should be clearly described in the introduction.*

We have added a sentence at page 3, lines 22-23 to introduce possible interactions of the CRF system with other brain systems implicated in social behavior. Also, in the “introduction” section both OXY and AVP systems are mentioned because our electrophysiology studies examined the effect of morphine upon the activity of OXY- and AVP-positive neurons.

Our interest in the PVN CRF-OXY/AVP network also stems from previous findings from our laboratory showing that genetic inactivation of the CRF_2 receptor eliminated both sociability deficits and increased hypothalamic OXY and AVP expression associated with long-term cocaine withdrawal in male mice (Morisot et al., 2018). Moreover, evidence suggests the implication of AVP systems in opiate effects. In particular, pharmacological antagonism of AVP-V1b receptors decreased the acquisition of morphine-induced conditioned place preference in male C57BL/6N mice housed with morphine-treated mice (Bates et al., 2018).

> *Throughout the manuscript, it seems that there is an assumption that ip morphine or antalarmin have specific effects on the PVN and that these mediate behavior - this is impossible to assume and there are many meaningful alternatives (for example, both MOR and CRF modulation of the raphe or accumbens are worth exploration). While it is up to the authors to conduct additional studies, a demonstration that the physiology findings are in fact specific to the PVN would greatly increase confidence that the pharmacology is localized here. Similarly, direct infusion of antalarmin to the PVN, or cell-specific manipulation of OT neurons (OT-cre mice with inhibitory dreadds) combined with morphine pre-exposure would really tie the correlative data together for a strong mechanistic interpretation.*

We agree that the suggested experiments would greatly increase the understanding of the brain mechanisms underlying the social behavior deficits induced by opiate substances. This

has been acknowledged at page 17, line 25 to page 18, line 6 of the present version of the manuscript.

> *Also in the introduction, the reference to shank3b mice is not the most direct evidence of oxytocin involvement in sociability. It may be helpful to point reviewers to studies with direct manipulation of these populations (Grinevich group, for example).*

At page 4, lines 4-6 of the "introduction" section, we have added a sentence to mention a seminal paper by the Grinevich group demonstrating an important role for OXY-expressing PVN parvocellular neurons in social behavior (Tang et al., 2020). Moreover, at page 4, lines 8-10 we have mentioned a recent study showing that targeted chemogenetic silencing of PVN OXY neurons in male rats impaired short- and long-term social recognition memory (Thirtamara Rajamani et al., 2024).

> *It would be helpful in the figures to indicate which panels contain male or female data.*

The sex of the mice is mentioned above each panel of the main and supplemental figures, except for the studies with CRF_1 receptor-deficient mice wherein only experiments carried out with male mice were illustrated. In the latter case, the sex (male) of the mice is mentioned in the related legend.

> *The discussion itself departs from the central data in a few ways - the passages suggesting that morphine produces a stress response and that CRF1 antagonists would block the stress state are highly speculative (although testable). The manuscript would have more impact if the sex-specific effects and alternative hypotheses were enhanced in the discussion.*

At page 16, line 12 to page 17, line 7 of the "discussion" section, we have suggested that interaction of the CRF system with other brain systems implicated in social behavior (i.e., OXY, dopamine) might underlie the sex-linked CR_1 receptor-mediated effects of morphine reported in our manuscript. Also, at page 15, line 19 to page 16, line 2 we have mentioned studies showing sex-linked CRF_1 receptor signaling and cellular compartmentalization that might be relevant to the present findings. Finally, to further support the notion of morphine-induced PVN CRF activity, at page 15, lines 4-6 we have mentioned a study suggesting that activation of presynaptic mu-opioid receptors located on PVN GABA terminals might reduce GABA release (and related inhibitory effects) onto PVN CRF neurons (Wamsteeker Cusulin et al., 2013). Nevertheless, we believe that more work is needed to better understand the role for the CRF_1 receptor in opiate-induced stress responses and activity of OXY and dopamine systems implicated in social behavior.

> ***Reviewer #3 (Recommendations for the authors):***
>
> *(1) You should provide justification for the doses selected for treatments and the route of administration for the CRFR1 antagonist, especially for females.*

This has been added at page 23, lines 6-13 of the present version of the manuscript. In particular, the doses and routes of administration for morphine and antalarmin used in the present study were chosen based on previous work from our laboratory. Indeed, the intraperitoneal administration of morphine (2.5 mg/kg) impaired social behavior in male and female mice, without affecting locomotor activity (Piccin et al., 2022). Moreover, the oral route of administration for antalarmin was chosen for its translational relevance, as it could be easily employed in clinical trials assessing the therapeutic value of pharmacological CRF_1 receptor antagonists.

> *(2) For the electrophysiology data you should include the number of cells per animal that were obtained. It appears that fewer cells from more females were obtained than in males and so the distribution of individual animals to the overall variance may be different between males and females.*

The number of cells examined and animals used in the electrophysiology experiments are reported above each panel of the related Figures 2, 3 and 4 as well as in the supplementary tables S1B and S1C. Overall, the number of cells examined in male and female mice was quite similar. Also, the number of male and female mice used was comparable. Standard errors of the mean (SEM) were quite similar across the different male and female groups (Fig. 2B and 2D), except for vehicle/morphine-treated male mice. Indeed, in the latter group a considerable number of cells displayed elevated firing responses to morphine, which accounted for the higher spread of the data. Accordingly, as mentioned above, the three-way ANOVA with sex (males *vs.* females), pretreatment (vehicle *vs.* antalarmin) and treatment (saline *vs.* morphine) as between-subjects factors revealed that male mice treated with vehicle/morphine showed higher firing frequency than all other male and female groups ($P<0.0005$). Finally, a similar pattern of firing frequency was observed also in neurons co-expressing OXY and AVP, wherein vehicle/morphine-treated male mice displayed higher SEM, as compared to all other male and female groups (Fig. 4C and 4F). Thus, except for vehicle/morphine-treated mice, distribution of the firing frequency data did not seem to be linked to the sex of the animal.

> *(3) You should consider using a nested analysis for the slice electrophysiology data as that is more appropriate.*

We thank the reviewer for this suggestion. However, after careful consideration, we have decided to keep the current statistical analyses. In particular, given the relatively low variability of our data, we believe that the use of parametric ANOVA tests is appropriate. Moreover, additional details supporting our choice are provided just above in our response to the comment #2.

> *(4) While it makes sense to not want to directly compare male and female data that results in needing to run a 4-way ANOVA, there are many measures, such as sociability, firing rate, etc., that if including sex as a factor would result in running a 3-way ANOVA and would allow for direct comparison of male and female mice.*

Please, see above our response to the same comment made by Reviewer 1. Notably, the results of our new statistical analyses including sex as a variable further support sex-linked effects of the CRF_1 receptor antagonist antalarmin upon morphine-induced sociability deficits and PVN neuronal firing. Nevertheless, we would like to keep the figures illustrating our findings as they are since it easily allows detecting the observed sex-linked results. Finally, we hope that this reviewer agrees with our choice, which is consistent with the wording of the title (i.e., “in male mice”).

> *(5) There are grammatical and phrasing issues throughout the manuscript and the manuscript would benefit from additional thorough editing.*

We appreciate this reviewer’s feedback. Thus, upon revising, we have carefully edited the manuscript with regard to possible grammatical and phrasing errors. We hope that our changes have made the manuscript clearer in order to facilitate readability by the audience.

> *(6) The discussion should be edited to include consideration of an explanation for the presence of the effect in male, but not female, mice more clearly. The discussion should*

> *also include some discussion as to why the distribution of cell types used in the electrophysiology recordings was different between males and females and whether the distribution of CRFR1 is different between males and females. Lastly, the authors need to include consideration of extrahypothalamic CRF and CRFR1 as a possible explanation for their effects. While they have PVN neuron recordings, the treatments that they used are brain-wide and therefore the possibility that the critical actions of CRFR1 could be outside the PVN.*

At page 15, line 11 to page 16, line 2 of the "discussion" section, we have suggested several mechanisms that might underlie the sex-linked behavioral and brain effects of CR_1 receptor antagonism reported in our manuscript. With regard to the distribution of cell types examined in the electrophysiology studies, at page 16, lines 6-9 we have mentioned a seminal review reporting sex-linked expression of PVN OXY and AVP in a variety of animal species that is similar to our results. Moreover, at page 18, lines 2-6 we mentioned that more studies are needed to examine PVN CRF_1 receptor expression in male and female animals, an issue that is still poorly understood. Finally, at page 16, line 12 to page 17, line 7 of the "discussion" section we also suggest that CRF_1 receptor-expressing brain areas other than the PVN, such as the BNST or the VTA, might contribute to the sex-linked effects of morphine reported in our manuscript. Thus, in agreement with this reviewer's suggestion, in the present version of the manuscript we have further emphasized the possible implication of CRF_1 receptor-expressing extrahypothalamic brain areas in social behavior deficits induced by opiate substances.

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