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# Rôle du système CRF dans les effets de récompense cérébrale et les troubles de la motivation induits par la morphine

Alessandro Piccin

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THÈSE PRÉSENTÉE  
POUR OBTENIR LE GRADE DE  
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**L'UNIVERSITÉ DE BORDEAUX**

ÉCOLE DOCTORALE DE LA VIE ET DE LA SANTÉ  
SPÉCIALITÉ: NEUROSCIENCES

Par Alessandro PICCIN

**ROLE OF THE CRF SYSTEM IN THE REWARDING AND  
MOTIVATIONAL EFFECTS OF MORPHINE**

Sous la direction de : Angelo CONTARINO

Soutenue le 18 Décembre 2018

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## **Titre :**

**Rôle du système CRF dans les effets de récompense cérébrale et les troubles de la motivation induits par la morphine**

## **Résumé :**

La neurobiologie à l'origine d'un comportement social des consommateurs d'opiacés et des personnes dépendantes reste largement méconnue, entravant le développement de nouveaux traitements efficaces contre les troubles de l'utilisation des opiacés. Cependant, des preuves précliniques récentes suggèrent que le système du facteur de libération de la corticotrophine (CRF) pourrait être impliqué.

Dans cette optique, nous avons utilisé la méthode des tâches à trois chambres (3-CH) ainsi qu'une méthode génétique et pharmacologique pour évaluer l'impact de l'administration de la morphine et du sevrage sur le comportement social des souris vis-à-vis de l'utilisation du système CRF.

Dans une première série d'expériences, nous avons constaté que les sujets mâles manifestaient un intérêt pseudo-social accru suscité par l'hostilité pendant le sevrage des opiacés, ce qui indiquait les effets à long terme de l'administration chronique de médicaments sur le fonctionnement social normal. Par ailleurs, les femelles ont manifesté moins d'intérêt social lors du sevrage aux opiacés, un phénotype qui reproduit plus facilement ce qui a été observé chez l'homme. Notamment, le déficit en récepteurs CRF1 a complètement sauvé ce dernier déficit social, ajoutant ainsi aux preuves croissantes liant le système CRF aux dysfonctionnements comportementaux induits par la substance.

Dans une deuxième série d'expériences, nous avons étudié plus largement les effets initiaux de la morphine sur les activités « naturellement » gratifiantes. Nous avons constaté qu'une seule et même administration aiguë d'une dose relativement faible du médicament suffisait à la fois à générer une récompense cérébrale et à induire de profonds déficits en intérêt social et en motivation liée à l'alimentation, fournissant ainsi des preuves expérimentales initiales du « détournement » du cerveau. Il est intéressant de noter que l'administration systémique d'antalarmine, antagoniste du récepteur de la CRF, a complètement inversé ce dernier déficit social, ce qui indique que le système CRF joue un rôle essentiel dans la médiation de la diminution de l'intérêt suscité par les substances pour des activités rémunératrices « naturellement » et renforce la notion de potentiel thérapeutique du ciblage de la CRF.

## **Mots clés :**

Récompense cérébrale et motivation ; drogues opiacées ; système CRF ; vulnérabilité au stress ; morphine ; pharmacologie.

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## **Title :**

**Role of the CRF system in the rewarding and motivational effects of morphine**

## **Abstract :**

The neurobiology underlying poor social behavior of opiate users and dependent individuals remains largely unknown, hampering the development of novel effective therapies for opiate use disorders. However, recent pre-clinical evidence suggests that the corticotropin-releasing factor (CRF) system might be involved.

In this light, we employed the three-chamber (3-CH) task and genetic/pharmacological approaches to assess the impact of morphine administration and withdrawal upon social behavior in mice with regard to the CRF system.

In a first set of experiments, we found that male subjects displayed increased hostility-driven pseudo-social interest during opiate withdrawal, indicative of long-lasting effects of chronic drug administration upon normal social functioning. On the other hand, female subjects displayed lower social interest during opiate withdrawal, a phenotype that more straightforwardly replicates what observed in humans. Notably, CRF1 receptor-deficiency completely rescued the latter social deficit, adding to the growing evidence linking the CRF system to substance-induced behavioral dysfunctions.

In a second set of experiments, we investigated the initial effects of morphine more widely on naturally rewarding activities. We found that a single, acute administration of a relatively low dose of the drug was sufficient to produce brain reward and at the same time to induce profound deficits in social interest and food-driven motivation, providing initial experimental evidence of "hijacking" of brain reward systems by substances of abuse. Interestingly, systemic administration of the CRF receptor antagonist antalarmin completely reversed the latter social deficit, indicating a critical role of the CRF system in mediating substance-induced decreased interest for "naturally" rewarding activities and strengthening the notion of a therapeutic potential for CRF-targeting pharmacological agents.

## **Keywords :**

Brain reward and motivation ; opioids ; CRF system ; stress vulnerability ; morphine ; pharmacology.

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Role of the CRF system in the rewarding and  
motivational effects of morphine

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Soutenue le 18 Décembre 2018

This thesis has been typeset using L<sup>A</sup>T<sub>E</sub>X.

[...]

Heroin, be the death of me  
Heroin, it's my wife and it's my life, haha  
Because a mainline into my vein  
Leads to a center in my head  
And then I'm better off than dead  
Because when the smack begins to flow  
I really don't care anymore  
About all the Jim-Jims in this town  
And all the politicians making crazy sounds  
And everybody putting everybody else down  
And all the dead bodies piled up in mounds  
Cause when the smack begins to flow  
And I really don't care anymore  
Ah, when that heroin is in my blood  
Heh, and that blood is in my head  
Then thank God that I'm as good as dead  
And thank your God that I'm not aware  
And thank God that I just don't care  
And I guess I just don't know  
Oh, and I guess that I just don't know

“Heroin”, written by LOU REED.  
The Velvet Underground & Nico, 1967.





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## ABBREVIATIONS

<b>+/+</b>	wild-type
<b>+/-</b>	heterozygous
<b>-/-</b>	knock-out
<b>3-CH</b>	three-chamber
<b>A</b>	animal
<b>ABB</b>	aggressive biting behavior
<b>ACTH</b>	adrenocorticotrophic hormone
<b>AMPA</b>	alpha-amino-3-hydroxy-5-methyl-isoxazole propionic acid
<b>ANOVA</b>	analysis of variance
<b>anta</b>	antalarmin
<b>APA</b>	american psychiatric association
<b>ASV-30</b>	antisauvagine-30
<b>AVP</b>	arginin vasopressin
<b>BNST</b>	bed nucleus of the stria terminalis
<b>BW</b>	body weight
<b>CeA</b>	central nucleus of the amygdala
<b>CNS</b>	central nervous system
<b>CPP</b>	conditioned place preference
<b>CRF</b>	corticotropin-releasing factor
<b>CRF-BP</b>	CRF-binding protein
<b>CRF<sub>1</sub></b>	CRF <sub>1</sub> receptor
<b>CRF<sub>2</sub></b>	CRF <sub>2</sub> receptor
<b>CRH</b>	corticotropin-releasing hormone
<b>DA</b>	dopamine
<b>DSM</b>	diagnostic and statistical manual of mental disorders
<b>EPM</b>	elevated plus maze
<b>EPS</b>	elevated platform stressor
<b>FDA</b>	food and drug administration
<b>FR</b>	fixed ratio
<b>GC</b>	glucocorticoid
<b>GPCR</b>	G-protein-coupled receptor
<b>GR</b>	glucocorticoid receptor
<b>Hip</b>	hippocampus

<b>HPA</b>	hypothalamic-pituitary-adrenal
<b>HPF</b>	highly palatable food
<b>Hyp</b>	hypothalamus
<b>i.c.v.</b>	intracerebroventricular
<b>i.p.</b>	intraperitoneal
<b>ITI</b>	inter-trial interval
<b>MDMA</b>	3,4-methylenedioxymethamphetamine
<b>MeA</b>	medial nucleus of the amygdala
<b>mor</b>	morphine
<b>mor 1.25</b>	morphine 1.25 mg/kg
<b>mor 2.5</b>	morphine 2.5 mg/kg
<b>MR</b>	mineralocorticoid receptor
<b>mRNA</b>	messenger RNA
<b>NAc</b>	nucleus accumbens
<b>NOR</b>	novel object recognition
<b>NPS</b>	new psychoactive substances
<b>NS</b>	non-stressed
<b>O</b>	object
<b>OXY</b>	oxytocin
<b>OD</b>	opiate use disorder
<b>OW</b>	opiate withdrawal
<b>OWD</b>	opiate withdrawal day
<b>PET</b>	positron emission tomography
<b>PFC</b>	prefrontal cortex
<b>POMC</b>	pro-opiomelanocortin
<b>PR</b>	progressive ratio
<b>PRE</b>	pre-conditioning test
<b>POST</b>	post-conditioning
<b>PR</b>	progesterone receptor
<b>PVN</b>	paraventricular nucleus of the hypothalamus
<b>sal</b>	saline
<b>SI</b>	social interaction
<b>SNP</b>	social novelty preference
<b>SOC</b>	sociability
<b>SON</b>	supraoptic nucleus
<b>SUDs</b>	substance use disorder
<b>TD</b>	treatment day
<b>THC</b>	tetrahydrocannabinol
<b>UCN1</b>	urocortin 1
<b>UCN2</b>	urocortin 2
<b>UCN3</b>	urocortin 3
<b>veh</b>	vehicle
<b>VMH</b>	ventromedial hypothalamus
<b>VTA</b>	ventral tegmental area
<b>WHO</b>	world health organization

## ABSTRACT

Opiate use disorders (OUDs) are chronic relapsing diseases with a major health and socio-economic impact. Like other substance use disorders, OUDs are characterized by a myriad of somatic, emotional-like, motivational, cognitive and social behavior deficits. Nevertheless, the brain mechanisms underlying the clinical features of OUDs remain poorly understood, hampering the development of novel effective therapies. The corticotropin-releasing factor (CRF) system, a major coordinator of behavioral, neuroendocrine and autonomic responses to stressors, might play a critical role in behavioral and brain alterations associated with OUDs. CRF-like peptides exert their actions through two subtypes of receptors, termed CRF<sub>1</sub> and CRF<sub>2</sub>. Using clinically-oriented laboratory animal models, previous studies reported differential, and often opposite, roles for the two CRF receptor subtypes in the effects of substances of abuse. Notably, genetic inactivation of CRF<sub>1</sub> or CRF<sub>2</sub> receptors respectively exacerbated or reduced somatic signs and cognitive deficits associated with relatively early or late opiate withdrawal phases. However, the role of the CRF system in the social behavior deficits induced by opiate administration and withdrawal remains largely unknown.

Herein, we employed the three-chamber (3-CH) task for sociability (i.e., preference for an unfamiliar conspecific *versus* an object) and social novelty preference (SNP, i.e., preference for a novel *versus* a familiar conspecific) in order to assess the impact of chronic morphine administration and withdrawal upon social behavior in mice. Surprisingly, we found that morphine withdrawal increased, instead of decreasing, the social interest towards an unfamiliar same-sex conspecific in male mice. Further ethological analyses revealed that morphine withdrawal also increased aggressive behavior in male mice. Notably, social interest and aggressive behavior followed a similar time-course and positively correlated with one another, suggesting a major role for aggressiveness in the apparent social interest displayed by opiate-withdrawn mice. The latter findings indicate that aggressive behavior might contribute to the social behavior dysfunctions associated with substance withdrawal. Finally, exposure to an ethological environmental stressor relatively long time after morphine discontinuation did not affect social behavior, indicating stress resilience in opiate-withdrawn mice.

Using the CRF<sub>1</sub> receptor-deficient mouse model, we also investigated the role of the CRF<sub>1</sub> receptor in the effects of opiate withdrawal upon social behavior. We found that CRF<sub>1</sub> receptor-deficiency increased social interest in substance-naïve male mice. Moreover, like the studies mentioned above, opiate withdrawal increased the interest for an unfamiliar conspecific as well, strengthening the notion of a possible aggressive-driven social approach in opiate-withdrawn male mice.

Drugs of abuse activate brain reward systems and strongly narrow behavior towards substance-seeking and substance-taking to the detriment of “natural” rewarding activities, such as social interaction and food intake. Thus, using the conditioned place preference (CPP), the 3-CH and the operant behavior paradigms, we assessed morphine effects upon brain reward, social behavior and motivation for food. We found that a single, acute administration of a relatively low morphine dose induced CPP, indicating activation of brain reward systems. However, the same drug dose strongly impaired social behavior and motivation for food in both male and female mice. Notably, morphine did not affect ambulation, olfaction or anxiety-like behavior, suggesting a selective substance-induced disruption of the rewarding and motivational properties of social behavior and food intake. Altogether, our findings indicate decreased interest for “natural” rewarding activities following exposure to a brain rewarding dose of morphine, providing initial experimental evidence of “hijacking” of brain reward systems by substances of abuse.

Then, to investigate the role of the CRF system in vulnerability of brain reward and motivation systems to opiate substances, we used the CRF<sub>1</sub> receptor-preferring antagonist antalarmin. Systemic administration of the compound fully rescued the deficits in social behavior, but did not affect the reduced motivation for food induced by morphine. Thus, at least to some extent, the latter results indicate a critical role for the CRF system in substance-induced decreases in “natural” rewarding activities, strengthening the notion of a therapeutic potential for CRF-targeting pharmacological agents.

**Key-words:** mice; social behavior; motivation; natural rewards; food; drugs of abuse; morphine; opiate withdrawal; vulnerability; stress; aggressive behavior; corticotropin-releasing factor; pharmacology.

Les troubles de l'utilisation des opiacés (OUDs, en anglais) sont des affections chroniques récidivantes ayant un impact socio-économique et de santé publique majeur. Les OUDs sont caractérisés par des déficits somatiques, affectifs, motivationnels, cognitifs et du comportement social, cependant les mécanismes cérébraux sous-jacents des caractéristiques cliniques des OUD restent peu connus, freinant le développement de thérapies efficaces. Le système du facteur de libération de corticotrophine (CRF), coordinateur majeur des réponses comportementales, neuroendocrines et autonomiques aux événements stressants, joue un rôle crucial dans les altérations cérébrales et comportementales associées aux OUD. Les peptides analogues à CRF exercent leurs actions via deux récepteurs : CRF<sub>1</sub> et CRF<sub>2</sub>. Avec l'utilisation de modèles cliniques d'animaux de laboratoire, des études ont démontré des rôles différentiels et souvent opposés pour les récepteurs CRF<sub>1</sub> et CRF<sub>2</sub> dans l'effet des substances d'abus. Il a notamment été démontré que l'inactivation génétique de CRF<sub>1</sub> ou CRF<sub>2</sub> peut respectivement exacerber ou réduire les déficits somatiques et cognitifs associés avec les différentes phases de sevrage aux opiacés.

Cependant, le rôle du système CRF dans les déficits de comportements sociaux induits par l'administration d'opiacés et le sevrage demeure largement inconnu. Ici nous avons utilisé un test à trois chambres (3-CH) pour la sociabilité (i.e., préférence pour un congénère inconnu vs. un objet) et la préférence de nouveauté sociale (i.e., préférence pour un congénère nouveau vs. un congénère familier) chez la souris afin d'évaluer l'impact d'une administration chronique de morphine et du sevrage sur le comportement social. De manière surprenante, chez les souris mâles le sevrage à la morphine augmentait l'approche sociale envers un congénère inconnu du même sexe. Cependant, des analyses plus approfondies ont révélé également une augmentation du comportement agressif. Avec une interruption de morphine, l'intérêt social pour un congénère inconnu et le comportement agressif sont corrélés et suivent une même évolution temporelle, suggérant un rôle majeur de l'agressivité dans le comportement social des souris sevrées aux opiacés. Finalement, l'exposition à un stress environnemental éthologique longtemps après l'interruption de morphine n'a pas altéré le comportement social indiquant une résilience au stress de ces souris.



L'utilisation de souris déficientes pour le récepteur CRF<sub>1</sub> nous a permis d'étudier le rôle de ce récepteur dans les effets de comportement social du sevrage à la morphine. La déficience pour le récepteur CRF<sub>1</sub> a augmenté le comportement social des souris naïves pour la substance. De plus, de la même manière que précédemment, le sevrage aux opiacés a augmenté l'intérêt pour un congénère inconnu, renforçant la notion d'une approche sociale supposément conduite par l'agressivité.

Les substances d'abus activent le circuit cérébral de la récompense et réduisent fortement le comportement aux seules recherche et prise de substance, au détriment d'activités "naturellement" récompensantes comme la interaction sociale et la prise de nourriture. Ainsi en utilisant les paradigmes de préférence de place conditionnée (CPP), de 3-CH et de comportement opérant, nous avons évalué les effets de la morphine sur la récompense cérébrale, le comportement social et la motivation pour la nourriture appétente. Nous avons mis en évidence qu'une administration aiguë et unique d'une dose relativement faible de morphine induisait un CPP, indiquant l'activation du circuit cérébral de récompense. Cependant, la même dose a fortement altéré le comportement social et la motivation pour la nourriture des souris. Ainsi, nos résultats indiquent un intérêt diminué pour les activités "naturellement" récompensantes après une exposition à une dose de morphine récompensante, apportant une évidence expérimentale de "détournement" du circuit cérébral de récompense par les substances d'abus.

Pour explorer le rôle du système CRF dans la vulnérabilité de la récompense cérébrale et le système de motivation aux opiacés, nous avons utilisé un antagoniste d'affinité préférentielle pour le récepteur CRF<sub>1</sub>, l'antalarmin. La administration systémique d'antalarmin a inversé complètement le déficit de comportement social mais n'a pas eu d'effet sur la diminution de la motivation pour la nourriture induite par la morphine. Ce dernier résultat indique donc un rôle critique du système CRF dans la réduction d'intérêt pour les récompenses "naturelles" induite par les substances d'abus, renforçant ainsi la notion de potentiel thérapeutique pour les agents pharmacologiques ciblant CRF.

**Mots-clés:** souris; sociabilité; motivation; récompense naturelle; nourriture; drogue; morphine; sevrage des opiacés; vulnérabilité; stress; comportement agressif; facteur de libération de la corticotrophine; pharmacologie.

# CHAPTER 1

## GENERAL INTRODUCTION

### 1.1 Drug dependence

The purpose of the present section is to briefly define drug dependence. First, a terminological explanation is given, followed by a quantitative picture of the problem. Then, diagnostic criteria and behavioral disorders related to drug use, abuse and dependence are presented, with insights from both clinical and pre-clinical research and related neurobiological notions.

#### 1.1.1 Presentation of the problem

##### 1.1.1.1 What does “drug dependence” mean?

Drug dependence is generally defined as a chronic, relapsing disorder that is comprised of three stages: preoccupation/anticipation, binge/intoxication, withdrawal/negative affect. After decades of research, these three stages are now conceptualized as feeding into one another, becoming more intense over time and ultimately leading to the pathological state known as addiction, which is usually portrayed as a collapsed vicious cycle (Figure 1.1). As an individual moves from being a “user” to “abuser” to “dependent”, a shift occurs from impulsivity to compulsivity and from positive reinforcement (i.e., recreational use of the drug due to its euphoric effect) [Di Chiara and Imperato, 1988, Wise and Bozarth, 1985] to negative reinforcement (i.e., “self-medication” use of the drug to avoid or relieve pre-existing or withdrawal-related aversive states) [Markou et al., 1998] driving motivated behavior. It is important to understand that the latter distinction has crucial therapeutic implications. For instance, the most successful treatment for an addictive disorder to date, methadone maintenance for heroin dependence, was developed based on the hypothesis that relieving withdrawal symptoms would diminish the desire to continue heroin use [Dole and Nyswander, 1965]. Importantly, the progression towards drug dependence involves alterations in brain circuitries that result in long-lasting drug-induced neuroplastic changes [Koob and Volkow, 2010]. When do these changes begin is still poorly understood, but, notably, they might occur even following pre-

natal exposure [McCarthy et al., 2014, Monnelly et al., 2018, Roos et al., 2014] or after the very first substance use [Walker et al., 2018, Alvarenga et al., 2010, Ungless et al., 2001]. In the past decades, thanks to major advances in pre-clinical research, critical neurotransmitters (i.e., gamma-aminobutyric acid, glutamate, dopamine, opioid peptides, serotonin, acetylcholine, endocannabinoids, corticotropin-releasing factor) and neurocircuits (i.e., ventral tegmental area, nucleus accumbens, extended amygdala, cerebellum, prefrontal cortex) underlying the pathological changes at each of these stages have been identified.

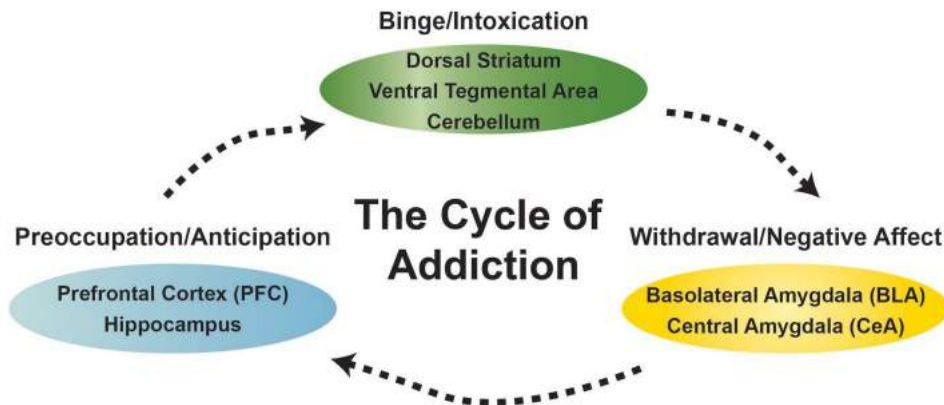


Figure 1.1 – Simplified diagram illustrating the three stages that characterize the so-called “cycle of addiction”. Behavioral states and brain areas characteristic of each phase are also reported [Herman and Roberto, 2015]

### 1.1.1.2 World epidemiological picture

Drug dependence represents a major public health issue. The Internet-driven globalization of trade witnessed during the last decade has been expanding and diversifying both the range of drugs and drug markets, affecting populations of industrialized and developing countries as never before. About 275 million people worldwide (approximately 5.6% of the global population aged 15-64) used drugs at least once during 2016 and 31 million of people using drugs suffered from substance use disorders (SUDs), meaning that their drug use was harmful to the point where they may needed treatment. Some 450,000 people died in 2015 as a result of drug use, according to the World Health Organization (WHO). Of those deaths, 167,750 were a direct result of SUDs (mostly overdoses), with opioids accounting for 76% of them and confirming themselves as the most harmful class of drugs to date. The rest were indirectly attributable to drug use and included deaths related to HIV and hepatitis C acquired through unsafe injection practices. More data from the World Drug Report 2018 show that we are also facing a potential supply-driven expansion of drug markets, with production of opium and manufacture of cocaine at the highest levels ever recorded. In particular, the non-medical use of prescription analgesic opioids is of increasing concern for both law enforcement authorities and public health professionals. Different analgesic opioids are misused in different regions. In North America, illicitly obtained fentanyl and its

analogs, mixed with heroin or other drugs (such as cocaine and MDMA), are driving an unprecedented number of overdose deaths: in 2016, 63,632 people died from a drug overdose in the United States, the highest number on record and a 21% increase from the previous year. Outside North America, the impact of fentanyl and its analogues is relatively low. In Europe, for example, the main opioids of concern remain heroin and morphine, but the non-medical use of methadone, buprenorphine and fentanyl has also been reported. In countries in West and North Africa and in the Near and Middle East, the non-medical use of tramadol, a popular pharmaceutical opioid used to treat moderate and moderate-to-severe pain, is emerging as a substance of abuse. This drug is not yet under international control and is perceived by recreational users as a way of boosting energy and improving mood. However, tramadol can produce physical dependence, with WHO studies showing that this dependence may occur when it is used daily for no more than a few weeks. In the meantime, more new psychoactive substances (NPS) are being synthesized and more are available than ever (a total of 803 NPS were reported in the period 2009-2017), with increasing reports of associated harm and fatalities. In addition, drug trafficking online using the darkweb continues to grow exponentially, despite successes in shutting down popular trading platforms like Hansa or AlphaBay, which featured more than 250,000 listings for illegal drugs and chemicals, had over 200,000 users and 40,000 vendors during their activity period. All these threats to health and well-being, as well as to security, safety and sustainable development, demand an urgent response and therefore pressure health-care systems and international communities to step up in responses to cope with these challenges.

### **1.1.1.3 Diagnosis criteria for drug dependence**

In the fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5), drug dependence is classified in the section “substance-related and addictive disorders” that also comprises behavioural addictions (i.e., sex, exercise or shopping addictions and gambling disorders). Substance-related disorders are divided into two groups: substance use disorders and substance-induced disorders. Each specific substance is addressed as a separate use disorder (e.g. opioid use disorder, pertinent to the subject of this thesis), but nearly all substance use disorders are diagnosed based on the same “A” criteria reported in Table 1.1. In contrast to the DSM-4TR, the notion of severity for substance use disorders (i.e., from mild to severe) is introduced in the DSM-5 and it is based on the number of criteria observed: the presence of 2-3 criteria is defined as mild, 4-5 criteria as moderate and 6 or more criteria as severe dependence. The DSM-5 also emphasizes the notion of remission when substance use disorder diagnosis is followed by a period of time where none of the diagnostic criteria have been met (with the exception of criterion A4, “Craving, or a strong desire/urge to use the substance,” which may be met). If patients met none diagnostic criteria for a period of at least 3 months, but less than 12 months, they are considered in early remission; for 12 months or longer periods, they are considered in sustained remission. Substance-induced disorders are specific for each class of drugs and refer to in-

toxication, withdrawal, and substance/medication-induced mental disorders, such as psychotic disorders, anxiety disorders, and neurocognitive disorders.

Table 1.1 DSM-5 diagnostic categories and “A” criteria for SUDs.

Category	Criteria
Impaired control	<ol style="list-style-type: none"> <li>1. The substance is often taken in larger amounts or over a longer period than was intended.</li> <li>2. There is a persistent desire or unsuccessful efforts to cut down or control the substance use.</li> <li>3. A great deal of time is spent in activities necessary to obtain or use the substance.</li> <li>4. Craving, or a strong desire/urge to use the substance.</li> </ol>
Social impairment	<ol style="list-style-type: none"> <li>5. Recurrent substance use resulting in a failure to fulfill major role obligations at work, school, or home (e.g., interference with work).</li> <li>6. Continued substance use despite having persistent or recurrent social or interpersonal problems caused or exacerbated by the effects of the substance (e.g., arguments with others about substance use).</li> <li>7. Important social, occupational, or recreational activities are given up/reduced because of substance use.</li> </ol>
Risky use	<ol style="list-style-type: none"> <li>8. Recurrent substance use in situations in which it is physically hazardous (e.g., driving under the influence of alcohol).</li> <li>9. The substance use is continued despite knowledge of having a persistent or recurrent physical or psychological problem that is likely to have been caused or exacerbated by the substance.</li> </ol>
Pharmacological criteria	<ol style="list-style-type: none"> <li>10. Tolerance, as defined by either of the following: a) a need for markedly increased amounts of the substance to achieve the desired effect; b) a markedly diminished effect with continued use of the same amount of the substance.</li> <li>11. Withdrawal, as manifested by either of the following: a) the characteristic withdrawal syndrome for the substance; b) the substance (or a closely related substance) is taken to relieve or avoid withdrawal symptoms.</li> </ol>

#### 1.1.1.4 Treatment of drug dependence

In the last few years, a large body of research studies investigated the neurobiological mechanisms underlying the effects of substances of abuse and the development of drug dependence, identifying stages of transition from recreational to sustained substance use based on individual genetic predisposition and drug-induced adaptations, up to the loss of control and fulfilment of the above-mentioned diagnostic criteria for intervention. In spite of these tremendous advancements, the number of medications approved by the Food and Drug Administration (FDA) for the treatment of drug dependence is still scarce [Chiamulera et al., 2017] (Table 1.2). Moreover, the efficacy of current treatments is limited, with relapse rates being extremely high within the first year and psychosocial interventions only modestly enhancing their effectiveness. At

the moment, the aim of pharmacotherapy is to improve the quality of life of patients by controlling and/or reducing the manifestation and the intensity of physical and negative affective-like signs and symptoms during drug withdrawal periods. Three types of pharmacotherapy are currently used for drug dependence: agonists, antagonists and metabolism modulators. On a brighter note, for the first time ever, the World Drug Report 2018 highlights the importance of gender- and age-sensitive drug policies, putting forward the particular needs and challenges of women and young people.

Table 1.2 FDA-approved pharmacotherapies for drug dependence.

<b>Pharmacotherapy</b>	<b>Clinical indication</b>	<b>Mechanism of action</b>
Disulfiram	Prevention of relapse to alcohol use	Ethanol metabolism inhibition
Methadone	Opioid substitution therapy	Full opioid receptors agonism
Nicotine replacement	Tobacco smoking substitution therapy	Full nicotinic receptors agonism
Naltrexone	Drinking reduction and prevention of relapse	Opioid receptors antagonism
Bupropion	Anti-craving drug; relapse prevention in ex-smokers	Dopamine and noradrenaline re-uptake blocker; nicotinic receptors antagonist
Buprenorphine	Opioid detoxification therapy	Partial opioid receptors agonism
Acamprosate	Relapse prevention in ex-alcoholics	Partial glutamate receptors agonism
Nalmefene	Drinking reduction	Partial opioid receptor agonism
Varenicline	Relapse prevention in ex-smokers	Partial nicotinic receptors agonism

### 1.1.2 Drug-induced behavioral disorders

Physical dependence is a state that develops as a result of the adaptation (i.e., tolerance) produced by a resetting of homeostatic mechanisms in response to repeated drug use [Koob, 2008a]. Drugs of abuse can affect numerous systems that previously were in equilibrium. These systems find a new equilibrium in the presence of inhibition or stimulation by a specific drug. A person in this adapted or physically dependent state requires continued administration of the substance to maintain this equilibrium. If administration of the drug is stopped abruptly, another imbalance follows and the affected systems must go again through a process of readjusting to a new equilibrium without the drug. The appearance of a substance withdrawal syndrome when substance administration is terminated is the only actual evidence of physical dependence and in the next few sections are described the marked changes in behavior that come with it. Withdrawal symptoms have at least two origins: (1) removal of the drug producing dependence and (2) central nervous system (CNS) hyperarousal owing to readaptation to the absence of the drug. Even

if withdrawal symptoms are characteristic for a given category of drugs, they share various commonalities and generally tend to be opposite in nature to the original effects produced by the drug before tolerance developed.

### 1.1.2.1 Somatic signs and symptoms

When drug-dependent individuals abruptly terminate or substantially reduce their drug consumption, various somatic signs appear in a temporally dynamic process [Heilig et al., 2010]. The physical manifestation of drug withdrawal usually begins 3-12 hours after the last drug administration and peaks in between 36 and 96 hours of drug abstinence, depending from the substance abused [West and Gossop, 1994]. Relevantly to the current work, the time-course of withdrawal from various popular opiate substances is schematized in Table 1.3. The opiate withdrawal syndrome reflects generalized nervous system hyperexcitability and is characterized by influenza-like symptoms, including fever (i.e. body temperature dysregulation, sometimes accompanied by hallucinations), excessive sweating (i.e., diaphoresis), shaking (i.e., tremor), increased sensitivity to pain, rapid heartbeat (i.e., tachycardia), increased blood pressure, muscle aches, nausea, vomiting, diarrhea and cramps. Dysphoric mood and signs of pathological craving for the drug are also present. During this phase, the focus of treatment strategies is therefore to control generalized hyperexcitability and, in the most severe cases, to prevent motor seizures and delirium tremens [Victor and Adams, 1953, Mayo-Smith, 1997]. Subsequently, increased anxiety, depressed mood and disturbed sleep (i.e., insomnia) grow strong in the subject, often accompanied by cyclic changes in weight, now expressed in the absence of the above-mentioned acute physical symptoms [Heilig et al., 2010].

Using laboratory animals, researchers have been able to model many of these classic withdrawal symptoms, including motor stereotypy, altered cardiovascular function, central and behavioral thermal dysregulation, diarrhea and body weight oscillations [Emmett-Oglesby et al., 1990]. For instance, rats and mice withdrawn from chronic opiate administration (both opioid receptor-antagonist precipitated and spontaneous) present a constellation of somatic signs, including jumping (i.e., escape attempt), paw tremors, wet dog shakes, head shakes, body stretches, diarrhea, chewing, teeth chattering, palpebral ptosis and eye twitches [Maldonado et al., 1992, Gold et al., 1994, Papaleo and Contarino, 2006, Papaleo et al., 2008b, Mucha et al., 1979, Bruijnzeel et al., 2007, Papaleo et al., 2007, Bozarth and Wise, 1984]. Moreover, quantitative measures of increased paw tremors and altered metabolic states exist for mice and rats undergoing alcohol withdrawal [Macey et al., 1996, Meert et al., 1992, Rasmussen et al., 2006, Crawshaw et al., 1994, Perez and De Biasi, 2015]. In addition, hyperlocomotion and paw tremors are exhibited by mice experiencing THC [Huang et al., 1998] and nicotine withdrawal [Damaj et al., 2003]. The study of such somatic outcomes is of considerable interest because withdrawal symptoms are among the major causes of failure when drug-dependent individuals try to quit the drug [West et al., 1989]. Therefore, therapeutic actions towards relief of somatic symptoms might aid in drug cessation.

Table 1.3 Time-course of withdrawal from various popular opiate substances.

Opiate substance	Withdrawal onset	Withdrawal peak	Withdrawal end
Fentanyl	3-5 h	8-12 h	4-5 days
Heroin	8-12 h	36-72 h	7-10 days
Methadone	36-72 h	96-144 h	14-21 days
Morphine	8-12 h	36-72 h	7-10 days
Tramadol	8-12 h	72-96 h	7-10 days

### 1.1.2.2 Negative affective-like states

Psychological changes are also key components of the drug withdrawal syndrome. Symptoms of both anxiety and depression, including irritability, dysphoria (i.e., state of unease or dissatisfaction) and anhedonia (i.e., reduced ability to experience pleasure or diminished interest in engaging in pleasurable activities) [APA, 2013, Haertzen and Hooks, 1969] emerge following termination of chronic drug use [Koob and Le Moal, 2008]. Importantly, data have shown that depressed mood is associated with a worse clinical outcome among substance-dependent patients [Hasin et al., 2002]. The occurrence of depression in drug-dependent individuals is more frequent than in the general population, with approximately 40% of people committing suicide having some record of drug abuse [Dragisic et al., 2015]. Indeed, suicidal tendencies have been described as common features of alcohol [Pompili et al., 2010, Bradvik and Berglund, 2003] or opiate dependence [Darke and Ross, 2002, Oquendo and Volkow, 2018]. At the same time, epidemiological data indicate that the incidence rate of drug abuse among depressed patients is substantially higher than in the general population [Davis et al., 2008]. However, it is unclear whether drug abuse increases the risk of depression and vice versa [Ferguson et al., 2009]. Clinical studies report onset of depression following smoking cessation in individuals with no history of mood disorders [Aubin, 2009] or long-term changes in depressive symptoms and sleeping patterns in abstinent smokers [Moreno-Coutino et al., 2007]. Depressed mood is also a significant co-morbid symptom of alcohol abuse [Martinotti et al., 2008]. MDMA use has been associated to the development of depressive symptoms and increased anxiety-like behavior in a number of clinical studies as well [McCardle et al., 2004, Sumnall and Cole, 2005, MacInnes et al., 2001].

Animal models of anxiety and depression have proven very useful in the understanding of comorbidity [Hughes, 2007], with rodent models of drug withdrawal usually showing increased anxiety-like behavior in the elevated plus maze, the light/dark box, the marble-burying and the social interaction tests [Kliethermes et al., 2004, Overstreet et al., 2002, Becker et al., 2016]. Moreover, anhedonia, another key symptom of clinical depression, is consistently modelled in rodents using reward-sensitive procedures, such as intracranial self-stimulation and sucrose preference paradigms. Anhedonia-like behavior has been reported in rodents withdrawn from nicotine [Epping-Jordan et al., 1998, Bevins and Besheer, 2006, Bruijnzeel et al., 2010a], ethanol [Bruijnzeel



et al., 2010b, Schulteis et al., 1995], psychostimulants [D’Souza and Markou, 2010, Galineau et al., 2005, Straiko et al., 2007] and opiates [Schulteis et al., 1994]. However, the latter aspect of drug dependence is better discussed in the next subsection as part of the altered motivational processes associated with substance administration and withdrawal.

### 1.1.2.3 Altered motivation/craving

Motivation is defined as the process by which organisms finalize their behavior to the control of the environment in relation to their needs. A basic aspect of this process involves learning the relationship between biologically significant stimuli and otherwise neutral stimuli that come to predict their occurrence. By this process, organisms approach useful goal-stimuli, avoid harmful ones and disregard those of no use. It is believed that altered motivational processes dramatically reduce the ability to overcome drug dependence [APA, 2013]. Accordingly, motivation for drugs (i.e., craving) is particularly high during drug withdrawal periods [Robinson and Berridge, 2003] and has been demonstrated to positively correlate with the intensity of the drug withdrawal syndrome [Janiri et al., 2005].

Laboratory studies provide compelling evidence in favor of dramatic changes in motivational processes in animals exposed to relatively large amounts of drugs of abuse, but often with contrasting results. Morphine and cocaine withdrawal increased self-administration of heroin and remifentanyl in rats and rhesus monkeys [Cooper et al., 2008, Gerak et al., 2009] and early studies reported that during opiate, cocaine or ethanol withdrawal or upon presentation of withdrawal-conditioned stimuli, operant responding for food or for sweet solutions was decreased in rats and monkeys [Goldberg and Gonzalez, 1976, Simpson and Annau, 1977, Carroll and Lac, 1987, Baldwin and Koob, 1993, Denoble and Begleiter, 1978]. Cessation of amphetamine treatment has been shown to induce reward deficits [Kitanaka et al., 2008], such as an elevation in the brain reward threshold [Paterson et al., 2000], decreased sucrose intake [Barr and Phillips, 1999, Der-Avakian and Markou, 2010a,b] or impaired sexual behavior [Barr et al., 1999]. Opiate-withdrawn monkeys showed increased heroin choice over food, suggesting an increase in the relative reinforcing efficacy of heroin, as compared to food [Negus, 2006]. Accordingly, human studies report decreased responsiveness to “naturally” rewarding stimuli, reduced food intake and a poor nutritional status in heroin-dependent individuals [Lubman et al., 2009, Santolaria-Fernandez et al., 1995, Zijlstra et al., 2009]. However, other studies reported heightened consumption of liquid or solid reinforcers and elevated desire for food in opiate-dependent individuals [Morabia et al., 1989, Weiss, 1982]. Accordingly, a study from our laboratory reported that cessation of chronic morphine administration sharply increases the motivation to obtain palatable food, as assessed by an operant behavior task [Rouibi and Contarino, 2012]. Notably, opiate withdrawal did not affect the ability to discriminate between rewarded and non-rewarded actions, or to learn a new operant task to obtain palatable food. Indeed, upon application of a reward contingency reversal paradigm, opiate-withdrawn mice were faster than control drug-naïve mice in learning the new operant task, most probably due to

a persistent elevation in motivation [Rouibi and Contarino, 2012], suggesting that opiate withdrawal might preserve motivation-driven cognitive function. Importantly, increased food-driven behavior lasted for at least 12 days after morphine discontinuation whereas opiate withdrawal-induced body weight loss returned to control levels already 24 hours after the last administration, suggesting independency between motivation and energy needs [Rouibi and Contarino, 2012]. In conclusion, using clinically-relevant experimental paradigms, the above-mentioned studies demonstrated dramatic increases in motivation in drug-withdrawn subjects, either directed towards the drug of abuse itself or other reinforcers. In this context, to better understand motivational shifts induced by drugs of abuse, in the Article 3 reported herein we investigated the acute initial effects of morphine administration upon motivation for food using a palatable food-driven operant behavior task.

#### 1.1.2.4 Cognitive signs

Substance abuse has been extensively demonstrated to impair learning and memory. For instance, alcoholic patients showed an estimate of 50-75% deficit in learning and memory tasks during abstinence periods [Parsons and Nixon, 1993]. Chronic use of opioids has been linked to reduced attention and memory processes [Bruera et al., 1989], with drug-abstinent individuals showing cognitive deficiencies in the field of working memory, executive function and fluid intelligence [Rapeli et al., 2006]. Moreover, measures of cognitive performance, such as delayed recall and verbal learning, were significantly poorer in individuals with a history of amphetamine use [McCardle et al., 2004]. Notably, the cognitive dysfunctions of amphetamine and opioid users were not recovered several years after drug withdrawal [Ersche et al., 2006]. Psychostimulant drug use has also been shown to induce cognitive dysfunctions [APA, 2013, Millan et al., 2012]. For instance, cocaine users displayed higher impulsivity, bad decision-making and memory deficits [Bolla et al., 2000, Kirby and Petry, 2004], as well as significant impairments in verbal memory and fluency [Kelley et al., 2005], visuospatial and concentration tasks [Berry et al., 1993]. Finally, chronic THC use resulted in impaired decision-making and spatial working memory ability [Bolla et al., 2002] up to one month following drug cessation [Schweinsburg et al., 2008b,a].

Several preclinical studies have been successful in modeling drug-induced cognitive dysfunction, revealing impaired learning and memory performance following both drug administration and withdrawal in the five-choice serial reaction time, the Morris water maze and the novel object recognition (NOR) tests [Morisot and Contarino, 2016, Morisot et al., 2014, Bisagno et al., 2002, Briand et al., 2008b, Mendez et al., 2008]. For instance, mice withdrawn from chronic ethanol treatment showed significant learning and memory deficits, as revealed by impaired acquisition and long-term retention in the T-maze, the foot-shock avoidance, the shuttle box active avoidance and the step-down passive avoidance tests [Farr et al., 2005]. Discontinuation of chronic morphine administration in mice resulted in impaired performance in the object recognition task [Morisot and Contarino, 2016, Rabbani et al., 2009]. Memory was also impaired after withdrawal from repeated MDMA administration in mice

[Nawata et al., 2010]. Moreover, cocaine-withdrawn rats showed a decrease in memory performance during a Y-maze and a two-lever operant paradigm [Amin et al., 2016]. Impaired novel object recognition was also observed in cocaine-treated mice or rats 2 to 6 weeks after drug discontinuation [Briand et al., 2008a, Steele and Southwick, 1985, Morisot et al., 2014].

### 1.1.2.5 Social behavior dysfunction

The association between impaired social behavior and substance abuse was explicitly stated in early raw propositions that “drunkards” fail in the area of social interest [Steele et al., 1985]. Later studies in humans confirmed that multiple drugs of abuse impair social interest, an evolutionary adaptive and powerfully rewarding behavior [Verdejo-Garcia, 2014, Preller et al., 2014b, Uekermann and Daum, 2008, Babor et al., 1976]. Even in individuals who are socially well integrated when they start using drugs, continued drug use can often lead to social exclusion, which in turn promotes continued drug use in accordance to the vicious cycle of addiction (Figure 1.1). Specifically, during early stages of drug use, the drug is typically taken in a recreational, impulsive manner. However, as dependence develops, drug use is thought to become increasingly compulsive. As drug use escalates and transitions into compulsive drug use occurs, these individuals typically become unable to function socially, ultimately facing social marginalization and exclusion, factors that promote further drug use [Brownell et al., 1986].

Relatively recently, these profound and often long-lasting changes in social interest have been successfully modeled in rodents [Blanco-Gandia et al., 2015]. For instance, 1 week after chronic drug administration, both morphine- and cocaine-treated mice failed to display social interest in the three-chamber task (schematized in Figure 1.2) [Morisot et al., 2018, Zanos et al., 2014], a phenomenon that could be observed up to 4 weeks of abstinence in opiate-withdrawn mice [Becker et al., 2016]. Moreover, the social deficit induced by chronic cocaine administration and withdrawal could be reinstated by exposure to a mild environmental stressor 5 weeks after drug discontinuation [Morisot et al., 2018]. Worth citing, a recent study demonstrated that rats with a history of heroin self-administration that were given the choice between rescuing their cage-mate (i.e., a naturally occurring pro-social behavior) or continuing to self-administer heroin, always preferred the latter option [Tomek et al., 2018]. Indeed, not only drug withdrawal, but also acute and continued drug administration are emerging as potentially disruptive when it comes to social interest. Indeed, in the current work we provide initial evidence that acute morphine administration strongly impairs social interest in both male and female mice (Article 3). Two neuropeptides have been shown to be highly implicated in many aspects of mammalian social behavior: oxytocin (OXY) and arginine vasopressin (AVP). OXY is produced in the hypothalamus and facilitates a wide variety of processes related to social behavior, including maternal behavior, trust, anxiolysis and sexual pair-bond formation [Anacker and Beery, 2013, Ross and Young, 2009, Young et al., 2008]. On the other hand, AVP activity has been associated with aggression and anxiety [Kelly and Goodson, 2014]. Growing evidence has been suggesting an important role for OXY in

drug addiction [Sarnyai and Kovacs, 2014]. Indeed, researchers have hypothesized that drugs of abuse stimulate the OXY system [Dumont et al., 2009] and that social interest and drug dependence might share common neural substrates [Young et al., 2011, Liu et al., 2011]. This idea is further supported by the above-mentioned clinical findings showing that drug-dependent individuals typically display antisocial traits and poor socially-conscious decision making [Dawe et al., 2004, McGregor et al., 2008]. However, although pre-clinical trials in rodents highlighted the potential of OXY in rescuing social behavior deficits [Zanos et al., 2014], clinical translational value has not been achieved yet [Woolley et al., 2016, 2017, Kosfeld et al., 2005].

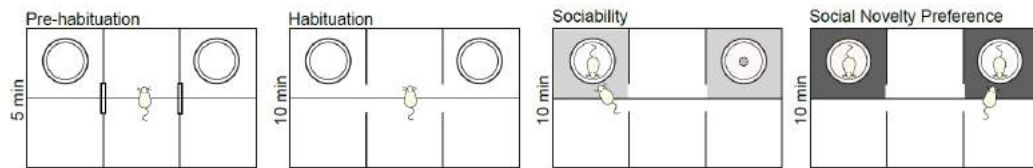


Figure 1.2 – Schematic illustration of the three-chamber (3-CH) task, widely employed in the work described in this thesis. During the pre-habituation phase, the subject mouse is confined to the central chamber for 5 min; then, the doors are opened and free exploration of the whole apparatus is allowed for 10 min (habituation phase). During the subsequent 10 min sociability phase, the subject mouse explores the entire apparatus with one wire cage containing an unfamiliar conspecific (social stimulus) and the other an unanimated object (unsocial stimulus). Preference for the social stimulus (time spent with the animal in the top half-chamber, light grey) is believed to reflect intact social interest. At last, during the 10 min social novelty preference phase, the subject mouse explores the entire apparatus with one wire cage containing the already met conspecific (familiar stimulus) and the other a novel unfamiliar conspecific (novel stimulus). Preference for the novel stimulus (time spent with the novel animal in the top half-chamber, dark grey) is believed to reflect intact discrimination abilities.

### 1.1.2.6 Vulnerability to stress

A central feature of drug addiction is the high rate of relapse during drug abstinence [Hunt et al., 1971, O’Brien and Gardner, 2005]. Over the years, many human studies have suggested that relapse is often provoked by stress [Khantzian, 1985, Kosten et al., 1986]. Indeed, substance abusers often cite stress as a reason for relapse into drug use [Ludwig and Wikler, 1974, Bradley et al., 1989, Wallace, 1989, McKay et al., 1995]. Vulnerability to stressful life events is indeed a hallmark of drug dependence that might persist long after cessation of drug intake and dramatically fuel key clinical features, such as deregulated up-shifted motivational states and craving [Preston and Epstein, 2011, Sinha, 2001]. Accordingly, exposure to stressors, such as food or sleep deprivation, extreme hyper- or hypothermia and drug withdrawal, has long been associated with increased drug craving [Childress et al., 1993].

Human evidence has led to the development of animal models to study the mechanisms underlying stress-induced relapse to drug-seeking and drug-taking. The first study to use a reinstatement model was published in 1995

using male and female rats that were trained to self-administer heroin for 12 hours a day for 12 days. In the latter study, acute exposure to 10 min of intermittent footshock stress immediately before the test reinstated heroin seeking after 1 or 2 weeks of extinction training and after an additional 4 to 6 weeks drug-free period [Shaham and Stewart, 1995]. Shortly after, various studies showed that the effect of intermittent footshock on reinstatement of heroin-seeking could be generalized to rats with a history of cocaine [Erb et al., 1996], alcohol [Le et al., 1998], nicotine [Buczek et al., 1999] and methamphetamine [Shepard et al., 2004] self-administration. Subsequently, exposure to other stressors was demonstrated to reinstate drug CPP after extinction in rats and mice: forced swim and restraint stress reinstated cocaine CPP in mice [Redila and Chavkin, 2008] and rats [Sanchez et al., 2003] and morphine CPP in rats [Li et al., 2013]. Moreover, systemic injections of the putative pharmacological stressors U50,488H [Redila and Chavkin, 2008] and yohimbine [Mantsch et al., 2010] reinstated cocaine CPP in mice. Finally, a 10 min exposure to an elevated platform stressor 1 hour prior testing was able to reinstate memory deficits in the novel object recognition task 5 to 8 weeks after cessation of either chronic cocaine [Morisot et al., 2014] or morphine [Morisot and Contarino, 2016] administration, up-shifted motivational states in a food-driven operant task (i.e., non-rewarded nose-pokes) 5 weeks after cessation of chronic morphine administration [Morisot et al., 2015] and social deficits 6 weeks after cessation of chronic cocaine administration [Morisot et al., 2018].

## 1.2 The corticotropin-releasing factor (CRF) system

The purpose of the present section is to briefly describe the CRF system. First, the anatomical distribution of its ligands and receptors is illustrated, followed by the description of its involvement in regulating stress responses via the hypothalamic-pituitary-adrenal (HPA) axis. Subsequently, the involvement of the CRF system in motivated behaviors is addressed, relatively to the subject of this thesis.

### 1.2.1 Anatomical complexity of the CRF system

The anatomical distribution of CRF in the brain suggests that this peptide not only acts as a key neuroendocrine stress mediator, but that is also able to regulate neuronal activity in a neuromodulatory fashion. In fact, CRF is expressed throughout the central nervous system (CNS), including the most limbic and cortical structures, where it has been shown to regulate the emotional and cognitive components of stress responses.

#### 1.2.1.1 The CRF family peptides

The mature and biologically active form of CRF is a 41 amino acid peptide generated by proteolytic cleavage of a 196 amino acid precursor that has been characterized in 1981 [Vale et al., 1981]. To date, the mammalian CRF family comprises three additional peptides: urocortin (UCN) 1, initially described in 1995 [Vaughan et al., 1995], followed by the discovery of UCN2 (or stresscopin-related peptide) and UCN3 (or stresscopin) shortly afterwards [Hsu and Hsueh, 2001, Lewis et al., 2001, Reyes et al., 2001]. CRF is most closely related to UCN1, sharing 43% amino acid homology, whereas CRF sequence identity with UCN2 and UCN3 is 34% and 26%, respectively [Dautzenberg and Hauger, 2002]. In comparison to CRF, UCN-expressing neurons are found in more discrete regions and nuclei of the CNS (Figure 1.3). All four neuropeptides have also been detected in the periphery: CRF mRNA has been detected in the adrenal gland, heart, gut, placenta, ovary, testis, lung and spleen in mice [Muglia et al., 1994]; UCN1 mRNA is abundant in the gastrointestinal tract and immune tissues such as the thymus and spleen in rats [Bittencourt et al., 1999]; UCN2 and UCN3 mRNA are expressed in the small intestine and skin in mice and recognized as modulators of centrally- and peripherally-controlled metabolic function [Kuperman and Chen, 2008, Li et al., 2003, Chen et al., 2004].

#### 1.2.1.2 The CRF receptors

CRF and urocortins signal through the activation of two, membrane-bound, G-protein-coupled receptors (GPCRs), named CRF<sub>1</sub> and CRF<sub>2</sub> receptor, which share 70% amino acid identity [Perrin et al., 1993, Chen et al., 1993, Chang et al., 1993, Vita et al., 1993]. CRF shows a much higher affinity for CRF<sub>1</sub>,

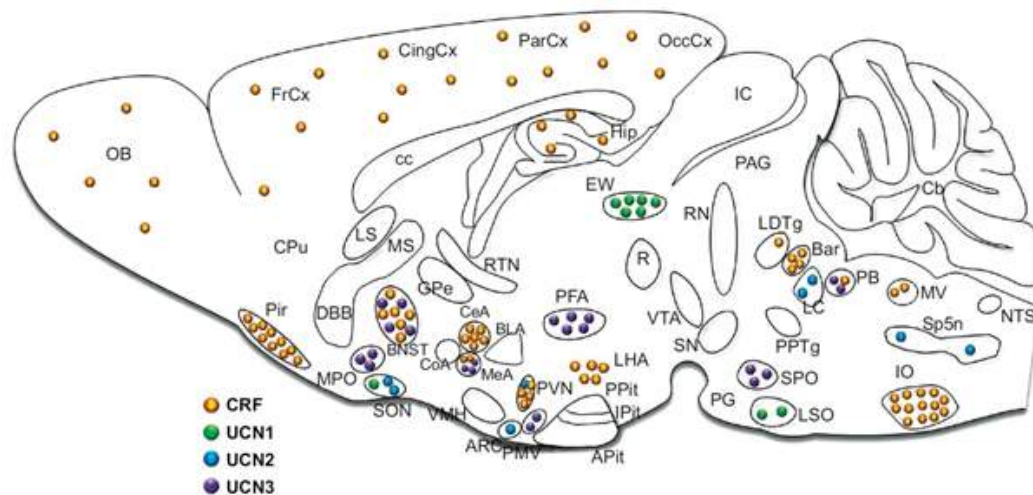


Figure 1.3 – Schematic illustration of the spatial distribution and relative expression of CRF family peptides in the mouse brain [Dedic et al., 2018]. Abbreviations: anterior pituitary (APit); arcuate nucleus: ARC; basolateral nucleus of the amygdala (BLA); bed nucleus of the stria terminalis (BNST); caudate putamen (CPu); central nucleus of the amygdala (CeA); cerebellum (Cb); cingulate cortex (CingCx); corticotropin-releasing factor (CRF); corpus callosum (cc); cortical nucleus of the amygdala (CoA); Barrington’s nucleus (Bar); diagonal band of Broca (DBB); Edinger Westphal nucleus (EW); frontal cortex (FrCx); globus pallidus (GPe); inferior colliculi (IC); inferior olive (IO); intermediate lobe of the pituitary (IPit); locus coeruleus (LC); lateral septum (LS); laterodorsal tegmental nucleus (LDTg); lateral hypothalamic area (LHA); lateral superior olive (LSO); medial nucleus of the amygdala (MeA); medial preoptic area (MPO); medial septum (MS); medial vestibular nucleus (MV); nucleus tractus solitarii (NTS); olfactory bulb (OB); occipital cortex (OccCx); parietal cortex (ParCx); parabrachial nucleus (PB); periaqueductal gray (PAG); perifornical area (PFA); piriform cortex (Pir); pontine gray (PG); posterior pituitary (Ppit); pedunculopontine tegmental nucleus (PPTg); premammillary nucleus (PMN); paraventricular nucleus of the hypothalamus (PVN); red nucleus (R); raphe nuclei (RN); reticular thalamic nucleus (RTN); superior colliculi (SC); substantia nigra (SN); supraoptic nucleus (SON); spinal trigeminal nucleus (Sp5n), superior paraolivary nucleus (SPO); urocortin 1 (UCN1); urocortin 2 (UCN2); urocortin 3 (UCN3) ventral medial hypothalamus (VMH); ventral tegmental area (VTA).

while UCN1 displays equal affinities for both receptors. UCN2 and UCN3, on the other hand, appear to be relatively selective ligands of CRF<sub>2</sub> [Perrin et al., 1995, Dautzenberg and Hauger, 2002, Hsu and Hsueh, 2001]. Several pharmacological agents are hypothesized to bind preferentially one or the other CRF receptor, such as the herein employed (Article 3) CRF<sub>1</sub> receptor-prefering antagonist antalarmin [Webster et al., 1996]. However, although some compounds show higher CRF<sub>1</sub> receptor *in vitro* binding affinity, as compared to the CRF<sub>2</sub> receptor [Ruhmann et al., 1998, Grace et al., 2007] (Table 1.5), neither behavioral nor CRF receptor activity studies exist yet to support the notion of receptor subtype-selectivity for the current available compounds. Moreover, based on the reported CRF<sub>1</sub>/CRF<sub>2</sub> receptor binding affinity ratios (ranging from 100 to 500), it cannot be excluded that

such compounds interact with both CRF receptor subtypes, at least at the behaviorally active doses usually employed in animal studies. Accordingly, intracerebroventricular (i.c.v.) administration of the CRF<sub>2</sub> receptor-preferring antagonist antisauvagine-30 (ASV-30) induced behavioral effects in mice lacking CRF<sub>2</sub> receptors, suggesting an interaction with CRF<sub>1</sub> receptors [Ruhmann et al., 1998]. Thus, pharmacological studies investigating the relative function of each of the two known CRF receptor subtypes should be considered with caution. On the other hand, genetically-engineered mouse models might provide more reliable information on the specific role of each receptor subtype. Indeed, CRF<sub>1</sub> and CRF<sub>2</sub> receptor-deficient mice show preserved CRF<sub>2</sub> and CRF<sub>1</sub> receptor activity, respectively [Contarino et al., 2000, Coste et al., 2000, Bale et al., 2000, Papaleo et al., 2008a]. Similarly to its main ligand CRF, CRF<sub>1</sub> receptor mRNA is found throughout the rodents CNS, including the cortex, cerebellum, limbic forebrain and anterior pituitary corticotropes, where its stimulation by CRF-like peptides initiates HPA axis activity [Van Pett et al., 2000, Kuhne et al., 2012]. The CRF<sub>2</sub> receptor displays a more confined and partially overlapping expression with the CRF<sub>1</sub> receptor, with high densities in the olfactory bulb, bed nucleus of the stria terminalis (BNST), lateral septum, ventromedial hypothalamic nucleus and dorsal raphe nucleus (Figure 1.4) [Van Pett et al., 2000, Chalmers et al., 1995, Lovenberg et al., 1995, Day et al., 2004, Lukkes et al., 2009].

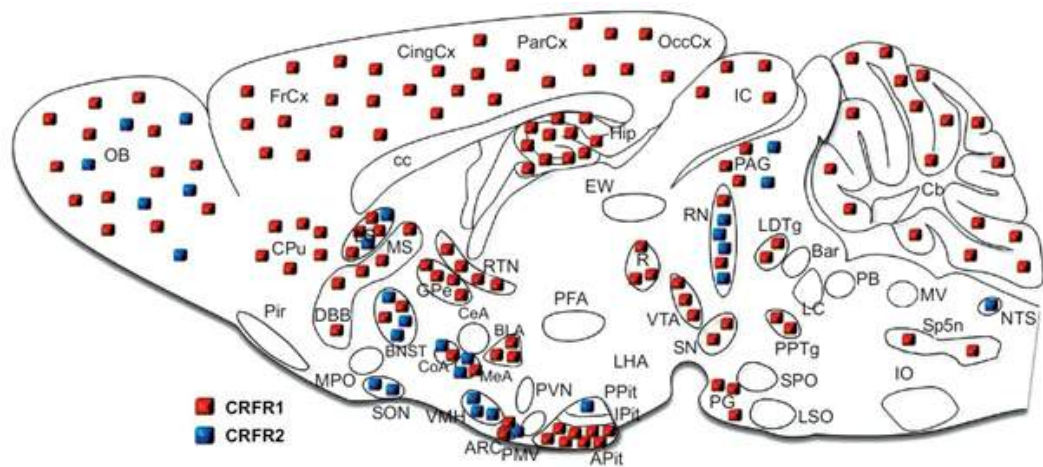


Figure 1.4 – Schematic illustration of the spatial distribution and relative expression of CRF<sub>1</sub> receptors (CRFR1) and CRF<sub>2</sub> receptors (CRFR2) in the mouse brain [Dedic et al., 2018]. Abbreviations: see Figure 1.2

### 1.2.1.3 The CRF-BP

The activity of CRF and UCN1 can be additionally regulated by the CRF-binding protein (CRF-BP) [Dautzenberg and Hauger, 2002, Seasholtz et al., 2002, 2001]. This 37-kDA protein is thought to act as an endogenous buffer, possibly by regulating the availability of active CRF and UCN1 (Figure 1.5) [Seasholtz et al., 2001, 2002, Behan et al., 1995]. *In situ* hybridization and



immunohistochemistry studies indicate that the CRF-BP is expressed predominantly in the cerebral cortex, amygdala, BNST and raphe nucleus in rats [Potter et al., 1992].

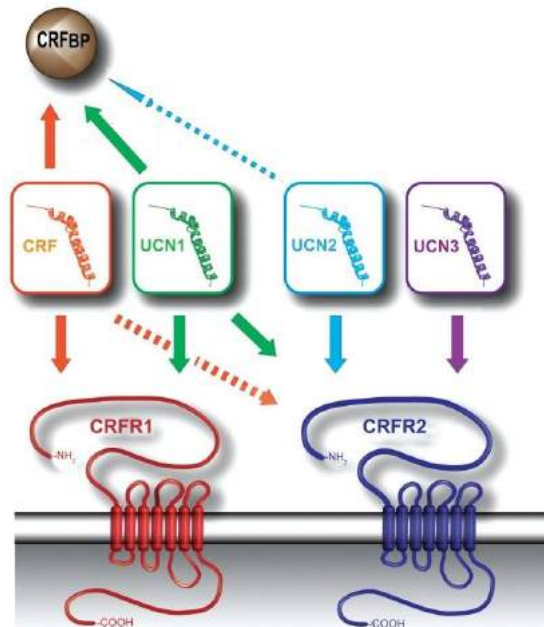


Figure 1.5 – CRF family members, their receptors and the CRF-binding protein [Dedic et al., 2018]. The arrows represent ligand-receptor or ligand-binding protein interactions. Dashed arrows indicate low-affinity binding, solid arrows indicate high-affinity binding. Abbreviations: corticotropin-releasing factor (CRF); CRF-binding protein (CRFBP); CRF<sub>1</sub> receptor (CRFR1); CRF<sub>2</sub> receptor (CRFR2); urocortin 1 (UCN1); urocortin 2 (UCN2); urocortin 3 (UCN3).

## 1.2.2 The CRF system and stress responses: the HPA axis

CRF (also referred to as corticotropin-releasing hormone or CRH) is the major physiological activator of the HPA axis and coordinates the neuroendocrine responses to stress. Perception of physical or psychological stress by an organism is followed by a series of events, including the release of CRF from parvocellular neuroendocrine neurons of the paraventricular nucleus of the hypothalamus (PVN). These neurons project via the external zone of the median eminence and release CRF into the hypophysial portal vasculature, which transports the neuropeptide to secretory corticotrope cells of the anterior pituitary, which express the CRF<sub>1</sub> receptor. The activation of CRF<sub>1</sub> receptors stimulates the release of ACTH and other pro-opiomelanocortin (POMC)–derived peptides [Vale et al., 1983]. ACTH, in turn, triggers the synthesis and release of glucocorticoids (GCs) from the adrenal cortex (cortisol in humans, corticosterone in rodents), which mediate numerous physiological and metabolic reactions and ultimately prepare the organism to deal with the stressful situation (Figure 1.6). These responses to GCs include cardiovascular

activation, energy mobilization, anti-inflammatory effects and suppression of reproductive and digestive functions [de Kloet, 2003, Sapolsky, 2000, Munck et al., 1984, Picard et al., 2014, Stahn and Buttgereit, 2008, Quax et al., 2013, Buttgereit et al., 2009]. In order to return the HPA axis to its normal state and to protect it from overshooting, GCs signal back via glucocorticoid (GR) and mineralocorticoid (MR) receptors at various feedback levels (e.g. pituitary, hippocampus and PVN), which ultimately inhibit the secretion of CRF and consequently ACTH. Noteworthy, the HPA axis is not exclusively activated during aversive stressful situations. In fact, the physiological stress-response to appetitive, rewarding stimuli can be as large as the response to negative stimuli. For instance, positive experiences such as a sexual encounters, wheel running and winning a fight (i.e., social victory) in rats have been shown to induce a similar degree of HPA axis activation as an aversive footshock, social defeat or restraint stress [Koolhaas et al., 1997, 2011].

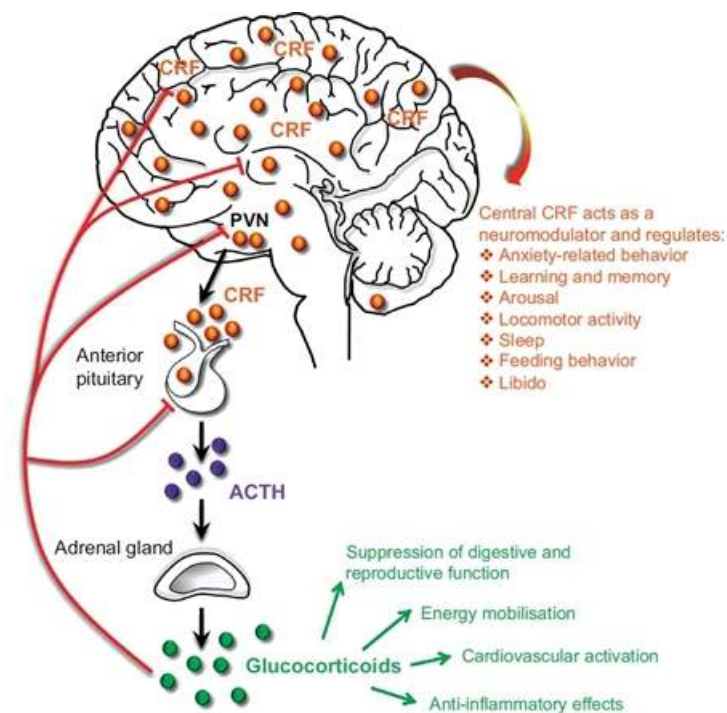


Figure 1.6 – CRF integrates neuroendocrine and higher-order behavioral responses by regulating peripheral HPA axis function and modulating synaptic transmission in the CNS [Dedic et al., 2018]. Abbreviations: adrenocorticotropic hormone (ACTH); corticotropin-releasing factor (CRF); paraventricular nucleus of the hypothalamus (PVN).

### 1.2.3 The CRF system and motivated behavior

Beyond activating aversive behaviors, stress also promotes some appetitive behaviors. For instance, food intake and relapse into drug-seeking in models of addiction are both increased by CRF-releasing stressors [Rouibi and Con-tarino, 2012, Shaham et al., 2000, Pecoraro et al., 2005]. However, besides the traditional association of CRF with aversive stress, some CRF brain systems

are activated also by positive rewards, even in the absence of stressors. For instance, CRF release is increased in the central nucleus of the amygdala (CeA) by food ingestion [Merali et al., 2004, 1998] and in the prefrontal cortex (PFC) by presentation of Pavlovian cues associated with food reward [Merali et al., 2004]. Similarly, sucrose ingestion normalizes CRF levels in the hypothalamus and in the amygdala in adrenalectomized rats [Laugero et al., 2001]. Most importantly, in the nucleus accumbens (NAc), CRF facilitates cue-elicited motivation for sucrose in rats [Pecina et al., 2006] and social bonding in prairie voles [Lim et al., 2007], behaviors believed to be mediated also by dopamine (DA) transmission [Aragona et al., 2006, Lex and Hauber, 2008]. Indeed, a wealth of experimental evidence highlights that motivated behavior is facilitated by activity of the mesolimbic DA projections from the ventral tegmental area (VTA) to the NAc [Salamone et al., 2009]. Accordingly, DA levels in the NAc are elevated during appetitive behavior [Roitman et al., 2004] and social interaction [Gunaydin et al., 2014], but, interestingly, also in response to a variety of stressors [Tidey and Miczek, 1996, Inglis and Moghaddam, 1999]. The latter studies suggest that mesolimbic VTA DA neurons are well positioned to mediate the interaction between stress and motivation. Accordingly, during stress exposure, CRF is released into the VTA in an activity-dependent manner [Wang et al., 2005]. Of major interest, Wanat et al. demonstrated that CRF acts on VTA DA neurons to attenuate the motivation to work for natural rewards [Wanat et al., 2013]. Specifically, using a progressive ratio (PR) operant behavior reinforcement schedule, the latter study first determined the amount of work (breakpoint) that rats were willing to make to obtain a food reward; then, to examine whether an acute stress modulated the motivation to work for the food reward in a CRF-dependent manner, rats received a bilateral intra-VTA injection of a non-selective CRF receptor antagonist (i.e.,  $\alpha$ -helical<sub>9-41</sub>) and underwent 20 minutes of acute restraint stress prior to be tested in the PR schedule of reinforcement. Stress exposure significantly reduced the breakpoint relative to prior baseline sessions, an effect that was blocked by the CRF receptor antagonist when administered into the VTA, *but not* in other brain regions [Wanat et al., 2013].

## 1.3 The CRF system and drug dependence

In the present section, the prominent role of the CRF system in drug dependence, compulsive-like drug self-administration and reinstatement of drug-seeking following cessation of substance intake is discussed. Related to the data presented in this thesis, antagonism of the CRF system as a potential therapeutic tool for drug dependence is also briefly discussed.

### 1.3.1 The CRF system and the acute effects of drugs of abuse

Most drugs with abuse potential, including opiates [Buckingham, 1982], amphetamine [Swerdlow et al., 1993], cocaine [Calogero et al., 1989], nicotine [Buckingham and Hodges, 1979], marijuana [Weidenfeld et al., 1994] and alcohol [Rivier et al., 1984] acutely activate the HPA axis via elevating hypothalamic production of CRF [Rivier et al., 2003, Sarnyai et al., 1992a]. Interestingly, blocking corticosterone production, by either adrenalectomy or pharmacological blockade of its synthesis, inhibited the acquisition of cocaine self-administration in rats [Goeders and Guerin, 1996]. The latter result suggested that hypothalamic CRF-induced HPA axis activation may be involved in the very onset of drug self-administration [Logrip et al., 2011], facilitating activity in brain reward and motivation circuits and, as a result, easing acquisition of drug-taking behavior [Piazza and Le Moal, 1997, Goeders, 1997]. Accordingly, functional antagonism of CRF neurotransmission has been shown to attenuate drug intake. Specifically, various CRF<sub>1</sub> receptor-preferring antagonists (i.e., antalarmin, MJL-1-109-2 and R121919) attenuated alcohol self-administration in rats undergoing acute withdrawal during an intermittent alcohol vapour exposure paradigm [Funk et al., 2007, Funk and Koob, 2007]. Moreover, non-selective CRF receptors antagonism (i.e., D-Phe CRF<sub>12-41</sub>) effectively decreased alcohol consumption in rats undergoing acute or protracted substance withdrawal [Valdez et al., 2002]. Similar results were observed in rats self-administering heroin and cocaine [Goeders and Guerin, 1996, Valdez et al., 2002, Specio et al., 2008].

### 1.3.2 The CRF system and drug-induced reward

The involvement of the CRF system in the rewarding effects of drugs of abuse has been poorly investigated, as compared to, for instance, its role in the stress-induced reinstatement of drug-seeking and drug-taking behavior. However, some studies employing conditioned place preference (CPP) procedures have provided initial evidence in favor of a role for the CRF system in the rewarding effects of cocaine [Lu et al., 2003, Contarino et al., 2017, Kreibich et al., 2009]. Indeed, intracerebroventricular (i.c.v.) infusion of a non-selective CRF receptors antagonist or of a CRF<sub>1</sub> receptor-preferring antagonist (i.e.,  $\alpha$ -helical CRF<sub>9-41</sub> or CP-154,526) prior to cocaine conditioning sessions blocked the acquisition of drug-induced CPP in rats [Lu et al., 2003]. Moreover, mice exposed to 6-min daily sessions of forced swim dur-

ing a week displayed potentiated cocaine-induced CPP that was blocked by administration of a CRF<sub>1</sub> receptor-preferring antagonist (i.e., antalarmin) immediately before each forced swim stress [Kreibich et al., 2009]. More recently, CRF<sub>1</sub> receptor-deficiency was shown to decrease the dose of cocaine needed to produce CPP [Contarino et al., 2017]. Moreover, in the same study, CRF<sub>1</sub> receptor-deficiency exacerbated stereotypy produced by relatively high cocaine doses, an effect that seemed to be independent from the HPA axis activity [Contarino et al., 2017]. With regard to the latter point, previous studies showed that the drastic reduction in corticosterone synthesis and levels by adrenalectomy does not affect CPP responses to cocaine in rats [Russo et al., 2003, Suzuki et al., 1995]. Finally, cocaine-induced CPP was inhibited by a CRF<sub>1</sub> receptor-preferring antagonist (i.e., antalarmin) in two different studies [Lu et al., 2003, Contarino et al., 2017]. Even more recently, it has been reported that CRF<sub>1</sub> receptor-preferring antagonism (i.e., CP-154,526) blocks the enhanced response to cocaine after social stress, as assessed by another CPP procedure [Ferrer-Perez et al., 2018]. Altogether, the above-mentioned results indicate a critical role for the CRF<sub>1</sub> receptor pathway in cocaine reward, independently from the closely related HPA axis activity.

### 1.3.3 The CRF system and withdrawal from drugs of abuse

While hypothalamic CRF may play a role in the very onset of drug self-administration, extrahypothalamic sources of CRF, particularly within the extended amygdala and other limbic structures, might be implicated in the negative reinforcement mechanisms associated with later stages of drug dependence [Koob, 2010]. Multiple lines of evidence have demonstrated that, while acute drug exposure yields a transient elevation of CRF expression in multiple brain regions [Zhou et al., 1996, Maj et al., 2003], chronic drug exposure results in an overactivation of the CRF system, which is central to the drug dependence and withdrawal phenotypes observed upon cessation of drug intake [Zorrilla et al., 2001, Zhou et al., 2003, Sommer et al., 2008, George et al., 2007, Caberlotto et al., 2004]. During the progression of drug dependence, drug exposure disrupts the HPA axis negative feedback mechanisms [Zhou et al., 1996], resulting in a blunted response [Koob and Kreek, 2007]. However, when the drug is subsequently withdrawn, CRF release in the extended amygdala increases, accompanied by somatic and psychological withdrawal signs [Richter and Weiss, 1999, Olive et al., 2002, Merlo Pich et al., 1995]. Particularly striking across multiple drugs of abuse is the elevation of CRF in the central nucleus of the amygdala (CeA) at various withdrawal time points, which could be observed in rats not only when assessing levels of messenger RNA (mRNA) expression [Caberlotto et al., 2004, Maj et al., 2003, Sommer et al., 2008] and protein content [Zorrilla et al., 2001], but also as an elevation of CRF released into the extracellular space [Rodriguez de Fonseca et al., 1997, Richter and Weiss, 1999, George et al., 2007]. Similar to the CeA, increased CRF release has also been observed in the lateral bed nucleus of the stria terminalis (BNST) during alcohol withdrawal in rats [Olive et al., 2002], suggesting an elevated

activation of CRF signaling throughout the whole structure of the extended amygdala. Some of the changes observed in CRF mRNA and peptide levels in pre-clinical studies are summarized in Table 1.4. The increase in CRF-like immunoreactivity observed in the amygdala 6 weeks after withdrawal from chronic alcohol or cocaine administration has been proposed to underlie the elevated anxiety-like behavior characteristic of advanced stages of addiction [Zorrilla et al., 2001]. In this light, administration of CRF receptor antagonists during multiple alcohol withdrawal periods had a long-lasting ability to prevent stress-induced anxiety-like behavior in rats [Breese et al., 2005b]. Moreover, elevated anxiety-like behavior and alcohol self-administration observed in rats after 4 or 6 weeks of withdrawal were reversed by a non-selective CRF receptors antagonist (i.e., D-Phe CRF<sub>12-41</sub>) [Valdez et al., 2002, 2003]. Relatively to the withdrawal-induced elevations of drug intake, data from CRF receptor knock-out mice support a prominent role for the CRF<sub>1</sub> receptor. Indeed, deletion of the CRF<sub>1</sub> receptor gene abolished drug dependence-induced elevations in the self-administration of alcohol [Chu et al., 2007] and opiate withdrawal-induced conditioned place aversion [Contarino and Papaleo, 2005]. However, CRF<sub>2</sub> receptor-deficient mice also showed no conditioned aversion to the environmental cues associated with both spontaneous and naloxone-precipitated opiate withdrawal, when compared to wild-type mice [Ingallinesi et al., 2012]. The latter results suggest the involvement of both CRF receptors in mediating dysphoria-like states. Similarly, the somatic signs associated with the withdrawal syndrome seem to involve both receptor subtypes as well. Specifically, CRF<sub>2</sub> receptors may regulate the effects of opiate withdrawal, which are largely absent in CRF<sub>2</sub> receptor knock-out mice [Papaleo et al., 2008b]. Accordingly, deletion of CRF<sub>1</sub> receptors increased the somatic signs of opiate withdrawal [Papaleo et al., 2007].

Table 1.4 Alterations in corticotropin-releasing factor (CRF) messenger RNA (mRNA) expression and protein levels throughout the various stages of drug dependence following exposure to cocaine, morphine or alcohol. Abbreviations: bed nucleus of stria terminalis (BNST); central nucleus of amygdala (CeA); hippocampus (Hip); hypothalamus (Hyp); paraventricular nucleus of hypothalamus (PVN); increase (↑); decrease (↓).

Stage	CRF	Cocaine	Morphine	Alcohol
Acute drug intake	mRNA	↑ PVN, CeA	↑ CeA	↑ PVN
	peptide	↓ Hyp, Hip	↑ Hyp, BNST	
Chronic drug intake	mRNA	↓ Hyp		↑ PVN
	peptide		↑ CeA, Hyp, ↓ BNST	↓ Hyp
Drug withdrawal	mRNA	↑ PVN	↑ PVN, CeA	↑ PVN
	peptide	↓ Hyp, CeA		↑ CeA

### 1.3.4 The CRF system and reinstatement of drug-seeking

Drug dependence is characterized by high rates of relapse even following prolonged periods of drug abstinence [Koob and Le Moal, 2001], which is of-

ten provoked by exposure to stressors [Sinha, 2001]. In laboratory animals, after acquisition and subsequent extinction of drug self-administration or conditioned place preference (CPP), reinstatement of drug-seeking can be induced by (1) re-exposure to a stimulus previously paired with the drug (cue-induced reinstatement), (2) priming injection of the drug (drug-induced reinstatement) and (3) exposure to a stressful event (e.g., intermittent unpredictable footshock, immobilization, food deprivation, social defeat, swim stress) or administration of pharmacological agents (e.g., yohimbine) [Erb et al., 1996, Kreibich et al., 2009, Shaham and Stewart, 1994, Shalev et al., 2000]. Initial evidence of the involvement of the CRF system in such phenomenon came from the observation that i.c.v. injections of a non-selective CRF receptors antagonist (i.e.,  $\alpha$ -helical CRF<sub>9-41</sub>) decreased footshock-induced reinstatement of heroin seeking in rats [Shaham et al., 1997]. Subsequently, several studies reported that i.c.v. injections of a similar non-selective CRF receptors antagonist (i.e., D-Phe CRF<sub>12-41</sub>) decreased footshock-induced reinstatement of drug-seeking in animals exposed to cocaine [Erb et al., 1998], alcohol [Le et al., 2000] or nicotine [Zislis et al., 2007]. The fact that systemic injections of a CRF<sub>1</sub> receptor-preferring antagonist (i.e., CP-154,526) were also able to decrease footshock-induced reinstatement of heroin-, cocaine- [Shaham et al., 1998] and ethanol-seeking [Le et al., 2000] suggested that these effects were mediated by the CRF<sub>1</sub> receptor. Moreover, CRF<sub>1</sub> receptor-preferring antagonism (i.e., CP-154,526) reversed cue-induced reinstatement of cocaine-seeking and attenuated cue- and drug priming-induced reinstatement of amphetamine-seeking in rats [Moffett and Goeders, 2007, Goeders and Clampitt, 2002], strengthening the idea that reinstatement of drug-seeking behavior is mainly a CRF<sub>1</sub> receptor-mediated process. In this light, i.c.v. injections of a CRF<sub>1</sub> receptor-preferring antagonist (i.e., R278995), but not of a CRF<sub>2</sub> receptor-preferring antagonist (i.e., astressin-2B), were able to decrease footshock-induced reinstatement of nicotine-seeking in rats [Bruijnzeel et al., 2009]. Footshock-induced morphine or cocaine CPP “reactivation” (i.e., a previously learned CPP that is no longer expressed and that is restored by exposure to a stressor) was decreased in rats by i.c.v. injection of a non-selective CRF receptor antagonist (i.e.,  $\alpha$ -helical CRF<sub>9-41</sub>), but not of a CRF<sub>2</sub> receptor-preferring antagonist (i.e., antisauvagine-30) [Lu et al., 2000, 2001]. Moreover, i.c.v. injection of a non-selective CRF receptor antagonist (i.e.,  $\alpha$ -helical CRF<sub>9-41</sub>) decreased food deprivation-induced reinstatement of heroin-seeking in rats [Shalev et al., 2006] and systemic administration of a CRF<sub>1</sub> receptor-preferring antagonist (i.e., antalarmin) decreased yohimbine-induced reinstatement of alcohol-seeking in rats [Ghitza et al., 2006, Marinelli et al., 2007]. Finally, additional evidence in favor of a role for extra-hypothalamic CRF in stress-induced reinstatement of drug-seeking came from the fact that i.c.v. injections of CRF reinstated heroin- [Shaham et al., 1997], cocaine- [Brown et al., 2009] and alcohol-seeking [Le et al., 2000] in rats. Overall, these findings point out to a critical role for the CRF system in stress- and cue-induced reinstatement of drug-seeking.

### 1.3.5 The CRF system: potential therapeutic target?

As previously highlighted, in terms of neurobiological consequences, neuropsychiatric disorders (e.g., autism, schizophrenia, depression, anxiety) and substance use disorders share a high level of comorbidity. In this light, the therapeutic potential of CRF receptor-specific antagonists relies in their long known antidepressant and anxiolytic activity [Bremner et al., 1997, Nemeroff et al., 1984, Zorrilla et al., 2013a]. Among all therapeutic candidates, CRF<sub>1</sub> receptor-preferring antagonists have long been considered by many to possess the strongest preclinical evidence, possibly due to their higher selectivity *in vitro*, as compared to CRF<sub>2</sub> receptor-preferring antagonists (Table 1.5).

Table 1.5 Antagonists of CRF receptors commonly used in animal models of drug dependence. Relative specificity for each CRF receptor subtype measured *in vitro* and expressed as inhibitor constant values ( $K_i$ ) is reported.

Compound	Activity	CRF <sub>1</sub> vs. CRF <sub>2</sub>
$\alpha$ -helical CRF <sub>9-41</sub>	non-selective CRF receptors antagonist	$K_i=35$ vs. 11nM
D-Phe CRF <sub>12-41</sub>	non-selective CRF receptors antagonist	$K_i=20$ vs. 50nM
antalarmin	CRF <sub>1</sub> receptor-preferring antagonist	$K_i=1.0$ vs. >10000nM
CP-154,526	CRF <sub>1</sub> receptor-preferring antagonist	$K_i=0.44$ vs. >10000nM
CP 376395	CRF <sub>1</sub> receptor-preferring antagonist	$K_i=12$ vs. >10000nM
LWH-63	CRF <sub>1</sub> receptor-preferring antagonist	$K_i=0.68-0.7$ at CRF <sub>1</sub>
MJL-1-109-2	CRF <sub>1</sub> receptor-preferring antagonist	$K_i=1.9$ at CRF <sub>1</sub>
MTIP	CRF <sub>1</sub> receptor-preferring antagonist	$K_i=0.22$ vs. >1000nM
NBI 35965	CRF <sub>1</sub> receptor-preferring antagonist	$K_i=4$ vs. >10000nM
R121919	CRF <sub>1</sub> receptor-preferring antagonist	$K_i=0.24$ vs. >1000nM
R278995	CRF <sub>1</sub> receptor-preferring antagonist	$K_i=53.2$ vs. >10000nM
antisauvagine-30	CRF <sub>2</sub> receptor-preferring antagonist	$K_i>100$ vs. 0.29nM
astressin-2B	CRF <sub>2</sub> receptor-preferring antagonist	$K_i>10000$ vs. 0.49nM

In the past two decades, the effects of the manipulation of the CRF system on critical features of drug dependence have been extensively studied (Table 1.6). Relatively to the symptoms of drug withdrawal, VTA-specific CRF<sub>1</sub> receptor-preferring pharmacological antagonism (i.e., antalarmin), as well as optogenetic silencing, have been shown to significantly alleviate nicotine withdrawal-induced anxiety in mice [Zhao-Shea et al., 2015]. Relatively to the stress-induced reinstatement of drug-seeking and drug-taking, systemic administration of various brain penetrant CRF<sub>1</sub> receptor-preferring antagonists consistently blocked relapse-like behavior triggered by stressors in a number of studies [Heilig and Koob, 2007, Le and Shaham, 2002, Mantsch et al., 2016, Zorrilla et al., 2013a]. For instance, knocking down CRF<sub>1</sub> receptors in the VTA with short hairpin RNAs blocked the food deprivation-induced reinstatement of cocaine-seeking in mice [Chen et al., 2014]. In a similar study, but using a different kind of stressor, administration of a CRF<sub>1</sub> receptor-preferring antagonist (i.e., CP-154,526) prior to a physical stressor (i.e., footshock) significantly attenuated the reinstatement of drug-seeking behavior in rats with



a history of cocaine or heroin self-administration [Shaham et al., 1998]. Intra-VTA CRF<sub>1</sub> (i.e., CP 376395) or CRF<sub>2</sub> receptor-preferring antagonism (i.e., astressin-2B) during social defeat prevented the subsequent escalation of cocaine self-administration in rats [Holly et al., 2016]. Moreover, intra-VTA CRF<sub>1</sub> receptor-preferring antagonism (i.e., NBI 35965) paired with simultaneous stimulation of CRF<sub>2</sub> receptors (i.e., urocortin 3) significantly reduced binge-like ethanol consumption in mice [Rinker et al., 2017]. Acute treatment with a nonpeptide CRF<sub>1</sub> receptor-preferring antagonist (i.e., LWH-63) inhibited ethanol self-administration in a subset of rats that responded to acute withdrawal from chronic alcohol vapor by increasing their alcohol intake [Sabino et al., 2006]. A CRF<sub>1</sub> receptor-preferring antagonist (i.e., CP 376395) administered peripherally or into the VTA reduced the escalation of cocaine self-administration after social defeat [Boyson et al., 2014]. Relevantly to reward, it has been reported that CRF<sub>1</sub> receptor-preferring antagonism (i.e., antalarmin and CP-154,526) inhibits the rewarding effects of cocaine [Lu et al., 2003, Contarino et al., 2017] and blocks the enhanced response to the drug after social stress, as assessed by CPP procedures [Ferrer-Perez et al., 2018].

Table 1.6 Effects of manipulation of the CRF system on critical features of drug dependence. Abbreviations: drug reward (Rwd); somatic signs of withdrawal (Smt); negative affective-like states (Naf); reinstatement of drug-seeking (Rst); urocortin 3 (UCN3); increase (↑); decrease (↓); not tested (NT).

Manipulation		Rwd	Smt	Naf	Rst
Synthesis of CRF-like peptides	CRF	NT	NT	↑	↑
	UCN3	↓	NT	↓	NT
Pharmacological blockade of CRF receptors	CRF <sub>1-2</sub>	↓	↓	↓	↓
Genetic disruption of CRF receptors	CRF <sub>1</sub>	↑	↑	↓	NT
	CRF <sub>2</sub>	NT	↓	↓	↓

Unfortunately, clinical translation of such pre-clinical results has not been achieved yet. The first CRF<sub>1</sub> receptor-preferring antagonist evaluated in humans (i.e., R121919) showed promising results relatively to depression-like symptoms [Zobel et al., 2000], but was followed by a series of studies yielding negative results in depression [Binneman et al., 2008], anxiety [Coric et al., 2010] and attention deficit [Kwako et al., 2015] disorders. Given the large body of pre-clinical research pointing out to the involvement of CRF<sub>1</sub> receptor-mediated signalling in the psychopathology of drug dependence, the limited therapeutic success achieved by these studies is somehow surprising. Various reasons may account for this. First of all, the dosage of CRF<sub>1</sub> receptor-preferring antagonist needed to obtain sufficient receptor occupancy remains unclear. Accurately determining the dose would require a specific CRF<sub>1</sub> ligand that is suitable for positron emission tomography (PET). Radioligands with PET imaging potential have been developed, but are still being characterized [Lodge et al., 2014]. Furthermore, since higher binding affinity is associated with increased antagonist efficacy, the dissociation kinetics of antagonists may be important for pre-clinical and clinical efficacy [Zorrilla et al., 2013a]. How-

ever, to conclude on a positive note, we are the first to our knowledge reporting a strong behavioral effect of CRF<sub>1</sub> receptor-preferring antagonism (i.e., antalarmin) on the acute effects of initial substance administration, a result that suggests a whole new therapeutic value for these pharmacological agents.



## AIM OF THE CURRENT WORK

Drug use, abuse and dependence are major health and socio-economic issues, characterized by profound social and motivational deficits that are believed to strongly contribute to the establishment and maintenance of compulsive drug-seeking and drug-taking, making abstinence difficult to sustain and relapse more likely to happen [Le Moal and Koob, 2007, Volkow et al., 2011]. However, the specific role of the CRF system in mediating such social and motivational alterations remains unknown.

Thus, by employing the three-chamber (3-CH) social task [Moy et al., 2004], a food-driven operant behavior paradigm, a single-exposure conditioned place preference (CPP) procedure, CRF<sub>1</sub> receptor-deficient mutant mice and the CRF<sub>1</sub> receptor-preferring antagonist antalarmin, we aimed at investigating:

- 1 the impact of chronic morphine administration and withdrawal upon social behavior in a time-course fashion (Article 1);
- 2 the impact of a mild environmental stressor upon social behavior following long-term opiate withdrawal (Article 1);
- 3 the role of the CRF<sub>1</sub> receptor in the social behavior alterations induced by chronic morphine administration and withdrawal (Article 2);
- 4 the impact of acute morphine administration on “natural” rewards, such as social interaction and food intake (Article 3);
- 5 the role of the CRF system in the behavioral alterations induced by acute morphine administration (Article 3).



## **Increased social interest and aggressive behavior in opiate-withdrawn mice.**

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## **2.1 Abstract**

### **2.1.1 Background and purpose**

In humans, decreased social interest is a major clinical feature of opiate use disorders (OUDs). However, no animal studies modeling the timeline of drug-induced social behavior deficits are available to date. In the present study, we assessed the short- and long-term effects of spontaneous opiate withdrawal upon social interest. In addition, we investigated whether exposure to an ethological environmental stressor affects social behavior after a relatively long drug-free period.

### **2.1.2 Experimental approach**

Two experiments were carried out. In the first experiment, mice were repeatedly tested in the three-chamber (3-CH) task 7, 21, 35 and 49 days following the interruption of chronic morphine administration. In the second experiment, after being exposed to a mild environmental stressor, mice were tested in the 3-CH task only once, 49 days following the interruption of chronic morphine administration. The 3-CH task allowed the investigation of social interest and discrimination abilities. Follow-up ethological measurements of aggressive behavior were also taken.

### **2.1.3 Key results**

Morphine withdrawal increased social interest towards an unfamiliar conspecific. However, ethological analysis also revealed increased aggressive behavior in opiate-withdrawn mice. Notably, social interest and aggressive behavior followed a similar time-course and positively correlated with one another. Moreover, following exposure to an environmental stressor, neither control nor long-term opiate-withdrawn mice displayed impaired social interest, indicating stress resilience.

### **2.1.4 Conclusions and implications**

The present findings demonstrate that spontaneous withdrawal from chronic morphine administration boosts social interest in mice tested in the 3-CH paradigm, as compared to saline-treated control mice. We herein suggest that this phenomenon is due to increased aggressive behavior, an unprecedented result that contributes to the clarification of the complex symptomatology of OUDs, suggesting a possible role for aggressiveness in social behavior dysfunctions. Moreover, we show that performances in the 3-CH paradigm are completely unaffected by exposure to a mild environmental stressor following chronic morphine treatment and spontaneous prolonged withdrawal, thereby providing the first experimental evidence of resilience to stress in opiate-withdrawn mice.

## 2.2 Introduction

Opiate use disorders (OUDs) are chronic relapsing diseases with a major health and socio-economic impact. Indeed, worldwide prevalence rates of OUDs range between 0.5-2% of the population aged 15–64 ([www.who.int](http://www.who.int)). Moreover, opiate substances account for about 76% of the deaths related to substances of abuse, highlighting the particularly harmful nature of these drugs ([www.unodc.org/wdr2018](http://www.unodc.org/wdr2018)). Studies also show an alarming rise in the recreational use of prescription analgesic opiates (e.g., morphine, fentanyl, oxycodone) among adolescents, indicating that the incidence rate of OUDs may dramatically increase in the next few years [EMCDDA, 2017]. OUDs are characterized by a myriad of somatic and non-somatic symptoms, such as severe influenza-like signs, negative affective-like states, altered motivational processes, impaired social behavior and a long-lasting vulnerability to stressors [APA, 2013, Preller et al., 2014a, Preston and Epstein, 2011, Sinha, 2001]. Currently, OUDs are treated mainly by substitutive opioid receptor agonists, such as methadone and buprenorphine [Nutt and Lingford-Hughes, 2008]. However, opiate “therapy” also induces dependence [EMCDDA, 2017]. Novel treatments for OUDs are thus urgently needed and their development heavily relies on a better understanding of these diseases.

Studies suggest that substance-induced deficits in social behavior, such as artificial sociability, antisocial traits and social isolation, strongly contribute to the establishment and maintenance of compulsive drug-seeking and drug-taking, making abstinence difficult to sustain and relapse more likely to happen [Babor et al., 1976, Le Moal and Koob, 2007, Volkow et al., 2011]. Nevertheless, the neural mechanisms underlying disruption of social behavior by substances of abuse remain poorly understood. In order to study the deficits in social behavior induced by opiate administration and withdrawal and related brain alterations, various laboratory animal models have been developed. In particular, studies using the social interaction (SI) [File and Hyde, 1978] or the three-chamber (3-CH) test [Moy et al., 2004] revealed decreased social investigation in morphine- and heroin-withdrawn rats and mice, which could be observed up to 49 days after discontinuation of opiate administration [Becker et al., 2016, Goeldner et al., 2011, Lalanne et al., 2017, Lutz et al., 2014, Zanos et al., 2014]. However, studies also reported enhanced social interaction in adult mice following morphine exposure during adolescence [Lutz et al., 2013], thereby highlighting the need for further investigations.

On the other hand, extensive evidence has been linking opiate withdrawal to increased aggressive behavior in rodents. Indeed, 48 hours after the removal of a subcutaneous morphine pellet [Kantak and Miczek, 1986, Tidey and Miczek, 1992] or cessation of chronic morphine treatment [Rodriguez-Arias et al., 1999], mice undergoing spontaneous opiate withdrawal exhibited heightened aggressive behavior (i.e., threats and attacks) in the resident-intruder paradigm, as compared to controls. Dependent rats undergoing spontaneous morphine withdrawal also displayed increased aggressive behavior 65-72 hours after the last drug administration [Lal et al., 1971]. Moreover, mice chronically administered with morphine displayed increased aggressive behavior up



to at least 15 days following naloxone-precipitated withdrawal [Felip et al., 2000]. Accordingly, human studies also report on the adverse social consequences of opiate use and withdrawal. Indeed, increased aggressive behavior, irritability and social isolation have been observed in dependent subjects following long-term self-administration of heroin and during periods of opiate abstinence [Babor et al., 1976, Gerra et al., 2004a,b].

In the present study, to assess the short- and long-term effects of spontaneous opiate withdrawal upon social behavior, we treated C57BL/6 male mice with increasing doses of morphine and repeatedly tested them in the 3-CH task throughout a relatively long period of time following discontinuation of substance administration. In addition, we investigated the effect of a relatively long opiate withdrawal period upon exposure to an ethological environmental stressor. We employed the 3-CH paradigm since it is believed to provide automated measures of social interest (i.e., sociability, or preference for an unfamiliar conspecific *versus* an unanimated object) and discrimination abilities (i.e., social novelty preference, or preference for a novel *versus* a familiar conspecific) that, differently from the SI paradigm, are independent from anxiety-like states or locomotor activity [Moy et al., 2013, Silverman et al., 2010]. Considering that opiate withdrawal has long been associated with increased aggressive behavior, in order to discern aggressiveness from harmless social interest [Kaidanovich-Beilin et al., 2011], throughout the various 3-CH tests we also examined the expression of aggressive behavior, as assessed by the number of bites and the latency to first bite. The latter approach allowed us to control for the expression of aggressive behavior in short- and long-term opiate-withdrawn mice, as well as for its correlation with social interest.

## 2.3 Materials and methods

### 2.3.1 Subjects

C57BL/6 male mice were purchased from Janvier Labs. The colony room ( $22\pm 2$  °C, relative humidity: 50–60%) was maintained on a 12 h light/dark cycle (lights on at 08h00). Mice were housed in groups of 4 in polycarbonate cages (29.5 x 11.5 x 13 cm; L x W x H) containing bedding and a cotton nestlet (SAFE, Augy, France). They had *ad libitum* access to standard laboratory food (3.3 kcal/g; SAFE, Augy, France) and fresh water. Mice were 4 weeks old upon arrival and 10-16 weeks old at the beginning of the experiments, with a body weight of 22-30 g ( $25\pm 0.2$  g; mean  $\pm$  S.E.M.). Animals were daily monitored for the presence of adverse effects of the experimental treatment and veterinary advice was sought if they displayed signs of distress. All studies were conducted in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and approved by the local Animal Care and Use Committee. The present study complied with the ARRIVE Guidelines [Kilkenny et al., 2010] and the BJP standard guidance for studies using animals [Curtis et al., 2015, McGrath and Lilley, 2015].

### 2.3.2 Three-chamber (3-CH) apparatus

The 3-CH apparatus was a rectangular box (60 x 40 x 20 cm, L x W x H) divided in 3 equal chambers and made of dark Plexiglas. Dividing transparent Plexiglas 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, 14 cm high with bars spaced 1 cm apart) in which a living mouse or an unanimated object could be placed.

### 2.3.3 3-CH testing protocol

Prior to the beginning of the experiments, animals were handled (1 min/day) during 3 consecutive days. The behavioral testing was conducted during the light phase of the 12 h light/dark cycle in a quiet dedicated room dimly illuminated (10 lux). The 3-CH task allowed the study of 1) sociability (i.e., preference for an unfamiliar conspecific *versus* an unanimated object) and 2) social novelty preference (i.e., SNP, preference for a novel *versus* a familiar conspecific). To reduce the number of animals used, mice were repeatedly tested in the 3-CH task at the various drug withdrawal time points (Experiment 1). Due to the limited number of animals that could be tested daily, behavioral studies investigating the effect of a relatively long opiate withdrawal period upon exposure to an ethological environmental stressor (Experiment 2) were identically carried out using 4 independent cohorts of mice. No statistical differences were found between the independent experiments and therefore the results were pooled. Within each animal cohort and across testing days, all the experimental conditions (i.e., treatment, stress) were pseudo-randomized. During the pre-habituation phase, the subject mouse was confined to the cen-

tral chamber for 5 min; then, the doors were opened and he could freely explore the 3 chambers and the empty wire cages for 10 min (habituation phase). During the subsequent 10 min sociability phase, the subject mouse was allowed to explore the entire apparatus with one wire cage containing an unfamiliar conspecific and the other an unanimated object. Ultimately, a 10 min SNP test was carried out, during which a novel unfamiliar conspecific was placed in the wire cage that previously contained the unanimated object, so that the subject mouse could choose between an already investigated familiar and a novel mouse. The unfamiliar conspecifics were substance-naïve C57BL/6 male mice age-matched with the subject animals, handled (1 min/day) and habituated to the wire cages (10 min/day) over the 3 days preceding the first 3-CH test. The position of the unfamiliar conspecific (i.e., left or right side chamber) was counterbalanced within each experimental group. Between each test, the apparatus was cleaned with water and the wire cages with 70% ethanol and water. The experiments were recorded on a video camera placed on top of the apparatus and measures obtained from a previously validated homemade video tracking software. In particular, the time spent by the subject mouse exploring the upper half part of the side chambers containing the wire cages (i.e., all 4 paws inside the half-chamber) was considered as a measure to assess his social interest and discrimination abilities. Moreover, to control for sedation or hyperactivity [Moy et al., 2013], the total distance travelled during each of the experimental phases (habituation, sociability and SNP) was recorded.

### 2.3.4 Morphine administration paradigm

Mice received intraperitoneal (i.p.) injections (10 ml/kg) of physiological saline or morphine hydrochloride (Francopia, Gentilly, France) every 12 h (08h00 - 20h00) for 6 consecutive days, as follows: day 1: 20 mg/kg; day 2: 40 mg/kg; day 3: 60 mg/kg; day 4: 80 mg/kg; day 5: 100 mg/kg; day 6: 100 mg/kg, only one injection in the morning. The latter treatment was chosen because it produced cognitive deficits and vulnerability to the same ethological environmental stressor employed herein in previous studies [Morisot and Contarino, 2016, Morisot et al., 2015]. Mice were weighed immediately before each injection and body weight changes calculated as percentage of the body weight recorded just prior to the first injection.

### 2.3.5 Experimental planning

Two different experiments were carried out. In Experiment 1, mice were repeatedly tested in the 3-CH task 7, 21, 35 and 49 days after the last injection (Figure 2.6A). In Experiment 2, mice were tested in the 3-CH task only once, 49 days after the last injection (Figure 2.6B). To assess vulnerability to stress, within each experimental group, approximately half of the mice were exposed to an elevated platform stressor (EPS) for 10 min and tested in the 3-CH task 1 h later; the other half of subject animals remained undisturbed in their home-cages (i.e., non-stressed, NS). The EPS was a square (10 x 10 cm) made of dark grey polypropylene and situated 40 cm above the floor. The stressing

procedure was carried out in a separate room under direct lighting of the animal (70 lux). Throughout the testing procedure, to control for the ability to detect social stimuli without loading on cognitive function, no inter-trial interval (ITI) was applied between the sociability and the SNP phases [Millan and Bales, 2013, Silverman et al., 2010].

### 2.3.6 Quantification of aggressive biting behavior (ABB)

In order to control for the expression of aggressive behavior, an observer blind to the experimental groups was trained to quantify the ABB occurring during the sociability phase for Experiment 1. Considered the physical characteristics of the apparatus, bites were distinguished into 3 different categories: (1) direct bites to the animal (e.g. biting the tail of the unfamiliar conspecific when it is sticking out of the cage); (2) focused bites to the animal's wire cage (e.g. biting the bars, the basement or the roof of the cage trying to directly reach the unfamiliar conspecific); (3) unfocused bites to the wire cage containing the unanimated object. In addition, the latency to first bite was evaluated, reflecting the time in between the opening of the doors and the first biting episode directed towards either the unfamiliar conspecific or its cage.

### 2.3.7 Statistical analysis

Each mouse was assigned a unique identification number that was used to conduct blind testing and data analysis. To prevent initial side preferences from biasing the results, only animals exploring the two half-chambers containing the wire cages for at least 60 cumulative sec and within a 20-80% ratio during the habituation phase were included in the study (inclusion criteria). For Experiment 1, a three-way analysis of variance (ANOVA) with Treatment (saline *versus* morphine) as a between-subjects factor and Side (either animal *versus* object or familiar *versus* novel) and Phase (either habituation *versus* sociability or sociability *versus* SNP) as within-subjects factors was used. For Experiment 2, a four-way ANOVA with Treatment (saline *versus* morphine) and Stress (non-stressed *versus* EPS-stressed) as between-subjects factors and Side (either animal *versus* object or familiar *versus* novel) and Phase (either habituation *versus* sociability or sociability *versus* SNP) as within-subjects factors was used. A two-way ANOVA was used to analyze body weight changes, with Treatment (saline *versus* morphine) as a between-subjects factor and Days as a within-subjects factor. Within treatments, the numbers of bites were compared using paired t-tests. Linear regression analysis were performed to explore putative correlations between the number of direct + focused bites (X axis) and the time spent with the unfamiliar conspecific during the sociability phase (Y axis). The accepted value for significance was  $P < 0.05$ . If main or interaction effects were significant, 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.

## 2.4 Results

### 2.4.1 Opiate withdrawal increases social interest

The intermittent administration of escalating doses of morphine induced a body weight loss (Treatment X Day effect:  $F_{4,88}=21$ ,  $P<0.0001$ ) that was evident starting 48 hours after the beginning of the treatment, before the third drug injection ( $P<0.001$ ; Figure 2.7A). However, upon OWD 7, saline- and opiate-withdrawn mice displayed similar body weight changes, indicating body weight recovery ( $P=0.10$ ; Figure 2.7A). Analysis of sociability measures on OWD 7 revealed a Phase X Side interaction effect ( $P<0.0001$ ; Table 1), but no Phase X Side X Treatment interaction effect ( $P=0.12$ ; Table 1). Indeed, during the sociability phase, saline- and morphine-withdrawn mice spent more time in the half-chamber containing the mouse, as compared to both the previous phase ( $P<0.001$ ; Figure 2.8A) and the opposite half-chamber containing the object ( $P<0.001$ ; Figure 2.8A), indicating that relatively early opiate withdrawal does not affect social behavior in mice. Analysis of sociability measures on OWD 21, however, revealed a Phase X Side X Treatment significant interaction effect ( $P<0.05$ ; Table 1). Indeed, during the sociability phase, saline-treated mice spent a similar amount of time in the half-chamber containing the mouse, as compared to both the previous phase ( $P=0.19$ ; Figure 2.8B) or the opposite half-chamber containing the object ( $P=0.15$ ; Figure 2.8B). The latter result indicates loss of social interest upon re-testing in saline-treated mice tested in the 3-CH task. In contrast, morphine-withdrawn mice still spent more time in the half-chamber containing the mouse, as compared to both the habituation phase ( $P<0.05$ ; Figure 2.8B) and the opposite half-chamber containing the object ( $P<0.001$ ; Figure 2.8B). This result indicates that opiate withdrawal increases the interest for an unfamiliar conspecific in morphine-withdrawn mice, as compared to saline-treated control mice. Finally, no Phase X Side ( $P=0.79$ ,  $P=0.26$ ; Table 1) or Phase X Side X Treatment ( $P=0.14$ ,  $P=0.47$ ; Table 1) interaction effects were observed on OWD 35 or 49, respectively, indicating loss of social interest also in opiate-withdrawn mice (Fig. 2.9A and 2.9B).

### 2.4.2 Opiate withdrawal increases aggressive behavior

On OWD 7, saline- and morphine-withdrawn mice showed similar levels of direct bites ( $P=0.13$ ), focused bites ( $P=0.28$ ), unfocused bites ( $P=0.42$ ) and latency to first attack ( $P=0.93$ ), indicating that relatively early opiate withdrawal phases do not affect aggressive behavior (Figure 2.8C). However, on OWD 21, morphine-withdrawn mice exhibited more direct bites than saline-withdrawn mice ( $P<0.05$ ; Figure 2.8D). Although not significant, morphine-withdrawn mice also showed more focused bites, as compared to saline-treated mice ( $P=0.06$ ; Figure 2.8D). On the other hand, the two groups did not differ in the number of unfocused bites ( $P=0.54$ ; Figure 2.8D) or latency to first attack ( $P=0.95$ ; Figure 2.8D). Interestingly, the number of bites directed towards the unfamiliar conspecific (direct + focused bites) positively correlated

with the time spent in the half-chamber containing the social stimulus during the sociability tests carried out on OWD 7 ( $r=0.76$ ,  $P<0.05$ ; Figure 2.8E) and OWD 21 ( $r=0.86$ ,  $P<0.001$ ; Figure 2.8F). In contrast, the latter two measures did not correlate in saline-withdrawn mice, neither on OWD 7 ( $r=0.20$ ,  $P=0.53$ ; Fig. 2.8E) or OWD 21 ( $r=0.42$ ,  $p=0.17$ ; Figure 2.8F). The latter results indicate that the increase in social interest observed in opiate-withdrawn mice on OWD 21 might be driven by augmented aggressiveness and that some signs of aggressive behavior might be present already on OWD 7, when no opiate withdrawal effect is observed upon social interest. Finally, during both OWD 35 and 49, no differences in aggressive behavior were noted between experimental groups (Fig. 2.9C and 2.9D), indicating a transient nature for opiate withdrawal-induced aggressiveness.

### 2.4.3 Opiate withdrawal does not affect SNP

Independently from the experience of drug administration and withdrawal, SNP performances were unaffected throughout all 4 tests (Phase X Side X Treatment interaction effects: OWD 7:  $P=0.45$ , OWD 21:  $P=0.07$ , OWD 35:  $P=0.20$ , OWD 49:  $P=0.45$ ; Table 2; Figure 2.10). Indeed, both saline- and morphine-withdrawn mice spent more time in the half-chamber containing the novel mouse, as compared to the opposite half-chamber containing the familiar mouse (OWD 7:  $P<0.001$ , OWD 21:  $P<0.05$ , OWD 35:  $P<0.05$ , OWD 49:  $P<0.05$ ; Figure 2.10). However, only on OWD 7 and 21 both groups spent more time in the half-chamber containing the novel mouse compared to the previous sociability phase ( $P<0.05$  and  $P<0.05$ ; Figure 2.10A and B). The latter result suggests that repeated testing in the 3-CH apparatus might result in a progressive decline in SNP as well. Finally, opiate withdrawal did not affect locomotor activity (Table 4). Indeed, throughout OWD 7 to 49, saline- and morphine-withdrawn mice travelled similar distances during the 3 experimental phases of the 3-CH task (Figure 2.11), excluding a role for locomotor activity in the social behavior differences described herein.

### 2.4.4 Stress does not impair social behavior in long-term opiate-withdrawn mice

To investigate whether long-term opiate-withdrawal associates with stress-induced vulnerability of social behavior, mice were treated with saline or morphine and tested in the 3-CH task 49 days after the last administration; within treatment, half of the mice were exposed to an elevated platform stressor (EPS) 1 h prior being tested. The intermittent administration of escalating morphine doses induced a body weight loss (Treatment X Day effect:  $F_{4,256}=71$ ,  $P<0.0001$ ) that was evident starting 48 hours from the beginning of the treatment, after the third injection ( $P<0.0001$ ; Figure 2.7B). However, in opiate-treated mice body weight was fully recovered on OWD 49 (Figure 2.7B). Neither treatment nor stress affected sociability (Table 3). Indeed, during the sociability phase, saline- and morphine-withdrawn mice spent more time in the half-chamber containing the mouse, as compared to both the habitua-

tion phase ( $P < 0.001$ ; Figure 2.12) or the opposite half-chamber containing the object ( $P < 0.001$ ; Figure 2.12), independently from the exposure to the EPS. Likewise, neither treatment nor stress affected SNP (Table 3). Indeed, a Phase X Side interaction effect ( $P < 0.0001$ ) revealed that during the SNP phase saline- and morphine-withdrawn mice spent more time in the half-chamber containing the novel mouse, as compared to both the sociability phase ( $P < 0.0001$ ; Figure 2.13) or to the opposite half-chamber containing the familiar mouse ( $P < 0.0001$ ; Figure 2.13), independently of exposure to the EPS. The latter results indicate that long-term opiate withdrawal does not affect social behavior in mice and that it is not associated with vulnerability to environmental stressors. Notably, sociability and SNP scores of mice tested for the first time at OWD 49 (Experiment 2; Figure 2.13) were similar to those observed in mice tested for the first time at OWD 7 (Experiment 1; Figure 2.8A), suggesting that repeated testing in the 3-CH apparatus might lead to a progressive habituation response. Finally, analysis of locomotor activity measures revealed an effect of the treatment ( $P < 0.05$ ), but no Treatment X Stress effect ( $P = 0.61$ ). In particular, opiate-withdrawn mice travelled less distance than saline-withdrawn mice, independently from the test phase considered (Phase x Treatment interaction effect:  $P = 0.77$ ; Table 5). The fact that, despite traveling significantly different distances, the two experimental groups displayed identical social interest and social recognition abilities, highlights the independence of social behavior measures from locomotion in the 3-CH paradigm.

## 2.5 Discussion

In the present study, we show that C57BL/6 adult male mice experiencing withdrawal from chronic morphine administration displayed higher social interest towards an unfamiliar conspecific in the three-chamber (3-CH) paradigm, as compared to their saline counterparts. Moreover, we demonstrate that chronic morphine administration and withdrawal increased aggressive biting behavior (ABB), in parallel to the boosted interest towards an unknown congener exhibited by the same mice. Throughout, preference for social novelty was preserved and did not differ between saline- and morphine-withdrawn mice, indicating unaltered olfactory function and ability to detect and discern social stimuli. Furthermore, following a rather long 49 days substance withdrawal period, neither opiate withdrawal nor exposure to a relatively mild stressor impaired social behavior, indicating resilience to stress in morphine-withdrawn mice.

To assess the impact of morphine withdrawal upon sociability and social novelty preference, mice were repeatedly tested in the 3-CH paradigm, starting from 7 days after the last administration and up to 49 days of abstinence, with a time interval of 14 days between each test. Substance-naïve and morphine-treated mice spent a similar amount of time investigating an unfamiliar conspecific during the first behavioral test, 7 days after the last administration. The latter result is in apparent contrast with the only other study available employing the 3-CH task and showing reduced sociability and social novelty preference following 7 days of spontaneous morphine withdrawal in male mice [Zanos et al., 2014]. One could argue that different experimental conditions may underlie the discrepancies between the two studies, such as minor changes in the cumulative amount of drug administered (920 mg/kg in their study and 700 mg/kg in ours) or different housing conditions (individual housing in their study and standardized group housing in ours). Nevertheless, unpublished data from our laboratory (not shown) indicate that neither higher cumulative amounts of drug (i.e., 1200 mg/kg) nor individual housing lead to social impairments in the 3-CH task at opiate withdrawal day 7. Moreover, our result is in line with previous studies conducted in both morphine- [Goeldner et al., 2011] and heroin-withdrawn male mice [Lutz et al., 2014] and showing unaffected social interest in the social interaction (SI) test after 7 days of opiate abstinence. However, these studies also reported decreased SI in withdrawn mice 28 and 49 days following the last drug administration, whereas we herein show unaffected social interest in non-stressed animals tested for the first time 49 days following the last morphine administration. This discrepancy highlights once again the profound differences between the 3-CH and the SI paradigms. Indeed, while their measures of SI differ from our measures of social interest, they corroborate reports from our laboratory showing increased anxiety-like behavior during long-term opiate abstinence (i.e., 37 days after the last administration) [Morisot et al., 2015]. During the second test (i.e., first re-test), at opiate withdrawal day 21, morphine-treated mice displayed a significantly stronger interest for the unknown congener, as compared to substance-naïve controls. The latter result suggests heightened social interest in morphine-treated mice



experiencing opiate withdrawal. During the third and fourth test (i.e., second and third re-tests), at opiate withdrawal 35 and 49, both experimental groups exhibited a profound loss of social interest, spending a similar amount of time with the unknown congener and the unanimated object. Independently from the treatment received, social novelty preference was unaffected throughout the whole experiment, despite a slight progressive decrease of social interest towards the novel congener was noticeable as well.

To our knowledge, we are the first to point out that repeated testing in the 3-CH apparatus leads to a strong behavioral habituation response. The only other evidence available of this phenomenon comes from the very first paper introducing the 3-CH paradigm [Moy et al., 2004] and showing a decrease in the time spent with the unknown congener and an increase in locomotor activity following re-testing. In our case, however, the progressive decrease in the interest for the task is not mirrored by a locomotor sensitization.

Exposure to a mild environmental stressor (i.e., the elevated platform) 49 days following the last administration did not affect social behavior in neither morphine-treated nor substance-naïve mice. This result differs from what happens in humans, where stress exposure is considered a major risk factor for relapse to drug-intake in former addicted individuals [Sinha, 2001]. Accordingly, previous laboratory animal studies showed that physical, psychological or pharmacological stressors reinstate substance-seeking behaviors and induce negative affective-like states, even after relatively long periods of withdrawal [Morisot and Contarino, 2016, Morisot et al., 2014, 2018, 2015, Breese et al., 2005a, Blatchford et al., 2005, Shaham et al., 2003]. Specifically, the same stressor has been previously shown to induce the re-emergence of recognition memory deficits in mice spontaneously withdrawing from chronic morphine administration [Morisot and Contarino, 2016, Morisot et al., 2014, 2015] and of sociability deficits in cocaine abstinent mice [Morisot et al., 2018]. The fact that the same environmental stressor did not induce social impairments in opiate-withdrawn mice, as compared to cocaine abstinent ones, might be explained by the existence of different mechanisms underlying the incubation of psychostimulant and opiate craving [Pickens et al., 2011]. For instance, during cocaine withdrawal, a robust increase and persistence in drug-seeking behavior is noticeable up to 60 days following the last drug exposure, as assessed by lever pressing for drug-paired cues in rats [Grimm et al., 2001]. When it comes to heroin, on the other hand, this behavior vanishes somewhere in between 25 and 60 days of withdrawal [Shalev et al., 2001]. Moreover, Becker et al. have shown that, after a 4-week abstinence period from chronic drug administration, morphine- and cocaine-treated mice exhibit clearly different behavioral profiles [Becker et al., 2016]. For instance, mice treated with morphine, *but not* cocaine, displayed decreased sociability in the 3-CH task, profound social interaction deficits, increased motor stereotypies and marble burying. Coherently, morphine abstinent mice showed increased Fos levels in anxiety-related brain structures (CeA, PCN) and decreased Fos expression in reward-related brain structures (VTA), whereas cocaine abstinent animals displayed reduced Fos staining in anxiety-related brain structures (CeA, PCN) and unaltered Fos levels in reward-related brain structures (VTA) [Becker et al., 2016]. Al-

together, their results provide strong evidence of the existence of profound differences between morphine and cocaine abstinent mice.

In addition, the current study provides further evidence of increased aggressive behavior in mice withdrawing from opiate substances and, most importantly, is one of the few available portraying this phenomenon during spontaneous withdrawing conditions [Rodriguez-Arias et al., 1999, Tidey and Miczek, 1992]. In humans, increased irritability and aggressiveness are considered a hallmark of heroin abstinence [Gerra et al., 2004b]. Unfortunately, such symptomatic profile has not been well characterized in animal models of spontaneous withdrawal. The vast majority of the studies available are naloxone-precipitated and focus on heightened aggressive behavior during acute (i.e., 48 hours) morphine withdrawal [Kantak and Miczek, 1986, 1988, Lal et al., 1971, Rodriguez-Arias et al., 1999, Tidey and Miczek, 1992], with only one study showing increased aggressiveness in relatively long-term (i.e., 14 days) abstinent mice experiencing naloxone-precipitated morphine withdrawal [Felip et al., 2000]. Thanks to the time-course fashion of our experimental planning, we were able to identify a specific window of time during spontaneous prolonged withdrawal when subjects experienced augmented will to engage in aggressive behavior. This window loosely extends from 7 to 35 days of abstinence, coherently including the timing of the study cited above and linking the latter results to a huge amount of existing literature. Notably, aggressive biting behavior (ABB) positively correlated with the social interest expressed by opiate-withdrawn mice in the 3-CH paradigm at both opiate withdrawal days 7 and 21. Only on the latter day, though, drug-withdrawing subjects displayed significantly higher ABB, as compared to controls, in parallel with the expression of significantly higher social interest, as described above. This increase in aggressiveness becomes non-significant again after 35 days and ultimately completely extinct after 49 days of abstinence.

The 3-CH task has proven several times to be able to measure social interest independently from locomotor alterations and anxiety-like states. For instance, ethanol withdrawal decreases the exploration of the 3-CH apparatus, indicative of increased anxiety-like behavior, without impairing sociability [Moy et al., 2013]; BTBR mice (i.e., mouse model of autism spectrum disorders) exhibit lower sociability without displaying significant differences in exploratory activity, as compared to C57BL/6 controls [Yang et al., 2012]; wild-type CRF<sub>2</sub> receptor mice display sociability deficits after 7 days of cocaine withdrawal without significant differences in the number of entries to the chambers of the apparatus [Morisot et al., 2018]. Even in the current study, morphine withdrawal induced significant hypolocomotion without altering social interest 49 days after the last administration. However, we herein show that aggressive behavior and social interest follow the same time course. By demonstrating that they are positively related at a behavioral level, we highlight that their development following drug exposure and withdrawal is shared. Noteworthy, although previously described as a possibility [Kaidanovich-Beilin et al., 2011], we are the first to our knowledge to have scored additional behaviors (i.e., ABB) in the 3-CH paradigm and to have done such thing in a time-course fashion. This approach turned out to be very interesting, since it made possi-

ble for us to unravel a possible role for aggressive behavior in the heightened interest for an unknown congener elicited by opiate withdrawal. In addition, our protocol did not require the use of additional cohorts of animals, pointing out practical and ethical advantages for future experimental designs. However, our studies also highlighted what could be considered a major flaw of the 3-CH paradigm: under specific circumstances (i.e., opiate withdrawal, male mice), increased aggressive behavior in such paradigm could be mistaken for social interest.

In conclusion, the present study demonstrates in an original way that spontaneous withdrawal from chronic morphine administration induces higher social interest in male mice tested in the 3-CH paradigm, as compared to saline-treated control mice. We herein suggest that this phenomenon is due to increased aggressiveness, as assessed by ethological measures of aggressive biting behavior (ABB), which we believe drives the withdrawing-subject towards the unfamiliar conspecific for reasons other than harmless social interest. Noteworthy, the increment in ABB is narrowed to a specific window of time, thereby suggesting the existence of peculiar oscillations in aggressive behavior during opiate-withdrawal, an unprecedented result that contributes to the clarification of the complex symptomatology of OUDs. Moreover, we show that performances in the 3-CH task are completely unaffected by chronic morphine treatment and exposure to a mild environmental stressor along spontaneous prolonged withdrawal, thereby providing the first evidence of stress resilience in opiate-withdrawn mice. Future research should better investigate long-term effects of spontaneous opiate withdrawal on both social and aggressive behavior, trying to unravel putative shared neurobiological mechanisms and keeping in mind the surprising results of this study when it comes to choosing paradigms, protocols and experimental animals.

## 2.6 Tables and figures

Variable	OW day 7		OW day 21		OW day 35		OW day 49	
	F <sub>1,22</sub>	p	F <sub>1,22</sub>	p	F <sub>1,22</sub>	p	F <sub>1,22</sub>	p
Treat	0.34	0.564	1.92	0.179	0.00	0.999	0.23	0.633
Phase	24.2	<0.0001	13.5	<0.05	0.67	0.422	1.79	0.195
Side	21.1	<0.001	14.3	<0.05	6.53	<0.05	1.96	0.176
Treat x Phase	0.01	0.914	0.12	0.733	0.01	0.933	1.14	0.297
Treat x Side	0.15	0.703	1.89	0.183	2.45	0.132	0.59	0.452
Phase x Side	20.9	<0.001	9.11	<0.05	0.07	0.789	1.36	0.256
Treat x Phase x Side	2.54	0.125	4.85	<0.05	2.28	0.145	0.54	0.472

Table 1, Figure 2.1 – Statistical analysis of social interest of drug-naïve and morphine-withdrawn mice (Treat) during the time-course experiment (Experiment 1). Social interest is evaluated comparing the time spent in the half-chamber with the unfamiliar conspecific during the sociability phase to both the same half-chamber during the previous habituation phase (Phase) and the opposite half-chamber (Side) containing the unanimated object. The accepted value for significance was  $P < 0.05$ .

Variable	OW day 7		OW day 21		OW day 35		OW day 49	
	F <sub>1,22</sub>	p	F <sub>1,22</sub>	p	F <sub>1,22</sub>	p	F <sub>1,22</sub>	p
Treat	0.29	0.595	0.14	0.711	0.35	0.560	0.58	0.452
Phase	13.6	<0.05	4.37	<0.05	3.80	0.064	1.43	0.244
Side	1.97	0.175	0.47	0.500	2.76	0.111	4.06	0.056
Treat x Phase	0.17	0.686	1.21	0.283	0.59	0.449	0.02	0.888
Treat x Side	0.10	0.756	0.15	0.295	1.84	0.188	0.25	0.621
Phase x Side	50.7	<0.0001	29.7	<0.0001	13.4	<0.05	4.46	<0.05
Treat x Phase x Side	0.58	0.453	3.55	0.073	1.75	0.199	0.60	0.446

Table 2, Figure 2.2 – Statistical analysis of discrimination abilities of drug-naïve and morphine-withdrawn mice (Treat) during the time-course experiment (Experiment 1). Discrimination abilities are evaluated comparing the time spent in the half-chamber with the novel unfamiliar conspecific to both the same half-chamber during the previous sociability phase (Phase) and the opposite chamber (Side) containing the already familiar conspecific. The accepted value for significance was  $P < 0.05$ .

Variable	OW day 49 NS/EPS			
	HAB/SOC		SOC/SNP	
	F <sub>1,61</sub>	p	F <sub>1,61</sub>	p
Treat	0.46	0.499	0.00	0.993
Stress	0.12	0.726	0.66	0.420
Phase	97.9	<0.0001	1.22	0.274
Side	60.2	<0.0001	28.6	<0.0001
Treat x Stress	0.19	0.666	0.01	0.907
Treat x Phase	3.07	0.085	0.57	0.451
Treat x Side	0.17	0.679	0.20	0.652
Stress x Phase	0.15	0.699	2.43	0.124
Stress x Side	0.63	0.429	0.06	0.811
Phase x Side	169	<0.0001	165	<0.0001
Treat x Stress x Phase	0.00	0.980	0.76	0.386
Treat x Stress x Side	0.80	0.375	0.87	0.355
Treat x Phase x Side	0.46	0.500	0.24	0.625
Stress x Phase x Side	0.30	0.589	0.05	0.830
Treat x Stress x Phase x Side	0.25	0.618	0.13	0.714

Table 3, Figure 2.3 – Statistical analysis of social interest and discrimination abilities of drug-naïve and morphine-withdrawn mice (Treat) during the single test experiment (Experiment 2) with appliance of a mild environmental stressor (Stress). Social interest and discrimination abilities are evaluated as described in Table 1 and 2, respectively. The accepted value for significance was  $P < 0.05$ .

Variable	OW day 7		OW day 21		OW day 35		OW day 49	
	F <sub>1,22</sub>	p	F <sub>1,22</sub>	p	F <sub>1,22</sub>	p	F <sub>1,22</sub>	p
Treat	0.34	0.56	0.90	0.35	0.11	0.74	0.70	0.41
Phase	7.87	<0.05	14.8	<0.001	24.4	<0.0001	32.1	<0.0001
Treat x Phase	1.15	0.29	0.69	0.41	1.25	0.27	0.87	0.36

Table 4, Figure 2.4 – Statistical analysis of locomotor activity displayed by drug-naïve and morphine-withdrawn mice (Treat) during the time-course experiment (Experiment 1). Locomotion is analyzed for habituation, sociability and social novelty preference (Phase). The accepted value for significance was  $P < 0.05$ .

Variable	OW day 49 NS/EPS	
	F <sub>1,61</sub>	p
Treat	8.66	<0.05
Stress	1.62	0.21
Phase	11.8	<0.001
Treat x Stress	0.26	0.61
Treat x Phase	0.08	0.77
Stress x Phase	2.10	0.15
Treat x Stress x Phase	0.83	0.36

Table 5, Figure 2.5 – Statistical analysis of locomotor activity displayed by drug-naïve and morphine-withdrawn mice (Treat) during the single test experiment (Experiment 2) with appliance of a mild environmental stressor (Stress). Locomotion is analyzed for habituation, sociability and social novelty preference (Phase). The accepted value for significance was  $P < 0.05$ .



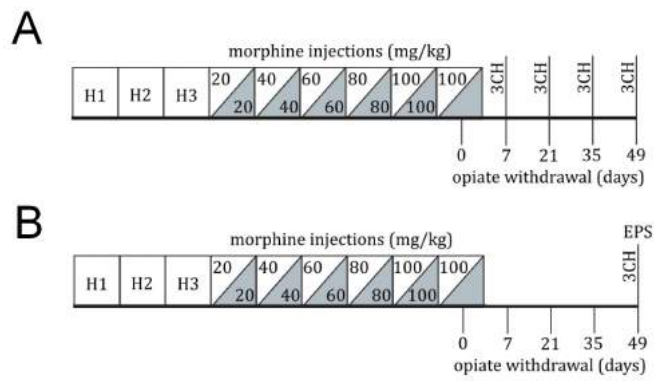


Figure 2.6 – **Experimental planning.** (A) Experiment 1: following 3 days of handling (H), mice were injected twice a day every 12 hours (white and grey standing for AM and PM) with either saline or increasing doses of morphine, before being repeatedly tested in the three-chamber (3CH) apparatus at 7, 21, 35 and 49 days of opiate withdrawal. (B) Experiment 2: following the same handling and administration procedures employed in Experiment 1, approximately half of the mice were exposed to an elevated platform stressor (EPS) 1 h prior being tested in the 3-CH apparatus only once, after 49 days of opiate withdrawal.

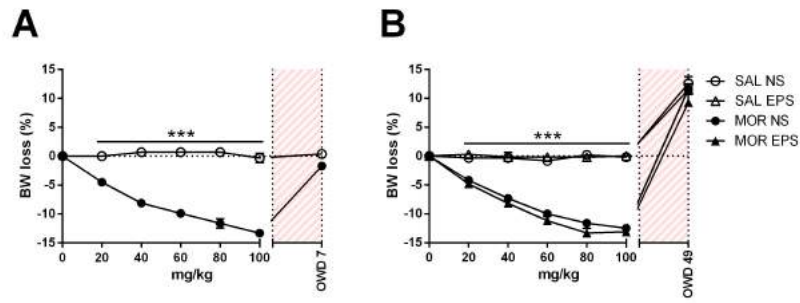


Figure 2.7 – **Body weight changes.** The intermittent administration of escalating morphine doses induced a body weight loss that become significant starting 48 hours after the beginning of the treatment, before the third drug injection . However, upon OWD 7 for Experiment 1 (A) and OWD 49 for Experiment 2 (B), respectively, saline- and opiate-withdrawn mice displayed similar body weight changes, indicating complete body weight recovery. Values represent mean  $\pm$  S.E.M. N=12-16 per experimental group. \*\*\*P<0.0001.

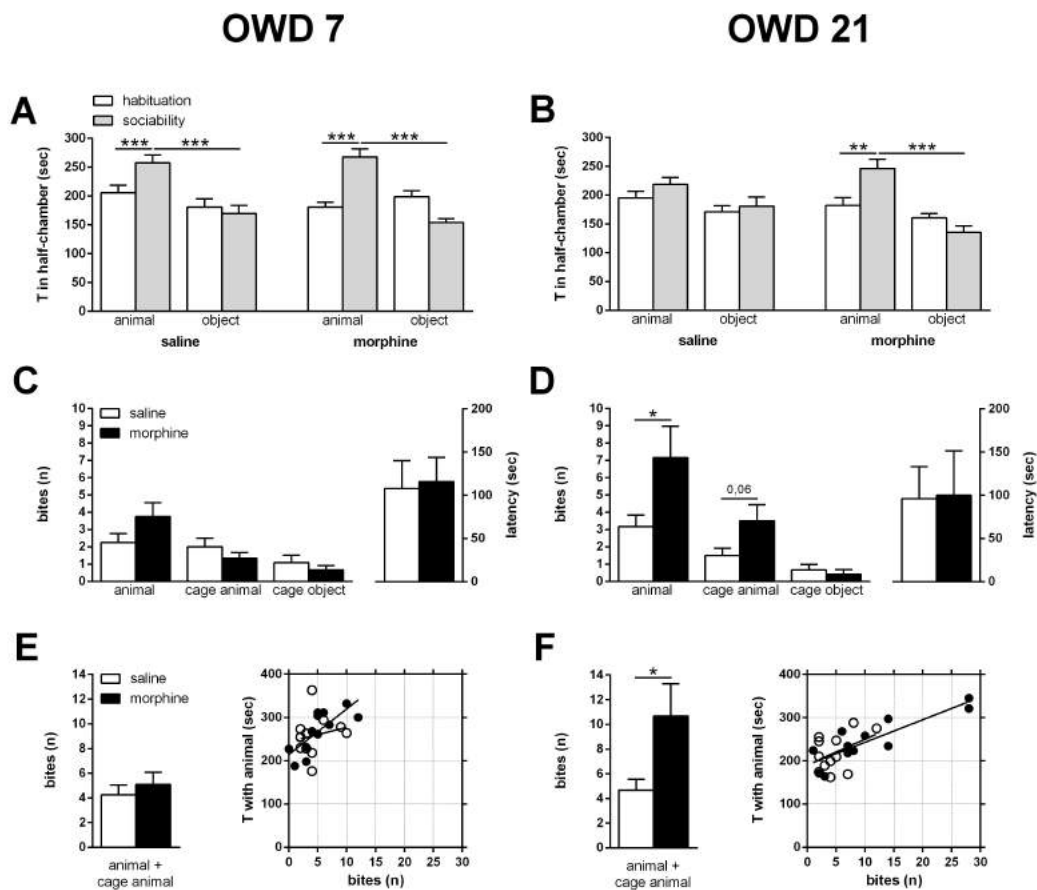


Figure 2.8 – Social interest and aggressive behavior (Experiment 1: OWD 7-21). At OWD 7, independently from the treatment received, mice spent significantly more time in the half-chamber with the unfamiliar conspecific (animal, light grey), as compared to both the same half-chamber during the previous habituation phase (animal, white) and the opposite half-chamber containing the unanimated object (object, light grey) (A). At OWD 21, just morphine-treated subjects displayed significant social interest (B). At OWD 7, saline- and morphine-withdrawn mice showed similar levels of biting behavior (C). At OWD 21, morphine-withdrawn mice exhibited significantly higher biting behavior towards the unfamiliar conspecific (animal), as compared to saline-treated control mice (D). The total number of bites directed towards the unfamiliar conspecific and his cage (animal + cage animal) correlated with the time spent in the half-chamber containing the social stimulus during the sociability tests carried out on both OWD 7 (E) and OWD 21 (F). Values represent mean  $\pm$  S.E.M. N=12 per experimental group. \* $P < 0.05$ , \*\* $P < 0.001$ , \*\*\* $P < 0.0001$ .

OWD 35

OWD 49

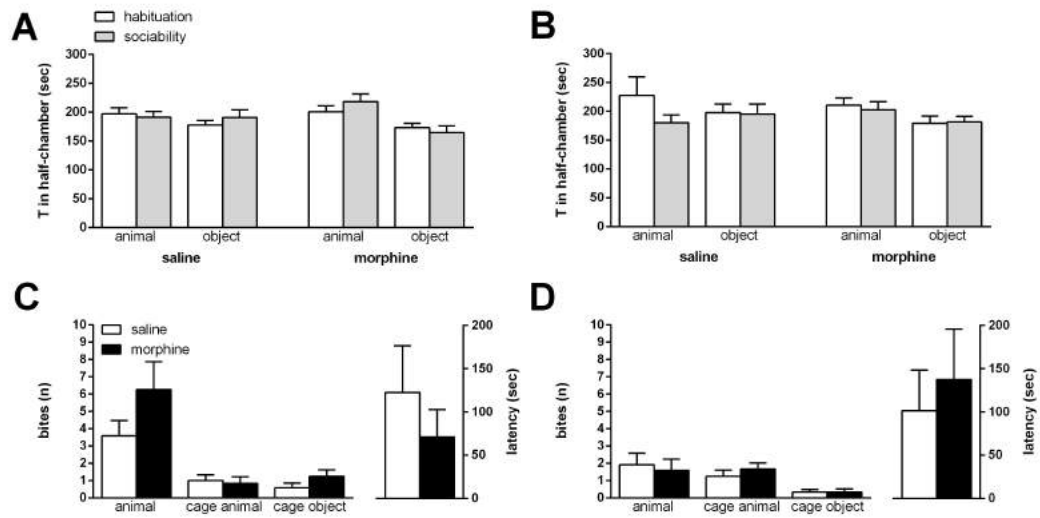


Figure 2.9 – **Social interest and aggressive behavior (Experiment 1: OWD 35-49)**. Independently from the treatment, at both OWD 35 (A) and OWD 49 (B) mice failed to spend more time in the half-chamber with the unfamiliar conspecific (animal, light grey), as compared to either the same half-chamber during the previous habituation phase (animal, white) or the opposite half-chamber containing the unanimated object (object, light grey). At both OWD 35 (C) and OWD 49 (D) saline- and morphine-withdrawn mice showed similar levels of biting behavior. Values represent mean  $\pm$  S.E.M. N=12 per experimental group.

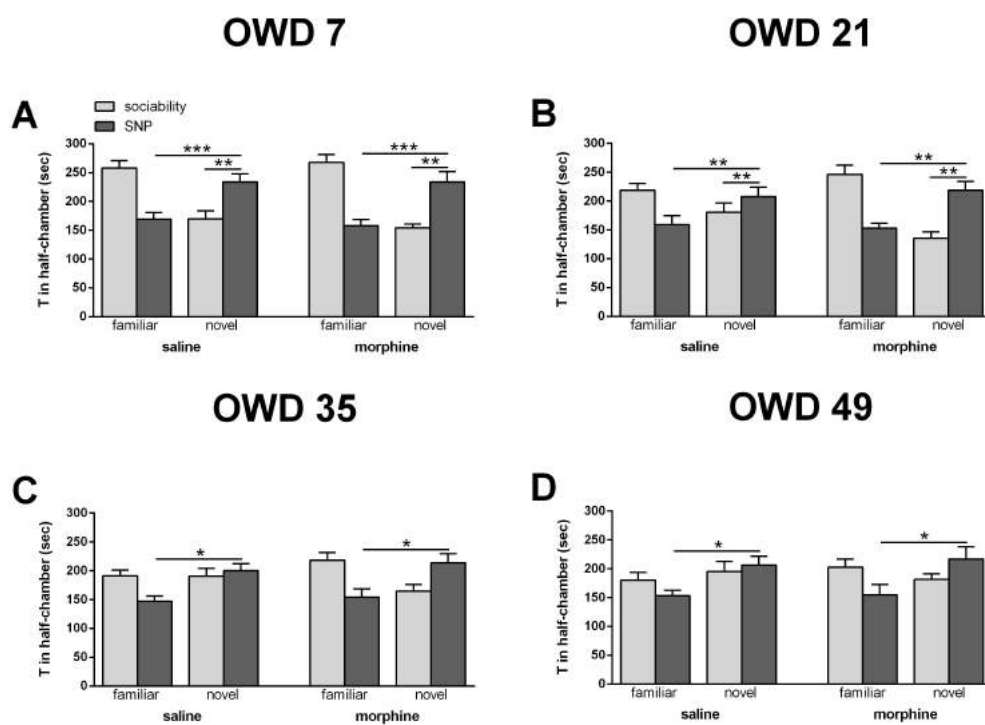


Figure 2.10 – **Social discrimination abilities (Experiment 1)**. Throughout all 4 testing days, mice always spent more time in the half-chamber with the novel unfamiliar conspecific (novel, dark grey), as compared to the opposite chamber containing the already familiar conspecific (familiar, dark grey). On OWD 7 (A) and OWD 21 (B), however, the time spent with the novel unfamiliar conspecific was significantly higher also when compared to the same half-chamber during the previous sociability phase (novel, light grey). Values represent mean  $\pm$  S.E.M. N=12 per experimental group. \*P<0.05, \*\*P<0.001, \*\*\*P<0.0001.

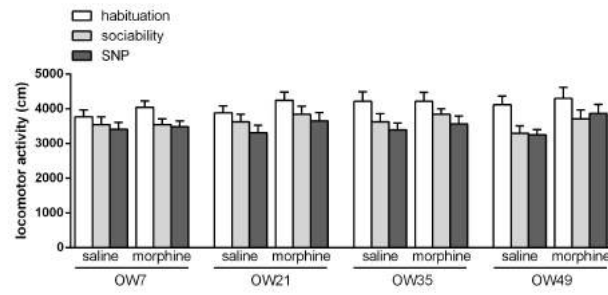


Figure 2.11 – **Locomotor activity (Experiment 1)**. Locomotor activity remained unaffected throughout the whole experiment, with no significant differences between neither phases nor testing days in both saline- and morphine-treated animals. Values represent mean  $\pm$  S.E.M. N=12 per experimental group.

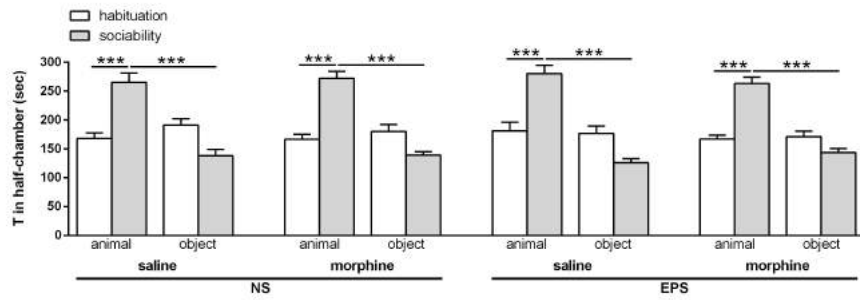


Figure 2.12 – **Social interest (Experiment 2)**. Independently from the treatment received (saline, morphine) or the appliance of a mild environmental stressor (NS, EPS), mice from all experimental groups spent more time in the half-chamber with the unfamiliar conspecific (animal, light grey), as compared to both the same half-chamber during the previous habituation phase (animal, white) and the opposite half-chamber containing the unanimated object (object, light grey). Values represent mean  $\pm$  S.E.M. N=15-16 per experimental group. \*\*\*P<0.0001.

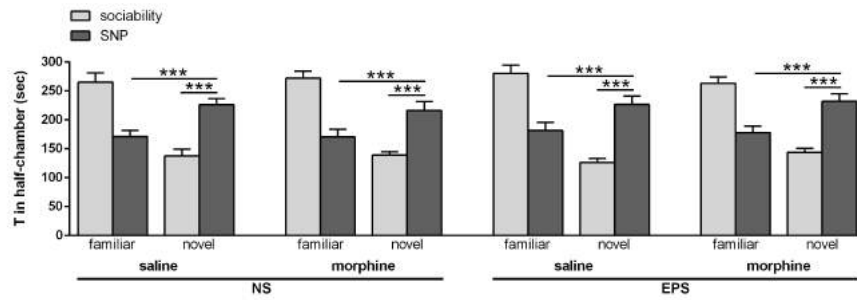


Figure 2.13 – **Social discrimination abilities (Experiment 2)**. Independently from the treatment received (saline, morphine) or the appliance of a mild environmental stressor (NS, EPS), mice from all experimental groups spent more time in the half-chamber with the novel unfamiliar conspecific (novel, dark grey), as compared to both the same half-chamber during the previous sociability phase (novel, light grey) and the opposite half-chamber containing the already familiar conspecific (familiar, dark grey). Values represent mean  $\pm$  S.E.M. N=15-16 per experimental group. \*\*\*P<0.0001.



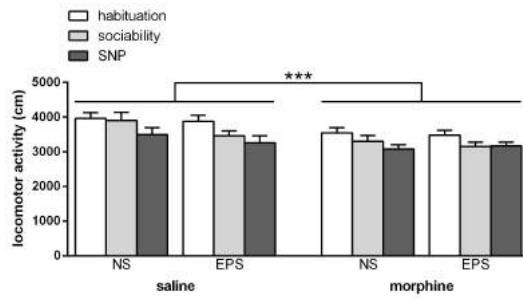


Figure 2.14 – **Locomotor activity (Experiment 2)**. Independently from the appliance of a mild environmental stressor (NS, EPS), morphine-treated mice travelled significantly less distance overall, as compared to saline-treated mice. Values represent mean  $\pm$  S.E.M. N=15-16 per experimental group. \*\*\*P<0.0001.

**Opiate withdrawal and CRF<sub>1</sub>  
receptor-deficiency increase social  
interest in mice.**

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## 3.1 Abstract

### 3.1.1 Background and purpose

In humans, poor social interest is a major clinical feature of opiate use disorders. The corticotropin-releasing factor (CRF) system mediates behavioral and neuroendocrine responses to stressors and might underlie the behavioral deficits associated with drug use, abuse and withdrawal. However, its implication in the social impairments induced by opiate substances remains unknown. CRF signaling is mediated by two CRF receptor subtypes, termed CRF<sub>1</sub> and CRF<sub>2</sub>. In the present study, we investigated the role for the CRF<sub>1</sub> receptor in social behavior following discontinuation of drug administration.

### 3.1.2 Experimental approach

Littermate wild-type (CRF<sub>1</sub><sup>+/+</sup>), heterozygous (CRF<sub>1</sub><sup>+/-</sup>) and knock-out (CRF<sub>1</sub><sup>-/-</sup>) mice were tested in the three-chamber task for sociability (i.e., preference for an unfamiliar conspecific *versus* an unanimated object) and social novelty preference (SNP, i.e., preference for a novel *versus* a familiar conspecific) 7 days following the interruption of chronic morphine administration.

### 3.1.3 Key results

Morphine withdrawal increased social interest towards an unknown congener in CRF<sub>1</sub><sup>+/+</sup> mice, as compared to their saline counterparts. Interestingly, partial and full CRF<sub>1</sub> receptor-deficiencies also increased social interest towards an unknown and a novel congener, independently from the experience of drug administration and withdrawal.

### 3.1.4 Conclusions and implications

The present findings suggest a central role for the CRF<sub>1</sub> receptor pathway and a minor role for the hypothalamic-pituitary-adrenal axis in social behavior deficits, independently from the experience of opiate administration and withdrawal, strengthening the notion of a therapeutic potential for CRF-targeting pharmacological agents.

## 3.2 Introduction

Opiate Use Disorders (OUDs) are a major health issue worldwide, especially due to the alarming rise in the recreational use of prescription analgesic opiate drugs (<https://www.unodc.org/wdr2018/index.html>). The key clinical features of OUDs are the extremely aversive somatic and negative affective-like states of the drug withdrawal syndrome [APA, 2013], a pathological motivation and irresistible desire for the drug, antisocial traits [Kalivas and Volkow, 2005, McGregor et al., 2008, Preller et al., 2014a, Young et al., 2008] and a long-lasting vulnerability to stressors [Preston and Epstein, 2011, Sinha, 2001]. Altogether, these elements make abstinence difficult to sustain and relapse more likely to happen [Le Moal and Koob, 2007, Stewart, 2008]. Currently, OUDs are treated mainly by substitutive opioid receptor agonists, such as methadone and buprenorphine [Nutt and Lingford-Hughes, 2008], which unfortunately also induce dependence [EMCDDA, 2017]. The development of effective new therapies is therefore urgently needed and heavily relies upon a better understanding of the brain mechanisms underlying such clinical features.

The corticotropin-releasing factor (CRF) system helps mediate the neuroendocrine and behavioral responses to stressful challenges and plays a well-established role in the regulation of the hypothalamic-pituitary-adrenal (HPA) axis. CRF might also be implicated in OUDs. For instance, early (8-48 h) morphine withdrawal is associated with increased CRF mRNA expression in the central nucleus of the amygdala (CeA) and the paraventricular nucleus of the hypothalamus (PVN), brain regions implicated in the effects of substances of abuse [Ingallinesi et al., 2012, Maj et al., 2003, Papaleo et al., 2007]. CRF signaling is mediated by two types of receptors, named CRF<sub>1</sub> and CRF<sub>2</sub> [Hauger et al., 2003]. Initial studies using pharmacological agents showed that CRF receptors antagonism attenuated either the somatic signs or the negative affective-like states of naloxone-precipitated opiate withdrawal [Heinrichs et al., 1995, Iredale et al., 2000, Lu et al., 2000, Stinus et al., 2005]. More recent studies, employing mouse models bearing genetic inactivation of only one type of CRF receptor, unveiled distinct, if not opposite, roles for CRF<sub>1</sub> and CRF<sub>2</sub> receptors in the behavioral consequences of opiate administration and withdrawal. For instance, CRF<sub>1</sub> or CRF<sub>2</sub> receptor-deficiency increases or decreases, respectively, the somatic signs and the recognition memory deficits induced by spontaneous morphine withdrawal in mice [Morisot and Contarino, 2016, Papaleo et al., 2007, 2008b]. Moreover, CRF<sub>2</sub> receptor-deficiency reduces the negative affective-like states of early morphine withdrawal phases and eliminates the stress-induced reemergence of recognition memory deficits and vulnerability of motivational states in mice undergoing long-term spontaneous morphine withdrawal [Ingallinesi et al., 2012, Morisot and Contarino, 2016, Morisot et al., 2015, Rouibi and Contarino, 2013].

In parallel, an increasing number of studies suggest a central but complex role for the CRF system in social behavior. For instance, intracerebroventricular (i.c.v.) administration of CRF has been shown to facilitate partner preference in male prairie voles [DeVries et al., 2002] and to decrease the

time of interaction with an unknown or a novel congener throughout a CRF<sub>1</sub> receptor-mediated mechanism in mice tested in the three-chamber paradigm, as demonstrated by employing a pharmacological approach (i.e., antalarmin) [Bagosi et al., 2017a,b]. In addition, transgenic CRF overexpression, urocortin 3- or CRF<sub>2</sub> receptor-deficiency have been shown to enhance social investigation and social memory, as assessed by the social interaction test and a social odor discrimination task (i.e., ability to discriminate between a tube filled with clean bedding and a tube filled with bedding belonging to sex- and age-matched familiar or unknown congeners) [Deussing et al., 2010, Kasahara et al., 2011]. However, more recently, mice bearing a targeted CRF<sub>2</sub> receptor-deficiency in the medial nucleus of the amygdala (MeA) were shown to exhibit an abnormally low preference for novel conspecifics in a three-chamber-based task [Shemesh et al., 2016], thereby highlighting the need for further investigations. Nevertheless, altogether these findings point out at the CRF system as a likely major player in altered social behavior.

However, the role of the CRF system in the social behavior deficits induced by drugs of abuse remains very little studied. To our knowledge, only one study to date has investigated the specific role of the CRF system in drug-induced deficits in social interest. In such study, Morisot et al. showed that CRF<sub>2</sub> receptor-deficiency eliminates the sociability deficit and vulnerability induced by withdrawal from chronic cocaine administration [Morisot et al., 2018]. Interestingly, deletion of the CRF<sub>2</sub> receptor not only rescued the behavioral deficit, but also prevented the stress-induced increased expression of oxytocin (OXY) in the supraoptic nucleus (SON) associated with long-term cocaine withdrawal. Accordingly, decreased OXY peptide levels in the hypothalamus, increased OXY receptor binding in the lateral septum and amygdala and decreased social behavior have also been shown in mice withdrawing from chronic morphine administration [Zanos et al., 2014].

In this context, the present study aimed at elucidating the role for the CRF<sub>1</sub> receptor in putative social behavior alterations induced by chronic morphine administration and spontaneous opiate withdrawal in mice. For this purpose, we employed the well-validated three-chamber paradigm, which is thought to reliably measure social interest, independently from emotional-like states [Moy et al., 2013, Silverman et al., 2010].

## 3.3 Materials and methods

### 3.3.1 Subjects

Male CRF<sub>1</sub> receptor wild-type (CRF<sub>1</sub><sup>+/+</sup>), heterozygous (CRF<sub>1</sub><sup>+/-</sup>) and knock-out (CRF<sub>1</sub><sup>-/-</sup>) mutant mice were generated on a mixed C57BL/6x129 background and derived from mating CRF<sub>1</sub><sup>+/-</sup> mice. Genotypes were identified by PCR analysis of tail DNA. The colony room (22±2 °C, relative humidity: 50–60%) was maintained on a 12 h light/dark cycle (lights on at 08h00). 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). They had ad libitum access to standard laboratory food (3.3 kcal/g; SAFE, Augy, France) and fresh water. The subjects were 11-23 weeks old at the beginning of the experiment, with a body weight of 23-33 g (27±0.3 g; mean ± S.E.M.). Animals were daily monitored for the presence of adverse effects of the experimental treatment and veterinary advice was sought if they 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. The present study complied with the ARRIVE Guidelines [Kilkenny et al., 2010] and the BJP standard guidance for studies using animals [Curtis et al., 2015, McGrath and Lilley, 2015].

### 3.3.2 Morphine administration paradigm

Each animal was handled (1 min/day) for 3 consecutive days before the experiment started. Afterwards, mice received intraperitoneal (i.p.) injections (10 ml/kg) of physiological saline or morphine hydrochloride (Francopia, Gentilly, France) every 12 h (08h00 - 20h00) for 6 consecutive days, as follows: day 1: 20 mg/kg; day 2: 40 mg/kg; day 3: 60 mg/kg; day 4: 80 mg/kg; day 5: 100 mg/kg; day 6: 100 mg/kg, only one injection in the morning. This treatment was chosen because it successfully produced cognitive deficits and vulnerabilities to stressors in previous studies [Morisot and Contarino, 2016, Morisot et al., 2015](Morisot et al., 2014a). Mice were weighed immediately before each injection and body weight (BW) changes calculated as percentage of the BW recorded just prior to the first injection.

### 3.3.3 Three-chamber apparatus

The three-chamber apparatus was a rectangular box (60 x 40 x 20 cm, L x W x H) made of dark Plexiglas and divided in 3 equal chambers. Dividing transparent Plexiglas 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, 14 cm high with bars spaced 1 cm apart) in which a living mouse or an unanimated object could be placed.

### 3.3.4 Three-chamber testing

Mice were tested in the three-chamber (3-CH) paradigm 7 days following the last administration. The testing was conducted during the light phase of the 12 h light/dark cycle in a quiet dedicated room dimly illuminated (10 lux). The 3-CH task allowed the study of 1) sociability (i.e. preference for an unfamiliar conspecific *versus* an unanimated object) and 2) social novelty preference (SNP, i.e. preference for a novel *versus* a familiar conspecific). Due to the breeding capacity and to the limited number of animals that could be tested daily, behavioral studies were identically carried out using 5 independent cohorts of mice. No statistical differences were found between the independent experiments and therefore the results were pooled. Within each cohort and across test days, all experimental conditions (i.e., genotype, treatment) were pseudo-randomized. During the pre-habituation phase, the subject mouse was confined into the central chamber for 5 min; then, the doors were opened and he could freely explore the three chambers and the empty wire cages for 10 min (habituation phase). 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 unanimated object. Ultimately, a 10 min SNP test was carried out, during which a novel mouse was placed in the wire cage previously containing the object, so that the subject mouse could now choose between the already investigated familiar and the novel mouse. The unfamiliar mice were substance-naïve C57BL/6 male mice age-matched with the subject mice, handled (1 min/day) and habituated to the wire cages (10 min/day) over the 3 days preceding their first employment. The position of the unfamiliar mouse (i.e., left or right side chamber) was counterbalanced within experimental groups. Between each test, the apparatus was cleaned with water and the wire cages with 70% ethanol and water. The experiments were recorded on a video system and the time measures obtained from a previously validated homemade video tracking software. In particular, the time spent by the subject mouse exploring the upper half part of the side chambers containing the wire cages was considered as a measure of social interest. Moreover, to control for sedation or hyperactivity [Moy et al., 2013], the total distance travelled during each of the experimental phases (habituation, sociability and SNP) was recorded.

### 3.3.5 Statistical analysis

Each mouse was assigned a unique identification number that was used to conduct blind testing and data analysis. To prevent initial side preferences from biasing the results, only subjects exploring both the half-chambers containing the wire cages for at least 60 cumulative sec and within a 20-80% ratio during the habituation phase were included in the study. A four-way analysis of variance (ANOVA) with Genotype (CRF<sub>1</sub><sup>+/+</sup> *versus* CRF<sub>1</sub><sup>+/-</sup> *versus* CRF<sub>1</sub><sup>-/-</sup>) and Treatment (saline *versus* morphine) as between-subjects factors and Side (either animal *versus* object or familiar *versus* novel) and Phase (either habituation *versus* sociability or sociability *versus* SNP) as within-subjects factors was used. A two-way ANOVA was used to analyze body weight changes,

with Treatment (saline *versus* morphine) as a between-subjects factor and Days as a within-subjects factor. The accepted value for significance was  $P < 0.05$ . If main or interaction effects were found, 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.



## 3.4 Results

### 3.4.1 Increased social interest in opiate-withdrawn CRF<sub>1</sub> wild-type mice

The intermittent administration of escalating doses of morphine induced a body weight loss (Treatment X Day effect:  $F_{4,296}=51.7$ ,  $P<0.0005$ ) that was evident starting 48 hours after the beginning of the treatment, before the third drug injection ( $P<0.0005$ ; Figure 3.3). This effect was independent from the genotype (Genotype X Treatment X Day:  $F_{8,296}=1.75$ ,  $P=0.09$ ). Upon day 7 of opiate withdrawal, saline- and morphine-treated mice displayed similar body weight changes, indicating body weight recovery ( $F_{1,76}=0.03$ ,  $P=0.87$ ; Figure 3.3).

Statistical analysis of time measures during the sociability phase revealed a Phase X Side X Genotype X Treatment significant interaction effect (Table 1,  $P<0.05$ ). Independently from the treatment received, all CRF<sub>1</sub><sup>+/+</sup> mice displayed significant social interest during the sociability phase (Figure 3.4), as revealed by more time spent in the half-chamber containing the unfamiliar conspecific, as compared to both the previous habituation phase (saline:  $P<0.05$ ; morphine:  $P<0.0005$ ) or the opposite half-chamber containing the unanimated object (saline:  $P<0.005$ ; morphine:  $P<0.0005$ ). Morphine-treated CRF<sub>1</sub><sup>+/+</sup> mice, however, spent significantly more time in the half-chamber containing the unknown congener, as compared to saline-treated CRF<sub>1</sub><sup>+/+</sup> control mice ( $P<0.0005$ ; Figure 3.4). The latter result indicates that experiencing opiate withdrawal increases the will to interact with an unknown conspecific.

### 3.4.2 Partial and full CRF<sub>1</sub> receptor-deficiency increase social interest

Independently from the treatment received, CRF<sub>1</sub><sup>+/-</sup> and CRF<sub>1</sub><sup>-/-</sup> mice displayed strong social interest during the sociability phase (Figure 3.4), spending significantly more time in the half-chamber containing the unfamiliar conspecific, as compared to both the previous habituation phase ( $P<0.0005$ ) or the opposite half-chamber containing the unanimated object ( $P<0.0005$ ). Interestingly, the time spent by saline-treated CRF<sub>1</sub><sup>+/+</sup> control mice interacting with the unknown mouse was significantly lower when compared to all these 4 experimental groups as well ( $P<0.0005$ , Figure 3.4). The latter result suggests that, independently from the experience of opiate withdrawal, inactivation of approximately half CRF<sub>1</sub> receptors is sufficient to induce a significant increase in social interest.

### 3.4.3 Partial and full CRF<sub>1</sub> receptor-deficiency increase social novelty preference

Statistical analysis of social novelty preference (SNP) measures revealed a Phase X Side X Genotype significant interaction effect (Table 1,  $P<0.05$ ). Independently from the treatment received, CRF<sub>1</sub><sup>-/-</sup> mice exhibited significant

social discrimination abilities during the SNP phase (Figure 3.5), spending more time in the half-chamber containing the novel unknown congener, as compared to both the previous sociability phase ( $P < 0.0005$ ) or the opposite half-chamber containing the already familiar mouse ( $P < 0.05$ ).  $CRF_1+/-$  mice also spent more time with the novel unfamiliar mouse (Figure 3.5), but just compared to the previous phase ( $P < 0.0005$ ), not to the opposite half-chamber ( $P = 0.62$ ). Similarly,  $CRF_1+/+$  mice also spent more time in the half-chamber with the novel unknown congener (Figure 3.5), but just compared to the previous phase ( $P < 0.05$ ), not to the opposite half-chamber ( $P = 0.86$ ). However, the time spent by  $CRF_1-/-$  mice with the novel unfamiliar conspecific is not significantly higher when compared to  $CRF_1+/-$  ( $P = 0.17$ ) or  $CRF_1+/+$  ( $P = 0.11$ ) mice. The latter result suggests that, independently from the experience of opiate withdrawal, full inactivation of  $CRF_1$  receptors slightly ameliorates social discrimination abilities.

A strong decrease in locomotion is observed in between phases (Table 2,  $P < 0.0005$ ), independently from the treatment (Table 2,  $P = 0.65$ ) or the genotype (Table 2,  $P = 0.05$ ). This effect probably reflects a progressive habituation of the mice to the apparatus and the focus on the introduction of subsequent stimuli. Independently from the treatment, an effect of genotype on locomotion is present (Table 2,  $P < 0.005$ ), with  $CRF_1-/-$  mice travelling longer distances as compared to both  $CRF_1+/-$  ( $P < 0.05$ ) and  $CRF_1+/+$  ( $P < 0.005$ ) mice (Figure 3.6). Moreover, independently from the genotype, there is an effect of the treatment (Table 2,  $P < 0.05$ ). Post-hoc analysis, however, failed to show a significant difference between saline- and morphine-treated animals ( $P = 0.08$ ).

## 3.5 Discussion

In the current study, by employing the three-chamber (3-CH) paradigm, we show that  $CRF_1^{+/+}$  adult male mice experiencing withdrawal from chronic morphine administration display higher social interest towards an unfamiliar conspecific, as compared to their saline counterparts. Furthermore, by employing a genetic approach, we show that partial or full deletion of  $CRF_1$  receptors also outcome in heightened social interest towards an unknown or a novel congener, independently from the experience of opiate withdrawal.

To assess the impact of opiate withdrawal and of  $CRF_1$  receptor-deficiency upon social interest, wild-type ( $CRF_1^{+/+}$ ), heterozygous ( $CRF_1^{+/-}$ ) and knock-out ( $CRF_1^{-/-}$ ) adult male mice were injected with either saline or morphine and tested in the 3-CH paradigm 7 days after the last drug administration. To mimic the clinical settings, mice were treated with intermittent escalating doses of morphine and opiate withdrawal-related alterations assessed following spontaneous withdrawal [O'Brien, 1996]. Using such a clinically-oriented paradigm, we found that, on opiate withdrawal day 7 (OWD7),  $CRF_1^{+/+}$  morphine-treated mice displayed significantly stronger social interest towards an unfamiliar conspecific, as compared to saline-treated  $CRF_1^{+/+}$  control mice. This result diverges sharply from what is usually observed in humans, where drug withdrawal is strongly linked to antisocial traits and social isolation [Kalivas and Volkow, 2005, McGregor et al., 2008, Preller et al., 2014a, Young et al., 2008]. It also differs from the only other study available investigating the effects of chronic morphine administration in the 3-CH paradigm and showing reduced social interest in drug-treated animals on OWD7 [Zanos et al., 2014]. However, our result fuels the already existing idea that, when testing adult male mice in the 3-CH paradigm, increased aggressiveness triggered by the experience of drug administration and withdrawal might interfere with social interest, thereby masking putative social behavior deficits. As a matter of fact, unpublished data from our laboratory show that aggressiveness (i.e., aggressive biting behavior) and social interest positively correlate at a behavioral level in C57BL/6 adult male mice tested in the 3-CH task at OWD 7 (see Article 1). In this light, it is worth considering that our inbred CRF strain is based on a C57BL/6 genetic background and that the cumulative amount of morphine (i.e., 700 mg/kg) and age-range of animals employed in the two studies are the same (i.e., 11-23 weeks old at the beginning of the experiment). It is therefore reasonable to hypothesize that increased aggressiveness induced by opiate withdrawal might underlie the significantly higher social interest displayed by morphine-treated  $CRF_1^{+/+}$  subjects when compared to their saline-treated counterparts.

On the other hand, the fact that  $CRF_1$  receptor-deficient mice, independently from the treatment received, displayed stronger social interest towards the unfamiliar conspecific, as compared to saline-treated  $CRF_1^{+/+}$  control mice, provides initial evidence of CRF-mediated mechanisms underlying social interest and related alterations. Accordingly, it has been recently suggested that CRF might affect social interest in a  $CRF_1$ -dependent manner. Specifically, intracerebroventricular (i.c.v.) administration of the CRF peptide de-

creased the time of interaction with the unfamiliar conspecific in male mice tested in the 3-CH task, an effect that was prevented by co-administration of the CRF<sub>1</sub> receptor-preferring antagonist antalarmin [Bagosi et al., 2017b]. Altogether, these results provide initial pharmacological and genetic evidence of a major role for the CRF<sub>1</sub> receptor pathway in mediating social interest and related dysfunctions. Importantly, existing literature excludes the possibility of increased aggressive behavior in CRF<sub>1</sub><sup>-/-</sup> mice being responsible for such phenomenon. Indeed, pharmacological antagonism of CRF<sub>1</sub> receptors has been shown to induce a higher latency to bite and lower lateral attack frequencies and chase durations in Syrian hamsters [Farrokhi et al., 2004] and rhesus monkeys [Habib et al., 2000]. In the study by Farrokhi et al., antagonism of CRF<sub>1</sub> receptors even enhanced the frequency and duration of olfactory investigation, indicating that decreased aggressiveness was not due to deficient social behavior [Farrokhi et al., 2004]. Moreover, CRF<sub>1</sub> receptor-deficient mice exhibited normal levels of isolation-induced intermale aggression in the resident-intruder paradigm, with a significantly lower percentage of attacks to the ventral portion of the mid-section of intruders by CRF<sub>1</sub><sup>-/-</sup> mice being the only significant difference between genotypes [Gammie and Stevenson, 2006].

Genetic inactivation of approximately 50% of CRF<sub>1</sub> receptors [Contarino and Papaleo, 2005, Schmidt et al., 2003] resulted in a behavioral phenotype similar to that observed in CRF<sub>1</sub><sup>-/-</sup> mice, indicating that even partial disruption of the CRF<sub>1</sub> receptor pathway is sufficient to boost social interest. Now, CRF<sub>1</sub><sup>+/-</sup> and CRF<sub>1</sub><sup>-/-</sup> mice greatly differ in both basal and stress-related hypothalamic-pituitary-adrenal (HPA) axis activity, with only CRF<sub>1</sub><sup>-/-</sup> mice showing deficient corticosterone responses to stress [Contarino and Papaleo, 2005, Smith et al., 1998, Timpl et al., 1998]. Therefore, the current data suggest that there is little-to-no involvement of the HPA axis in mediating social dysfunctions. This result is in line with previous works showing that CRF<sub>1</sub><sup>+/-</sup> and CRF<sub>1</sub><sup>-/-</sup> mice often display similar behavioral responses. For instance, they both lack the negative motivational states that accompany opiate withdrawal [Contarino and Papaleo, 2005] and they both lead to anxiolytic-like effects in the elevated plus maze (EPM) test [Trimble et al., 2007], which are known to be independent from the HPA axis activation [Muller et al., 2003, Papaleo et al., 2007, Refojo et al., 2011, Smith et al., 1998]. On the other hand, CRF<sub>1</sub><sup>+/-</sup> and CRF<sub>1</sub><sup>-/-</sup> mice differently experience the somatic signs of opiate withdrawal [Papaleo et al., 2007], with just CRF<sub>1</sub><sup>-/-</sup>, not CRF<sub>1</sub><sup>+/-</sup> mice, displaying heightened jumps, wet dog shakes and diarrhea 8 hours after the cessation of chronic morphine administration. Altogether, these data support a model that links plasma corticosterone deficiencies to somatic responses, *but not* to social, affective and anxiety-like outcomes of drug administration and withdrawal.

In line with what witnessed during the sociability phase, when compared to CRF<sub>1</sub><sup>+/+</sup> mice, CRF<sub>1</sub><sup>-/-</sup> mice displayed a stronger preference for social novelty as well, an effect that was independent from the treatment received. On the other side, partial genetic disruption of CRF<sub>1</sub> receptors (i.e., CRF<sub>1</sub><sup>+/-</sup>) just slightly ameliorated the preference for a novel unknown congener. This is not the first evidence of the expression of intermediate behavioral outcomes

relatively to  $CRF_1+/-$  mice. For instance,  $CRF_1+/-$  mice showed levels of alcohol withdrawal-induced anxiety-like behavior that were intermediate between those detected in  $CRF_1+/+$  and  $CRF_1-/-$  mice [Timpl et al., 1998]. Supporting the idea that  $CRF_1$  receptors might play a major role in mediating preference for social novelty, it has been recently demonstrated that administration of a  $CRF_1$  receptor-preferring antagonist (i.e., antalarmin) could rescue the deficit in social novelty preference induced by central administration of the CRF peptide in male mice [Bagosi et al., 2017a].

Finally, on a different note,  $CRF_1-/-$  mice travelled a significantly longer distance, as compared to both  $CRF_1+/-$  and  $CRF_1+/+$  mice. It has long been demonstrated that activation of CRF receptors modulates locomotor activity under various conditions [Dunn and Berridge, 1990]. For instance,  $CRF_1$  receptor-deficiency has been shown to eliminate the locomotor activation induced by CRF administration [Contarino et al., 2000]. It could be argued that such ambulatory alterations underlie the differences in social interest observed in the current work. However, morphine-treated  $CRF_1+/+$  mice and both saline- and morphine-treated  $CRF_1+/-$  mice display increased social interest while travelling the same distance of saline-treated  $CRF_1+/+$  mice. The latter result is exemplary of the independence of the two measurements in the current scenario and excludes ambulatory alterations to be responsible for the social behavioral differences described above.

In conclusion, the present study shows that spontaneous withdrawal from chronic morphine administration induces higher social interest in  $CRF_1+/+$  mice, as compared to controls. Based on the data presented in Article 1, we suggest that opiate withdrawal-induced increased aggressive behavior underlies such phenomenon. Moreover, we show that, independently from chronic morphine treatment and spontaneous prolonged withdrawal, partial ( $CRF_1+/-$ ) or full ( $CRF_1-/-$ ) disruption of major components of the stress-responsive system outcomes in higher social interest as well, compared to saline-treated  $CRF_1+/+$  control mice. The latter findings provide initial evidence of a major role for the  $CRF_1$  receptor pathway and of a minor role for the HPA axis in mediating social interest and related deficits.

### 3.6 Tables and figures

Variable	Sociability OWD7		SNP OWD7	
	F	P	F	p
Geno	F <sub>2,76</sub> =0.11	0.90	F <sub>2,76</sub> =0.68	0.51
Treat	F <sub>1,76</sub> =1.98	0.16	F <sub>1,76</sub> =0.00	0.99
Phase	F <sub>1,76</sub> =214	<0.0005	F <sub>1,76</sub> =1.99	0.16
Side	F <sub>1,76</sub> =101	<0.0005	F <sub>1,76</sub> =48.6	<0.0005
Geno X Treat	F <sub>2,76</sub> =0.56	0.57	F <sub>2,76</sub> =0.80	0.45
Geno X Phase	F <sub>2,76</sub> =0.41	0.67	F <sub>2,76</sub> =1.04	0.36
Geno X Side	F <sub>2,76</sub> =2.43	0.09	F <sub>2,76</sub> =1.43	0.25
Treat X Phase	F <sub>1,76</sub> =1.17	0.28	F <sub>1,76</sub> =1.23	0.27
Treat X Side	F <sub>1,76</sub> =0.09	0.77	F <sub>1,76</sub> =0.04	0.83
Phase X Side	F <sub>1,76</sub> =292	<0.0005	F <sub>1,76</sub> =131	<0.0005
Geno X Treat X Phase	F <sub>2,76</sub> =1.11	0.37	F <sub>2,76</sub> =0.74	0.48
Geno X Treat X Side	F <sub>2,76</sub> =1.37	0.26	F <sub>2,76</sub> =0.80	0.45
Geno X Side X Phase	F <sub>2,76</sub> =4.94	<0.05	F <sub>2,76</sub> =4.59	<0.05
Treat X Side X Phase	F <sub>1,76</sub> =4.91	<0.05	F <sub>1,76</sub> =2.96	0.09
Geno X Treat X Side X Phase	F <sub>2,76</sub> =4.04	<0.05	F <sub>2,76</sub> =1.28	0.29

Table 1, Figure 3.1 – Statistical analysis of social interest (i.e., time measures of the sociability phase) and social discrimination abilities (i.e., time measures of the social novelty preference phase, SNP) displayed by drug-naïve and morphine-withdrawn (Treat) CRF<sub>1</sub>+/+, CRF<sub>1</sub>+/- and CRF<sub>1</sub>-/- (Geno) mice at opiate withdrawal day 7 (OWD7). Social interest is described as the time spent in the half-chamber with the unfamiliar conspecific, as compared to both the same half-chamber during the previous habituation phase (Phase) and the opposite half-chamber (Side) containing the unanimated object. Social discrimination is described as the time spent in the half-chamber with the novel unknown congener, as compared to both the same half-chamber during the previous sociability phase (Phase) and the opposite half-chamber (Side) containing the already known familiar congener. The accepted value for significance was P<0.05.

Locomotor activity OWD7		
Variable	F	P
Geno	$F_{2,76}=7.22$	<0.005
Treat	$F_{1,76}=4.31$	<0.05
Phase	$F_{2,152}=63.9$	<0.0005
Geno X Treat	$F_{2,76}=0.48$	0.62
Geno X Phase	$F_{4,152}=2.42$	0.05
Treat X Phase	$F_{2,152}=0.43$	0.65
Geno X Treat X Phase	$F_{4,152}=1.83$	0.13

Table 2, Figure 3.2 – Statistical analysis of locomotor activity displayed during the habituation, sociability and social novelty preference (SNP) phases (Phase) by drug-naïve and morphine-withdrawn (Treat)  $CRF_{1+}/+$ ,  $CRF_{1+}/-$  and  $CRF_{1-}/-$  (Geno) mice at opiate withdrawal day 7 (OWD7). The accepted value for significance was  $P < 0.05$ .

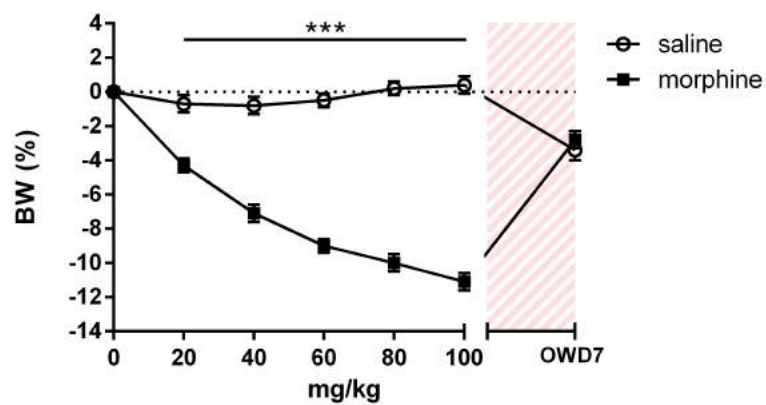


Figure 3.3 – **Body weight changes.** The intermittent administration of escalating morphine doses induced a body weight (BW) loss that became significant 48 hours after the beginning of the treatment, as assessed before the third drug injection. However, upon opiate withdrawal day 7 (OWD7), saline-treated controls and opiate-withdrawn mice displayed similar body weight changes, indicating complete body weight recovery. N=39-43 per treatment group. Values represent mean  $\pm$  S.E.M. \*\*\*P<0.0005.



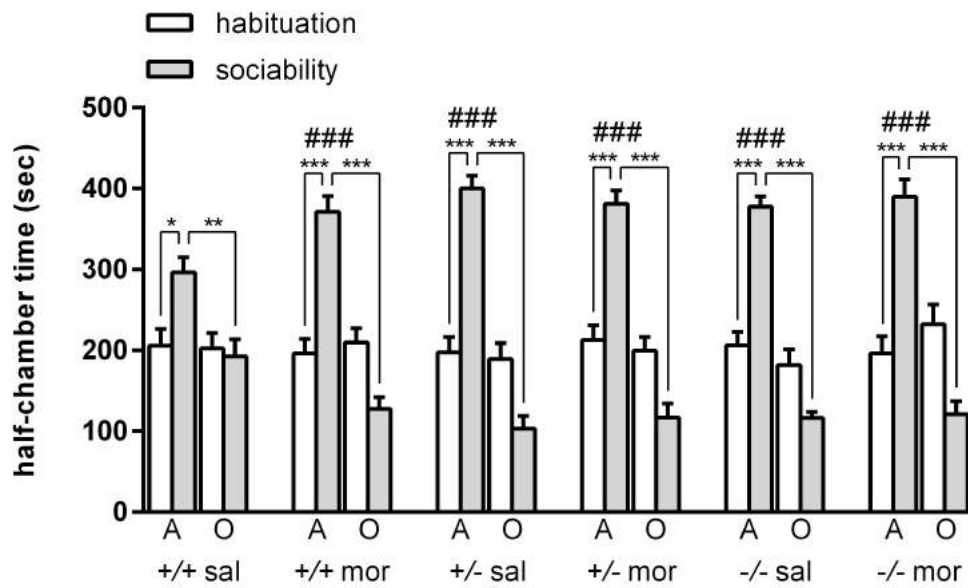


Figure 3.4 – **Social interest.** Statistical analysis of habituation and sociability measures revealed a Geno X Treat X Side X Phase significant interaction effect. Post-hoc individual group comparisons showed that all experimental groups (n=6) displayed social interest. However, morphine-treated (mor) CRF<sub>1</sub> wild-type (+/+) mice spent significantly more time with the animal (A), as compared to saline-treated (sal) CRF<sub>1</sub> wild-type mice. Moreover, independently from the experience of drug administration and withdrawal, CRF<sub>1</sub> heterozygous (+/-) and knock-out (-/-) mice also spent significantly more time with the animal, as compared to saline-treated wild-type mice. Social interest is expressed as the time spent by the subject mouse in the half-chamber with the animal (A, light grey), as compared to both the same half-chamber during the previous habituation phase (A, white) and the opposite half-chamber containing the unanimated object (O, light grey). N=12-15 per experimental group. Values represent mean  $\pm$  S.E.M. \*P<0.05, \*\*P<0.005, \*\*\*P<0.0005. ###P<0.0005 (# = as compared to the time spent with the animal during the sociability phase by saline-treated wild-type controls).

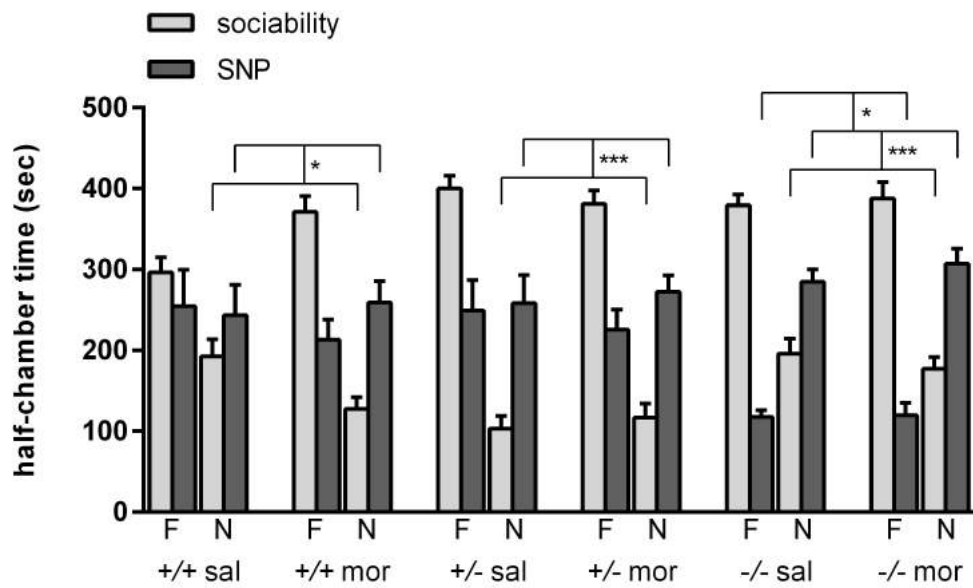


Figure 3.5 – **Social discrimination abilities.** Statistical analysis of sociability and SNP measures revealed a significant Geno X Side X Phase significant interaction effect. Post-hoc individual group comparisons showed that all experimental groups (n=3) displayed social discrimination abilities. Independently from the treatment received (sal/mor), CRF<sub>1</sub> wild-type (+/+) mice spent significantly more time with the novel animal (N), but just compared to the previous phase. CRF<sub>1</sub> heterozygous (+/-) mice exhibited a similar preference, but to a higher extent. CRF<sub>1</sub> knock-out (-/-) mice also spent significantly more time with the novel animal, but not just compared to the previous phase, also to the opposite half-chamber containing the familiar animal (F). Social discrimination is expressed as the time spent by the subject mouse in the half-chamber with the novel animal (N, dark grey), as compared to both the same half-chamber during the previous sociability phase (N, light grey) and the opposite half-chamber containing the familiar animal (F, dark grey). N=12-15 per experimental group. Values represent mean ± S.E.M. \*P<0.05, \*\*\*P<0.0005.

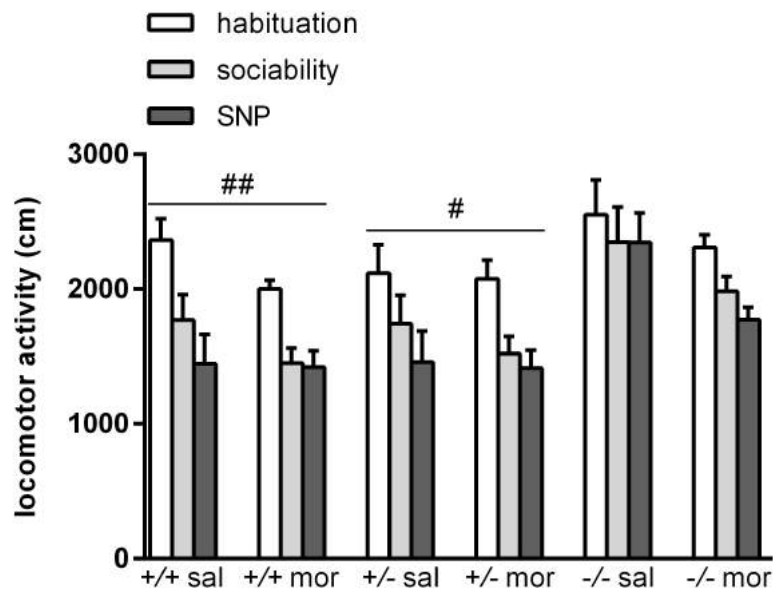


Figure 3.6 – **Locomotor activity.** Independently from the treatment received (sal/mor), the total distance travelled by CRF<sub>1</sub> knock-out (-/-) mice was significantly higher, as compared to both heterozygous (+/-) and wild-type (+/+) mice. N=12-15 per experimental group. Values represent mean ± S.E.M. #P<0.05, ##P<0.005 (# = as compared to CRF<sub>1</sub> knock-out mice).

**Morphine decreases social interest and  
the motivation to obtain highly  
palatable food in mice: role of the  
CRF system.**

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## 4.1 Abstract

### 4.1.1 Background and purpose

Drugs of abuse activate brain reward systems and strongly narrow behavior towards substance-seeking and substance-taking to the detriment of “natural” rewarding activities, such as social interaction or food intake. The corticotropin-releasing factor (CRF) system, a major coordinator of behavioral, neuroendocrine and autonomic responses to stressors, might play a critical role in mediating behavioral and brain alterations associated with drug intake.

### 4.1.2 Experimental approach

Using the three-chamber (3-CH) task and a food-driven operant behavior paradigm, we assessed the effects of relatively low doses of morphine upon social behavior and motivation for highly palatable food (HPF) in both male and female mice. Moreover, using a single-exposure conditioned place preference (CPP) procedure, we tested the rewarding properties of the most effective morphine dose. Finally, we employed the CRF<sub>1</sub> receptor-preferring antagonist antalarmin to assess the involvement of the CRF system in the alterations induced by acute drug administration.

### 4.1.3 Key results

A single intraperitoneal injection of morphine (2.5 mg/kg) sharply impaired social behaviour and motivation to obtain HPF in a gender-independent manner. Notably, morphine did not affect locomotion, olfaction or anxiety-like behaviour, suggesting a specific effect upon the hedonic properties of the “natural” reinforcers in question. Interestingly, the same morphine dose induced CPP, indicating activation of brain reward systems. Systemic administration of the CRF<sub>1</sub> receptor-preferring antagonist antalarmin completely reversed the social behavior deficit, *but not* the decreased motivation for food, induced by acute drug administration.

### 4.1.4 Conclusions and implications

Altogether, our findings indicate decreased interest for “natural” hedonic activities following exposure to a brain rewarding morphine dose, providing initial experimental evidence of “hijacking” of brain reward and motivation systems by opiate substances. Moreover, we demonstrate that the CRF system might be, at least in part, implicated in such phenomenon, strengthening the notion of a therapeutic potential for CRF-targeting pharmacological agents.

## 4.2 Introduction

Drugs of abuse act on brain reward systems, although the brain evolved to respond to “natural” rewards, such as food and social interaction [Kelley and Berridge, 2002]. It has been postulated that “artificial” stimulation of brain reward systems by drugs of abuse triggers neuroadaptations within these systems, altering reward processing so that the value of the drug is enhanced and, concurrently, the value of “natural” rewards is reduced [Koob and Le Moal, 1997, Volkow et al., 2011]. Accordingly, human studies reported decreased responsiveness to “natural” reinforcers across a range of psychophysiological measures (i.e., self-ratings, facial electromyography, startle-elicited postauricular reflex, event-related potentials) [Lubman et al., 2009], reduced food intake [Lubman et al., 2009] and defective brain activation (as assessed by functional magnetic resonance imaging) in response to pleasant non-drug-related stimuli in heroin-dependent individuals [Zijlstra et al., 2009].

Using laboratory animals, researchers have been able to model some of these phenomena. For instance, early studies reported that during opiate, cocaine or ethanol withdrawal or upon presentation of withdrawal-conditioned stimuli, operant responding for food or for sweet solutions was decreased in rats and monkeys [Goldberg and Gonzalez, 1976, Simpson and Annau, 1977, Denoble and Begleiter, 1978, Carroll and Lac, 1987, Baldwin and Koob, 1993]. Similarly, cessation of amphetamine administration was shown to induce reward deficits in various animal models [Kitanaka et al., 2008]. For instance, amphetamine-treated rats exhibited an elevation in the brain reward threshold [Paterson et al., 2000], decreased sucrose intake [Barr and Phillips, 1999, Der-Avakian and Markou, 2010a,b] and impaired sexual behavior [Barr et al., 1999]. Moreover, opiate-withdrawn monkeys showed increased heroin choice over food, suggesting an increase in the relative reinforcing efficacy of the drug, as compared to food [Negus, 2006]. In addition, a recent study reported that rats with a history of heroin self-administration given the opportunity to rescue a cage-mate from confinement (i.e., a naturally occurring rewarding pro-social behavior) and continue to self-administer the drug, preferred the latter option [Tomek et al., 2018]. On the other hand, depending upon dose, route of administration, employment of food- deprivation procedures and time after injection at which testing occurs, acute morphine administration was shown to either suppress or enhance food intake [Carr, 1984, Gulati et al., 1991, Jalowiec et al., 1981, Leshem, 1988, Sanger and McCarthy, 1980]. Finally, acute administration of various psychostimulants [Daza-Losada et al., 2009, Maldonado and Navarro, 2001, Navarro and Maldonado, 1999, Slamberova et al., 2015, 2010] and of relatively low doses of morphine [Slamberova et al., 2016] was shown to acutely reduce social investigation in the social interaction paradigm in adult male mice and rats. Unfortunately, the latter paradigm assesses mostly anxiogenic-like activity during agonistic encounters, rather than providing measures of social interest that are independent from anxiety-like states or locomotor activity, like the three-chamber paradigm does [Moy et al., 2013, Silverman et al., 2010].

Besides brain reward systems, drugs of abuse also activate brain stress

systems, such as the corticotropin-releasing factor (CRF) system [Briand and Blendy, 2010, Koob, 2008b]. For instance, cocaine increases the expression and the extracellular levels of CRF in the central nucleus of the amygdala (CeA), a brain region underlying both the effects of drugs of abuse and stress responses [Maj et al., 2003, Richter et al., 1995]. Besides, extrahypothalamic brain CRF systems mediate the behavioral effects of drugs of abuse. Indeed, CRF receptor antagonists eliminated alcohol self-administration in rats undergoing both acute withdrawal [Funk and Koob, 2007, Funk et al., 2007] and protracted abstinence [Valdez et al., 2003]. In addition, CRF receptor antagonists attenuated the escalation of intravenous cocaine [Goeders and Guerin, 1996, Specio et al., 2008] and heroin self-administration [Greenwell et al., 2009] and effectively and consistently reduced the stress-induced reinstatement of drug-seeking behavior in mice and rats [Erb et al., 1998, Kriebich et al., 2009, Le et al., 2000, Shaham et al., 1997, Shaham and Stewart, 1994, Shalev et al., 2000, Zislis et al., 2007]. However, despite extensive research supporting the role of CRF in drug addiction [Koob, 2008b], its specific involvement in the “hijacking” of brain reward systems by drugs of abuse remains to be elucidated. Nevertheless, mice bearing a specific genetic mutation for one of the two CRF receptor subtypes, termed CRF<sub>1</sub> and CRF<sub>2</sub>, have provided initial evidence of the involvement of the CRF system in drug-induced reward alterations. For instance, recent studies from our laboratory have shown that CRF<sub>1</sub> receptor-deficient mice display altered sensitivity to the rewarding effects of cocaine, as assessed by conditioned place preference (CPP) responses [Contarino et al., 2017], and that CRF<sub>2</sub> receptor-deficiency rescues the social behavior deficits induced by chronic cocaine administration in mice [Morisot et al., 2018]. The latter results provide initial evidence that the CRF system might underlie the “hijacking” of brain reward systems described above.

Thus, the goal of the current work was to build a reliable model of reduced interest for “natural” rewards following exposure to a small, rewarding dose of morphine and to investigate the putative role of the CRF system in mediating such phenomenon.

## 4.3 Materials and methods

### 4.3.1 Subjects

C57BL/6 male and female mice were generated in our local animal facility and derived from mating mice originally purchased from Janvier Labs. Subject animals were housed in groups of 2-4 and kept on a 12 h light/dark cycle (lights on from 08h00 to 20h00) in a colony room maintained under standard laboratory conditions (temperature of  $22\pm 1^\circ\text{C}$ , relative humidity of 50-60%). Standard laboratory food (3.3 kcal/g; SAFE, Augy, France) and fresh water were available *ad libitum*. Mice were 12-20 weeks old at the beginning of the experiments, with a body weight comprised between 23-32 g ( $27\pm 0.3$  g; mean  $\pm$  S.E.M.) for the males and 18-25 g ( $22\pm 0.3$  g; mean  $\pm$  S.E.M.) for the females. Animals were monitored on a daily basis and veterinary advice was sought if any signs of distress were noticed. All studies were conducted in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and approved by the local Animal Care and Use Committee. All efforts were made to minimize animal discomfort and to reduce the number of animal used.

### 4.3.2 Morphine administration paradigm

Mice received an intraperitoneal (i.p.) injection (10 ml/kg) of either physiological saline or morphine hydrochloride (Francopia, Gentilly, France) dissolved in physiological saline in the volume of 1.25 mg/kg or 2.5 mg/kg.

### 4.3.3 Antalarmin administration paradigm

A vehicle solution (94% physiological saline, 1% NaOH, 5% HCl; pH=5) or antalarmin (TOCRIS, Lille, France) dissolved in vehicle solution in the volume of 20 mg/kg was administered orally (per os, 10 ml/kg) 1 hour before the i.p. injection. During this time span, mice were kept in their home cage.

### 4.3.4 Three-chamber (3-CH) testing

The impact of acute morphine administration upon social behavior was investigated with the 3-CH task [Moy et al., 2004]. The 3-CH apparatus was a rectangular box (60 x 40 x 20 cm, L x W x H) divided in 3 equal chambers and made of dark Plexiglas. Dividing transparent Plexiglas walls had small square 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) in which a living mouse or an unanimated object could be placed. Prior to being tested, each animal was handled (1 min/day) during 3 consecutive days. The experiments were conducted during the light phase of the 12 h light/dark cycle (from 10h00 to 15h00) in a dedicated quiet room under dim light conditions (10 lux). Immediately after being injected with either saline, morphine 1.25 mg/kg or



morphine 2.5 mg/kg, subjects were first confined in the central chamber for 5 min in order to get used to the testing conditions (pre-habituation phase). Subsequently, they were allowed to explore the whole apparatus for 10 min (habituation phase) prior being challenged with a preference task. The 3-CH paradigm allowed testing for: 1) sociability (i.e., preference for an unfamiliar conspecific *versus* an unanimated object); 2) intact olfaction (i.e., preference for scented cotton *versus* unscented cotton). The unfamiliar conspecifics were substance-naïve mice of the same sex and age-matched with the subject animals, handled (1 min/day) and habituated to the wire cages (10 min/day) over the 3 days preceding the test. The scented stimulus was a cotton nestlet taken from a cage of three sex- and age-matched mice 24 h following its placement in the cage. After being cleaned of residual bedding, the shredded cotton was placed inside a wired column (3 cm diameter, 12 cm high) and under one wire cage as an olfactory stimulus. A novel cotton nestlet manually shredded with gloves was placed inside an identical wired column and under the opposite wire cage as control stimulus. Across test days, all experimental conditions (i.e., treatment) were pseudo-randomized and the position (i.e., left or right side chamber) of the unfamiliar conspecific or the scented cotton counterbalanced within each experimental group. The experiments were recorded with a camera placed on top of the apparatus and the time spent exploring its various sections and the locomotor activity scored by a previously validated home-made video tracking system. Time spent engaging socially (i.e., social interest) was defined as the time spent by the subject animal with all four paws in the half-chamber containing the social stimulus. To prevent initial side preferences or insufficient exploratory behavior from biasing the results, only animals exploring each side half-chamber for no more than 80% of the 10 min habituation phase and for at least 1 cumulative min were included in the study.

#### 4.3.5 Elevated plus-maze

The impact of acute morphine administration on anxiety was investigated with the elevated plus-maze (EPM) test. The apparatus was made of black Plexiglas and consisted of two open arms (30 x 5 cm, L x W) and two enclosed arms of the same size with walls 30 cm high. The arms were elevated 30 cm above the ground and extended from a central platform (5 x 5 cm). All testing was conducted during the light phase of the light/dark cycle (from 10h00 to 15h00) in a dedicated quiet room under relatively dim light conditions (30 lux). In order to match the timings of the 3-CH test and avoid possible shifts in the effects of the drug, the subjects were positioned in the EPM apparatus 20 min after being injected intraperitoneally, thereby matching the middle of the sociability phase. Mice were individually tested for 5 minutes in the EPM apparatus. In particular, each mouse was placed onto the center platform facing an open arm to initiate the test session. Between each session, the apparatus was carefully cleaned with water. Behaviors scored were the number of open and closed arms entries and the time spent on the various sections of the apparatus. Arm entries were defined as entry of all four paws into the arm.

At the end of the test, the number of entries into and the time spent on the open arms were expressed as a percentage of the total number of arm entries and test duration, respectively. The closed and the total number of entries were taken as indices of ambulatory activity. The experiments were recorded with a video system and behaviors scored by a trained observer blind to the treatments.

### 4.3.6 Operant behaviour

The impact of acute morphine administration on the motivation for and the intake of highly palatable food (HPF) was investigated through an operant behavioural paradigm. Each operant behavior apparatus (22 x 14 x 20 cm, L x W x H) was equipped with dim light sources and with two nose-poke holes (1 cm in diameter, 8.5 cm apart, 2.5 cm from the grid floor) mounted at the opposite ends of the same wall, each equipped with infrared photo-beams connected to a computer (Imetronic, Pessac, France). Nose-poking into one of the two holes, i.e., the active hole, resulted in food pellet delivery whereas nose-poking into the other hole, i.e., the inactive hole, had no consequences. Centered between the nose-poke holes was a food trough situated 2 cm from the grid floor; food pellet delivery occurred when the photo-beam of the active nose-poke hole was interrupted for at least 500 msec. We used 20 mg palatable food pellets (5-TUL, 3.4 kcal/g; PMI Nutrition International, LLC, St. Louis, MO, USA), which were delivered by an automated dispenser situated outside the apparatus. Photo-beams allowed monitoring of food trough visits and an additional food pellet was not delivered until a food trough visit (removal of the previously delivered food pellet), thereby allowing resolution of food-directed behavior at the unit of an individual food pellet. The wire grid floor of the cage allowed the passage of uneaten food pellets to a sliding drawer, making storage impossible and allowing evaluation of food spillage. Each apparatus was also equipped with two series of photo-beams that served to record horizontal and vertical ambulatory activity. Prior to the beginning of the experiment, each mouse was handled for 1 minute during 3 consecutive days. Starting on the day after the last handling session, mice were daily confined to the apparatus for a 1 hour test (15h00-16h00). Within gender, half of the mice were assigned either the left or the right nose-poke hole as the active hole. A fixed ratio (FR)-1 reinforcement schedule was initially applied for either 6 (operant behavior) or 12 (operant behavior with antalarmin) consecutive days, i.e., one nose-poke resulting in the delivery of one food pellet. Then, FR-3 and a FR-6 reinforcement schedules were each applied for 3 consecutive days, during which 3 or 6 active nose-pokes produced the delivery of one food pellet, respectively. Food pellets obtained, food pellets spilt and extra-pokes (non-reinforced active nose-pokes) made during each FR phase were also calculated. The mice were then switched to a progressive ratio (PR)-2 reinforcement schedule for the rest of the experiment, i.e., the number of active nose-pokes required to obtain each successive food pellet was progressively increased by 2. A within-subject design was employed to test the effects of the various morphine doses. Following 3 PR-2 baseline days, every mouse was injected with saline, morphine 1.25

mg/kg and morphine 2.5 mg/kg, with either 1 (operant behavior) or 2 (operant behavior with antalarmin) injection-free additional PR-2 baseline days in between injection days. The mice were weighed just before each injection and body weight changes calculated as percentage of the body weight recorded just prior to the first injection.

#### **4.3.7 Single-exposure conditioned place preference**

Morphine reward was examined using the conditioned place preference (CPP) paradigm, one of the most employed behavioural models of drug reward in rodents. The CPP apparatus consisted of a rectangular Plexiglas box (42 x 21 x 21 cm, L x W x H) divided by a central partition into two chambers of equal size (20 x 20 x 20 cm, L x W x H). During the test sessions, an aperture (4 x 4 cm) in the central partition allowed the mice to enter both chambers of the apparatus. One chamber had dark grey walls and a smooth grey floor whereas the other had vertical white and black striped (2 cm) walls and a slightly rough white floor. Light intensity inside the CPP apparatus was 60 lux. Transparent Plexiglas lids allowed the recording of the experiment. During the three days preceding the experiment, each mouse was handled daily for 1 minute. On experimental day 1, mice were individually placed into a CPP chamber and, following the first entry into the opposite chamber, allowed to freely explore the apparatus for 20 minutes (pre-conditioning test, 12h00). On experimental day 2, drug-treated mice received saline in the morning (09h00) and morphine in the afternoon (15h00), injected intraperitoneally just before being confined in the assigned chamber for 30 min (conditioning). Control mice received saline prior both trials. On experimental day 3, post-conditioning tests were performed as the pre-conditioning tests by individually placing each mouse in the CPP chamber paired with saline during the afternoon conditioning trial. The pre- and post-conditioning sessions were recorded on a video system and the time spent in each chamber and the locomotor activity scored by a video tracking system. Time spent in a chamber was considered when all four paws were situated inside that chamber. An unbiased CPP procedure was used throughout the experiment, i.e., within a group, half of the mice were confined to the preferred and the other half to the non-preferred CPP chamber, as determined from the pre-conditioning test results. Moreover, within experimental groups, care was taken to balance drug assignment between the two CPP chambers.

#### **4.3.8 Statistical analysis**

Each mouse was assigned a unique identification number that was used to conduct blind testing and data analysis. A three-way analysis of variance (ANOVA) with Treatment (sal, mor 1.25, mor 2.5) as a between-subjects factor and Phase (habituation, sociability) and Side (animal, object or scented cotton, unscented cotton) as within-subjects factors was used to analyze the results of the 3-CH social and olfactory tasks. A two-way ANOVA with Treatment (sal, mor 1.25, mor 2.5) as a between-subjects factor and Phase (habituation, sociability) as a within-subjects factor was used to examine locomotor activity

in the 3-CH social and olfactory tasks. A one-way ANOVA with Treatment (sal, mor 1.25, mor 2.5) as a between-subjects factor was used to examine the percentage of entries made into the open arms, the percentage of time spent in the open arms, the number of closed arm entries and the total number of arm entries during the EPM test. A repeated measure ANOVA with Treatment (sal, mor 1.25, mor 2.5) as a within-subjects factor was used to analyze active nose-pokes, food pellets ingested, discrimination indexes and locomotor activities on the treatment days of the operant behaviour task. A four-way ANOVA with Pre-treatment (veh, anta) and Treatment (sal, mor) as between-subjects factors and Phase (habituation, sociability) and Side (animal, object) as within-subjects factors was used to analyze the results of the 3-CH social task with antalarmin. A three-way ANOVA with Pre-treatment (veh, anta) and Treatment (sal, mor) as between-subjects factor and phase (habituation, sociability) as within-subjects factor was used to examine locomotor activity in the 3-CH social task with antalarmin. A repeated measure ANOVA with Treatment (veh/sal, veh/mor, anta/sal, anta/mor) as a within-subjects factor was used to analyze active nose-pokes, food pellets ingested, discrimination indexes and locomotor activities on the treatment days of the operant behaviour task with antalarmin. The accepted value for significance was  $P < 0.05$ . If main or interaction effects were significant, the Newman-Keuls post-hoc test was employed for individual group comparisons. Statistical analyses were performed using the STATISTICA software (Version 10). Data graphs were created using GraphPad Prism.

## 4.4 Results

### 4.4.1 Acute administration of morphine induces deficits in social interest

Statistical analysis of sociability measures revealed a Phase X Side X Treatment significant interaction effect in both male ( $P < 0.05$ ; Table 1) and female ( $P < 0.005$ ; Table 1) mice. Post-hoc individual group comparisons indicated that, independently from the gender, mice treated with morphine in the volume of 2.5 mg/kg failed to spend more time in the half-chamber containing the unfamiliar conspecific, as compared to both the previous phase ( $P = 0.69$  for males,  $P = 0.19$  for females) or the opposite half-chamber ( $P = 0.64$  for males,  $P = 0.38$  for females). Moreover, drug-treated mice spent significantly less time in the half-chamber containing the animal when compared to saline-treated control mice ( $P < 0.005$  for males;  $P < 0.0005$  for females; Figure 4.4A and 4.4C). Altogether, these results are indicative of decreased social interest in both male and female mice administered with morphine in the volume of 2.5 mg/kg. In contrast, independently from the gender, saline-treated control mice spent more time in the half-chamber containing the unfamiliar mouse, as compared to both the habituation phase ( $P < 0.0005$  for males,  $P < 0.0005$  for females; Figure 4.4A and 4.4C) or the opposite half-chamber containing the unanimated object ( $P < 0.0005$  for males,  $P < 0.0005$  for females; Figure 4.4A and 4.4C), indicative of strong social interest in both male and female saline-treated control mice. Male mice injected with morphine in the volume of 1.25 mg/kg also spent more time in the half-chamber containing the unfamiliar mouse, as compared to both the previous phase ( $P < 0.05$ ; Figure 4.4A) or the opposite half-chamber ( $P < 0.05$ ; Figure 4.4A), indicative of intact social interest. On the other side, female mice injected with the same dose of morphine failed to spend more time in the half-chamber containing the unfamiliar conspecific, as compared to both the habituation phase ( $P = 0.06$ ) or the opposite half-chamber ( $P = 0.07$ ), indicative of decreased social interest. Moreover, female mice administered with morphine in the volume of 1.25 mg/kg spent significantly less time in the half-chamber containing the animal when compared to saline-treated control mice ( $P < 0.05$ ). Statistical analysis of locomotor activity measures revealed no effect of the treatment ( $P = 0.16$  for males,  $P = 0.22$  for females; Table 1; Figure 4.6A and 4.6B) independently from the test phases, as revealed by a non-significant Phase X Treatment interaction effect ( $P = 0.25$  for males;  $0.07$  for females; Table 1).

### 4.4.2 Morphine-induced deficits in social interest are independent from olfactory impairments

In male mice, statistical analysis of sociability measures revealed a Phase x Side x Treatment significant interaction effect ( $P < 0.05$ , Table 2). Indeed, i.p. administration of morphine in the volume of 2.5 mg/kg, *but not* 1.25 mg/kg, significantly reduced the time spent with the scented cotton, as compared to saline-treated controls. Despite this, post-hoc individual group comparisons

also indicated that all 3 experimental groups spent significantly more time in the half-chamber containing the scented cotton, as compared to both the habituation phase (saline:  $P < 0.0005$ ; morphine 1.25 mg/kg:  $P < 0.0005$ ; morphine 2.5 mg/kg:  $P < 0.05$ ; Figure 4.5A) or the opposite half-chamber containing the unscented cotton (saline:  $P < 0.0005$ ; morphine 1.25 mg/kg:  $P < 0.0005$ ; morphine 2.5 mg/kg:  $P < 0.05$ ; Figure 4.5A), indicative of intact ability to discriminate in male mice. In female mice, no significant effect of the treatment was noted (Phase x Side x Treatment:  $P = 0.40$ , Table 2), with all animals spending significantly more time in the half-chamber containing the scented cotton, as compared to both the habituation phase ( $P < 0.0005$ ) or the opposite half-chamber containing the unscented cotton ( $P < 0.0005$ ), as revealed by post-hoc tests following a significant Phase X Side significant interaction effect ( $P < 0.0005$ , Table 2). The latter result is indicative of intact ability to discriminate in female mice (Figure 4.5B).

#### **4.4.3 Morphine-induced deficits in social interest are independent from anxiety-like states**

Statistical analysis of elevated plus-maze (EPM) measures revealed no effect of the treatment. In particular, i.p. administration of morphine did not affect either the percentage of time spent in the open arms ( $F_{2,29} = 1.37$ ,  $P = 0.27$  for males;  $F_{2,29} = 0.12$ ,  $P = 0.88$  for females) or the percentage of open arm entries ( $F_{2,29} = 2.22$ ,  $P = 0.13$  for males;  $F_{2,29} = 0.25$ ,  $P = 0.78$  for females), indicative of unaltered anxiety in both male (Figure 4.6A) and female (Figure 4.6B) mice. The number of closed arm entries ( $F_{2,29} = 0.07$ ,  $P = 0.93$  for males;  $F_{2,29} = 0.76$ ,  $P = 0.48$  for females) and of total arm entries ( $F_{2,29} = 0.15$ ,  $P = 0.86$  for males;  $F_{2,29} = 0.36$ ,  $P = 0.70$  for females) were not affected by morphine administration as well, indicative of unaltered locomotion in both male (Figure 4.6C) and female (Figure 4.6D) mice.

#### **4.4.4 Acute administration of morphine induces deficits in the motivation for and in the intake of highly palatable food (HPF)**

Both male and female mice undergoing fixed ratio (FR) reinforcement schedules acquired and displayed consistent levels of food-motivated behaviour in the operant paradigm. Statistical analysis of operant behavior measures during treatment days (TD, pulled) revealed a significant effect of the treatment in the number of active nose-pokes ( $F_{2,20} = 4.81$ ,  $P < 0.05$  for males;  $F_{2,22} = 5.36$ ,  $P < 0.05$  for females) and food pellets consumed ( $F_{2,20} = 6.33$ ,  $P < 0.05$  for males;  $F_{2,22} = 6.85$ ,  $P < 0.005$  for females). Specifically, i.p. administration of morphine in the volume of 2.5 mg/kg, *but not* 1.25 mg/kg, significantly reduced the number of active nose-pokes ( $P < 0.05$  for males, Figure 4.7A;  $P < 0.05$  for females, Figure 4.8A) and of food pellets consumed ( $P < 0.05$  for males, Figure 4.7B;  $P < 0.005$  for females, Figure 4.8B), indicative of impaired motivation for and intake of HPF. No significant effect of the treatment on the discrimination index was noted ( $F_{2,20} = 1.60$ ,  $P = 0.23$  for males;  $F_{2,22} = 1.94$ ,  $P = 0.17$  for

females), indicative of intact ability to distinguish between active and inactive nose-pokes in both male (Figure 4.7C) and female (Figure 4.8C) mice. No significant effect of the treatment on horizontal activity (i.e., back and forth, measured in light beams) was noted ( $F_{2,20}=1.11$ ,  $P=0.35$  for males;  $F_{2,22}=2.91$ ,  $P=0.08$  for females), indicative of unaltered locomotor activity in both male and female mice (Figure 4.9A). A significant effect of the treatment on vertical activity (i.e., rearings, measured in light beams) was noted in female mice ( $F_{2,22}=5.43$ ,  $P<0.05$ ). Specifically, i.p. administration of morphine in the volume of 2.5 mg/kg, but not 1.25 mg/kg, significantly reduced the number of rearings ( $P<0.05$ , Figure 4.09B), indicative of impaired “exploratory behavior” in female mice. No significant effect was noted in male mice ( $F_{2,20}=2.09$ ,  $P=0.15$ ), despite a strong decrease in the number of rearing was noticeable as well.

#### 4.4.5 Morphine reward assessed by single-exposure conditioning place preference

Male mice administered with morphine during the afternoon conditioning session displayed rewarding responses to the drug. In particular, during the post-conditioning test, mice administered with the 2.5 mg/kg morphine dose, *but not* mice administered with saline, spent significantly more time in the drug-paired chamber of the CPP apparatus, as compared to the pre-conditioning test ( $F_{1,19}=5.28$ ,  $P<0.05$ ; Figure 4.10).

#### 4.4.6 CRF antagonism fully recovers the deficits in social interest induced by morphine

Statistical analysis of sociability measures revealed a Phase X Side X Pre-treatment X Treatment significant interaction effect in male mice ( $P<0.05$ , Table 3). Post-hoc individual group comparisons indicated that not just control subjects pre-treated with vehicle and treated with saline (veh/sal), but also subjects pre-treated with antalarmin and treated with morphine in the volume of 2.5 mg/kg (anta/mor) displayed significant social interest. Indeed, they spent more time with the unfamiliar conspecific, as compared to both the previous phase ( $P<0.05$  for veh/sal;  $P<0.005$  for anta/mor; Figure 4.11A) and the opposite half-chamber ( $P<0.05$  for veh/sal;  $P<0.05$  for anta/mor; Figure 4.11A). On the other side, subjects pre-treated with vehicle and treated with morphine (veh/mor) failed to spend more time in the half-chamber containing the unfamiliar conspecific, as compared to both the previous phase ( $P=0.91$ ) or the opposite half-chamber ( $P=0.77$ ). Moreover, veh/mor mice spent significantly less time in the half-chamber containing the animal when compared to veh/sal ( $P<0.0005$ ; Figure 4.11A), thereby replicating the results shown in Figure 4.4A. Surprisingly, veh/mor mice spent significantly less time in the half-chamber containing the animal when compared to anta/mor as well ( $P<0.0005$ ; Figure 4.11A), indicative of rescued social interest in morphine-treated male mice pre-treated with antalarmin. According to the statistics employed, subjects pre-treated with antalarmin and treated with saline (anta/sal) also failed

to display social interest, spending similar time in the half-chamber with the unknown congener, as compared to both the previous phase ( $P=0.82$ ) and the opposite half-chamber ( $P=0.49$ ). In addition, the time spent by anta/sal mice with the animal was significantly lower when compared to veh/sal mice ( $P<0.05$ ; Figure 4.12A). Statistical analysis of locomotor activity measures revealed no effect of the pre-treatment ( $P=0.88$ ; Table 3), but a significant effect of the treatment ( $P<0.05$ ; Table 3) that is dependent on the test phases, as revealed by a significant Phase X Treat interaction effect ( $P<0.05$ ; Table 3). Indeed, morphine-treated male mice travelled a longer distance during the sociability phase, as compared to saline-treated mice ( $P<0.05$ ; Figure 4.13). In female subjects, systemic pre-treatment with the CRF<sub>1</sub> receptor-preferring antagonist antalarmin induced freezing behavior in morphine-treated mice (anta/mor), forcing us to exclude almost all the animals in this experimental group (4/5) according to the inclusion criteria discussed in the Materials and methods section. However, statistical analysis of the remaining experimental groups (pre-treatment not considered) revealed a Phase X Side X Treatment significant interaction effect ( $P<0.05$ ; Table 3). Indeed, saline-treated mice spent more time in the half-chamber with the unfamiliar conspecific, as compared to both the previous phase ( $P<0.0005$ ; Figure 4.11B) and the opposite half-chamber ( $P<0.0005$ ; Figure 4.11B), while morphine-treated mice spent similar time in the half-chamber with the unfamiliar conspecific, as compared to both the previous phase ( $P=0.21$ ) and the opposite half-chamber ( $P=0.31$ ). Moreover, the time spent by morphine-treated mice with the animal was significantly lower when compared to saline-treated mice ( $P<0.005$ ; Figure 4.11B), thereby replicating the results shown in Figure 4.4B.

#### **4.4.7 CRF antagonism does not recover the deficits in motivation for and intake of HPF induced by morphine**

Both male and female mice undergoing fixed ratio (FR) reinforcement schedules acquired and displayed consistent levels of food-motivated behaviour in the operant paradigm. Statistical analysis of operant behavior measures during treatment days (TD, pulled) revealed a significant effect of the treatments in the number of active nose-pokes ( $F_{3,33}=8.53$ ,  $P<0.0005$  for males;  $F_{3,33}=23.3$ ,  $P<0.0005$  for females) and food pellets consumed ( $F_{3,33}=11.0$ ,  $P<0.0005$  for males;  $F_{3,33}=27.8$ ,  $P<0.0005$  for females). Specifically, independently from the per os pre-treatment, i.p. administration of morphine in the volume of 2.5 mg/kg significantly reduced the number of active nose-pokes ( $P<0.005$  for males, Figure 4.13A;  $P<0.0005$  for females, Figure 4.14A) and of food pellets consumed ( $P<0.005$  for males, Figure 4.13B;  $P<0.0005$  for females, Figure 4.14B), thereby replicating the results shown in Figure 4.7 and Figure 4.8. The latter results also indicate no effect of antalarmin on morphine-induced motivational and intake deficits. No significant effect of the treatments on the discrimination index was noted ( $F_{3,27}=0.17$ ,  $P=0.97$  for males;  $F_{3,27}=2.40$ ,  $P=0.09$  for females), indicative of intact ability to distinguish between active and inactive nose-pokes in both male (Figure 4.13C)



and female (Figure 4.14C) mice. No significant effect of the treatments on horizontal activity (i.e., back and forth, measured in light beams) was noted ( $F_{3,33}=0.59$ ,  $P=0.63$  for males;  $F_{3,33}=2.21$ ,  $P=0.10$  for females), indicative of unaltered locomotor activity in both male and female mice (Figure 4.15A). A significant effect of the treatment on vertical activity (i.e., rearings, measured in light beams) was noted in both male ( $F_{3,33}=11.4$ ,  $P<0.0005$ ) and female ( $F_{3,33}=14.1$ ,  $P<0.0005$ ) mice. In particular, independently from the pre-treatment, i.p. administration of morphine in the volume of 2.5 mg/kg significantly reduced the number of rearings ( $P<0.0005$ ), indicating impaired “exploratory behavior” in both male and female mice (Figure 4.15B).

## 4.5 Discussion

In the present study, we investigated the initial effects of morphine on “natural” rewards in mice. We found that, independently from the gender, acute administration of relatively low doses of the drug induces profound deficits in social interest, motivation for and intake of highly palatable food (HPF). Notably, we demonstrated that these phenomena are not due to altered locomotor activity, olfactory dysfunctions, impaired stimuli discrimination or anxiety-like states. Once selected the most effective dose of morphine (2.5 mg/kg), we subsequently revealed its rewarding properties with an original single-exposure conditioned place preference (CPP) procedure. Then, by employing a pharmacological approach, we investigated the involvement of the CRF system in the behavioral deficits induced by the selected dose of the drug. Aside replicating all of the previously obtained results, pharmacological blockade of CRF receptors was able to fully recover the drug-induced deficits in social interest, *but not* the deficits in motivation for and intake of HPF.

To assess the impact of relatively low doses of morphine (1.25-2.5 mg/kg) upon social interest, mice were tested in the three-chamber (3-CH) task immediately after intraperitoneal (i.p.) administration of the drug. Independently from the gender, acute administration of the 2.5 mg/kg dose of morphine strongly impaired social interest, as compared to saline-treated controls. Specifically, drug-treated mice failed to spend more time in the half-chamber containing the unfamiliar conspecific, as compared to both the previous habituation phase and the opposite half-chamber containing the unanimated object. In male subjects, although not significant, blunted social interest was noticeable after injection of the 1.25 mg/kg dose of morphine, suggesting a dose-dependent effect of the drug. In female subjects, however, both morphine doses significantly impaired social interest, indicating a gender difference. To date, relatively few studies have investigated the initial impact of substances of abuse upon social behavior. For instance, psychostimulants such as methamphetamine, amphetamine, cocaine and MDMA have been shown to acutely suppress social interaction (SI) in adult male mice [Maldonado and Navarro, 2001, Navarro and Maldonado, 1999] and rats [Daza-Losada et al., 2009, Slamberova et al., 2015, 2010]. Moreover, morphine (2.5-5 mg/kg) has been shown to acutely reduce particular patterns of SI in adult male rats, such as mutual sniffing, allo-grooming, following and genital investigation [Slamberova et al., 2016]. However, to our knowledge, the present study is the first to date showing reduced social interest following acute administration of a substance of abuse in the 3-CH paradigm, which, unlike the SI test, provides measures of social interest that are independent from anxiety-like states [Moy et al., 2013, Silverman et al., 2010]. Nevertheless, we carried out further studies to provide additional evidence of unaltered anxiety-like behavior in drug-treated subjects by testing our animals in the elevated plus-maze (EPM), a thoroughly validated rodent model for the study of the anxiolytic-like or anxiogenic-like properties of pharmacological agents [Cole et al., 1995, Hogg, 1996]. Independently from the gender, none of the two doses of morphine employed herein resulted in altered anxiety-like states, as assessed by the percentage of time or

entries made into the open arms of the EPM apparatus [Pellow et al., 1985, Rodgers et al., 1997]. Previous works have shown that acute i.p. injections of morphine (5-6 mg/kg) produced anxiolytic-like effects in rats tested in the EPM [Koks et al., 1999, Rezayof et al., 2009, Zarrindast et al., 2005]. However, in agreement with the present findings, no anxiolytic-like effects of morphine were observed in mice administered with drug doses lower than 10 mg/kg [Shin et al., 2003].

One original aspect of the current study is the set-up of a 3-CH task to test the effects of morphine upon olfaction, based on evidence of adult male mice displaying similar interest towards an unfamiliar conspecific and a scented cotton in the same behavioral paradigm [Ryan et al., 2008]. Accordingly, in the current study, delivering a discrete social olfactory cue (i.e., scented cotton, a shredded cotton nestlet left for 24 h in a cage with three same-sex age-matched unknown congeners) in the 3-CH task was sufficient to elicit interest in drug-naïve male and female mice. Interestingly, the amount of time spent by saline-treated mice with the scented cotton nestlet during the olfactory task was undiscernible from the amount of time spent by saline-treated mice with the unfamiliar conspecific during the social task, strengthening the importance of olfactory stimuli in driving mouse behavior [Arakawa et al., 2008, Hurst et al., 2001]. Notably, during the olfactory task, both male and female mice treated with the 2.5 mg/kg dose of morphine displayed a reliable preference for the scented cotton, as compared to the unscented one. The latter result is indicative of unaltered olfaction-driven stimuli discrimination in morphine-treated mice. Moreover, the fact that mice administered with the drug displayed interest towards the animal-scented cotton, *but not* towards the animal itself, represents a powerful indication that the non-living stimulus, despite representing a strong olfactory cue, does not embody all the requirements needed to be considered a real “social” stimulus (e.g., visual, tactile and auditory cues).

To assess the impact of relatively low doses of morphine (1.25-2.5 mg/kg) upon motivation for and intake of HPF, mice were trained for the acquisition of a food-driven operant task. Specifically, the impact of morphine administration was assessed using a progressive ratio (PR) schedule of reinforcement, which is known to reliably measure the motivational properties of a reinforcer and/or the motivational state of an animal [Arnold and Roberts, 1997, Hodos, 1961]. Since we used a within-subject experimental design, to control for residual effects of the drug, a PR-2 schedule was applied also during the injection-free operant tests performed in between treatment days. Independently from the gender, acute administration of the 2.5 mg/kg dose of morphine significantly impaired the motivation for and the intake of HPF, as revealed by a reduced number of active nose-pokes and of food pellets ingested. However, the ability to discriminate between active and inactive nose-pokes (i.e., discrimination index) was unaffected by the drug, indicating preserved cognitive function. Acute administration of the 1.25 mg/kg dose of morphine had no effect on either of the two measures. It could be argued that the initial hypophagic response observed herein is due to the sedative properties of the drug, since doses of morphine ranging between 5-10 mg/kg have been reported to cause an

initial phase of about 1 h of inhibition of all activities (i.e., freezing behavior) in single-housed mice [Hecht and Schiorring, 1979]. However, our measures of horizontal activity (i.e., back and forth) showed no significant differences between experimental groups, thereby excluding putative sedative effects of the drug. Accordingly, an early study reported that lower doses of morphine (0.3-2.5 mg/kg) do not modify locomotor activity in group-housed adult male mice [Marcais et al., 1981]. Interestingly, the latter study also showed an inhibition of spontaneous climbing behavior following injection of morphine (1.25 mg/kg), in line with the strong decrease in vertical activity (i.e., rearing) observed herein. Relevantly to the decreased motivation to obtain food in drug-administered subjects, rhesus monkeys acutely administered with morphine (0.1-5.6 mg/kg) 15 min before the testing session displayed a significant dose-dependent decrease in food-reinforced operant behavior [Schulze and Paule, 1991]. Moreover, male rats acutely administered with morphine (3 mg/kg) exhibited decreased response rates to incremental food-based repeated acquisition procedures (i.e., incremented sequential responses on lever presses required to obtain food) without showing impairments in the accuracy of discrimination [Paule and McMillan, 1984]. Interestingly, acute morphine administration and morphine withdrawal seem to have opposite effects upon food-driven operant behavior. Indeed, morphine withdrawal significantly increased motivation for and intake of HPF in male mice tested in a similar operant behavior paradigm [Rouibi and Contarino, 2012]. Notably, in the latter study, the increased motivation for food displayed by morphine-withdrawn mice was paralleled by decreased horizontal activity and unaffected vertical activity, once again excluding a role for locomotion and/or exploration in the motivational properties of drugs of abuse.

To assess the rewarding properties of morphine, we used the CPP paradigm, a behavioral test extensively employed to investigate the rewarding effects of drugs of abuse and other reinforcing stimuli in rodents [Calcagnetti and Schechter, 1994, Contarino et al., 2002, Hoffman, 1989, Tzschentke, 1998]. However, in contrast to the majority of studies carried out to date with repeated drug administrations and conditioning sessions protocols, herein we used a single-exposure (i.e., one conditioning trial) CPP procedure. The latter approach has been previously employed to assess the rewarding effects of drugs of abuse. For instance, single-exposure CPP has been reported for nicotine [Spina et al., 2006], morphine [Bardo and Neisewander, 1986, Fenu et al., 2006], cocaine [Crooks et al., 2010] and more recently alcohol [Grisel et al., 2014]. The single-exposure CPP is of major interest for several reasons. First of all, it is less time consuming and avoids excessive manipulation (i.e., fewer injections), thereby reducing stress and discomfort of the animals. Secondly, allows the study of the very initial positive or negative reinforcing effects of the drug upon behavior [Bozarth, 1987], ruling out potential consequences of repeated drug exposures upon neuronal signalling [Ungless et al., 2001]. Notably, the present findings might have higher scientific significance, as compared to prior results obtained with the same experimental approach. Indeed, besides administering a much lower dose of the drug (i.e., 2.5 mg/kg instead of 8 mg/kg) through a different administration route (i.e., intraperitoneal instead

of intravenous) in another animal model (i.e., mice instead of rats), we employed a more reliable CPP paradigm (i.e., an unbiased CPP protocol instead of a systematic association of the drug with the non-preferred compartment). Despite these major differences with previous works, we were able to demonstrate a significant preference for a context paired with a single exposure to morphine in male mice. Future experiments will address the question if female mice display morphine reward using the same experimental approach.

To assess the involvement of the CRF system in the social and motivational deficits induced by acute administration of a rewarding dose of morphine, we employed a pharmacological approach. In male mice, per os administration of the CRF<sub>1</sub> receptor-preferring antagonist antalarmin completely rescued the profound deficits in social interest induced by the drug, as assessed by the 3-CH task. This result indicates a central role for the CRF system in mediating substance-induced impairments in social behavior. Notably, antalarmin did not affect locomotion, indicating a specific effect upon social behavior. However, pre-treatment with antalarmin induced freezing behavior in morphine-treated, *but not* in saline-treated, female mice. The mechanisms underlying the latter effect remain totally unknown. However, the same dose of antalarmin employed herein (20 mg/kg, per os) did not produce freezing behavior in female mice undergoing opiate withdrawal [Papaleo et al., 2007] or male mice treated with cocaine [Contarino et al., 2017], suggesting a gender-dependent impact of antalarmin upon the acute effects of opiate drugs.

An increasing number of studies suggest a central but complex role for the CRF system in the modulation of social behavior. For instance, male mice bearing a targeted CRF<sub>2</sub> receptor-deficiency in the medial nucleus of the amygdala (MeA) exhibited abnormally low preference for novel conspecifics in a 3-CH-based task [Shemesh et al., 2016]. Moreover, intracerebroventricular (i.c.v.) administration of CRF has been shown to decrease the time of interaction with an unfamiliar or a novel conspecific in male mice tested in the 3-CH task, an effect that was reverted by i.c.v. co-administration of antalarmin [Bagosi et al., 2017a,b]. In the present study, however, the rescue of morphine-induced deficits in social interest by systemic administration of the latter compound might have higher clinical value.

To our knowledge, only another study to date has investigated the role of the CRF system in the social behavior deficits induced by drugs of abuse. In such study, Morisot et al. showed that CRF<sub>2</sub> receptor-deficiency eliminates the sociability deficits and vulnerability induced by withdrawal from chronic cocaine administration [Morisot et al., 2018]. Thus, the present findings provide additional evidence of CRF-mediated mechanisms underlying the deleterious effects of drugs of abuse upon social behavior. Nevertheless, antalarmin failed to rescue the decreased nose-poking or pellet intake that followed acute morphine administration, suggesting that the CRF system is not involved in the initial food-driven motivational deficits induced by opiate drugs. The differential effects of the CRF<sub>1</sub> receptor-preferring antagonist upon morphine-induced social or motivational deficits remain to be elucidated. A wealth of experimental evidence indicates that motivated behavior is facilitated by the activity of the mesolimbic dopamine (DA) projections that from the ventral tegmental

area (VTA) extend to the nucleus accumbens (NAc) [Salamone et al., 2009]. In this context, Bariselli et al. have recently shown that inhibition of VTA-DA neurons attenuates the exploration of non-familiar conspecifics and interferes with the reinforcing properties of social interaction in male mice, as assessed by the 3-CH task and a conditioned place preference (CPP) procedure, respectively [Bariselli et al., 2018]. Even more interestingly, Wanat et al. have demonstrated that CRF acts in the VTA to attenuate the motivational properties of “natural” rewards. Specifically, rats undergoing acute restraint stress or injected with CRF into the VTA exhibited reduced motivation for food under a PR reinforcement schedule, an effect that was blocked by bilateral intra-VTA injection of a CRF receptors antagonist [Wanat et al., 2013]. At last, an additional plausible player in this complex equation is the hypothalamic neurohormone oxytocin (OXY). Indeed, aside from its long-known pro-social [Bartz et al., 2010, Ishak et al., 2011, Kosfeld et al., 2005] and hypophagic effects [Lawson et al., 2015, Ott et al., 2013, Thienel et al., 2016, Spetter et al., 2018], it has been recently demonstrated that OXY release in the VTA is necessary to elicit social reward [Hung et al., 2017]. Thus, future studies might investigate the role of VTA-DA neurons and hypothalamic OXY circuits, as well as their interaction with the CRF system, in the behavioral effects described herein.

In conclusion, the present results indicate that, independently from the gender, acute administration of a relatively small rewarding dose of morphine in mice strongly decreases the interest for “natural” rewards, such as social behavior and the motivation to obtain HPF. These findings provide initial experimental evidence of “hijacking” of brain reward systems by substances of abuse, blending in and enriching fundamental assumptions of addiction neuroscience models. We propose that the latter phenomenon is mediated, at least in part, by the CRF system, thereby strengthening the notion of a therapeutic potential for CRF-targeting pharmacological agents. This notion acquires additional clinical relevance when considering that treatment approaches that have recently gained major importance, such as cognitive behavioral therapy, rely on the emotional responsiveness and social abilities of drug users [Moos, 2007], which are dramatically impaired in drug-dependent individuals [Preller et al., 2014b]. In addition, we set-up a novel CPP assay of initial sensitivity to the rewarding effects of morphine and presented an original 3-CH-based olfactory task that might be useful to understand critical features of substance use disorders.

## 4.6 Tables and figures

3-CH social task		Sociability males		Sociability females	
Variable	F	P	F	P	
Treat	F <sub>2,31</sub> =6.27	<0.05	F <sub>2,31</sub> =8.65	<0.005	
Phase	F <sub>1,31</sub> =45.3	<0.0005	F <sub>1,31</sub> =40.4	<0.0005	
Side	F <sub>1,31</sub> =10.9	<0.005	F <sub>1,33</sub> =14.7	<0.005	
Treat X Phase	F <sub>2,31</sub> =0.48	0.62	F <sub>2,31</sub> =1.41	0.26	
Treat X Side	F <sub>2,31</sub> =1.07	0.35	F <sub>2,31</sub> =0.85	0.44	
Phase X Side	F <sub>1,31</sub> =8.44	<0.05	F <sub>1,31</sub> =20.7	<0.0005	
Treat X Phase X Side	F <sub>2,31</sub> =5.10	<0.05	F <sub>2,31</sub> =8.01	<0.005	
3-CH social task		Locomotion males		Locomotion females	
Variable	F	P	F	P	
Treat	F <sub>2,31</sub> =1.97	0.16	F <sub>2,31</sub> =1.57	0.22	
Phase	F <sub>1,31</sub> =25.8	<0.0005	F <sub>1,31</sub> =0.38	0.54	
Treat X Phase	F <sub>2,31</sub> =1.45	0.25	F <sub>2,31</sub> =2.83	0.07	

Table 1, Figure 4.1 – Statistical analysis of social interest (sociability) and locomotion displayed by saline-treated and morphine-treated (Treat) male and female mice during the 3-CH social task. Social interest is described as the time spent by the subject animal in the half-chamber with the unfamiliar conspecific, as compared to both the same half-chamber during the previous habituation phase (Phase) and the opposite half-chamber (Side) containing the unanimated object. The accepted value for significance was  $P < 0.05$ .

3-CH olfactory task Variable	Sociability males		Sociability females	
	F	P	F	p
Treat	F <sub>2,31</sub> =1.34	0.28	F <sub>2,29</sub> =3.24	0.05
Phase	F <sub>1,31</sub> =85.9	<0.0005	F <sub>1,29</sub> =80.1	<0.0005
Side	F <sub>1,31</sub> =19.7	<0.0005	F <sub>1,29</sub> =8.72	<0.05
Treat X Phase	F <sub>2,31</sub> =0.09	0.91	F <sub>2,29</sub> =2.28	0.12
Treat X Side	F <sub>2,31</sub> =1.82	0.18	F <sub>2,29</sub> =1.01	0.38
Phase X Side	F <sub>1,31</sub> =35.5	<0.0005	F <sub>1,29</sub> =20.6	<0.0005
Treat X Phase X Side	F <sub>2,31</sub> =5.39	<0.05	F <sub>2,29</sub> =0.95	0.40

Table 2, Figure 4.2 – Statistical analysis of olfactory discrimination (sociability) displayed by saline-treated and morphine-treated (Treat) male and female mice during the 3-CH olfactory task. Olfactory discrimination is described as the time spent by the subject animal in the half-chamber with the scented cotton, as compared to both the same half-chamber during the previous habituation phase (Phase) and the opposite half-chamber (Side) containing the unscented cotton. The accepted value for significance was  $P < 0.05$ .



3-CH social task	Sociability males		Sociability females	
Variable	F	P	F	P
Pre-Treat	F <sub>1,23</sub> =3.13	0.09	NP	NP
Treat	F <sub>1,23</sub> =2.83	0.11	F <sub>1,11</sub> =27.2	<0.0005
Phase	F <sub>1,23</sub> =15.9	<0.005	F <sub>1,11</sub> =41.1	<0.0005
Side	F <sub>1,23</sub> =11.9	<0.005	F <sub>1,11</sub> =3.97	0.07
Pre-Treat X Treat	F <sub>1,23</sub> =25.9	<0.0005	NP	NP
Pre-Treat X Phase	F <sub>1,23</sub> =0.11	0.74	NP	NP
Pre-Treat X Side	F <sub>1,23</sub> =3.23	0.09	NP	NP
Treat X Phase	F <sub>1,23</sub> =0.03	0.87	F <sub>1,11</sub> =5.97	<0.05
Treat X Side	F <sub>1,23</sub> =0.60	0.45	F <sub>1,11</sub> =0.41	0.54
Phase X Side	F <sub>1,23</sub> =9.36	<0.05	F <sub>1,11</sub> =8.82	<0.05
Pre-Treat X Treat X Phase	F <sub>1,23</sub> =10.0	<0.005	NP	NP
Pre-Treat X Treat X Side	F <sub>1,23</sub> =6.76	<0.05	NP	NP
Pre-Treat X Phase X Side	F <sub>1,23</sub> =2.93	0.10	NP	NP
Treat X Phase X Side	F <sub>1,23</sub> =0.99	0.33	F <sub>1,11</sub> =9.32	<0.05
Pre-Treat X Treat X Phase X Side	F <sub>1,23</sub> =6.07	<0.05	NP	NP
3-CH social task	Locomotion males			
Variable	F		P	
Pre-Treat	F <sub>1,23</sub> =0.02		0.88	
Treat	F <sub>1,23</sub> =6.64		<0.05	
Phase	F <sub>1,23</sub> =4.46		<0.05	
Pre-Treat X Treat	F <sub>1,23</sub> =0.07		0.80	
Pre-Treat X Phase	F <sub>1,23</sub> =0.85		0.37	
Treat X Phase	F <sub>1,23</sub> =5.95		<0.05	
Pre-Treat X Treat X Phase	F <sub>1,23</sub> =0.05		0.83	

Table 3, Figure 4.3 – Statistical analysis of social interest (sociability) and locomotor activity displayed by saline-treated and morphine-treated (Treat) male and female mice during the 3-CH social task with antalarmin (Pre-Treat). Social interest is described as the time spent by the subject animal in the half-chamber with the unfamiliar conspecific, as compared to both the same half-chamber during the previous habituation phase (Phase) and the opposite half-chamber (Side) containing the unanimated object. The accepted value for significance was  $P < 0.05$ .

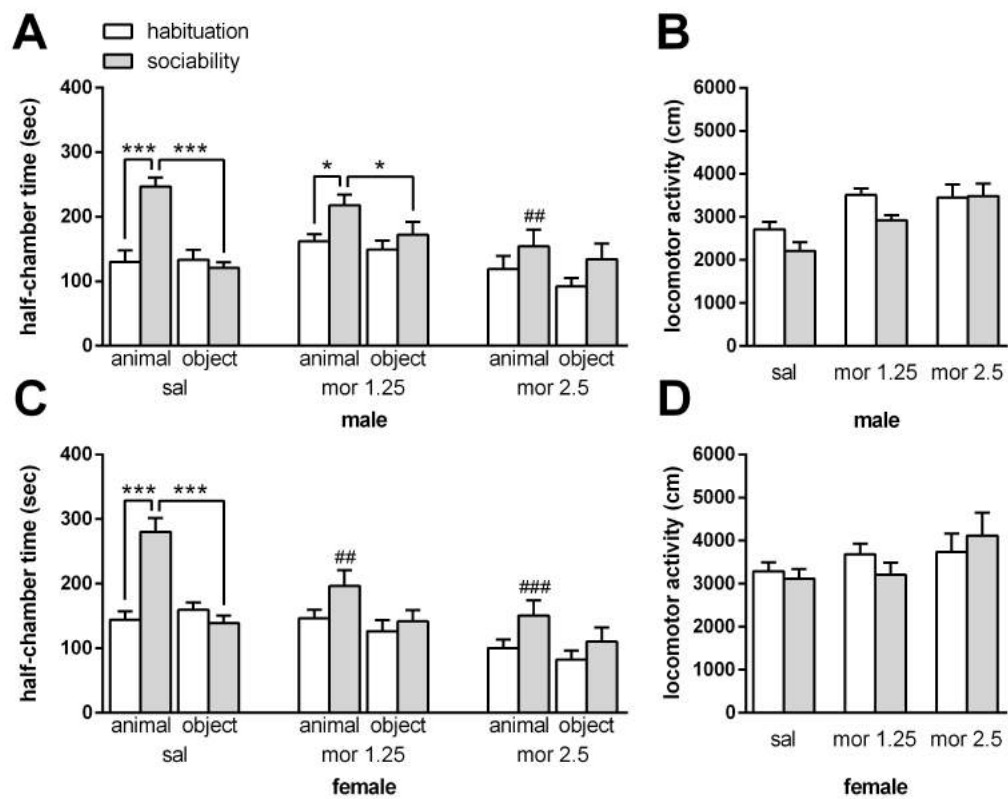


Figure 4.4 – **3-CH social task and relative locomotor activity.** Social interest displayed by male (A) and female (B) mice in the 3-CH social task after treatment with either saline (sal), morphine 1.25 mg/kg (mor 1.25) or morphine 2.5 mg/kg (mor 2.5). Social interest is expressed as the time spent by the subject mouse in the half-chamber with the unfamiliar conspecific (animal, grey), as compared to both the same half-chamber during the previous habituation phase (animal, white) and the opposite half-chamber containing the unanimated object (object, grey). Independently from the gender, animals treated with morphine in the dose of 2.5 mg/kg spent less time with the unfamiliar conspecific, as compared to saline-treated controls. (C) In female subjects, animals treated with morphine in the dose of 1.25 mg/kg also spent less time with the unfamiliar conspecific, as compared to saline-treated controls. Distance travelled by male (B) and female (D) mice during the two phases of the 3-CH social task. No significant differences were noted between experimental groups. Values represent mean  $\pm$  S.E.M. N=9-13 per experimental group. \* $P < 0.05$ , \*\*\* $P < 0.0005$ . ## $P < 0.005$ , ### $P < 0.0005$  (# = as compared to the time spent with the animal during the sociability phase by saline-treated controls).

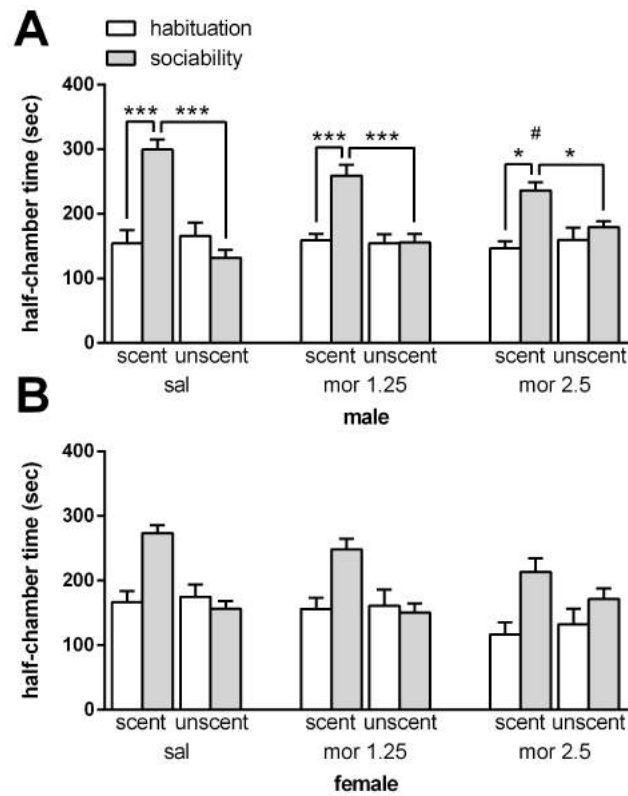


Figure 4.5 – **3-CH olfactory task**. Olfactory discrimination abilities displayed by male (A) and female (B) mice in the 3-CH olfactory task after treatment with either saline (sal), morphine 1.25 mg/kg (mor 1.25) or morphine 2.5 mg/kg (mor 2.5). Olfactory discrimination is expressed as the time spent by the subject mouse in the half-chamber with the scented cotton (scent, grey), as compared to both the same half-chamber during the previous habituation phase (scent, white) and the opposite half-chamber containing the unscented cotton (unscent, grey). (A) In male subjects, animals treated with morphine in the dose of 2.5 mg/kg spent less time with the scented cotton, as compared to saline-treated controls. Despite this, all experimental groups displayed intact olfactory discrimination abilities. (B) In female subjects, all animals displayed intact olfactory discrimination abilities, with no significant differences noted between experimental groups. Values represent mean  $\pm$  S.E.M.  $N=9-12$  per experimental group. \* $P<0.05$ , \*\*\* $P<0.0005$ . # $P<0.05$  (# = as compared to the time spent with the scented cotton during the sociability phase by saline-treated controls).

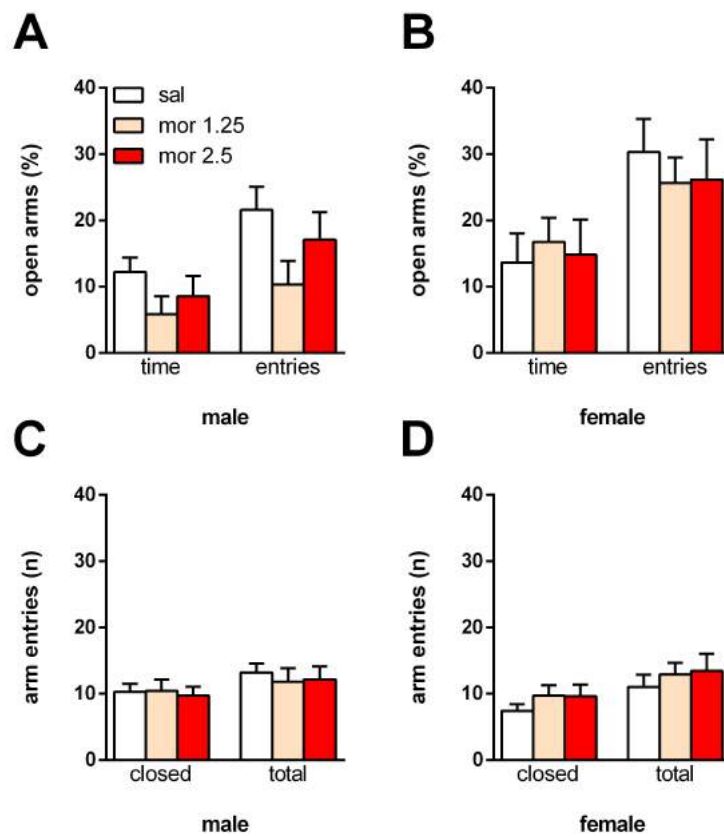


Figure 4.6 – **Elevated plus-maze test.** Percentage of time spent on and percentage of entries made into the open arms by male (A) and female (B) mice after treatment with either saline (sal), morphine 1.25 mg/kg (mor 1.25) or morphine 2.5 mg/kg (mor 2.5). Number of closed and total (closed plus open) arm entries made by male (C) and female (D) mice. No significant differences were noted between experimental groups. Values represent mean  $\pm$  SEM. N=10-11 per group.

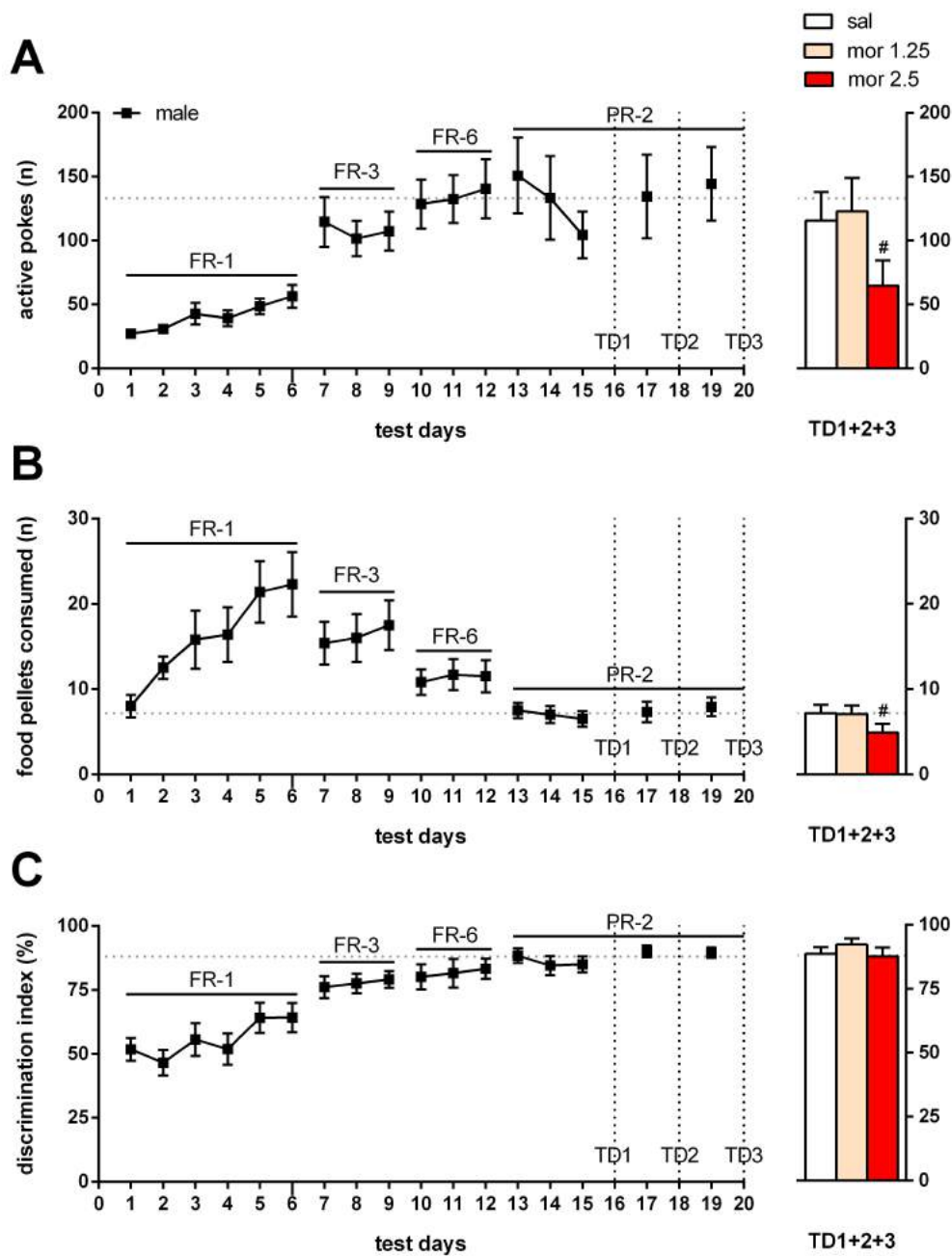


Figure 4.7 – **Operant behavior (male)**. Evolution of active nose-poking (A), daily food pellets intake (B) and discrimination index (C) of male mice undergoing fixed (FR) and progressive ratio (PR) reinforcement schedules. On treatment days (TD) 1, 2 and 3, mice were administered with saline (sal), morphine 1.25 mg/kg (mor 1.25) or morphine 2.5 mg/kg (mor 2.5). The higher dose of morphine induced a significant decrease in the number of active pokes (A) and of food pellets consumed (B) on a stable PR-2 reinforcement schedule. Values represent mean  $\pm$  SEM. N=11 per group. #P<0.05 (# = as compared to saline-treated control mice).

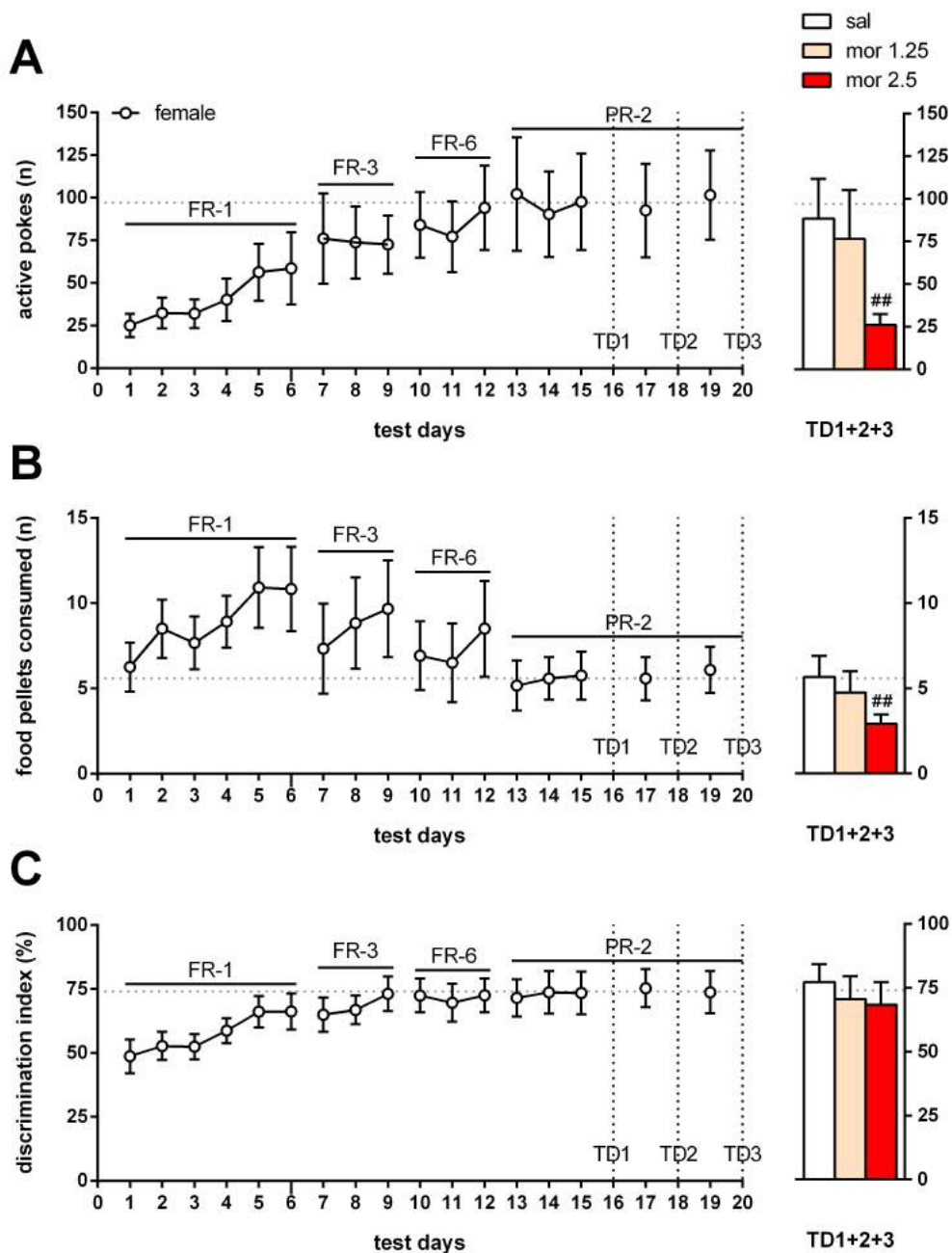


Figure 4.8 – **Operant behavior (female)**. Evolution of active nose-poking (A), daily food pellets intake (B) and discrimination index (C) of female mice undergoing fixed (FR) and progressive ratio (PR) reinforcement schedules. On treatment days (TD) 1, 2 and 3, mice were administered with saline (sal), morphine 1.25 mg/kg (mor 1.25) or morphine 2.5 mg/kg (mor 2.5). The higher dose of morphine induced a significant decrease in the number of active pokes (A) and of food pellets consumed (B) on a stable PR-2 reinforcement schedule. Values represent mean  $\pm$  SEM. N=12 per group. ##P<0.005 (# = as compared to saline-treated control mice).

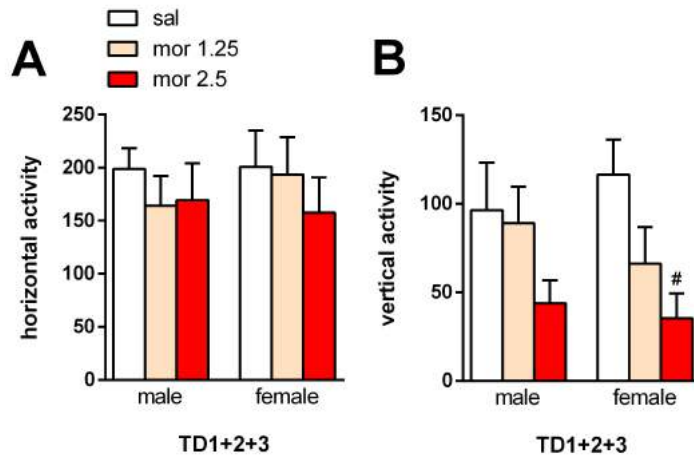


Figure 4.9 – **Locomotor activity (operant behavior)**. (A) Horizontal activity (i.e., back and forth, measured in photo beams) of male and female mice the day of the treatment (TD), when they were administered with saline (sal), morphine 1.25 mg/kg (mor 1.25) or morphine 2.5 mg/kg (mor 2.5). No significant differences were noted between experimental groups. (B) Vertical activity (i.e., rearings, measured in photo beams) of male and female mice the day of the treatment (TD). In female subjects, the higher dose of morphine induced a significant decrease in vertical activity. Values represent mean  $\pm$  SEM. N=11-12 per group. # $P < 0.05$  (# = as compared to saline-treated control mice).

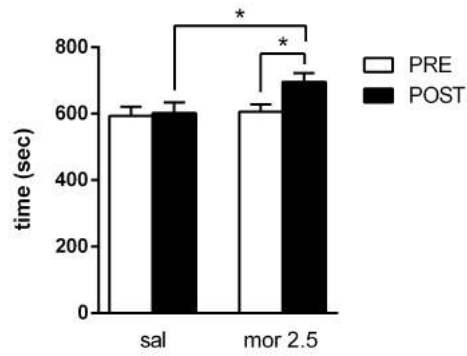


Figure 4.10 – **Single-exposure conditioned place preference.** Time spent in the drug-paired chamber of the conditioned place preference apparatus during the pre- and post-conditioning tests. Mice injected with morphine 2.5 mg/kg (mor 2.5) during the afternoon conditioning session displayed a significant increase in the time spent in the drug-paired chamber. Values represent mean  $\pm$  SEM. N=10-11 per group. \*P<0.05.



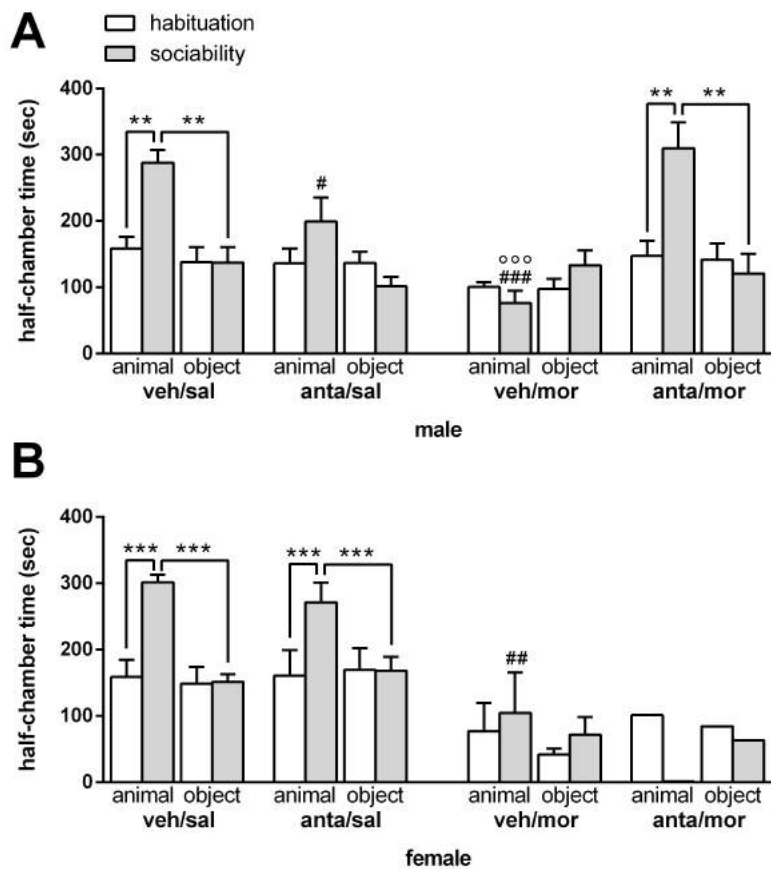


Figure 4.11 – **3-CH social task with antalarmin.** Social interest displayed by male (A) and female (B) mice in the 3-CH social task after pre-treatment with either vehicle (veh) or antalarmin (anta) and treatment with either saline (sal) or morphine 2.5 mg/kg (mor). Social interest is expressed as the time spent by the subject mouse in the half-chamber with the unfamiliar conspecific (animal, grey), as compared to both the same half-chamber during the previous habituation phase (animal, white) and the opposite half-chamber containing the unanimated object (object, grey). (A) In male subjects, veh/mor mice spent less time with the unfamiliar conspecific, as compared to both veh/sal and anta/mor mice. (B) In female subjects, anta/mor mice displayed freezing behavior. Nevertheless, veh/mor mice spent less time with the unfamiliar conspecific, as compared to veh/sal mice. Values represent mean  $\pm$  S.E.M. N=4-7 per experimental group. \* $P < 0.05$ , \*\* $P < 0.005$ , \*\*\* $P < 0.0005$ . ## $P < 0.005$ , ### $P < 0.0005$  (# = as compared to the time spent with the animal during the sociability phase by veh/sal). °°° $P < 0.0005$  (° = as compared to the time spent with the animal during the sociability phase by anta/mor).

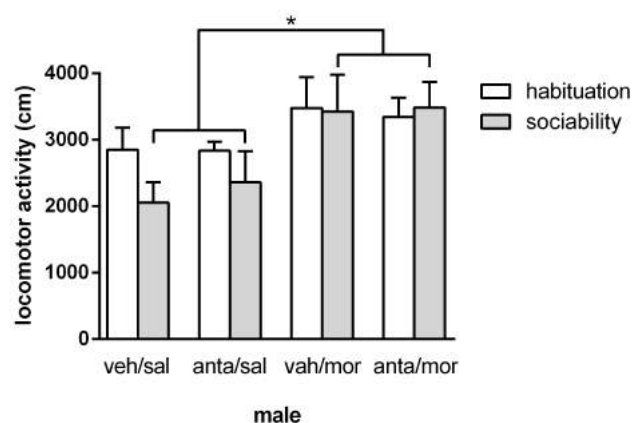


Figure 4.12 – **Locomotor activity (3-CH social task with antalarmin)**. Distance travelled by male mice during the two phases of the 3-CH social task after pre-treatment with either vehicle (veh) or antalarmin (anta) and treatment with either saline (sal) or morphine 2.5 mg/kg (mor). Independently from the pre-treatment, morphine-treated mice travelled a longer distance during the sociability phase, as compared to saline-treated mice. Values represent mean  $\pm$  SEM. N=9-13 per group. \*P<0.05.

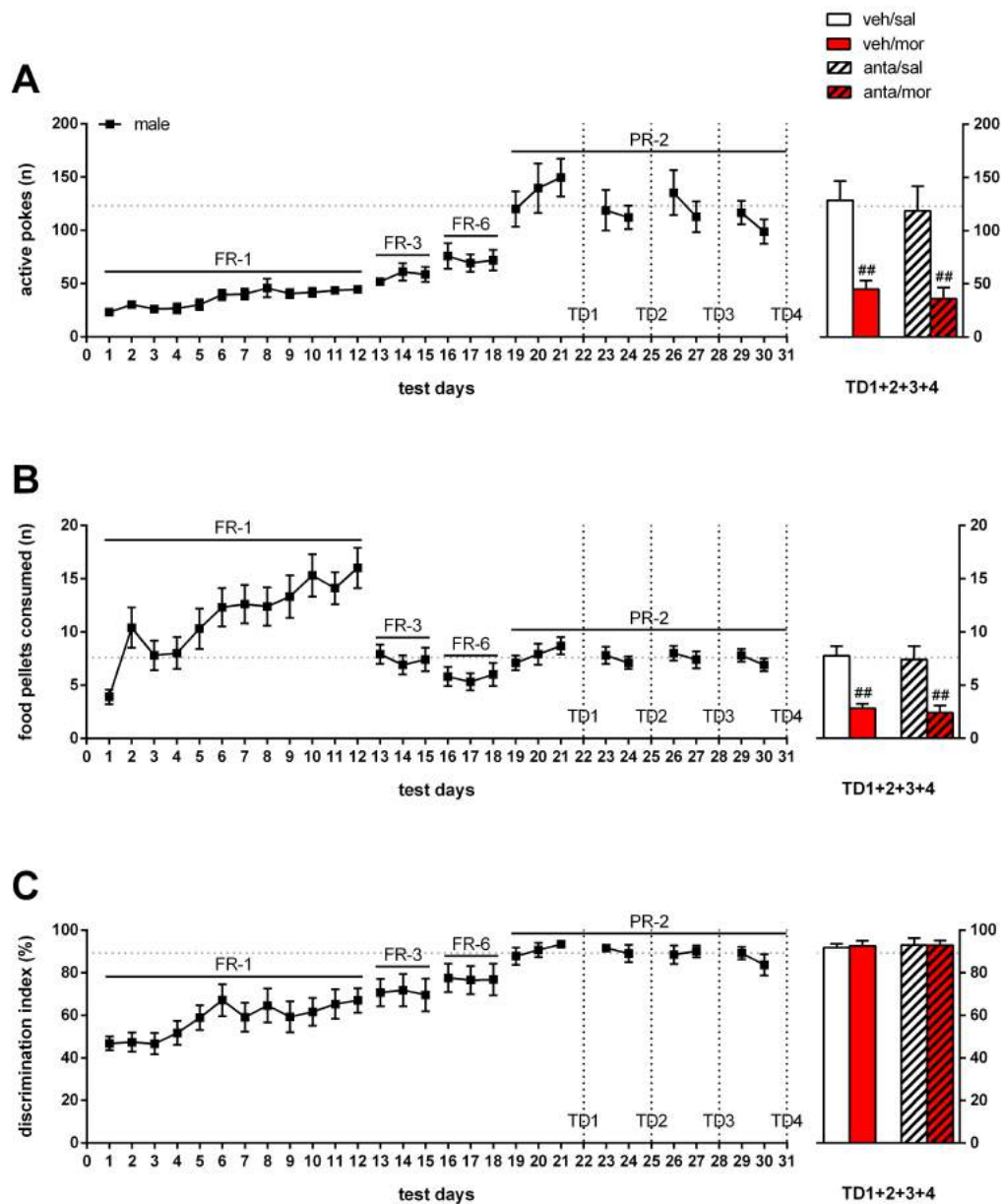


Figure 4.13 – **Operant behavior with antalarmin (male)**. Evolution of active nose-poking (A), daily food pellets intake (B) and discrimination index (C) of male mice undergoing fixed (FR) and progressive ratio (PR) reinforcement schedules. On treatment days (TD) 1, 2 and 3, mice were pre-treated with either vehicle (veh) or antalarmin (anta) 1 h before being administered with either saline (sal) or morphine 2.5 mg/kg (mor) and placed in the operant chambers. Independently from the pre-treatment, morphine-treated mice displayed a significant decrease in the number of active pokes (A) and of food pellets consumed (B) on a stable PR-2 reinforcement schedule. Values represent mean  $\pm$  SEM. N=12 per group.  $###P < 0.005$  ( $\# =$  as compared to saline-treated control mice).

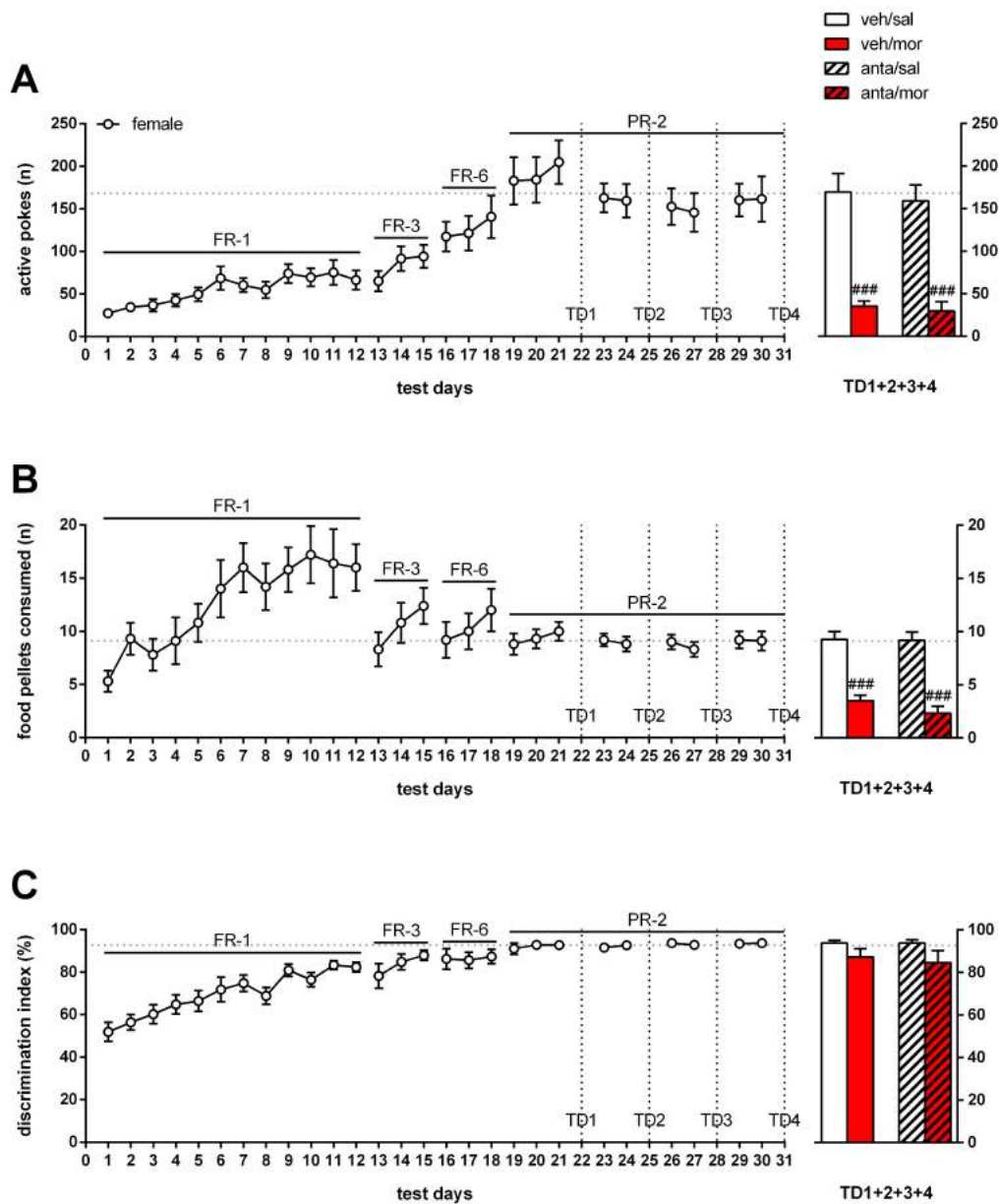


Figure 4.14 – **Operant behavior with antalarmin (female)**. Evolution of active nose-poking (A), daily food pellets intake (B) and discrimination index (C) of female mice undergoing fixed (FR) and progressive ratio (PR) reinforcement schedules. On treatment days (TD) 1, 2 and 3, mice were pre-treated with either vehicle (veh) or antalarmin (anta) 1 h before being administered with either saline (sal) or morphine 2.5 mg/kg (mor) and placed in the operant chambers. Independently from the pre-treatment, morphine-treated mice displayed a significant decrease in the number of active pokes (A) and of food pellets consumed (B) on a stable PR-2 reinforcement schedule. Values represent mean  $\pm$  SEM. N=12 per group. ###P<0.0005 (# = as compared to both saline-treated experimental groups).

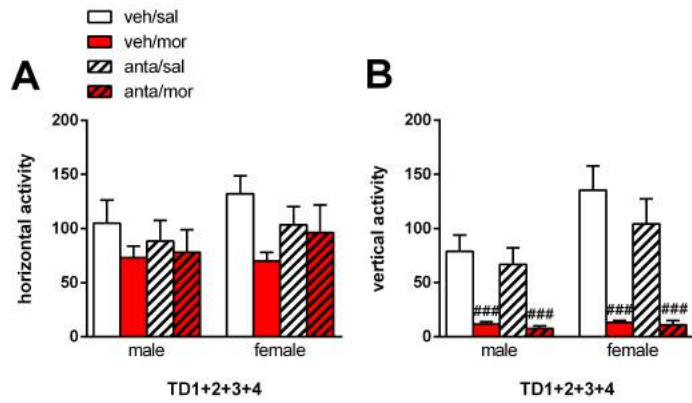


Figure 4.15 – **Locomotor activity (operant behavior with antalarmin)**. (A) Horizontal activity (i.e., back and forth, measured in photo beams) of male and female mice the day of the treatment (TD), when they were pre-treated with either vehicle (veh) or antalarmin (anta) 1 h before being administered with either saline (sal) or morphine 2.5 mg/kg (mor) and placed in the operant chambers. No significant differences were noted between experimental groups. (B) Vertical activity (i.e., rearings, measured in photo beams) of male and female mice the day of the treatment (TD). Independently from the gender and the pre-treatment, morphine-treated mice displayed decreased vertical activity. Values represent mean  $\pm$  SEM. N=12 per group. ###P<0.0005 (# = as compared to both saline-treated experimental groups).

## 5.1 Summary of present results

The aim of the present work was to assess vulnerability/resilience to opiate substances and to investigate the role for the corticotropin-releasing factor (CRF) system in the short- and long-term behavioral outcomes of opiate administration and withdrawal in mice.

To begin with, we investigated the effect of chronic morphine administration and withdrawal on social behavior in a previously unreported time-course fashion. Using the three-chamber (3-CH) test for sociability and social novelty preference, we initially found that opiate-withdrawn mice displayed significantly higher social interest following discontinuation of morphine administration, as compared to drug-naïve control mice. However, follow-up ethological analyses revealed increased aggressive behavior in opiate-withdrawn mice. Notably, social interest for an unfamiliar conspecific and aggressive behavior followed a similar time-course and strongly correlated one with each other, suggesting a possible role for aggressiveness in social behavior dysfunctions. Moreover, following exposure to an environmental stressor relatively long time after morphine discontinuation, neither drug-naïve control nor long-term opiate-withdrawn mice displayed impaired social interest for an unfamiliar conspecific, indicating stress resilience.

Using the CRF<sub>1</sub> receptor-deficient mouse model, we also tested the role for the CRF<sub>1</sub> receptor in opiate withdrawal-induced changes in social behavior. Likewise the studies mentioned above, we found that opiate withdrawal increased social interest in wild-type mice, as compared to saline-treated control mice. Moreover, CRF<sub>1</sub> receptor-deficiency increased social interest, independently from drug administration and withdrawal, providing initial evidence in favor of a major role for the CRF<sub>1</sub> receptor pathway in social behavior. In addition, CRF<sub>1</sub> heterozygous mice displayed a behavioral profile similar to CRF<sub>1</sub> knock-out mice. The latter finding suggests a minor role for the HPA axis in mediating social behavior of drug-naïve or opiate-withdrawn mice.

Then, we investigated the effect of relatively small doses of morphine on social behavior and motivation for and intake of highly palatable food (HPF).

In both male and female mice, we found that a single, acute administration of morphine strongly impaired social behavior, motivation for and intake of HPF, as assessed by the 3-CH test and a food-driven operant behavior task, respectively. Notably, morphine did not affect ambulatory activity, olfaction or anxiety-like behavior, suggesting a specific effect upon the hedonic/motivational properties of social behavior and HPF. Subsequently, we assessed the rewarding effects of the morphine dose producing social behavior and motivation deficits and found that it could induce conditioned place preference (CPP) after just a single drug conditioning session. The latter results indicated that the dose of morphine producing social behavior and motivation deficits also induced activation of brain reward systems. Altogether, these findings indicate decreased interest for “natural” rewards following exposure to a morphine dose that produces brain reward, providing initial and original experimental evidence of “hijacking” of brain reward systems by substances of abuse.

To investigate the role of the CRF system in the vulnerability of brain reward systems to opiate substances, we then employed a pharmacological approach. We found that systemic administration of the CRF<sub>1</sub> receptor-preferring antagonist antalarmin completely reversed the social behavior, *but not* the motivation and intake deficits induced by acute morphine administration. Thus, to some extent, the latter results indicate a critical role for the CRF system in mediating the drug-induced decrease in the interest for “natural” rewards, thereby strengthening the notion of a therapeutic potential for CRF-targeting pharmacological agents.

## 5.2 How does chronic morphine administration and withdrawal affect social interest?

In humans, deficits in social behavior, such as artificial sociability, antisocial traits and social isolation, are hallmarks of substance use disorders (SUDs) [Kessler et al., 1997, Regier et al., 1990, Mariani et al., 2008]. These deficits are believed to strongly contribute to the establishment and maintenance of compulsive drug-seeking and drug-taking, making abstinence difficult to sustain and relapse more likely to happen [Babor et al., 1976, Le Moal and Koob, 2007, Volkow et al., 2011].

In animals, withdrawal from chronic drug administration has been shown to decrease social interest in the 3-CH task in a number of studies [Becker et al., 2016, Morisot et al., 2018, Zanos et al., 2014] and the reemergence of social behavior deficits following exposure to an ethological environmental stressor has also been recently observed [Morisot et al., 2018]. However, throughout our investigation of the impact of opiate withdrawal upon social behavior using the same behavioral paradigm (i.e., the 3-CH task), morphine-treated mice always spent either a similar or a higher amount of time interacting with the unfamiliar congener, as compared to saline-treated control mice. In addition, following a relatively long drug-free period, exposure to an environmental stres-

sor did not affect social behavior. Our results, insofar as unexpected, indicate unaffected or heightened social interest induced by chronic morphine treatment and spontaneous prolonged opiate withdrawal and resilience to stress. In the present studies, we suggested that the increase in social interest was due to augmented aggressiveness, as revealed by ethological measurements of increased aggressive biting behavior (ABB). Indeed, we believe that aggressive behavior might drive opiate-withdrawn mice towards the unfamiliar conspecific for reasons other than social interest. Noteworthy, the increment in ABB is narrowed to a specific time window, thereby indicating oscillations of aggressive behavior during opiate withdrawal, a novel result that could contribute to the understanding of the complex symptomatology of SUDs.

### **5.2.1 Could the alterations induced by chronic morphine administration and withdrawal be generalized to other drugs of abuse?**

Over the last decades, a unitary view of drug dependence has emerged, nurtured by influential theories highlighting shared psychological processes and neurobiological substrates across different drugs of abuse. Although these theories have allowed significant advances in the field, they fail to take into account important differences between drugs of abuse, especially when it comes to opiate *versus* psychostimulant drugs. For instance, using a battery of several behavioral tests, a recent study [Becker et al., 2016] investigated the outcome of chronic drug administration and of withdrawal from various drugs of abuse (i.e., cocaine, morphine, nicotine, THC, alcohol) in adult male mice. Mice undergoing a 28-day withdrawal from morphine, nicotine, THC and alcohol, *but not* cocaine, exhibited deficient social interaction, motor stereotypes and exacerbated anxiety-like behavior, associated with increased Fos stimulation of anxiety-related regions (i.e. CeA, PVN) and lowered Fos stimulation of reward-related regions (i.e., VTA). Most importantly, when tested in the 3-CH task, morphine-withdrawn, *but not* cocaine-withdrawn mice, failed to spend significantly more time in the chamber containing the unfamiliar conspecific, as compared to the unanimated object, indicating impaired social interest. Accordingly, the percentage of time in close contact ratio was significantly reduced in morphine-withdrawn, *but not* in cocaine-withdrawn mice [Becker et al., 2016]. Moreover, in a recent study from our laboratory [Morisot et al., 2018], a deficit in social interest was detected 7 days after the cessation of chronic cocaine administration, a result that differ from the present findings obtained with morphine administration and withdrawal. In addition, in the latter study, exposure to an environmental stressor was able to induce the reemergence of social behavior deficits in long-term cocaine-withdrawn mice, whereas in the present study morphine-withdrawn mice displayed stress resilience. In this context, it is noteworthy to mention that the same stressor applied in the present and previous studies could induce the reemergence of recognition memory deficits and up-shifted motivational states in long-term opiate-withdrawn mice, as assessed in the novel object recognition (NOR) and operant behavior tasks, respectively [Morisot and Contarino, 2016, Morisot



et al., 2015]. Thus, overall, these findings suggest a dissociation between social behavior and cognitive function.

### 5.2.2 Opiate withdrawal and increased aggressive behavior: a gender issue?

Female mice routinely employed in pre-clinical studies are relatively non-aggressive, as compared to male mice [Scott, 1966]. Consequently, the existing literature regarding aggressive behavior in mice is almost entirely male-based, from the study of territorial aggression [Crawley et al., 1975, Miczek and O'Donnell, 1978] and dominance [Wang et al., 2011] to the outcomes of social provocation [Fish et al., 1999], from the effects of social isolation [Guidotti et al., 2001] to substance-induced aggression [Fish et al., 1999, Miczek and de Almeida, 2001, Miczek et al., 1998], with the only exception being the studies of maternal aggression [Noirot et al., 1975, Lonstein and Gammie, 2002]. Moreover, research on genetics and neurobiological substrates underlying aggressive behavior investigates offensive behaviors, which are observed only in adult male mice. For instance, it has been demonstrated that GABAergic projections from the basal forebrain to the lateral habenula modulate the rewarding aspects of behavior displayed by adult aggressor male mice [Golden et al., 2016] and that this feature might underlie the addiction-like properties of aggressive behavior [Golden et al., 2017]. Another recent study has shown that male, *but not* female mice, respond positively to optogenic stimulation of progesterone receptor-expressing (PR+) neurons in the ventrolateral subdivision of the mouse ventromedial hypothalamus (VMHvl), which are believed to mediate territorial aggression [Yang et al., 2017]. The latter studies suggest that using female mice might have led to different results in the experiments described in the present work (Article 1 and Article 2), probably unraveling the effects of drug administration and withdrawal on non-aggressive social behavior. Indeed, preliminary data from our laboratory indicate that, unlike male mice, chronic morphine administration and withdrawal induce sociability deficits in female CRF<sub>1</sub> wild-type mice. Interestingly, this impairment seems to be rescued by CRF<sub>1</sub> receptor-deficiency (Figure 5.1). We are thus carrying out additional studies to confirm the latter findings. Our new results might bring novel evidence in favor of gender-related issues in the investigation of the social behavior deficits induced by opiate administration and withdrawal.

### 5.2.3 Compensatory mechanisms in CRF receptor-deficient mutant mice

Inducing null mutations provides a powerful technique to investigate gene function, especially when no selective pharmacological agents are available. However, inactivation of a gene function may be associated to compensatory mechanisms [El-Brolosy and Stainier, 2017, Tautz, 1992]. Indeed, CRF<sub>1</sub> and CRF<sub>2</sub> receptor-deficiencies are associated with changes in the expression of CRF-like peptides. For instance, CRF<sub>1</sub> knock-out mice have increased CRF mRNA in the PVN [Smith et al., 1998] and CRF<sub>2</sub> knock-out mice have in-

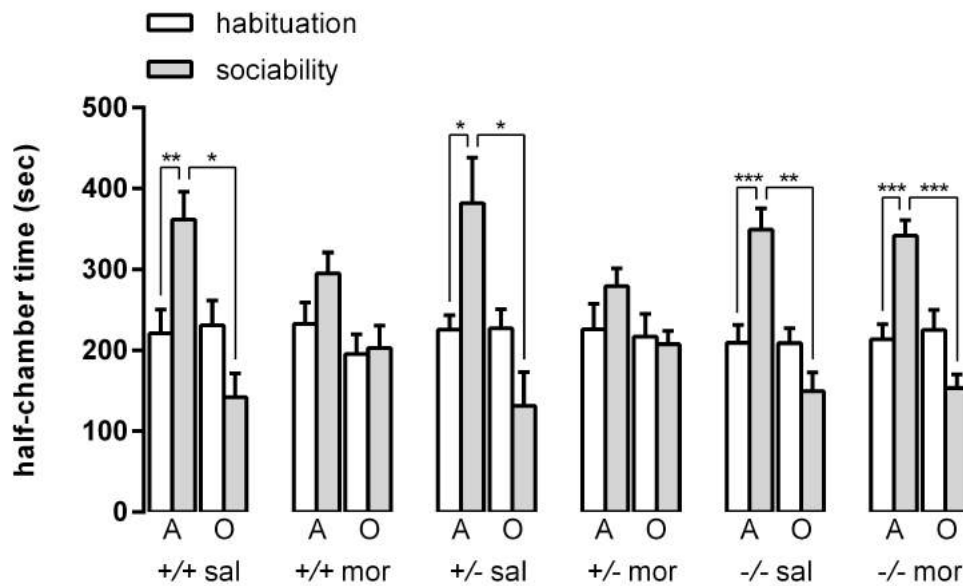


Figure 5.1 – Social interest displayed in the 3-CH task by CRF<sub>1</sub> receptor wild-type (+/+), heterozygous (+/-) and knock-out (-/-) adult female mice on opiate withdrawal day 7. This preliminary data suggest that full CRF<sub>1</sub> receptor-deficiency eliminates social behavior impairments induced by morphine (mor) administration and withdrawal. Social interest is expressed as the time spent exploring the half-chamber containing the animal (A) during the sociability phase, as compared to both the same half-chamber during the previous habituation phase and the opposite half-chamber containing the object (O) during the sociability phase. N=8-13/group. Values represent mean ± S.E.M. \*P<0.05, \*\*P<0.005, \*\*\*P<0.0005 (paired t-test within-group comparisons).

creased UCN1 mRNA in the Edinger-Westphal nucleus and the CeA and augmented CRF mRNA in the CeA [Weninger et al., 1999, Bale et al., 2000]. Thus, it cannot be excluded that compensatory mechanisms in CRF<sub>1</sub> receptor knock-out mice might have contributed to the behavioral effects observed in the present work (Article 2). Local infusions into discrete brain regions of lentiviral-based systems of RNA interference for the CRF<sub>1</sub> or the CRF<sub>2</sub> receptor might address the latter issue in future studies.

### 5.3 How does acute morphine administration affect “naturally” rewarding activities?

Most drugs of abuse produce subjective feelings of well-being and euphoria in humans, which are thought to contribute to the drugs' potential to be used or abused [Fischman and Foltin, 1991, de Wit and Griffiths, 1991]. Accordingly, drug-induced changes in mood or subjective states have long been the primary indicators used by the Food and Drug Administration (FDA) to assess the likelihood of abuse for new medications [Balster and Bigelow, 2003, Carter and Griffiths, 2009]. Drugs of abuse have been hypothesized to “hijack”

brain reward systems, strongly narrowing behavior towards substance-seeking and substance-taking to the detriment of “natural” rewarding activities, such as social interaction and food intake [Meyer et al., 2016]. However, the study of the “hijacking” properties of drugs has been impeded, at least in part, by the absence of reliable animal models able to assess the initial sensitivity to the reinforcing properties of substances of abuse, an important endophenotype in the trajectory toward excessive drug-taking behavior and drug dependence [Lambert et al., 2006]. Indeed, not only behavioral and neurochemical sensitization [Jackson and Nutt, 1993, Vanderschuren et al., 2001], but also gene expression [Le Foll et al., 2005] and neuronal plasticity [Ungless et al., 2001] have been shown to occur already after the first drug exposure, possibly promoting long-lasting drug-conditioning and drug-seeking behavior [Ciccocioppo et al., 2004].

In both animals and humans, studies on the objective responses to reinforcing stimuli provide valuable insights into brain reward function [Kelley and Berridge, 2002]. For instance, in rodents, some “natural” reinforcers, such as sucrose, elicit a characteristic facial liking reaction [Grill and Norgren, 1978]. Unfortunately, no measure of initial subjective reward perception exists for drugs of abuse in rodents and, even if such approach were useful in studying initial reactions, it is questionable whether such methodology would reliably assess reward for drugs taken by administration routes other than oral [Kiefer et al., 1990]. However, this concept of initial “liking” could be embodied by single-exposure conditioned place preference (CPP) procedures, where the preference for a context paired with a single drug intake is evaluated. In this context, the current work provides original evidence that acute administration of a brain rewarding dose of morphine sharply decreases the interest for “natural” rewarding activities, like social interaction and food intake. Moreover, following assessment of locomotion, olfaction and anxiety-like behavior, we suggest a specific effect of the drug upon the hedonic/motivational properties of these “natural” reinforcing stimuli.

### **5.3.1 Could the alterations induced by acute morphine administration be generalized to other drugs of abuse?**

Single drug exposure conditioned place preference has been documented for nicotine [Spina et al., 2006], morphine [Bardo and Neisewander, 1986, Fenu et al., 2006], cocaine [Crooks et al., 2010, Runegaard et al., 2017] and most recently for ethanol [Grisel et al., 2014]. Moreover, studies in mice and rats have shown that a single exposure to almost all drugs of abuse can induce behavioral sensitization. As a matter of fact, a single injection of cocaine [Valjent et al., 2010], amphetamine [Frussa-Filho et al., 2004, Chinen et al., 2006], morphine [Valjent et al., 2010, Vanderschuren et al., 2001] or ethanol [Fukushiro et al., 2010] enhances the locomotor stimulation produced by the subsequent administration of the drug hours, days or weeks later, which is potentiated when the locomotor-stimulating effect of the priming injection is paired with the test environment [Chinen et al., 2006]. Nevertheless, different drugs of

abuse might diversely affect “natural” rewarding activities. For instance, in a recent study in humans [Spronk et al., 2016], acute cannabis administration was accompanied by slower reaction times and impaired accuracy, while cocaine yielded quicker reaction times and improved accuracy. The latter result strengthens the notion that, despite common neurobiological mechanisms, the behavioral outcomes of different drugs of abuse are most often drug-specific.

## 5.4 Valuable methodological advancements of the current work

From a methodological perspective, we invested a considerable amount of time and energy to advance the current interpretation of results obtained with the 3-CH paradigm. First of all, by considering the time spent in just the top half-chambers of the apparatus (containing the unfamiliar conspecific or the unanimated object), we provided a more reliable measure of social behavior, as compared to the previously standard and most often used whole-chamber measurements. Secondly, by developing and validating a home-made video-track system, we provided automated measures of the behavior, thus avoiding possible human biases or errors. Furthermore, taking into consideration also the results obtained during the habituation phase of the 3-CH task and by introducing inclusion/exclusion criteria, we considerably increased the reliability of the findings obtained. Moreover, by employing a more rigorous statistical analysis that takes into account the behavioral dynamics across the different phases of the 3-CH test (i.e., three- or four-way ANOVA including the measures obtained during the habituation phase), we increased by far the biological significance of our results. With regard to the latter point, it should be mentioned that most of the 3-CH studies carried out to date used simple Student’s t-tests to compare the time spent by the tested animal with the unfamiliar conspecific or the unanimated object. Furthermore, unlike prior studies [Moy et al., 2004], we are the first to our knowledge to point out that repeated testing in the 3-CH task leads to a strong behavioral habituation response and to score additional behaviors (i.e., ABB), an issue previously described just as a possibility [Kaidanovich-Beilin et al., 2011]. Notably, the latter approach might have evidenced what could be considered a major flaw of the 3-CH paradigm, i.e., under specific conditions (i.e., opiate withdrawal, male mice) increased aggressive behavior expressed during this test could be erroneously interpreted for social interest.

## 5.5 Future perspectives

How does morphine administration modify neural circuitries, leading to decreased interest for “natural” reinforcers? In the current work, we show that systemic pre-treatment with the CRF<sub>1</sub> receptor-preferring antagonist antalarmin is able to completely rescue the profound deficits in social interest induced by acute injection of morphine, thereby providing initial evidence in

favor of the involvement of the CRF system in the above-mentioned “hijacking” properties of drugs of abuse. However, although some CRF antagonists preferentially bind the CRF<sub>1</sub> or the CRF<sub>2</sub> receptor *in vitro* (i.e., antalarmin), it cannot be excluded that they interact with both CRF receptor subtypes *in vivo*, at least at the behaviorally active doses usually employed [Zorrilla et al., 2013b]. Thus, to clarify the specific role for the CRF<sub>1</sub> or the CRF<sub>2</sub> receptor in morphine-induced social behavior deficits, future studies could be carried out using lentiviral-based systems of RNA interference [Lebow et al., 2012, Sztainberg et al., 2010]. In this context, the ventral tegmental area (VTA) would be a likely target region of interest, since this brain region has long been implicated in the rewarding and motivational properties of “natural” rewards, including social interaction, and substances of abuse [Bariselli et al., 2016, Kalivas and Volkow, 2005]. Our hypothesis is that lentiviral-induced disruption of VTA CRF<sub>1</sub> receptor-mediated (or CRF<sub>2</sub> receptor-mediated) neurotransmission might eliminate the social behavior deficits induced by acute administration of morphine. With regard to the life-long whole-body CRF<sub>1</sub> or CRF<sub>2</sub> receptor-deficient mouse models, lentiviral-based systems of RNA interference could allow the assessment of the specific role for each CRF receptor subtype in discrete brain regions. Moreover, the latter genetic approach might rule out possible confounding factors linked to the lack of CRF receptors throughout development. The result of such studies might shed new light on the neural mechanisms underlying substance use disorders and promote research-based pre-clinical and clinical trials aimed at evaluating the therapeutic potential of CRF receptor subtype-selective pharmacological agents.

Recent optogenetic studies revealed that activity dynamics of VTA-nucleus accumbens (NAc) projections might encode and predict key features of social interaction [Gunaydin et al., 2014]. Dopamine (DA) neurons arising from the VTA and projecting to the NAc have long been implicated in the reinforcing actions of substances of abuse [Nestler, 2005, Wanat et al., 2009, Wise and Morales, 2010]. In the context of the “hijacking” phenomenon described herein, investigating the relative impact of “natural” and “artificial” reinforcers in such anatomical pathway would be hence very intriguing. Interestingly, it has been shown that a single *in vivo* exposure to cocaine induces long-term potentiation of alpha-amino-3-hydroxy-5-methyl-isoxazole propionic acid (AMPA) receptor-mediated currents at excitatory synapses onto DA cells in the VTA [Ungless et al., 2001]. The latter result indicates that a prominent form of synaptic plasticity can be elicited by a single *in vivo* exposure to a drug of abuse and therefore be involved in the early stages of the development of drug dependence. It is likely that a single injection of morphine also induces plasticity in the VTA, since overexpression of the AMPA receptor subunit GluR1 in the VTA increases the stimulant and rewarding properties of morphine [Carlezon et al., 1997]. Therefore, it would be interesting to assess the electrophysiological impact of the morphine dose employed herein (2.5 mg/kg) and examine whether DA neurons are implicated in the behavioral effects described in the current work (Article 3).

Accumulating evidence indicates that oxytocin (OXY) plays a crucial role in social and affiliative behaviors. Indeed, deleting OXY or the OXY re-

ceptor gene results in dramatic social behavior deficits in mice [Sala et al., 2013, Takayanagi et al., 2005, Winslow and Insel, 2002, Pobbe et al., 2012]. Moreover, changes in the OXY system have been observed following drug administration. For instance, withdrawal from chronic cocaine or morphine administration decreases OXY levels in the hypothalamus and the hippocampus and increases OXY receptor-binding in the lateral septum and the amygdala [Sarnyai et al., 1992b, Georgiou et al., 2016, Zanos et al., 2014]. Moreover, treatment with OXY may decrease cocaine self-administration and cue-induced reinstatement of cocaine-seeking behavior in rats [Leong et al., 2017]. Furthermore, following exposure to a stressful event, long-term cocaine-withdrawn mice displayed elevated expression of OXY in the supraoptic nucleus (SON), an effect that was abolished by CRF<sub>2</sub> receptor-deficiency [Morisot et al., 2018]. Nevertheless, the role for the CRF system in social behavior deficit and brain OXY changes induced by acute administration of drugs of abuse remains poorly understood. Therefore, by using the *in-situ* hybridization technique routinely employed in our laboratory [Morisot et al., 2018, 2014, 2015, Ingallinesi et al., 2012, Papaleo et al., 2007], it would be of great interest to investigate the expression of OXY in the paraventricular (PVN) and the supraoptic (SON) nuclei of the hypothalamus, which are major sources of OXY in the brain that also express both CRF receptor subtypes [Young et al., 2005, Van Pett et al., 2000]. Finally, single-dose or continuous treatment with OXY have been reported to improve social interest in animal models of autism spectrum disorder [Sala et al., 2011, Teng et al., 2016, Hara et al., 2017, Harony-Nicolas et al., 2017, Penagarikano et al., 2015, Meziane et al., 2015]. Thus, it would be interesting to test the effects of OXY administration (e.g., intranasal) upon the social behavior deficits induced by acute morphine administration. The latter studies might pave the way to the development of novel pharmacological therapies aimed at preventing the initial fall into drug abuse in the trajectory towards drug dependence.



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