N° d'ordre: 4434

THÈSE

PRÉSENTÉE A

L'UNIVERSITÉ BORDEAUX 1

ÉCOLE DOCTORALE DE LA VIE ET DE LA SANTÉ

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POUR OBTENIR LE GRADE DE

DOCTEUR

SPÉCIALITÉ: NEUROSCIENCES

Dépendance aux drogues opiacées : focus sur le système *corticotropin-releasing factor*

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Soutenue le 21 Décembre 2011

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Université Bordeaux 1
Les Sciences et les Technologies au service de l'Homme et de l'environnement

THESIS

DEFENDED AT THE

UNIVERSITY OF BORDEAUX 1

ÉCOLE DOCTORALE DE LA VIE ET DE LA SANTÉ

By Khalil Adnane ROUIBI

FOR

PhD DEGREE

NEUROSCIENCES

Opiate dependence: focus on the corticotropin-releasing factor system

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Defended on December 21, 2011

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À mes parents qui m'ont soutenu et qui ont toujours cru en moi

Résumé-Abstract

RESUME

La prise illicite de drogues opiacées est un problème majeur de santé publique dans le monde. Malgré un vaste corpus de littérature scientifique traitant du problème de la dépendance aux opiacés, peu d'études ont abouti à un traitement efficace. L'apparition du syndrome de *sevrage aux opiacés* (SAO) suite à l'arrêt de la prise d'opiacés est considérée comme élément clef dans la vulnérabilité associée à la rechute de la prise d'opiacés. En effet, le syndrome de SAO est caractérisé par différentes altérations comportementales et neurobiologiques en réponse au stress qui sont déterminantes dans le phénomène de dépendance aux opiacés. Le système *corticotropin-releasing factor* (CRF) est un coordinateur central des circuits de réponse au stress par l'intermédiaire de ses deux récepteurs le: CRF_1 et CRF_2. L'objectif de cette thèse est de déterminer le rôle du récepteur CRF_2 dans l'apparition de certaines composantes des états affectifs négatifs impliquées dans la rechute de la prise d'opiacés lors du sevrage.

Tout d'abord, nous avons abordé dans le *chapitre I* une description des études les plus représentatives du rôle du système CRF dans la dépendance aux drogues avec une attention particulière à la littérature traitant des drogues opiacées.

Dans le *chapitre II* nous avons démontré par une série d'expériences conduite chez des souris invalidées au récepteur CRF_2 ($CRF_2^{-/-}$), que la délétion génétique du récepteur CRF_2 éliminait les états dysphoriques et anhédoniques ainsi que les altérations moléculaires provoquées par le SAO sans détériorer les réponses neuroendocriniennes qui sont primordiales lors des adaptations aux situations stressantes associées au sevrage.

De nombreuses données cliniques et précliniques montrent que le SAO induit des altérations motivationnelles vis-à-vis des récompenses. Dans le *chapitre III* nous avons trouvé que les souris $CRF_2^{-/-}$ entrainées dans une procédure de tâche opérante dirigée vers l'obtention d'une nourriture palatable, montraient une diminution des troubles motivationnels induits par le SAO.

Plusieurs rapports montrent que chez l'Homme, les évènements stressants apparaissant lors du sevrage provoquent une rechute de la consommation d'alcool ou de drogues. Dans le *chapitre IV* nous avons développé un modèle murin qui montrait un rétablissement de recherche de nourriture palatable suite à une procédure de stress appliquée pendant une période de SAO. Par ailleurs, nous avons observé un dimorphisme sexuel du rôle du récepteur CRF_2 dans le rétablissement de recherche de nourriture palatable, suite au stress, longtemps après un SAO.

Les résultats de ce travail de thèse nous permettent de mettre en avant le récepteur CRF_2 comme possible cible thérapeutique dans le traitement de la dépendance aux drogues opiacées.

Mots-clefs: dépendance aux opiacés; le systéme *corticotropin releasing factor*; voie du récepteur CRF_2; sevrage; affect négatif; anhédonie; motivation; rechute; stress ; souris

ABSTRACT

Opiate illicit use represents one of the most severe sanitary problems throughout the world. Even though a vast body of literature has been focusing on the critical problem of the opiate dependence, there are still currently no reliable safe and effective treatments. Among humans, the emergence of the opiate withdrawal (OW) syndrome after cessation of opiate intake is considered as a key motivational element that lead to the vulnerability to opiates relapse. Therefore, the OW is characterized by a various alteration of the behavioral and neurobiological homeostasis responses to stress which are determinants in opiate dependence. The corticotropin-releasing factor (CRF) system is the major coordinator of stress-responsive circuitry. Through its two receptors CRF_1 and CRF_2, the CRF system has recently emerged as major contributor in the development of components of the OW syndrome. The aim of this thesis is to determine the role of CRF_2 receptor in components of the negative affective states implicated in the OW syndrome.

First of all, we deal with a description of the role of the CRF system in drug dependence with a particular attention to the literature relevant to the opiate drugs (*Chapter I*).

In *chapter II*, the behavioral and biological experiments were conducted in CRF_2 receptor-deficient mice ($CRF_2^{-/-}$). In particular, we reported that genetic deletion of the CRF_2 receptor eliminates dysphoria, ahhedonia and molecular alterations elicited by OW without impairing brain, neuroendocrine and autonomic stress-coping responses to withdrawal.

Thus, multiple data in both human and animal literature showed motivational alterations to opiates and natural rewards during OW. *The chapter III* would be dedicated to addressing the role of CRF_2 receptor during OW on the motivational properties toward natural reward. Using behavioral approaches of operant responding to highly palatable food (HPF), we found that $CRF_2^{-/-}$ reduces motivational disorders elicited by OW.

Finally, it has been reported in human literature that the stress is one of the critical factors leading individual-addicts to drug seeking and relapse during alcohol and drug withdrawal. Accordingly, preclinical studies mimicking the human stressful events showed alcohol and drug-seeking relapse during abstinence. *The chapter IV* would deal with a description of mouse model of stress-induced food reinstatement seeking behavior during prolonged OW. Furthermore, we reported a gender dimorphism in the role of the CRF_2 receptor in the stress-induced reinstatement of HPF seeking behavior long-lasing after opiate treatment.

As discussed in the *chapter V* these findings underscore the importance of CRF_2 receptor as possible effective treatment of the critical problem of opiate dependence.

Keywords: Opiate dependence; corticotropin releasing factor system; CRF_2 receptor pathway; withdrawal; negative affect; anhedonia; motivation; relapse; stress; mouse

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Abbreviations

ABBREVIATIONS

Ach	acetylcholine
ACTH	adrenocorticotropic hormone
ANOVA	analysis of variance
AS-30	anti-sauvagine-30
BWT	black-white test
BNST	bed nucleus of stria terminalis
BP	breakpoint
BW	body weight
cAMP	cyclic adenosine monophosphate
CeA	central nucleus of the amygdala
CNS	central nervous system
CPA	conditioned place aversion
CPP	conditioned place preference
CRF	corticotropin-releasing factor
CRFR	corticotropin-releasing factor receptor
CTL	control
DSM	diagnostic and statistical manual
DYN	dynorphin
EMCDDA	european monitoring centre for drugs and drug addiction
EPF	elevated platform
EPM	elevated plus-maze
FR	fixed ratio
HPA	hypothalamic-pituitary-adrenal axis
HPF	highly palatable food
ICSS	intracranial self-stimulation
i.p.	intra-peritoneal
i.v.	intra-ventricular
KOR	κ-opioid receptor
LC	locus coereleus
LD	light-dark
mRNA	messenger ribonucleic acid
nAChRs	nicotinic acetylcholine receptors
NE	norepinephrine
NOR	novel object recognition

Nacc	nucleus accumbens
NaccSH	nucleus accumbens shell
NK	neurokinine
OF	open field test
OW	opiate withdrawal
PR	progressive ratio
PVN	paraventricular nucleus
SAO	sevrage aux opiacés
s.c.	subcutaneously
SCF	standard chow food
SPO OW	spontaneous opiate withdrawal
TH	tyrosine hydroxylase
TTX	tetrodotoxin
UCN	urocortin
UNODC	united nations office on drugs and crime
URAB	ultra-rapid antagonist blockade
VTA	ventral tegmental area
WHO	world health organization

Chapter I

INTRODUCTION

Drug dependence is a major public health problem. With the globalization of trade during the last decades, the illicit drug use witnessed an intensive development that has affected populations of both industrialized and developing countries. Opioids are the most widespread drugs among drug users (UNODC, 2010). According to a report of The World Health Organization, opioid dependence represents a major cost to society, it results many health consequences including higher risks of premature death, HIV and hepatitis B and C contamination (WHO, 2004).

One of the most insidious features of Human opioid dependence is the development of the OW syndrome which is comprised of severe influenza-like somatic signs and symptoms and negative affective states include anxiety, depression, anhedonia, brain reward deregulation, and motivational disorders (APA, 2000; Cheetham et al., 2010; Hatzigiakoumis et al., 2011; Janiri et al., 2005; Koob and Le Moal, 2001; O'brien, 1996; Volkow et al., 2002).The escape from the extremely stressful OW signs and symptoms is one of the motivating factors in the drug-seeking and drug-taking behavior, thus strongly contributing to the recreational use of opioids and perpetuation of the opioid dependence cycle (Kenny et al., 2006; Lu et al., 2005).

The CRF system is a major component of stress-responsive circuitry (Heinrichs et al., 1994). In mammals, CRF signaling is mediated by two receptor pathways, named CRF_1 and CRF_2 (Hauger et al., 2003). Genetically engineered mouse models have allowed the identification of opposite roles for the two known CRF receptor pathways in behavioral and neuroendocrine responses to stressors (Bale and Vale, 2004; Contarino and Gold, 2002). Moreover, using clinically relevant mouse paradigms (Papaleo and Contarino, 2006), the genetic deletion of the CRF_1 receptor fully eliminated behavioral and brain correlates of negative affect but exacerbated somatic components of OW (Contarino and Papaleo, 2005; Papaleo et al., 2007b). In net contrast, genetic deletion of the CRF_2 receptor decreased the somatic expression of OW (Papaleo et al., 2008). Overall, these prior researches suggest a complex physiopathological role for the CRF system in opiate dependence and withdrawal.

In light of the above, the *chapter I* would firstly deal with a description of opioid dependence and somatic and negative affective states elicited by OW. Drug dependence is considered as a stressful condition which suggests a large contribution of brain stress system in the somatic and negative emotional states associated with drug dependence. The second part of this *chapter* would be dedicated to investigate the role of the CRF system in the negative reinforcing effects of drugs and the drug seeking and relapses during abstinence, with a particular attention to the literature relevant to the opioid drugs.

1. Opiate drug dependence

1.1. Opioids

Opiates, from "*opos*", the Greek word of juice, were defined as a natural alkaloids drug containing or derived from extracts of the milky juice of the *Papaver somniferum* (van Ree et al., 1999). The poppy plant is originated in lower *Mesopotamia* and is actually cultivated principally in Himalayans countries (Meyers, 2007). To collect opium, small vertical incisions are applied from base to summit of the head of poppy. The chemical composition of the milky exudates capsule seed of *Papaver somniferum* is more complex; Opium contains 20 alkaloids (About 25% of the mass of opium) including morphine, papaverine, codeine, thebaine, noscapine, etc, as well as other compounds (Remberg et al., 2008).

The term opioids include natural (opiate), semi-synthetic or synthetic drugs which act on opiate receptors (Jaffe and Martin, 1990; Martin, 1967 cited by Koob and Le Moal, 2006). This term include also antagonists of opioids receptor and endogenous ligands named β-endorphins, enkephalins and dynorphins (Jaffe and Martin, 1990). In 1976, Martin and colleagues discovered several types of opioid receptors named: μ, κ and σ (Martin et al., 1976). Contemporaneously, other group characterizes a further type of opioid named δ (Lord et al., 1977). Additional researches revealed that σ-type receptor is non opioid in nature, leaving three main types of opioid receptor μ, δ and κ (Mannalack et al., 1986). Importantly, there seems to be some affinity between the different endogenous opioid ligands and the opioid receptor types: β-endorphins for μ, enkephalins for δ and dynorphins for κ (van Ree et al., 1999).

1.2. Brief history of opioids

The first descriptions of poppy date back to antiquity. The archaeological indications presupposed that the opium was used in *Mesopotamia*, which is considered as the birthplace of "*Sumero-Akkadian*" civilisation (Meyers, 2007). A Sumerian tablet, discovered in *Uruk* and dating from approximately four thousand years, mentions that poppies consisted of the monosyllables *Gill* and *Hull* and has the meaning "*the plant which brings the enjoyment*" (Meyers, 2007). In antique Egypt the Papyrus of "*Ebers*", dating from the 15th century B.C, is a collection of medicinal plants, which describes the effects of the opium on the body (Meyers, 2007). Avicenna, an Arabic doctor of the middle ages, describes in his most famous work "*Cannon*", the analgesic effects of the opium and uses it with his patients during surgeries and amputations of members (Hijazi, 1984). In 1700, Dr John Jones made the description of the symptoms of OW after cessation of opium intake in his book "*The Mysteries of opium Revealed*". In 1806, the isolation of the alkaloid morphine and his description was made by a young German pharmacist named Friederich Wilhelm Adam Sertürner (Haas, 1995). A few

decades later, the opium war between China and the United Kingdom (1839-1842) was declared as to predict the beginning of sanitary problems connected to the use of opioids for the next centuries (Brownstein, 1993). Actually, opioids are the most generalized and used drugs in the world.

1.3. Epidemiology of opiate drug dependence

The non medical use of opioids represents a major public health problem. Among illicit drugs, opioids are the most widely used in the world (Koob and Le Moal, 2006; UNODC, 2010). The globally opioids use is estimated between 12.8 and 21.9 million people in 2008, with the prevalence ranging between 0.3% and 0.5% of the world's population aged 15-64 (UNODC, 2010). In Europe, the prevalence of opiate use was estimated between 3.6 and 4.4 cases per 1000 population aged 15-64 years; this is corresponding to 1.35 million individuals (EMCDDA, 2010). Heroin is the most commonly abused opioids as 11 million people worldwide use this drug (UNODC, 2010). In the United States, the estimated cost of heroin dependence was $21.9 billion in 1996 (Mark et al., 2001).

Illicit use of opioids is often accompanied with multiple social and health problems, including crime, suicide, violence, death from opiate overdose and various infections with HIV, tuberculosis, hepatitis, etc (Soyka et al., 2011; WHO, 2004). In the medical field, the use of agonist medication such as methadone, codeine, oxycodone, etc., for the treatment of opiate dependence can lead to drift and illicit use of these substances (Soyka et al., 2011).

1.4. Definition of dependence

The term "*dependence*" is explicitly used in the DSM-IV or ICD-10 to refer to the combination of physical and psychological syndromes of drug dependence. Nevertheless, some addiction medicine specialists employ frequently the term *"addiction"*.

Drug dependence is a chronically relapsing disorder characterized by a compulsive drug self-administration, loss of control in limiting intake and habitual repetition of drug consuming regardless of the social and health adverse consequences (APA, 2000; Koob and Le Moal, 2006). The ICD-10 defines dependence as "*a cluster of physiological, behavioral, and cognitive phenomena in which the use of a substance or a class of substances takes on a much higher priority for a given individual than other behaviors that once had greater value*."

Dependence occurred after a gradual process of repeated drug administration. It comprises three stages:

A. Preoccupation/anticipation

B. Binge/intoxication

C. Withdrawal/negative affects

The early stage of drug dependence is marked by the impulsivity. This latter is defined as an increasing sense of arousal or tension before committing an impulsive act of pleasure, gratification there may or may not be a feeling of regret or guilt following the act (APA, 2000). In opposite, compulsive disorder is defined by the perseveration in the face of adverse consequences (Koob et al., 2004) **(Fig. 1)**. Importantly, a switch from impulsivity (at the early stages) to compulsivity (at the terminal stages) is a common brand of the drug dependence (Koob, 2008).

Koob G.F (2004) reported that in drug-addicts, *"the transition from impulsive behavior to compulsive behavior is established when a shift occurs from a positive reinforcement to a negative reinforcement"*. During drug withdrawal, the negative affective states such as chronic irritability, emotional pain, dysphoria, stress, alexithymia and loss of motivation drive the negative reinforcement of dependence (Koob, 2009).

The drop of reward threshold during drug abstinence leads to the establishment of an *anti-reward system* which is defined as "*a processes put in place to limit reward and maintain apparent functional stability*" (*Allostasis*) (Koob and Kreek, 2007). The deregulation of reward set point during allostatic state contributes to the persistent vulnerability to relapse during protracted abstinence and also requires the brain stress systems during compulsive stage (Koob and Le Moal, 2001). It is manifested by a decrease in reward function, implicating a various panel of molecules such as dopamine, serotonin and opioids peptides. But also the activation of the element of brain stress system including, CRF, dynorphin (DYN), norepinephrine and other neurotransmitters (Koob and Le Moal, 2001).

In the medical field, the emergence of OW syndrome after the discontinuation, the drastic reduction or abrupt cessation of opiate consumption is considered as a key motivational element that leads to the vulnerability to drug relapse and maintaining dependence (APA, 2000; Koob and Le Moal, 2006; O'brien, 1996). The OW syndrome is characterized by the manifestation of physical and psychological signs and symptoms (APA, 2000). These conditions are relieved by re-administration of the same opiate drug or of another drug with similar pharmacological action. In the following section we review some of physical and psychological components of the OW syndrome among Human and animal.

Impulse Control Disorders

Positive Reinforcement

Compulsive Disorders

Negative Reinforcement
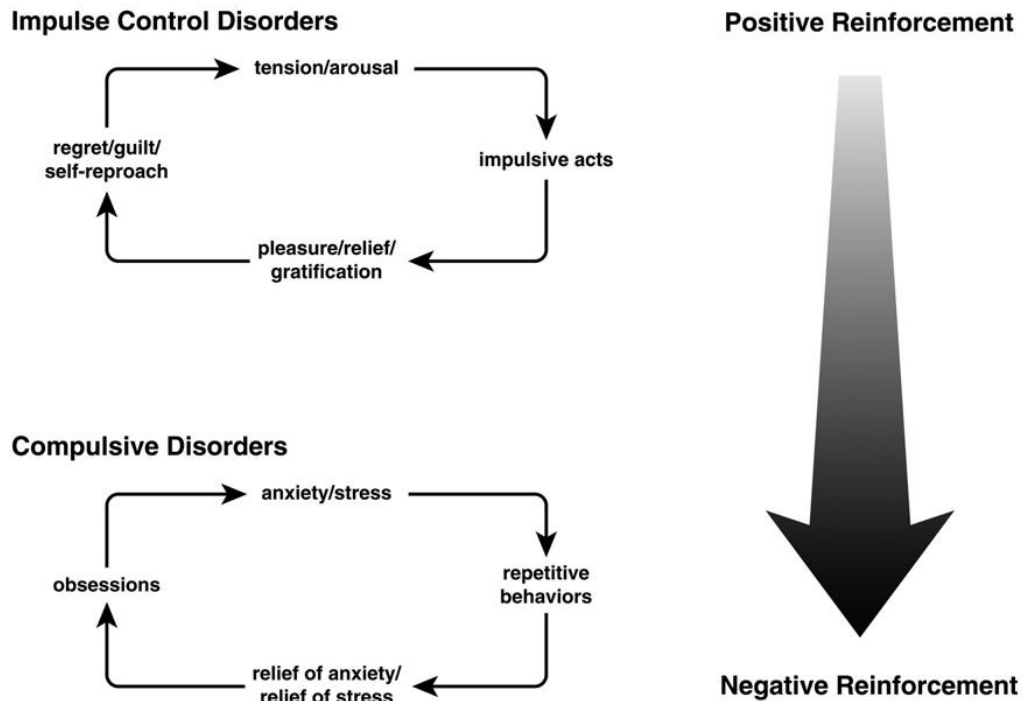
Fig. 1 "Stages of impulse and compulsive disorder cycles related to the source of reinforcement. In the first stage increasing arousal occurs before the impulsive act, with pleasure, gratification, or relief during the act; following the act there may or may not be regret or guilt. In compulsive stages, there are recurrent and persistent thoughts causing stress and anxiety followed by repetitive behaviors (compulsions) that are aimed at preventing or reducing distress (DMS IV). Positive reinforcement (pleasure/gratification) is more related to impulse control disorder while negative reinforcement (relief of anxiety or relief of stress) is more closely associated with compulsive disorders" (Koob, 2004).

2. Opiate withdrawal syndromes

2.1. Human opiate withdrawal syndrome

2.1.1. Somatic and negative affective syndromes

Among humans forced or self-imposed opiate abstinence, following a chronic opiate use, results in the manifestation and the development of withdrawal syndrome including numerous of aversive somatic signs and negative affective states (APA, 2000; Jaffe, 1990; O'brien, 1996). It has been proposed that the emergence of physical signs accompanied with negative affective states may severely motivate opiate-seeking behavior and compulsive opiate intake through negative reinforcement mechanisms (Kenny et al., 2006; Koob and Le Moal, 1997). Somatic signs refer to an altered physiological state induced by intermittent administration and increasing opiate doses. The continued use of the opiates prevents the emergence of varied somatic signs during OW such as: yawning, lacrimation, rhinorrhea, abdominal cramps, perspiration, gooseflesh, tremor, dilated pupils, anorexia, nausea, emesis, diarrhea, vomiting, restlessness, insomnia, weight loss, dehydratation, hyperglycemia, elevations of temperature and blood pressure, alteration of pulse rate, etc (APA, 2000; Koob and Le Moal, 2006).

The *American Psychiatric Association (APA)* specifies that the following symptoms must be present in order to meet diagnostic criteria for OW:

A. Either the individual has (1) stopped using (or has reduced the amount of) opioids, (2) or an opioid has been administered.

B. Three or more symptoms develop after the abstinence for few minutes or days after criterion A:

(1) Dysphoric (negative) mood

(2) Nausea or vomiting

(3) Muscle aches

(4) Runny nose or watery eyes

(5) Dilated pupils, goosebumps, or sweating

(6) Diarrhea

(7) Yawning

(8) Fever

(9) Insomnia.

C. The withdrawal symptoms must cause significant distress to the individual or impairment in functioning (socially, at work, or any other important area).

D. The OW symptoms cannot be due to a medical condition or other mental disorder.

The appearance of the early somatic symptoms of OW and peak of intensity depends on the pharmacokinetics and duration of action of opiate agonist, respectively (Koob and Le Moal,

2006; Soyka et al., 2011). For morphine, withdrawal symptoms appear after 14-20 h and peak at 26-48 h; concerning heroin symptoms appear after 6-12 h and peak at 48-72 h. Among the patients without treatment, the course of the majority of syndromes ends in 5-10 days after the last dose of morphine and heroin (APA, 2000; Koob and Le Moal, 2006). However, other signs in detoxified subjects including hyperthermia, mydriasis, increased blood pressure and respiratory rate, persist for a month after opiate cessation during a period of time termed "*protracted abstinence*" (Koob and Le Moal, 2006).

Opiate addicts with past experience of relapse reported in medical field that symptoms, such as depressed mood, irritability, anhedonia, dysphoria and anxiety, precede the renewed opiate intake (APA, 2000; Koob and Le Moal, 2006; O'brien, 1996). In addition, these symptoms persist weeks and months after the last injection (APA, 2000; Dole et al., 1966; Martin and Jasinski, 1969) and highlight the long-term vulnerability to opiate relapse during the drug-free period. Moreover, clinical studies indicate a higher contribution of the negative affect in the drug relapse than that of the somatic syndrome during long-term abstinence (Henningfield et al., 1987; Jasinski et al., 1985 cited by Schulteis and Koob, 1994). Profound neurobiological changes during long-term abstinence may explain the impact of the negative affect in drug relapse. The abnormal activation of the brain stress systems is considered as a key element which leads to the emergence of negative affect (Koob, 2008). For example, in heroin addicts, anxiety and depression was higher on the 3rd day of withdrawal and progressively decreased time-dependently in 10 and 30-days of protracted withdrawal (Shi et al., 2009). Interestingly, positive correlations between cortisol levels and depression on one hand and cortisol levels and anxiety on the other hand were observed, revealing a relationship between hypothalamic pituitary adrenal (HPA)-axis impairment and negative affect (Shi et al., 2009).

Particularly, a gender difference was observed in the psychiatric disorders related to stress. In fact, some authors reported a higher prevalence of depression and anxiety in women than in men which is attributed to greater sensitivity of stress-responsive system (Frew and Drummond, 2007; Kessler et al., 1993; Young et al., 2007). Also, compared to healthy individuals, prospective studies in opiate addicts showed more frequent anorexia in women than men (Santolaria-Fernandez et al., 1995). Finally, naloxone-induced increases in the stress hormone cortisol are higher in women than in men (Uhart et al., 2006). Others, reported that females rats exhibited hypersensitivity of CRF brain stress system responses than males rats which may explain the higher vulnerability of women to psychiatric pathologies showed in medical field (Bangasser et al., 2010; Bangasser et al., 2011). However, despite multiple adverse consequences of mental disorders and stress vulnerability elicited by OW, women showed a more rapid progression to medical treatment (Kerr et al., 2005; Unger et al., 2010).

2.1.2. Medication

The main goals of OW pharmacotherapy consist of (Soyka et al., 2011):

A. The enhancement of the transition to a drug-free period by the suppression and/or minimizing the major somatic and affective signs especially during the first days of OW.

B. The prevention of the relapse during initiate and long-term abstinence.

To day, the substitution of opiates by a gradual reduction of doses of μ-opioid-receptor agonist drugs is the most frequently strategy employed in the management of OW (Kleber et al., 2007; Nigam et al., 1993; Ziedonis et al., 2009). Because the methadone has a relative long elimination half-life (24h-36h), this molecule is largely employed in the medical field to manage symptoms of OW (Ward et al., 1996 cited by Gonzalez et al., 2004). The Levo-Alpha-Acethyl-Methadol (LAAM) is a derivative of methadone with a longer duration action than methadone (longer elimination half-life and slower onset of action). In particular, the LAAM suppress early OW signs and symptoms for >72h, which decreases strongly the frequencies of methadone intake (Gonzalez et al., 2004). The partial opiate receptor agonist buprenorphine is efficient in reducing symptoms of withdrawal and shortening the length of detoxification especially when it is combined with naloxone or naltrexone (Umbricht et al., 1999).

Another treatment is the use of the clonidine and lofexidine, an α_2-adrenergic agonists acting on the noradrenergic activity, which reduce sympathetic flow linked to OW. Clinical observations showed that methadone or buprenorphine mixed with clonidine or lofexidine are most effective in the opiate detoxification (Meader, 2010). The clonidine reduces symptoms such as insomnia, rhinorrhoea, lacrimation and distress (Charney et al., 1981; Greenstein and Siegal, 1997; Washton and Resnick, 1980) but neither alters nor decreases the time course of OW (Kleber et al., 1987). However, clonidine decreases the time course of anxiety (Kleber et al., 1987). Other physicians explored alternative methods such as the *Rapid antagonist blockade* by naloxone or naltrexone introducing during clonidine or lofexidine treatment. This procedure shortens the duration of OW without increasing patient's discomfort (Gowing et al., 2009).

Finally, the *ultra-rapid antagonist blockade* (URAB), combining naltrexone and clonidine, is a recent approach which aims to detoxify patients in 48 hours (Loimer et al., 1990; Loimer et al., 1988 cited by Gonzalez et al., 2004). The patients were given naltrexone under general anaesthesia and intensive medical treatment. This method is controversial because a significant risk of death in clinical setting can be provoked by the detoxification under anesthesia and incubation (significant risk of vomiting) (Pfab et al., 1999). In addition, residual OW symptoms persist during months after URAB detoxification (Cucchia et al., 1998; Scherbaum et al., 1998). Others reported that, 7 months after URAB, only 10% of patients continued to maintain a clinical naltrexone therapy and the others relapsed to an illicit opiate use (Rabinowitz et al., 1997).

Most importantly, the majority of the physicians associate the pharmacological treatment with a psychosocial intervention as the combination of the two therapies is more effective than the pharmacological therapy alone (Amato et al., 2008).

Altogether, the pharmacotherapy improves the patient's quality of life by controlling and/or reducing the appearance and the intensity of the somatic and the affective components of the OW syndrome. Furthermore, one of major advantage of pharmacotherapy is the management of OW syndrome under controlled and sanitized conditions. Indeed, the clinical control of the dependence decreases the risk of overdose and also limits the intravenous drug use which is responsible for HIV, hepatitis and tuberculosis infections and other sexually transmitted diseases (Reimer et al., 2011). However, despite these benefits, the pharmacotherapy does not resolve the problem of the of multiple somatic and affective signs persistence long-term after an opiate cessation. Even worse, the addictive potential of opioid agonist medications may simply transform the addictive potential toward illicit opiates in addicts. The consequence of this condition is the maintaining of dependence and a drift of pharmacotherapy initial goal which is the progressive withdrawal of opioids. Another limitation of the pharmacotherapy in the treatment of opiate dependence that must be considered, concerns the vulnerability of some population categories to pharmacotherapy molecules. For example, the pharmacotherapy treatment of opiate-addicts pregnant women should be avoided during the first trimester of pregnancy (Lingford-Hughes et al., 2004). Treatment is also difficult to conduct in the case of newborn babies who exhibit a particular OW signs, after birth, termed *neonatal abstinence syndrome* (Suffet and Brotman, 1984). Patients with comorbid depressive disorders taking antidepressant treatment present a higher risk of pharmacological interactions (Katz et al., 2010 cited by Soyka et al., 2011).

2.2. Opiate withdrawal syndrome in laboratory animals

2.2.1. Experimental procedure of dependence and opiate withdrawal induction

The first experimental step in the emergence of OW states in animal models of dependence is the induction of opiate dependence using various procedures including intra-peritoneal (i.p.) or subcutaneous (s.c.) repeated drug injection, subcutaneous drug pellet implantation delivery of drug solution by osmotic minipumps or catheters implantation or adding drug to food and drink.

In animals, constant infusion of drug by osmotic minipumps, or diffusion of drug by subcutaneous morphine pellet, provoke a constant level of morphine in the blood. In fact, this is a different condition of clinical setting when the dependence is the result of intermittent injections of drug doses. In particular, when heroin is injected intravenously, heroin enters rapidly in blood and has half-life of only 3 min, then it is converted rapidly to 6-monoacethylmorphine. After, heroin level decreases rapidly, reaching the limit of detection in 30

min (Koob and Le Moal, 2006). The OW emerges spontaneously after the drug cessation (Houshyar et al., 2003; Papaleo and Contarino, 2006). However, a large body preclinical studies, showed a privileged use of precipitated withdrawal by μ-opioid antagonist. In the context of human illicit opiate use, dependence is instigated by intermittent injections of increasing doses of opiates and withdrawal emerges spontaneously in the absence of an antagonist. Likewise, the induction of OW by catheter implantation in the animal under anesthesia may influence the emergence of OW syndrome. In addition, the impact of anesthesia in animals under pentobarbital or halothane molecule on the emergence and the kinetics of OW somatic signs and affective states are not known. In intracranial administration procedure, the effect of implantation of cannula without drug in the brain on the incidence of wet dog shakes and escape attempts was reported. e.g. the incidence of wet dog shake in cannula-implanted morphine-withdrawn rats is 80%, whereas, the incidence in unoperated morphine-withdrawn rats was 52% (Wei et al., 1973). Prior studies showed different consequences of procedure inducing withdrawal on the somatic signs and negative affect. These various methods of dependence and withdrawal induction make the comparison between different studies in animals difficult and may represent a limitation in the interpretation of results. To better mimic opiate use patterns in humans in our laboratory, we privilege to treating animals with repeated intermittent and increased opiate doses. Then, the OW emerges spontaneously after cessation of the opiate regimen.

2.2.2. Somatic syndromes of opiate withdrawal

Several animal models were proposed in order to understand the neurobiological basis of OW syndrome. The measures of OW among rodents include specific somatic signs such as jumping, paw tremor, wet dog shake, escape attempts, lacrimation, abdominal constriction, teeth chattering, body stretch, diarrhea, etc (Gold et al., 1994; Houshyar et al., 2003; Langerman et al., 2001; Papaleo and Contarino, 2006). In rodents, most of OW signs are observed by placing the animal individually into transparent Plexiglas cylinders for a determined period of time **(Photo. 1)** (Cicero et al., 2002; Houshyar et al., 2003; Papaleo and Contarino, 2006).

Photo. 1 Apparatus for the observation of the opiate withdrawal signs. Mice were individually placed into transparent Plexiglas cylinders and observed for the occurrence of OW signs during the following 30 min.

In rodents, body weight (BW) loss and suppress feeding behavior during the dark phase of the light:dark cycle are also markers of OW following intermittent morphine treatment (Boghossian et al., 2001; Cicero et al., 2002; Houshyar et al., 2003; Papaleo and Contarino, 2006; Thornhill et al., 1976). However, the duration of food intake alterations are briefer than BW alterations, after the last injection of morphine (Houshyar et al., 2003; Langerman et al., 2001). In rats, the caloric efficiency (mg weight gain / kcal ingested) is reduced during morphine withdrawal and is associated with numerous of physiological and behavioral adaptations that are similar to those observed during chronic stress (Akana et al., 2001). Stressors (Akana et al., 1999; Gomez and Dallman, 2001; Gomez et al., 2002) and morphine treatment (Houshyar et al., 2003) reduced plasma, insulin and leptin concentration indicating an increased sympathetic activity. The implication of the CRF in the deregulation of energy balance elicited by OW was also reported (Houshyar et al., 2003; Smagin et al., 1999). For example, the intracerebral administration of the CRF induces a decrease of the BW, food intake and caloric efficiency (Smagin et al., 1999). It is known that morphine withdrawal reduced energy balance and alters the HPA-axis and the CRF mRNA expression in multiple brain regions (Fisher et al., 1982; Houshyar et al., 2003). In addition, the application of acute stress enhanced the hypothalamic CRF, (adrenocorticotropin hormone) ACTH and corticosterone during the early 12 hr of morphine withdrawal (Houshyar et al., 2003). Furthermore, the analysis of the CRF peptide in several brain regions regulating namely the behavioral, autonomic and neuroendocrine responses to stress suggested that opiate dependence represents a severe stressful condition. In fact, morphine administrations alter CRF activity in the bed nucleus of stria terminalis (BNST), paraventricular nucleus (PVN) and Barrington's nucleus (Houshyar et al., 2003).

Brain area and neurotransmitters involved in the somatic signs of opiate withdrawal:

Several investigations in rodents explore the role of different brain regions involved in the expression of physical sign of OW. Early work showed that the intracerebral administration of naloxone in the medial thalamus junction exacerbates wet dog shake and escape attempts in morphine-withdrawn male rats (Wei et al., 1973). Likewise, the intracerebral administration of the methynaloxonium-precipitated OW, in the locus coeruleus (LC), specifically exacerbates the motor component of abstinence such as jumping, rearing and hyperactivity, in morphine-withdrawn male rats (Maldonado et al., 1992). The LC is a brain area which is the major source of the noradrenergic neurones of the central nervous system (Koob, 2008). This brain region possesses high density of opiate receptors, particularly of the μ and κ subtype (Tempel and Zukin, 1987). The brain noradrenergic system has been hypothesized to play an important role in opiate dependence and withdrawal (Koob, 2008). In fact, the role of the LC in the expression of somatic withdrawal signs was confirmed by a series of experiments in morphine-withdrawn rats which exhibited somatic signs after intracerebroventricular administration of methylnaloxonium

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in this region (Maldonado and Koob, 1993). Then, the electrolytic lesion in the LC of these animals attenuated the somatic signs of OW such as mastication, rearing, piloerection, hyperactivity, ptosis and eye twitch after precipitated OW. Furthermore, multiple data showed perturbations of the expression of several neurotransmitters implicated in the somatic component of OW syndrome such as norepinephrine, dopamine or CRF, etc in several brain regions (**Table. 1**) (Acquas et al., 1991; Acquas and Di Chiara, 1992; Aghajanian, 1978; Houshyar et al., 2003; Papaleo et al., 2007b; Tokuyama et al., 2001). In particular, increased behavioral signs of OW, observed in rats, correlated positively with increased electrical neuronal activity of the LC (Rasmussen et al., 1990). In addition, an increased of the glutamate and the norepinephrine in this nucleus was also reported (Tokuyama et al., 2001). Finally, the administration of the clonidine in the LC eliminates the OW, showing an additional evidence of the implication of noradrenergic mechanisms in the OW syndrome (Koob et al., 1992).

Others studies carried on rats, showed that the lesion of the amygdala (Calvino et al., 1979) and of that the raphe nuclei (Blasig et al., 1976) eliminates jumping but do not act on the others signs during OW. The peripheral administration of naloxone induces diarrhea in morphine-withdrawn male rats (Wei et al., 1973). However, the intracerebral administration of the methynaloxonium, which is unable to cross the brain-blood barrier does not exhibit diarrhea, salivation, lacrimation or rhinorrhea which suggests a peripheral control of these signs (Maldonado et al., 1992). In addition, the selective peripheral administration of opioid antagonist resulted in the expression of diarrhea, proving a role of local intestinal opioid receptors during OW (Bianchetti et al., 1986).

In a recent study, a series of experiments was conducted in mice using behavioral and biological approaches to determine the role of the the nucleus accumbens (Nacc) in the physical component of OW syndrome (Zachariou et al., 2006). DeltaFosB is transcripted in the Nacc during opiate dependence. Zachariou and colleagues argued that the DeltaFosB, via its action on DYN expression, in the nucleus accumbens is implicated in the somatic behavioral profile observed among opiate-dependent mice.

As a conclusion, the animal studies of OW symptoms lead to the characterization of multiple behavioral signs which may be controlled by various sites of the central nervous system. The LC, thalamus and amygdala mediate a large panel of OW signs. Other signs such as wet dog shakes were controlled by the hypothalamus. The opiate receptors in the peripheral system seem to mediate certain signs such as diarrhea and lacrimation.

Table. 1 Brain regions and neurotransmitters implicated in somatic signs and negative affective states of opiate withdrawal syndrome. ⬇ decreased; ⬆ increased.

Opiate withdrawal syndrome

Affective component		Somatic component	
Brain area	**Neurotransmitter**	**Brain area**	**Neurotransmitter**
Nucleus accumbens	Dopamine[a] ⬇ Dynorphine[d and g] ⬆	*Locus coeruleus*	Norepinephrine[b] ⬆ Glutammate[c] ⬆
Cerebral cortex	Dopamine[i] ⬇	*Periaqueductal gray*	Encephaline[k] ⬆
amygdala	Norepinephrine[j] ⬆ CRF[g and h] ⬆	*Hypothalamus*	CRF[d, f and h] ⬆ Norepinephrine[e] ⬆ Dopamine[e] ⬇

References: [a](Di Chiara, 2005; Di Chiara and Imperato, 1988); [b](Rasmussen et al., 1990); [c](Tokuyama et al., 2001); [d](Contarino and Papaleo, 2005); [e](Martinez-Pinero et al., 1994); [f](Houshyar et al., 2003); [g](Ingallinesi et al., 2011); [h](McNally and Akil, 2002); [i](Espejo et al., 2001); [j](Smith and Aston-Jones, 2008); [k](Frenois et al., 2002). (Adapted from: Ingallinesi, 2008).

2.2.3. Negative affective-like components of opiate withdrawal

Animal models of the negative affect comprise: Dysphoria, increases in sensitivity to anxiety-like behavior, suppression of locomotors activity, suppression of operant responding for drug and natural reward, anhedonia-like states, brain reward deficit, etc. (Carlezon and Chartoff, 2007; Haertzen and Hooks, 1969; Lieblich et al., 1991; Schulteis and Koob, 1994; Stinus et al., 2000).

A. *Dysphoria* associated with OW was examined in the *conditioned place aversion* (CPA) apparatus **(Photo. 2)**. Rodents avoid environmental cues repeatedly paired with spontaneous or precipitated OW states (Bechara et al., 1995; Rothwell et al., 2009; Stinus et al., 1990b). Most importantly, the aversive stimulus to environment and cue, associated to the OW experiencing, can also persists long-term after the last injection of opiates in rats and monkeys with the absence of the somatic signs (Azar et al., 2004; Baldwin and Koob, 1993; Goldberg et al., 1971). The maintenance of dysphoric states of long-term withdrawal persists even in the absence of opioid receptor occupancy (Koob, 2006) and could have a stronger impact than that of somatic signs on opiate-seeking and relapse (Koob and Le Moal, 2001; Liu and Schulteis, 2004). It is also suggested that the profound neurobiological adaptations of the central nervous system during OW participate to the vulnerability to opiate relapse.

To identify the brain regions involved in the aversive states of precipitated OW, morphine dependent rats were administered with methylnaloxonium in different brain sites (Stinus et al., 1990a). Then, these animals were conditioned in an environment associated to precipitated withdrawal. Interestingly enough, high doses of methylnaloxonium (1000-2000 ng) produce place aversion when injected in the lateral ventricle, periaqueductal gray, amygdala, ventral tegmental area and medial dorsal thalamus (Stinus et al., 1990b) while, low doses of methylnaloxonium (250-500 ng) produced place aversion only when injected in the Nacc, periaqueductal gray and amygdala (Stinus et al., 1990b). It is known that the Nacc mediates the motivational properties of natural rewards and opioid drugs (Di Chiara and North, 1992; Shippenberg et al., 1992; Widnell et al., 1996). Furthermore, biological and synaptic adaptive changes in the Nacc during OW were also reported (Dong et al., 2007; Georges et al., 2000; Walters et al., 2000). Acute administration of all major drugs, including opioids, induces an elevation of the extracellular levels of dopamine in the shell of the Nacc (NaccSH) (Pontieri et al., 1995). The repeated administration of opioids can elicit an opposite reaction within the same neurobiological mechanisms which are responsible of the rewarding effects of opioids *"within-system adaptation"*. This provides an evidence of a *within-system adaptations* (Solomon, 1980) consisting of a primary molecular alterations by acute administration of opioids in the Nacc which would be adapted to produce the opposing motivational aspect of opioids during OW (Koob and Bloom, 1983). In opponent process theory, positive affective (*Processes A*) and

negative affect (*process B*) were hypothesized to be controlling the central nervous system through mechanisms that would reduce the intensity of emotional states (Solomon and Corbit, 1974). The *process A* consists of a positive hedonic responses and occurs rapidly after drug intake. Conversely, the *process B* reflects a negative affect that appear after the end of *process A*. Otherwise, *"the opposing action of opioids can also appears after the recruitment of distinct neurobiological mechanisms, one not involved in the acute reinforcing effects of the opioids but engaging in the opposing effects of the reinforcing effect of opioids between-system adaptation"* (Koob and Kreek, 2007).
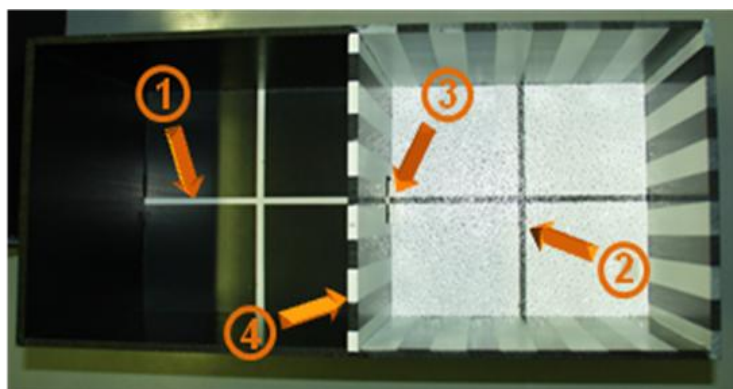
Photo. 2 Conditioned place aversion/preference apparatus. Consists of a rectangular Plexiglas box divided by a central partition **(4)** into two chambers of equal size **(1, 2)**. One compartment has black walls and a smooth Plexiglas floor **(1)**, whereas the other compartment has vertical black and white striped walls and a slightly rough floor **(2)**. During the test sessions, an aperture in the central partition **(3)** allows the mice to enter both sides of the apparatus, whereas during the conditioning trials the individual compartments are closed off from each Other. Transparent Plexiglas lids allow observation of the animal's behavior on a video monitor connected to a camera placed above the apparatus.

B. *Anhedonia* is defined as *"the inability to experience pleasure"* (Ribot, 1896). More recently, anhedonia is defined as *"a condition in which the capacity of experiencing pleasure is totally or partially lost"* (APA, 2000). The development of the anhedonia is more related to psychiatric disorders such as depression and anxiety and to the drug dependence (APA, 2000; Hatzigiakoumis et al., 2011; Janiri et al., 2005). Heroin dependent individuals exhibited decreased responsiveness to pleasant (non-drug-related) stimuli which is attributed to anhedonia (Lubman et al., 2009; Zijlstra et al., 2009).

Therefore, several authors reported that anhedonia is a part of the general malaise linked to the OW contributing as a major factor involved in the opiates relapse (Koob and Le Moal, 2001; Volkow et al., 2002). This is consolidated by clinical studies showing negative correlation between anhedonia and protracted abstinence in opiate-dependent patients (Janiri et al., 2005).

In animals, stress-induced decrease in the preference for highly palatable food (HPF) has been validated as a behavioral index of anhedonia-like behavior in rats (Papp et al., 1991; Willner et al., 1987). Some data indicate attenuated preferences for sweet saccharin solutions in morphine-withdrawn rats (Lieblich et al., 1991).

C. *Reward deregulation* is an important component of the negative states which drives the motivation for drugs and contributes to the dependence (Koob and Le Moal, 1997). Elevation of *Intracranial self-stimulation* (ICSS) threshold is believed to reflect a functional deficit of the brain reward system. For example, reward alterations occur when physical signs of cocaine and nicotine withdrawal are not manifested (Kenny and Markou, 2005; Markou and Koob, 1992).

In ICSS procedure, animals perform an operant task to obtain a positive reinforcement current delivered via an electrode implanted in the medial forebrain bundle at the level of lateral hypothalamus **(Fig. 2)** (Olds and Milner, 1954). Olds and Travis (1960) were the first who described attenuate response rate of reward threshold after morphine regimen (Olds and Travis, 1960). In rats, spontaneous or precipitated (with naloxone μ-opioid antagonist) fentanyl withdrawal induced an elevation of brain reward threshold in rats, as assessed in an animal model of ICSS (Bruijnzeel et al., 2006). This elevation of reward threshold decreases progressively during 2 days after the last injection of fentanyl to recover a normal baseline threshold level (Bruijnzeel et al., 2006). Similarly, using ICSS, in rats self-administer heroin for 23 hours/day, the opioid receptor antagonist naloxone produced an elevation of the brain reward threshold, indicating a profound brain reward system deficits (Kenny et al., 2006). In agreement with previous studies, other researchers reported a naloxone dose-dependent elevation of the brain reward threshold in morphine-withdrawn rats (Liu and Schulteis, 2004; Schulteis et al., 1994). In contrast, a lowering of reward threshold after daily short exposure (1 hr) to heroin self-administration was observed (Kenny et al., 2006) suggesting an enhanced functionality of the

brain reward system in limited access to heroin self-administration. In morphine-pretreated rats, a significantly greater preference has been shown for a morphine-paired environment than that of saline-paired one, in *conditioned place preference* (CPP) paradigm **(Photo. 1)**, 2 or 5 weeks after chronic morphine treatment (Harris and Aston-Jones, 2001, 2003). The latest data suggests an enhancement of brain reward system in long-term opiate dependence. Similarly, other authors using the CPP apparatus have demonstrated that, contrarily to non morphine-pretreated rats, morphine-withdrawn ones develop a place preference at lower doses suggesting a sensitization to the rewarding properties of morphine (Shippenberg et al., 1996). Furthermore, the analysis of the *c-fos* protein induction on brain areas linked to conditioned or affective properties of OW was revealed. More specifically, compared to the control group, morphine conditioned rats displayed a greater induction of this protein during morphine withdrawal in the anterior cingulate cortex, Nacc core and, ventral lateral BNST, and central and basolateral amygdala nuclei (Harris and Aston-Jones, 2003). Thus, opiate pretreatment induces a sensitization to the rewarding effects of morphine which is mediated by changes in neuronal excitability reflected by the *c-fos* protein induction. In opposite to the studies using ICSS procedure, these latter results suggest a progressive development of the reinforcing effects of opioids provoked by a sensitization of brain reward system following chronic exposition to opioids.

Taken together, these reports revealed a complex impact of OW on the brain reward systems which is difficult to interpret in terms of methodological differences used in the brain reward threshold measure.
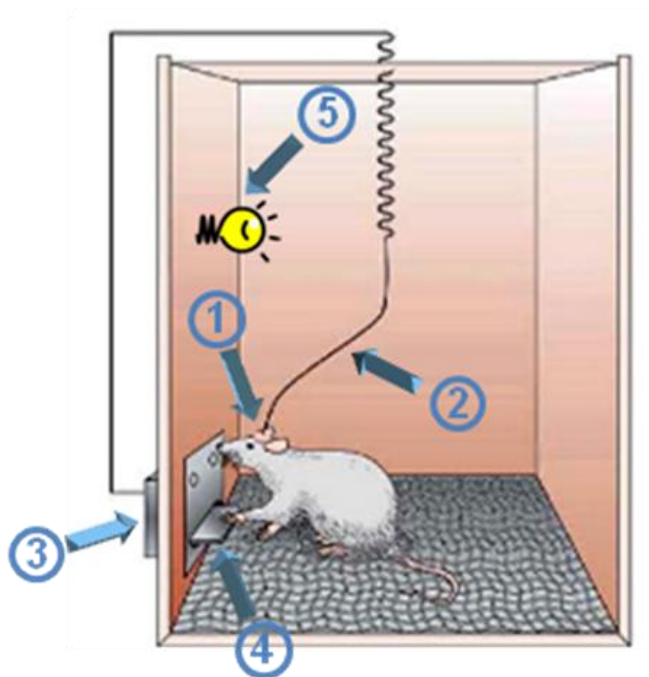
Fig. 2 Intracranial self-stimulation apparatus. A mouse with a monopolar electrode **(1)** permanently implanted into the medial forebrain bundle. A flexible wire lead **(2)** plugs into the electrode and connects the animal to the electrical stimulator **(3)**. The lever-pressing **(4)**, results in brain stimulation reward associated to a light signal **(5)**. (Adapted from: Bear, 2001).

D. *Disruption of motivation* to drug and natural rewards is a marker of the negative emotional state elicited by OW (Dalley et al., 2005; Koob and Le Moal, 2008). It is widely accepted in literature that addicts individuals develop a practically an exclusive interest for the drug-related activities and neglect the other pleasures of life such as food, sex, social activities, etc (APA, 2000; ICD, 2010; Koob and Le Moal, 2001; Negus, 2006; Robinson and Berridge, 2003). However, clinical evidences provide divergent findings about the motivational disorders associated with drug dependence and withdrawal. For instance, human studies show reduced food intake, poor nutritional status and decreased responsiveness to "natural" rewarding stimuli in heroin dependent individuals (Lubman et al., 2009; Santolaria-Fernandez et al., 1995; Zijlstra et al., 2009). In contrast, other studies report heightened preference for and/or consumption of liquid or solid sweets, elevated craving for sweet foods and unaltered nutritional status in opiate-dependent individuals (Morabia et al., 1989; Weiss, 1982).

As far as animals, the motivational measures of OW have included the disruption of trained operant behavior for opiates or palatable food **(Photo. 3)** (Koob et al., 1992). For example, in rats, spontaneous morphine withdrawal decreased the motivation to work for sweet solution in progressive ratio (PR) paradigm (Zhang et al., 2007). Dependence in rats, instigated with subcutaneous morphine pellet (Harris and Aston-Jones, 2003) or naloxone μ-opioid antagonist (Schulteis et al., 1994), provokes a severe OW states which decreases responding for food in fixed ratio (FR) reinforcement schedule.

Molecular studies showed that DYN is a peptide implicated in the palatable food intake and in the severity of dysphoric states associated with OW (Houshyar et al., 2004; Lightman and Young, 1988). Some authors exhibited a major role of DYN in the modulation of preference for sugar during OW. Precipitate withdrawal reduces the consumption of sucrose in wild-type mice but not in genetically invalidated DYN ones (Hayward et al., 2006). Others reported a higher level of pro-dynorphin in the brain following palatable food intake (Welch et al., 1996). Using brain dialysis coupled to high-performance liquid chromatography, Di Chiara and Imperato (1988) reported a decreased dopamine release in the Nacc, after the administration of κ-receptor agonists (Di Chiara and Imperato, 1988). Also, during OW, DYN induced a decrease of dopamine release in the Nacc which leads to the alteration of food-driven operant behavior (Di Chiara, 2005). This latter behavior (linked to biological alteration of DYN and dopamine neurotransmission) is considered as a key component of the negative affect developed during OW **(Table. 1)**.
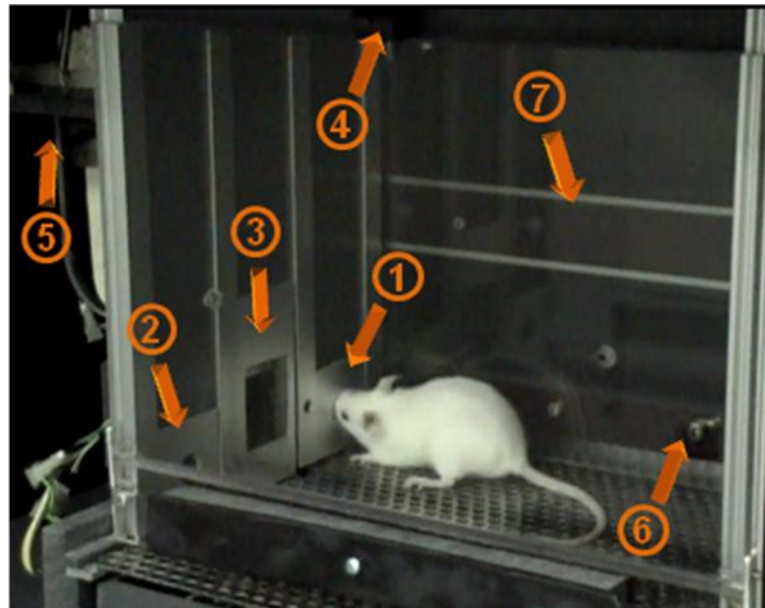
Photo. 3 Food operant conditioning. Apparatus is equipped with two nose-poke holes mounted at the opposite ends of the same wall of the apparatus **(1, 2)**, each equipped with infrared photo-beams connected to a computer. Nose-poking into one of the two holes, i.e., the active hole **(1)**, results in food pellet delivery and light signal **(4)** whereas nose-poking into the other hole **(2)**, i.e., the inactive hole, has no consequences. Centered between the nose-poke holes is a feeding-trough for the delivery of food pellets **(3)**. A food pellet delivery occurs when the photo-beam of the active nose-poke hole is interrupted. The food pellets are delivered by an automated dispenser **(5)**. The food trough is equipped with photo-beams that allow monitoring of food trough visits. Apparatus is also equipped with 2 series of infrared photo-beams that serve to record horizontal **(6)** and vertical **(7)** ambulatory activity.

In contrast with data above-mentioned, a recent rat study has shown a progressive increase of motivation for food during the time intervals occurring between repeated morphine injections (Cooper et al., 2010). Along the same line, using the natural reward incentive test, other authors explored the effects of abstinence 14 days after a cessation of morphine dose regimen and noticed an increase of high-fat food seeking and sexual pursuits in rats (Nocjar and Panksepp, 2007). Authors reported that sugar consumption decreases the severity of OW signs after naloxone-precipitated withdrawal in morphine-withdrawn rats (Jain et al., 2004) which represent a possible explanation of increase food-driven operant behavior. Indeed, palatable food and sweet solutions induce a release of beta-endorphin (Dum et al., 1983; Yamamoto et al., 2000), a down-regulation of DYN mRNA expression (Levin and Dunn-Meynell, 2002) and an increase of dopamine D_1 and μ-opioid receptor binding in several brain regions which may contribute to counteract the unease elicited by OW.

The divergence of results between studies already mentioned highlights the importance of procedural manipulation during the operant behavior. Food restriction is one of these procedures which are used frequently to enhance learning in food-driven operant behavior. However, in non opiate-withdrawn animals, food restriction alters brain opioid receptor signaling, making the comprehension of the role of opioid system in the food driven-operant behavior more complicated (Berridge, 1991; Wolinsky et al., 1994 cited by Papaleo et al., 2007a). Furthermore, the food restriction can also sensitize the brain reward systems via opioid receptor mechanisms in hypothalamus (Carr and Papadouka, 1994). As a matter of a fact, we cannot exclude the influence of food restriction during OW, which might limit the interpretation of the previous finding.

The exploration of the brain substrates implicated in the reinforcing effects of opioids designates the Nacc as a major protagonist of the positive reinforcement of opioids in non-dependent animals (Koob et al., 1992). In fact, non-dependent rats self-administer opioids directly in the Nacc (Goeders et al., 1984). This behavior was completely abolished by the administration of methylnaloxonium in the same region (Vaccarino et al., 1985). Interestingly, in morphine-dependent rats, the administration of methylnaloxonium in the Nacc induces disruption of food-driven operant behavior (Koob et al., 1989). These latter studies suggest that the opioid receptors of the Nacc does not only play an important role in the positive reinforcement effect during the acute administration of opioids, but also in the aversive effects of opioids developed during dependence (Koob et al., 1992; Koob et al., 1989).

2.2.4. Gender differences in the expression of opiate withdrawal

Among humans, recent evidences showed that female gonadal hormone estrogen may facilitate drug intake in women by interacting with brain stress system and reward system (Anker

and Carroll, 2011). Accordingly, in rats, administration of estrogen increases the acquisition and the reinstatement of drug-seeking behavior in female (Anker and Carroll, 2011; Larson et al., 2007). Furthermore, stressful events are one of the major factors leading to drug seeking and relapse during period abstinence (Kreek et al., 2009). Several data showed that the major components of brain stress system such as CRF system and neuroepinephrine system in the Locus coereleus (LC) are more sensitive to stressors in female than male rats (Bangasser et al., 2010; Bangasser et al., 2011). In fact, several authors hypothezised that the higher compromised stress adaptations in female, compared to male, may underlie their vulnerability to drug relapse and dependence (Broome et al., 2010; Hudson and Stamp, 2011).

Spontaneous withdrawal provoked by discontinuation of morphine treatment resulted in higher levels of somatic OW in male compared to female rats and mice (Cicero et al., 2002; Papaleo and Contarino, 2006). Although, this gender differences in rats and mice, were hidden when the OW was induced by naloxone (Blum et al., 1976; Cicero et al., 2002; el-Kadi and Sharif, 1994; Kest et al., 2001). More precisely, after morphine cessation, male exhibited precocious increases in wet dog shakes and paw tremor behaviors than female mice (Papaleo and Contarino, 2006) Moreover, male rodents exhibited a higher level of BW loss than female rodents for the same morphine regimen (Boghossian et al., 2001; Cicero et al., 2002; Papaleo and Contarino, 2006). Along with previous study, male rats showed a higher global score of OW signs and a higher BW loss than female rats (Cicero et al., 2002). However, in the latter study, the naloxone-precipitated OW eliminates the gender differences. In rats, gender differences have been found to be involved in the regulation of pain (Islam et al., 1993; Loyd and Murphy, 2006) and the arousal (Devidze et al., 2008) during morphine and methadone withdrawal, respectively.

Biological molecular studies of dependence showed good evidences of a gender effect during OW. Furthermore, endogenous opioid system exerts an inhibitor effect on HPA-axis in humans (Schluger et al., 1998) and rodents (Eisenberg, 1980; Zhou et al., 2005). More precisely, beta-endorphin which colocalize with CRF in the PVN of the hypothalamus, acts on the μ-opioid receptor and induces an inhibition of the CRF neuronal activity (Nikolarakis et al., 1987; Pilcher and Joseph, 1984; Zhou et al., 2006). Interestingly, the hypothalamic RNAm expression of the μ-opioid receptor decreases after morphine treatment in the case of female Guinea pig (Ronnekleiv et al., 1996). However, studies in male rats, showed an enhancement (Zhou et al., 2006) or a no alteration of the μ-opioid receptor RNAm expression in the same brain region during OW (Leri et al., 2006). Other hypothesized that the increased activity of orexin in the lateral hypothalamus plays an important role in opiate-withdrawal related behavior (Georgescu et al., 2003; Zhou et al., 2006). Genetic deletion of orexin in mice reduced physical or somatic signs elicited by OW (Georgescu et al., 2003). In addition, an orexin RNAm expression elevation was showed in male but not in female rats during methadone withdrawal (Devidze et al., 2008).

3. Corticotropin-releasing factor system

3.1. Corticotropin-releasing factor peptide family, binding sites and anatomical distribution

The extensive researches carried out on both humans and animals during the last decades attest the fundamental role of the CRF family peptides and receptors in neuroendocrine, physiological and behavioral responses to stressful events. Since the isolation and the identification of CRF peptide in 1981 (Vale et al), the discovery of diverse and selective function of CRF system was prospected through the study of the multiple actions of different ligands and receptors distributed heterogeneously in the brain and peripheral system (Vale et al., 1981 cited by Sarnyai et al., 2001). Subsequently, a new peptide called urocortin (UCN) was discovered in mammals using molecular biological approaches (Vaughan et al., 1995) after the identification of urotensin in fish (Lederis et al., 1982) and sauvagine in frogs (Montecucchi and Henschen, 1981). The latest research of a new member of CRF family leads to the cloning of stresscopin and stresscopin-related peptide from human DNA (Hsu and Hsueh, 2001) and urocortin$_2$ (UCN$_2$; Reyes et al., 2001) and urocortin$_3$ (UCN$_3$; Lewis et al., 2001) from mouse DNA.

CRF and UCN have diverse roles on the physiological and behavioral stress response by acting, with high affinity, via two G protein-coupled 7 transmembrane domain receptor subtype termed CRF$_1$ and CRF$_2$ (Dautzenberg and Hauger, 2002). In addition to the CRF$_1$ and CRF$_2$ receptors, CRF and UCN bind to a CRF-binding protein which modulates the stress response by limiting CRF receptors activation (Potter et al., 1992; Takahashi, 2001; Takahashi et al., 2001).

3.1.1. CFR peptides

In mammals, CRF is 41-amino acid polypeptide. Human and rats CRF are identical but differ from ovine CRF by seven amino acids (Dautzenberg and Hauger, 2002). Immunocytochemical studies showed a higher concentration of CRF in the PVN of the hypothalamus and extra hypothalamic region as limbic area include: amygdala, BNST and brain stem nuclei include: LC and nucleus of solitary tract involved in stress response (Sawchenko et al., 1993 cited by Sarnyai et al., 2001). UCN, which is a second mammalian CRF-like peptide, possesses 40-amino acid and is identified in human, sheep, rats and mouse (Dautzenberg and Hauger, 2002). The term urocortin refers to its sequence similarity in carp urotensin (63%, "uro") and mammalian CRF (45%, "cort"; Koob, 2009). UCN showed ~ 45 % of homology to human CRF. UCN$_1$ binds both CRF$_1$ and CRF$_2$ receptors and has different neuroanatomical distribution than the CRF (Hauger et al., 2003). UCN$_{2 and 3}$ are endogen selective CRF$_2$ agonist and have different neuroanatomical, pharmacological and distribution profile (Spina et al., 1996). UCN like immunoreactivity is found in brain area such as the Edinger-Westphal locus and the hypothalamus (Dautzenberg and Hauger, 2002). However, peripheral expression of UCN is more

important and localized in thymus, gastrointestinal tract, testis, cardiac myocytes, thymus, spleen and kidney (Kageyama et al., 1999).

The neuropeptide CRF has an important role in the response of body to stressors. *Stress* has been defined as "*anything which causes alterations of psychological homeostatic process*" (Burchfield, 1979). The psychological homeostasis refers to the maintenance of normal mood states in the individual at rest (Burchfield, 1979; Engel, 1953). The psychological alterations are accompanied with changes in physiological homeostasis responses to stress (Schachter and Singer, 1962). The central administration of CRF in animals displays a similar behavioral response to stressors (Dunn and File, 1987). However, the administration of antalarmin CRF_1 receptor antagonist reverses this effect (Zorrilla et al., 2002). Thus, the CRF has the capacity to stimulate the synthesis and the secretion of ACTH from the pituitary, and initiate pituitary-adrenal responses to stress by release of glucocorticoids, by the adrenal gland, in the bloodstream (Marti et al., 1999; Owens and Nemeroff, 1991; Rivier et al., 1982) (**Fig. 3**). Besides, the role of the CRF on the HPA-axis, CRF also controls a variety of biological phenomena such as food intake (Brady et al., 1990; Heinrichs and Richard, 1999) and energy balance (Rothwell, 1990).

Fig. 3 Schematic representation of the hypothalamic-pituitary-adrenal axis. CRF drives the hypothalamic-pituitary adrenal axis by stimulating the synthesis and the secretion of ACTH from the pituitary, and initiates pituitary-adrenal responses to stress by release of glucocorticoids, by the adrenal gland, in the bloodstream. CRF activates the sympathetic system by actions in the brainstem and mediates arousal and behavioral responses to stressors by actions in the amygdala, other basal forebrain regions, and ventral midbrain such as the VTA. (Koob, 2010; River et al; 1982; Marti et al, 1999; Owens and Nemeroff, 1991).
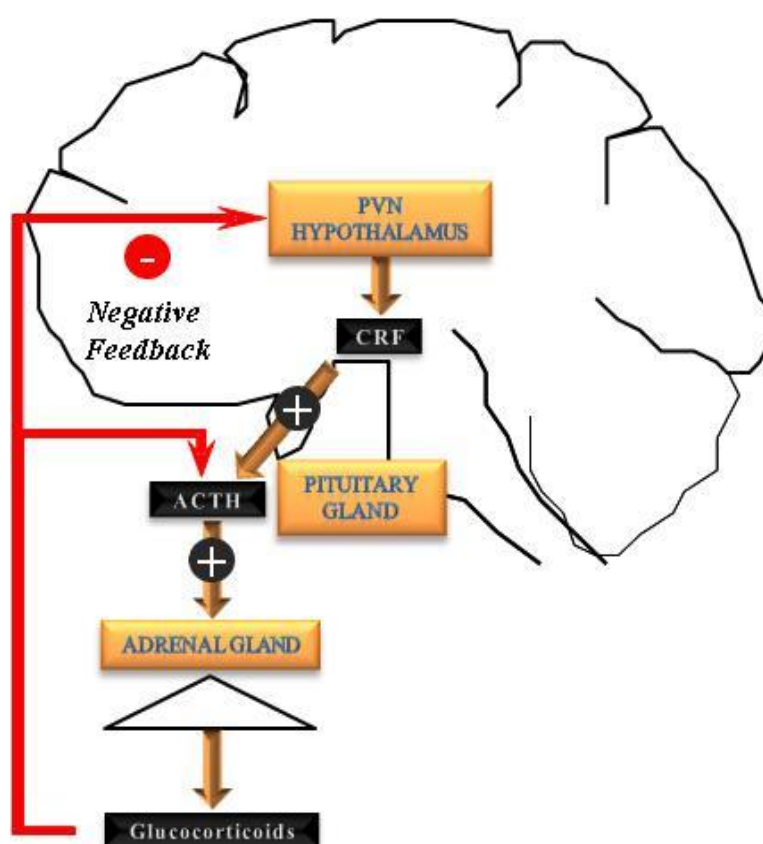
3.1.2. CRF receptors

CRF_1 and CRF_2 receptors are, respectively, a 415- 446 and 397- 438 amino-acid protein. They present 70% of amino acid homology with a higher homology in the intracellular and transmembrane domain (80-85% amino acid identity) than extracellular domains (<60% amino acid identity (Dautzenberg and Hauger, 2002). The CRF_1 receptor has no known polymorphism while CRF_2 receptor exists in four forms named: $CRF_{2\alpha}$, $CRF_{2\alpha\text{-tr}}$, $CRF_{2\beta}$ and $CRF_{2\delta}$ (Chalmers et al., 1995; Kostich et al., 1998; Lovenberg et al., 1995; Perrin et al., 1995)

The CRF_1 receptor in mammals was abundant in brain and the pituitary gland located at the base of brain. More specifically, the expression of CRF_1 receptor RNAm expression was found in anterior pituitary, cerebral cortex, cerebellum, amygdala, hippocampus and the olfactory bulbs (Chalmers et al., 1995; Sanchez et al., 1999) (**Fig. 4**). In addition, CRF_1 receptor RNAm is expressed in hypothalamus and locus coereleus (Dautzenberg and Hauger, 2002). The peripheral expression of CRF_1 receptor RNAm was not abundant and was localized in testis, ovary and adrenal gland. However, a peripheral role of the CRF_1 receptor in the pregnancy and inflammation was reported (Dautzenberg and Hauger, 2002; Karteris et al., 2001; Uzuki et al., 2001). CRF_2 receptor in mammals was expressed in the lateral septum and the BNST, the medial and posterior cortical nuclei of the amygdala, the ventromedial nucleus of hypothalamus, the mesencephalic raphe, the interpeduncular nuclei and the olfactory bulbs (Van Pett et al., 2000) (**Fig. 4**). $CRF_{2\alpha}$ receptor variant is expressed in brain regions whereas $CRF_{2\beta}$ receptor is expressed in the choroids plexus and the periphery tissues such as cardiac myocytes, gastrointestinal tract, lung, ovary and skeletal muscle (Lovenberg et al., 1995).

The pharmacological characterization of the CRF receptors reveals a strong divergence in the profile between CRF_1 and CRF_2 receptors. The CRF binds with a high affinity to the CRF_1 receptor and with low affinity to the CRF_2 receptor (Sarnyai et al., 2001). Compared to CRF peptide, the non mammalian CRF peptide (urotensin and sauvagine) and the mammalian UCN peptide ($UCN_{1, 2 \text{ and } 3}$) bind with up to 100 fold higher affinities to the CRF_2 receptor (Hauger et al., 2003). Because CRF_2 receptor is highly selective for UCN_1 over CRF, Hauger and colleagues (2003) proposed the renaming of CRF_2 receptor as the urocortin receptor.

The binding of the CRF receptors agonists to the extracellular domains of CRF_1 and CRF_2 receptor provokes a transformation in membrane conformation. This transformation induces an increasing affinity for the G_s Protein leading to the stimulation of adenylyl cyclase and activation of protein kinase A which increases the levels of cAMP. The protein G_q is another second messenger which could be implicated in another cascade signaling via phospholipase C. Finally, the G-protein receptor Kinase is involved in the desensitization and internalization of CRF_1 receptor via phosphorylation of C-terminus.
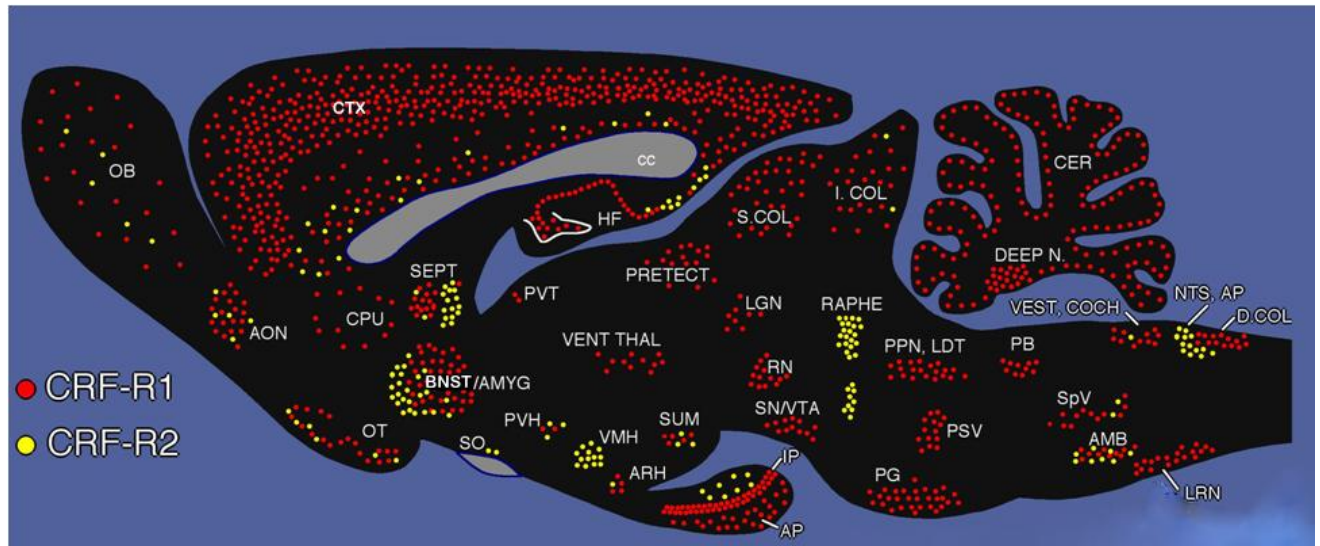
Fig. 4 CRF receptor distribution in rodent brain. Schematic drawing of sagittal section through the rat brain shows the distribution and relative density of mRNA's for CRFR$_1$ and CRFR$_2$. The CRFR$_2$ transcripts show a more restricted distribution that is largely non-overlapping with that of CRFR$_1$. **Abbreviations:** AMB nucleus ambiguus; AMYG amygdala; AON anterior olfactory nucleus; AP anterior lobe of pituitary gland; ARH arcuate nucleus of the hypothalamus; BNST bed nucleus of the stria terminalis; cc corpus callosum; CER cerebellum; COCH cochlear nuclei; CTX cortex; CPU caudoputamen; D.COL dorsal column nuclei; DEEP N. Deep nuclei of the cerebellum; HF hippocampal formation; I.COL. inferior colliculus; IP intermediate lobe of pituitary gland; LDT laterodorsal tegmental nucleus; LGN lateral geniculate nucleus; LRN lateral reticular nucleus; NTS nucleus of the solitary tract; OB olfactory bulb; OT olfactory tubercle; PB parabrachial nucleus; PG pontine gray; PPN pedunculopontine nucleus; PRETECT pretectal region; PSV principal sensory nucleus of the tegmental nerve; PVH paraventricular nucleus of the hypothalamus; PVT paraventricular nucleus of the thalamus; RN red nucleus; SEPT septal region; S.COL. superior colliculus; SN substantia nigra; SO supraoptic nucleus; SpV spinal trigeminal nucleus; SUM supramammillary nucleus; VENT THAL ventral thalamus; VEST vestibular nuclei; VMH ventromedial nucleus of the hypothalamus; VTA ventral tegmental area. (adapted from: Van Pett et al., 2000; Contarino and Gold, 2002).

3.2. CRF system and stress

Early works demonstrate that CRF_1 receptor activation is associated with increased stress responsiveness (Koob and Heinrichs, 1999) while the CRF_2 receptor activation is linked with a decrease in feeding behavior and stress responsiveness (Pelleymounter et al., 2000; Spina et al., 1996). However the role of CRF_2 receptor in decreasing the stress response is controversial because some studies showed a hyper-response to stress or no noticeable change in stress-related behavior which depends on the neuropharmacological probe and the dose used and brain site targeted (Fekete and Zorrilla, 2007; Ho et al., 2001; Koob and Zorrilla, 2010; Zhao et al., 2007).

In male and female mice, the physiological response to acute stress leads to an increase of ACTH and corticosterone secretion (Smith et al., 1998). The phenotype of mice deficient for CRF_1 receptor ($CRF_1^{-/-}$) displays no increase of ACTH and corticosterone plasma after forced-swim stress (Timpl et al, 1998) or physical-restraint stress (Smith et al., 1998). Moreover, the $CRF_1^{-/-}$ mice, tested in *elevated plus-maze* (EPM) and *light-dark* (LD) paradigms, have shown the display of a reduced anxiety-like response (Smith et al., 1998). Contrary to the wild-type mice, $CRF_1^{-/-}$ mice entered more frequently and spent more time in the open arms of EPM apparatus as well as spent more time in the light compartment of the LD apparatus (Smith et al., 1998; Timpl et al., 1998). The EPM and LD tests quantify exploration of open arms of the EPM apparatus, relative to closed arms, and lit compartment of the LD apparatus. Overall, an increase of time spent in open arms and lit compartment of EPM (Pellow G et al, 1985) and LD (Crawley and Goodwin, 1980), respectively, reflects a marked decrease of anxiety-like behavior in rodents **(Table. 2)**.

The genetic elaboration of mice deficient for CRF_2 receptor ($CRF_2^{-/-}$) have permitted for the identification of opposite roles of CRF_2 receptor in behavioral and neuroendocrine responses to stressors **(Table. 2)**. Immuno-histological analysis of pituitary of this phenotype mouse showed no differences in structures of pituitary and adrenal gland (Bale et al., 2000). In addition, normal and similar basal levels of ACTH and corticosterone were observed in wild-type and $CRF_2^{-/-}$ female and male mice (Bale et al., 2000). Nevertheless, after physical-restraint stress, the peaks of ACTH and corticosterone levels were achieved in a fast way in $CRF_2^{-/-}$ than wild-type mice indicating a hyper-sensitive response of the HPA axis to stress in $CRF_2^{-/-}$ mice (Bale et al., 2000). Interestingly, $CRF_2^{-/-}$ mice showed an opposite profile of anxiety-like response compared to $CRF_1^{-/-}$ mice. In fact, compared to wild-type mice, both male and female $CRF_2^{-/-}$ mice spent less time and reduced entries in the open arms of EPM apparatus (Bale et al., 2000). Whereas, the results of LD test revealed no difference of anxiety-like behavior state between wild-type and $CRF_2^{-/-}$ mice in the latter study.

Table. 2 Anatomical, neuroendocrinological and behavioral caracteristics of the genetic deletion of the CRF receptors in mice.

Abbreviations: **(+)** increased; **(-)** decreased; **(0)** no effect; **ND** not determined; **BWT** black-white test; **OF** open field test.

	$CRF_1^{-/-}$	$CRF_2^{-/-}$
Adrenal medulla (diameter)	(-)[a]	(0)[h]
Glomerulosa	(0)[b]	(0)[h]
Fasciculata	(-)[b]	(0)[h]
Reticularis	(0)[b]	(0)[h]
Medulla	(0)[b]	(0)[h]
ACTH in basal condition	(0)[a and b]	(0)[h]
Increased ACTH after forced-swim stress	(-)[a]	ND
Increased ACTH after physical-restraint stress	(-)[b]	(+)[g and j]
Corticosterone in basal condition	(-)[a and b]	(0)[h]
Increased corticosterone after forced-swim stress	(-)[a]	ND
Increased corticosterone after physical-restraint stress	(-)[b]	(+)[h and g]
CRF peptide expression		
PVN	(+)[a and b]	(0)[h]
Amygdala	(+)[a]/(0)[b]	(+)[h]
Hippocampus	(+)[a]	(+)[k]
Cerebral cortex	(+)[a]	ND
UCN (Edinger-Westphal nucleus)	ND	(+)[h]
$CRFR_{1 and 2}$ expression (brain and pituitary gland)	(0)[a and b]	(0)[h, i and g]
Anxiety-like states (LD[a and h], EPM[d and h], OF[h] and BWT[d])	(-)	(+)
Depresion-like states (Forced-swim test)	ND	(-)[f]
Spatial recognition memory (Y-maze test)	(-)[c and d]	ND
Social memory (social discrimination test)	ND	(+)[l]
Locomotor activity	(+)[a and e]	(0)[i]
CRF treatment-increased locomotor activity	(-)[e]	ND
Feeding in basal condition	(0)[i]	(0)[h]
Feeding after food-restriction stress	ND	(-)[h]
CRF treatment-suppressed food intake	(0)[e]	ND
Weight gain in basal condition	(0)[i]	(0)[h and i]
Weight gain after food restriction stress	ND	(0)[h]

References: [a](Timpl et al., 1998), [b](Smith et al., 1998), [c](Heinrichs, 1999), [d](Contarino et al., 1999a), [e](Contarino et al., 2000), [f](Bale and Vale, 2003), [g](Coste et al., 2000), [h](Bale et al., 2000), [i](Ingallinesi et al., 2011 ; Rouibi et Contarino, unpublished data), [j](Preil et al., 2001), [k](Todorovic et al., 2009), [l](Deussing et al., 2010).

Among humans, the implication of an exaggerated neuronal activity of the CRF system can be considered as a marker in the etiology of human stress-related disorders such as depression and anxiety (Holsboer, 1999). The role of chronic hypersecretion of the CRF peptide in depressive patients was hypothesized (Nemeroff, 1996). This latter track is consolidated by clinical and post mortem data showing HPA-axis impairment and high concentration of the CRF in the cerebrospinal fluid of severely depressed suicide victims (Nemeroff, 1996; Plotsky et al., 1998; Wong et al., 2000). A normalization of the HPA-axis activity was observed after a successful antidepressant treatment in a patient with severe depression profile (Arborelius et al., 1999). Post mortem studies has presented a down regulation of the CRF_1 receptor in the frontal cortex (Hucks et al., 1997) and no difference in the mRNA levels for CRF receptors in anterior pituitaries (Hiroi et al., 2001).

Altered activity of brain CRF systems was also observed in other cognitive disorders. e.g. in Alzheimer's diseases, decreases of the CRF peptide and increases of the CRF receptors were observed in the parietal, temporal and the occipital cortex suggesting an implication of the CRF system in the memory impairments (Bissette et al., 1985; De Souza et al., 1986). Similarly, in Parkinson's diseases a decrease of the CRF peptide was observed in the same brain regions (de Souza, 1988). Finally, the genetic polymorphism in the CRF receptors has been attributed to a variety of stress-related psychiatric disorders. Overall, genetic studies reported an association between CRF_1 receptor polymorphism with depression, suicide (Wasserman et al., 2009), alcohol dependence (Chen et al., 2010; Treutlein et al., 2006), obesity (Challis et al., 2004) and acute posttraumatic symptoms (Amstadter et al., 2011). It is also reported an association of polymorphism in gene regulating the CRF_2 receptor with obesity (Challis et al., 2004) and with suicidal behavior in bipolar disorder (De Luca et al., 2007).

The higher prevalence of stress-related disorders in drug addicts and some analogy between stress-related disorders and drug dependence highlighted a central role of the CRF system in drug dependence. In the following section we review several investigations about the role of the CRF system in drug dependence and withdrawal.

3.3. CRF system and drug

The drug dependence is considered as a stressful condition, which suggests the major contributions of the brain stress systems in the negative affect and somatic syndrome linked to withdrawal.

The CRF system is one of the main physiological and behavioral coordinator of stress response (Contarino and Gold, 2002). The CRF in PVN of hypothalamus plays an essential role in the pituitary response to stress (Zorrilla and Koob, 2004). The increases of CRF synthesis and release were also observed in the amygdala as a response to physical and psychological stressors (Bale and Vale, 2004; Zorrilla and Koob, 2004). Most of drugs of abuse activate the HPA-axis which leads to drug acquisition and seeking. The activation of HPA-axis and the extra-hypothalamic brain stress systems is as well a main characteristic of cocaine, opiate, nicotine and alcohol abuse. It is hypothesized to contribute to the drug relapse. Furthermore, CRF antagonism block anxiogenic-like effects of acute drug withdrawal and excessive drug intake associated with dependence and stress-induced reinstatement during abstinence (Breese et al., 2005; Funk and Koob, 2007; Kreek and Koob, 1998; Valdez et al., 2003).

The role of CRF system in syndromes of drug dependence can be explored through animal models of the different stages of the dependence cycle (Koob, 2008):

Firstly, the role of CRF and his binding family during craving/anticipation stage can be explored with animal models of reinstatement of drug, drug-associated stimuli or stress induced drug behavior seeking during period of drug abstinence (Shaham et al., 2003).

Secondly, dependence is characterized by a general pattern of excessive intake concomitant to behavioral and endocrine responses to stress during alcohol and drug-free period (Koob, 2008; Valdez and Koob, 2004). Much evidence shows an implication of CRF system in the negative affect linked with this pattern of alcohol and drug-intake (Baldwin et al., 1991; Contarino and Papaleo, 2005; Overstreet et al., 2004; Rassnick et al., 1993; Schulteis et al., 1998).

Finally, the role of CRF system in the somatic and negative affect of the withdrawal stage can be explored by using the animal models of CPA, ICSS, food and drug motivation in operant behavior, etc, following the precipitated or spontaneous withdrawal.

The chronic use of drug is associated with hyper-responsiveness to stressors which compromised stress and reward brain systems. The CRF system constitutes a key element of the exaggerated stress response during stages of drug dependence which contributes to the drug seeking and dependence. In the following sections, we would review the studies supporting this idea.

3.3.1. CRF system and drug seeking and reinstatement

In the case of humans, drug relapse and craving were associated with stressful life conditions (Kosten et al., 1986; Kreek and Koob, 1998; Kreek et al., 2009; McFall et al., 1992; Shiffman et al., 1985; Sinha and O'Malley, 1999). In laboratory animals, human stress situations were recreated using a panel of stressors that followed a period of chronic drug intake or self-administration (Shaham et al., 2000). The application of stress such as footshock, restraint, food restriction, etc during abstinence leads to the reinstatement of extinguished lever-pressing behavior. Reinstatement could be also induced after an acute drug administration or exposition to non-drug cues (Lu et al., 2003). In animal models of drug-reinstatement seeking behavior, animals are previously trained to self-administer drugs by pressing levels or nose-poking to receive intravenous drug infusions in operant chamber. Thereafter, drug was substituted by saline or by disconnecting the infusion minipumps to extinct drug-reinforced behavior. The ability of the stress, drug or related cues to reinstate drug seeking behavior is determined under extinction phase (Shalev and Kafkafi, 2002; Stretch and Gerber, 1973; Stretch et al., 1971).

Thus, it has been reported that stress such as footshock or restraint stress, reinstates seeking for alcohol and drugs during withdrawal (Ahmed and Koob, 1997; Ahmed et al., 2000; Buczek et al., 1999; Erb et al., 1996; Martin-Fardon et al., 2000; Shaham and Stewart, 1995a, b). For example, in rats, daily long-access to heroin self-administration (11 hr) is characterized by progressive increase of heroin intake which is not observed in daily short-access (1 hr). In heroin long-access groups of rats, intermittent footshock stress (1 mA; 0.5 sec on, with a mean off period of 40 sec) induces a reinstatement of heroin-seeking after a long period of drug-free extinction (during extinction phase drug was substituted by saline) (Ahmed et al., 2000). Similarly, 9 days after extinction of the CPP induced by 4mg/kg of morphine regimen, rats showed CPP reinstatement elicited by footshock stress or by 0.25 mg/kg doses of morphine or amphetamine (Wang et al., 2000). Others showed that rats, that have been trained to self-administer cocaine (10 mg/kg per infusion, i.v.) during 3hr sessions for 12 days, displayed a reinstatement for acute dose of cocaine (2.0 mg/kg, i.v.) and footshock stress (10 min, 0.5 mA, 0.5 s on, and mean off period of 40 sec) after a long period of drug-free extinction (4-6 weeks) (Erb et al., 1996). Similarly, in rats, the nicotine withdrawal is associated with a noticeable increase of anxiety-like behavior displayed in EPM after 7 days of nicotine treatment (0.1 mg/kg/day) (Irvine et al., 2001). Following intermittent footshock stress (5 and 15 min, 0.8 mA), rats that have been trained to self administer nicotine (0.03 mg/kg per infusion, 14 days) reinstated nicotine-seeking behavior 5-15 days after a nicotine cessation (Buczek et al., 1999).

Clinical evidences showed a high correlation between stressful life events and alcohol dependence (Brown et al., 1995; Cooper et al., 1990; Cooper et al., 1992; McFall et al., 1992; Sinha and O'Malley, 1999). During the abstinence period, reinstatement of responding to alcohol

in rats, previously extinguished, was also observed after the application of stress or neutral stimuli that have been paired with alcohol self-administration or that predict alcohol consumption (Ciccocioppo et al., 2001; Katner and Weiss, 1999; Liu and Weiss, 2002; Martin-Fardon et al., 2000).

Although, it is clear that stress increases drug intake in drug addicts and in laboratory animals. However the processes by which stress affects drug-motivated behavior are still not understood. Since CRF has a similar pattern in drug reinstatement and drug seeking than stress-induced reinstatement, several reports suggest an important contribution for the CRF and his ligands in the negative reinforcement of alcohol and drugs during dependence. In addition, stress-induced reinstatement occurs independently from stress-induced HPA-axis activation (Erb et al., 1998; Shaham et al., 1997) suggesting a central role of brain CRF system in drug seeking and relapse. Several data indicated that CRF system and especially CRF_1 receptor and extrahypothalamic CRF functions may represent a new target in the pharmacotherapy of drug withdrawal and stress induced drug-seeking behavior during drug abstinence. To reinforce this idea, it has been noticed that intermittent footshock-stress, in rats, reinstates extinguished alcohol-seeking behavior after a long period of drug abstinence (Le et al., 2000). This reinstatement was attenuated by the administration of the non selective CRF receptor antagonists d-phe-$CRF_{(12-41)}$ and CP-154,526. Interestingly, d-phe-$CRF_{(12-41)}$ receptor antagonist is less effective in the attenuation of the alcohol-seeking behavior reinstatement induced by footshock than CP-154,526 (Schulz et al., 1996). This latter is more selective for CRF_1 receptor than d-phe-$CRF_{(12-41)}$. These data suggest a greater role of CRF_1 receptor than CRF_2 in the stress induced alcohol-relapse seeking behavior. The latter study showed also that the removal of corticosterone by adrenalectomy does not affect the reinstatement of alcohol-seeking behavior induced by footshock. Interestingly, adrenalectomized rats with corticosterone pellets (50 mg/kg per day) displayed no effect on the reinstatement of alcohol-seeking behavior after stress proving an extrahypothalamic role of the CRF (Le et al., 2000). Others reported that the CRF antagonisms by d-Phe-$CRF_{(12-41)}$ does not block reinstatement of alcohol-seeking behavior induced by cue (Liu and Weiss, 2002). However, the non selective opioid antagonist naltrexone blocks the cue but not the stress-induced alcohol-seeking behavior reinstatement (Ciccocioppo et al., 2001; Ciccocioppo et al., 2003; Liu and Weiss, 2002). This thus suggests two different neuronal mechanisms involved in the reinstatement of alcohol behavior seeking.

There is an evidence of a major role of CRF system in stress-induced cocaine reinstatement. CRF system has also a strong implication in the stress-induced cocaine seeking during withdrawal. Both, Footshock stress and acute injection of cocaine provoke reinstatement of cocaine-seeking behavior after a prolonged drug-free period (Erb et al., 1996, 1998). The role of the CRF and corticosterone was examined in rats having been trained to self-administer cocaine

(1.0 mg/kg/infusion, i.v) for 10-14 days and then exposed to footshock or acute cocaine dose (20 mg/kg, i.p.) after 5-14 daily extinction sessions. In these animals, the footshock-stress reinstates cocaine-seeking in both intact and adrenalectomized rats with corticosterone supplementation but not in adrenalectomized rats (Erb et al., 2006). While the CRF receptor antagonist d-Phe-CRF$_{(12-41)}$ blocked footshock-induced reinstatement in both intact and in adrenalectomized rats with corticosterone supplementation indicating a basal corticosterone, secretion required for a reinstatement of cocaine-seeking induced by stress (Erb et al., 2006). Reinstatement after acute cocaine injection is minimally attenuated by CRF antagonism or adrenalectomy with corticosterone implantation (Erb et al., 2006). In the same line, after an extinction of cocaine self-administration (1.0 mg per kg per infusion) in rats, the intracranial injection of CRF associated with contextual conditioning results in a reinstatement of self-administration during cocaine withdrawal (Erb et al., 2006). Some authors explore more precisely the role of CRF$_1$ receptor in reinstatement of cocaine seeking behavior during withdrawal. In rats, the pretreatment with the CRF$_1$ receptor antagonist CP-154,526 (15 and 30 mg/kg, s.c.) attenuates the cocaine reinstatement induced by footshock stress after 14 days of extinction (Shaham et al., 1998). In separate experiments, animals injected with CP-154,526 exhibited a normal response to sucrose solution which would eliminate the possibility of disturbance of the learning and cognitive process in animals by the CP-154,526 (Shaham et al, 1998).

Thus, stress induces release of CRF in the ventral tegmental area (VTA) which is a brain region implicated in cocaine relapse and seeking (Wang et al., 2007). The intra-VTA administration of the antagonist of the CRF$_2$ receptor antisauvagin-30 (AS-30), completely blocks the footshock stress-induced reinstatement of cocaine seeking in rats previously trained to self-administer cocaine (Wang et al., 2007). In opposite to a study above-cited (Shaham et al., 1998), further experiments showed that the antagonism of CRF$_1$ receptor by intra-VTA administration of the NBI-27914 or the R121919, has no impact on the footshock stress-induced cocaine seeking reinstatement behavior (Wang et al., 2007). Intriguingly, a very recent study conducted on rats showed that bilateral administration of the CRF (250 or 500 ng/side) in VTA induced reinstatement of cocaine-seeking behavior after long-access to cocaine but not after short-access (Blacktop et al., 2011). Most importantly, the intra-VTA CRF-induced reinstatement was blocked by the administration of the CRF$_1$ receptor antagonist antalarmin (500 ng/side) or CP-376395 (500 ng/side) but not by the administration of CRF$_2$ receptor antagonist astressin-2B (500 ng or 1 μg/side) or AS-30 (500 ng/side) (Blacktop et al., 2011). This study sheds the light on additional investigations to determine the implication of CRF receptors in the cocaine-seeking behavior linked to stressful events.

The BNST and CeA are brain regions involved in the behavioral and neurophysiological response to stress mediated by the CRF system (Davis et al., 1997; Koob, 2009; Makino et al.,

1994; Richter and Weiss, 1999). The BNST is a brain area rich in CRF receptors, terminals and cell bodies (Erb and Stewart, 1999). One source of the CRF in the BNST arises from a CRF projection originating from the CeA (Sakana et al, 1986; Koob and Le Moal, 2006). Using a similar procedure than the one previously described, the rats pretreated with intra-BNST administration of CRF receptor antagonist d-Phe-CRF$_{(12-41)}$ (10 or 50 ng/side) showed attenuation of reinstatement of cocaine-seeking induced by footshock stress (Erb and Stewart, 1999). Conversely, the infusion of the CRF in the BNST induced reinstatement of cocaine-seeking (Erb and Stewart, 1999). Similar manipulations in the amygdala had no effects of d-Phe-CRF$_{(12-41)}$ (50 or 500 ng/side) or CRF (100 or 300 ng/side) on cocaine-seeking (Erb and Stewart, 1999). Functional lesions of the CeA-BNST pathway by blocking neurotransmission from the amygdala by sodium channel blocker (TTX, 0.25 ng) and injection of d-Phe-CRF$_{(12-41)}$ (50 ng/side) in the BNST in the opposite hemisphere reduce significantly reinstatement of cocaine-seeking induced by footshock stress (Erb et al., 2001). This result indicates a fundamental role of the CeA and suggests an important contribution of other regions with reciprocal connection with the CRF in stress-induced cocaine-seeking behavior. To support this latter notion, norepinephrine originating from the ventral noradrenergic pathways and projecting to the BNST and CeA, play an important role in the reinstatement of cocaine-seeking behavior (Leri et al., 2002; Shalev et al., 2002). The β_1 adrenergic antagonist betaxolol and the β_2 adrenergic antagonist ICI 118 551 infused into the CeA decrease dose-dependently footshock-induced cocaine-seeking behavior reinstatement (Leri et al., 2002). The release of norepinephrine in the BNST, in response to stressors, may be involved in the negative reinforcement of protracted abstinence and lead to drug-seeking (Aston-Jones and Harris, 2004). Overall, the BNST and CeA have an evident role in interconnection between CRF system and other neuronal pathways such as norepinephrine on the reinstatement of cocaine-seeking behavior.

Similarly to the experiences aforementioned, rats were trained to self-administer nicotine (0.03 mg/kg/infusion) under FR-5 reinforcement schedule, during 14 days. Then, nicotine was substituted by saline during the extinction phase and footshock stress (8 shocks in a 10-min time-period, 0.8 mA, 1 s shocks, mean off period 37 s) was then applied to reinstate nicotine-seeking behavior (Zislis et al., 2007). The footshock stress induces reinstatement of nicotine-seeking behavior which is reversed by the administration of respectively the d-Phe-CRF$_{(12-41)}$ (25 µg, i.c.v.) or clonidine (40 µg/kg/1ml, s.c.) 15 and 30 min before the test (Zislis et al., 2007).

Finally, In rats trained to self-administer heroin (100 µg/kg/infusion, i.v.) for 12-14 days, footshock stress (15 or 30 min, 0.5 mA) or acute heroin dose (0.25 mg/kg, s.c.) induced reinstatement of heroin-seeking behavior during the drug-free period (4-8 days of extinction sessions consisting to substitute heroine by saline (Shaham et al., 1997). Acute injections of the CRF peptide (0.3 and 1.0 µg, i.c.v.) and the CRF antagonist α-helical CRF (3 and 10 µg, i.c.v.)

reinstate and attenuate, respectively, the heroin-seeking behavior induced by heroin administration or footshock stress (Shaham et al., 1997). Most importantly, the CRF$_1$ antagonist CP-154,526 antagonist (15 and 30 mg/kg, s.c.) attenuate the reinstatement of heroin-seeking behavior induced by intermittent footshock stress (10 or 15 min, 0.5 mA) suggesting a crucial role of the CRF$_1$ receptor in heroin relapse induced by stress (Shaham et al., 1998). In addition, Shaham and colleagues showed that inactivation of both CeA and BNST with TTX completely blocks reinstatement of heroine-seeking behavior (Shaham et al., 2000). Similar results were observed in cocaine reinstatement suggesting a fundamental role of the CRF pathways of these brain regions in drug relapse.

3.3.2. CRF system and excessive drug intake

In humans, the development of drug dependence is characterized by a kind of excessive consumption pattern, loss of control over its consumption and the development of tolerance (DSM). In the case of alcohol excessive intake, the negative reinforcement states, associated with dependence, reinforces the drinking behavior to avoid the negative physical and psychological consequences of abstinence (Koob and Le Moal, 2006). Both clinical and preclinical studies have shown increased alcohol intake following stress (de Wit et al., 2003; Sillaber et al., 2002). It is suggested that ethanol intake reduces stress which, in its turn, is reinforced by the excessive and progressive increase of ethanol consumption. This pattern of excessive alcohol intake represents one of the major contributors to alcohol relapse during the period of abstinence. This pattern particularly leads to the maintenance of the alcohol dependence cycle despite the health and social deleterious consequences in alcohol-dependent individuals (APA, 2000; Koob, 2008; Valdez et al., 2002). Progressive increases and excessive alcohol intake are linked to CRF system deregulation and anxiety-like behavior (Koob, 2008). Supporting this, in animals, chronic exposure to alcohol leads to a progressive increase of alcohol-self administration (O'Dell et al., 2004; Valdez et al., 2002) and anxiety-like behavior displayed in EPM which is associated to enhanced CRF signaling in extra-hypothalamic brain regions (Valdez et al., 2002).

Using behavioral and pharmacological approaches, multiple investigations in animal models showed an important contribution of CRF and its binding sites in the motivational effect of alcohol during abstinence linked to excessive increases in alcohol intake (Funk et al., 2007; Koob, 2008; Valdez et al., 2002). Accordingly, functional antagonism of CRF neurotransmission attenuates the excessive alcohol intake (Funk and Koob, 2007; Funk et al., 2006; Funk et al., 2007; Valdez et al., 2002). In particular, CRF antagonist attenuates ethanol self-administration (Funk et al., 2007) and CRF receptor antagonism using d-Phe-CRF$_{(12-41)}$ (i.c.v. microinjection) completely eliminates the excessive ethanol consumption in dependent rats but not in non-

dependent rats (Valdez et al., 2002). Using intermittent ethanol vapor exposure paradigm during 4 weeks, the specific antagonism of the CRF_1 receptor using antalarmin, MJL-1-109-2 and R121919, showed reduced ethanol self-administration in male rats (Funk et al., 2006). In the same study, the three antagonists dose-dependently attenuated the ethanol self-administration and had no effect on non-dependent animals. Similar results were observed in heroin and cocaine dependent rats (Goeders and Guerin, 2000; Specio et al., 2008). The antagonism of CRF_1 receptor by MJL-1-109-2 and R121919 decreased heroin self-administration in rats during daily long-access (Greenwell et al., 2009). Also, systemic administration of the non peptides CRF_1 antagonists: antalarmin and MPZP reversed the enhancement of cocaine self-administration during extend-access (Specio et al., 2008). Indeed, antalarmin decreases the cocaine intake after a short daily access of cocaine self-administration while MPZP decreases cocaine intake after short and long daily access of cocaine self-administration (Specio et al., 2008). The MPZP decreased the cocaine intake at lower dose in long access group than short access indicating a hypersensitivity of the CRF system after long exposure to cocaine (Specio et al., 2008). The rats trained undergoing an alternating schedule of food reinforcement (FR-10 schedule) and cocaine self-administration (0.125, 0.25, or 0.5 mg/kg/infusion, FR-4 schedule of reinforcement) the CRF_1 receptor antagonist CP-154,526 (10–40 mg/kg, i.p.) decreases i.v. cocaine self-administration without altering the lever pressing for food (Goeders and Guerin, 2000).

The role of the CRF_2 receptor seems to be less important in the ethanol-associated behavior than CRF_1 receptor, suggesting a minor implication of this receptor in modulating alcohol dependence. In fact, even the genetic deletion of the CRF_2 receptor in mice model does not alter the preference or total intake of ethanol during 24 hr compared to wild-type mice. However, in 2 hr limited access to 7.5 and 10% of ethanol, the $CRF_2^{-/-}$ exhibited an increase in ethanol consumption, which was not the case for the wild-type mice (Sharpe et al., 2005). It is suggested that chronic or variable stress accentuates the role of the CRF_2 receptor (Sharpe et al., 2005; Valdez et al., 2002; Valdez et al., 2003). In the case of the study cited above (Sharpe et al., 2005), limited access can be considered as a stressful condition explaining the difference of ethanol intake between the two genotypes indicating a hypersensitivity of the CRF_2 receptor to additional stressful conditions in alcohol dependence.

3.3.3. CRF system and drug withdrawal

A. Alcohol

The withdrawal from alcohol is typified by a profound anxiety, dysphoria, and depression on the other hand and severe physical signs such as sweating, increased heart rate, nausea, fever and convulsion on the other hand (APA, 2000; Becker, 2000; Koob and Heinrichs, 1999). Some of the negative affective symptoms among humans can persist a long time after the chronic ethanol

cessation. Early work showed that 82% of alcoholic subjects reported an alleviation of anxiety after alcohol use (Hershon, 1977). Besides, antidepressants are particularly efficient to counteract this anxiety linked to alcohol withdrawal (Hershon, 1977).

The molecular exploration in the brain of alcohol-withdrawn rats exhibited a hyperactivation of the CRF system associated with an anxiety-like state (Bruijnzeel and Gold, 2005) and an increase of the CRF in brain areas implicated in anxiety-like behavior such as the CeA and BNST (Merlo Pich et al., 1995; Walker et al., 2003). In fact, ethanol withdrawal-induced increase of the CRF levels in the BNST can be reversed by ethanol intake (Olive et al., 2002). Furthermore, behavioral studies reported that alcohol-withdrawn rats showed a higher anxiety displayed by a reduced exploration of open arm in the EPM test (Kliethermes et al., 2004), a reduced social interaction with another congener (File et al., 1991), an increase in acoustic startle reactivity test (Macey et al., 1996) and an increase of anxiety parameter in the LD apparatus (Kliethermes et al., 2004). Similarly, after 16 days of chronic intake of liquid containing 8.7% of alcohol, withdrawn rats exhibited anxiogenic-like responses in the EPM (Baldwin et al, 1991). Basically, the percentage time of open arm exploration decreases during ethanol withdrawal. Thus, this effect was reversed by the i.c.v administration of 25 micrograms of the non specific CRF antagonist α-helical CRF (Baldwin et al., 1991). In alcohol withdrawn rats, others researches showed no administration effect of low doses of the α-helical CRF (250 ng; i.c.v) on the decrease of open arm exploratory which was displayed in the EPM test. However, for the same animals, the administration of the same dose of antagonist in the CeA reverses the anxiogenic-like effect (Rassnick et al., 1993). The ability of the α-helical CRF to reverse the decreases open arm exploration was not due to the stimulatory effect of this antagonist, because the alcohol withdrawal alone decreases the locomotor activity also in alcohol-withdrawn non-treated rats (Rassnick et al., 1993). This latter study proves a central role of CRF in the CeA in negative reinforcement of alcohol withdrawal. Using CRF_1 receptor knockout mice, Timpl et al., (1998) suggest an important role of the CRF_1 receptor in anxiety-like behavior provoked by alcohol withdrawal. In this study, the alcohol dependence was instigated by 20% of alcohol in liquid solution during 18 days. Then, the alcohol solution was removed and replaced by water. Subsequently, 12 hr after, mice were tested in the open field and LD apparatus. Compared to alcohol-naïve wild-type mice, alcohol-withdrawn wild-type mice showed an anxiogenic-like profile displayed by a decreasing activity in the open field exploratory test and a higher latency to enter in the lit compartment of LD apparatus. Interestingly, the alcohol-withdrawn knockout mice showed an anxiolytic-like profile displayed by more entries and time spent in the lit compartment of LD apparatus (Timpl et al., 1998).

B. Cocaine

Cocaine withdrawal is associated with a few relative physical signs such as motor hyperactivity and tremors, but also with a considerable number of negative affective symptoms such as dysphoria, depression, anxiety, insomnia, craving for drug, profound anhedonia, etc (Koob and Le Moal, 2006). 83% of cocaine addicts showed a marked anxiety and dysphoria during withdrawal (Gawin and Kleber, 1986). Anxiety and depression associated to episodes of craving and withdrawal are the major factors that maintain cocaine seeking and relapse (Koob and Le Moal, 2006).

It is supported that the CRF system pathways play an essential role in anxiety and depression developed during cocaine withdrawal which lead to recurrent episodes of cocaine craving and relapse (Goeders and Guerin, 1996a, b; Richter and Weiss, 1999; Sarnyai et al., 1995). Other scientists suggest a crucial role of the CRF in the aversive and anxiogenic effects of cocaine withdrawal. This is consolidated by the fact that repeated self-administration of cocaine increases the release of the CRF in the CeA (Richter et al., 1995; Richter and Weiss, 1999). In addition, the anxiety-like behavior generated by central administration of CRF is blocked by administration of CRF receptor antagonism (Britton et al., 1985). Moreover, in rats, intracranial injection of the CRF receptor antagonist blocks cocaine withdrawal-induced anxiety-like behavior (Basso et al., 1999). Finally, the adrenalectomy blocks the acquisition and maintenance of cocaine self-administration showing a good evidence of the role of corticosterone in cocaine reinforcement (Goeders and Guerin, 1996a).

C. Nicotine

Among humans, the abrupt cessation of tobacco smoking is characterized by the emergence of negative affective states such as depression, anxiety, irritability and difficulties to concentration which provide a strong motif for the continuation of smoking (APA, 2000; Kassel et al., 2003). Clinical observations reported that patients with major depression and anxiety symptoms are twice as likely to smoke cigarettes as healthy patients (Lasser et al., 2000). The nicotine withdrawal is associated with weight gain which is the consequence of increases in food intake (Ogden, 1994; Stamford et al., 1986). Typically, several evidences also show that nicotine withdrawal is associated with increasing rewarding properties of palatable food (Lerman et al., 2004; Stamford et al., 1986).

In rats, antagonism of nicotinic acethylcholine receptors (nAChRs) increases the somatic withdrawal signs and the brain reward threshold during the ICSS procedure (Bruijnzeel and Markou, 2004; Epping-Jordan et al., 1998). Similar patterns observed in alcohol, cocaine and opioids withdrawal were also observed during nicotine withdrawal such as an increase of the corticosterone levels during acute nicotine withdrawal (Benwell and Balfour, 1979) as well as an

increase of the CRF mRNA expression in the CeA after precipitated withdrawal (Marcinkiewcz et al., 2009). This pattern reflects an alteration of the brain stress system which contributes to the negative reinforcement and rewarding deficiency that is the source of nicotine-seeking and relapse (Barr and Markou, 2005).

In a recent study, the role of the CeA, BNST and Nacc in the alterations of brain reward mediated by the CRF system was studied (Marcinkiewcz et al., 2009). As it is the case with dependence to alcohol (Merlo Pich et al., 1995) or cocaine (Richter and Weiss, 1999), the nicotine precipitated-withdrawal also increases the extracellular CRF levels in the CeA (George et al., 2007). In nicotine-dependent rats (9 mg/kg per day of nicotine delivered by osmotic minipumps during 28 days), the ICSS procedure displayed brain reward deficit after the precipitation of nicotine withdrawal by nAChRs antagonist mecamylamine (3 mg/kg, s.c., injection started 6 days after implantation of nicotine minipumps). Interestingly, the administration of d-phe-CRF$_{(12-41)}$ (500 ng per site, unilateral dose) into the CeA and the NaccSH prevented the elevation of brain reward threshold caused by mecamylamine. Nevertheless, the injection of d-phe-CRF$_{(12-41)}$ in BNST does not reverse the elevation of brain reward threshold induced by a precipitated nicotine withdrawal (Marcinkiewcz et al., 2009). Furthermore, The d-phe-CRF$_{(12-41)}$ (i.c.v.) administered alone or with mecamylamine has no effect in brain reward threshold in non-dependent rats (Marcinkiewcz et al., 2009). The latest study represents an indication of a distinct role of the CeA and the BNST in nicotine withdrawal. Finally, the antagonism of the CRF receptor in the BNST has no effect on nicotine-withdrawal (Marcinkiewcz et al., 2009).

D. Opioids

As described in the previous section, OW syndrome appears after drastic reduction, discontinuation or abrupt cessation of opioids administration or after an administration of opioid antagonists. In rodents, OW is related with physical signs (Houshyar et al., 2003; Langerman et al., 2001; Papaleo and Contarino, 2006; Way et al., 1969), profound anxiety-like behavior and aversive states (Stinus et al., 2005), alteration of brain reward threshold (Bruijnzeel et al., 2005), alteration of motivational properties of food (Cooper et al., 2010; Harris and Aston-Jones, 2003; Zhang et al., 2007) and place aversion induced by the opioid antagonist (Stinus et al., 2000). The role of the CRF system in the negative reinforcement of OW is known through a series of clinical observations and animal experimental studies. The withdrawal of opioids activates the CRF stress system which may contribute to the expression of somatic signs and also increase risk of anxiety and depressive states. A progressive change of HPA-axis function and a sensitization of the CRF system were reported during the shift from acute to chronic opiate dependence (Koob, 2008). Therefore, clinical study showed that opiate-addicts exhibited an impairment of

HPA-axis and a hyperresponsiveness of the cortisol negative feedback system that regulates the HPA-axis (Koob, 2006).

Several studies showed the implication of the CRF and binding sites in the opioid dependence. In rats, spontaneous morphine withdrawal instigated by an intermittent and increasing dose of morphine associated with acute restraint stress, showed alterations of ACTH and corticosterone secretion 12 hr after morphine cessation (Houshyar et al., 2004). In the same study, morphine withdrawal, 12 hr after last injection of 8 days of morphine treatment, decreases CRF mRNA expression in the CeA and increases it in the PVN, Barrington's nucleus and the parvocellular part of PVN. Using *in situ hybridization histochemistry* analysis of brains of rats, other scientists found an up-regulation of CRF mRNA expression in the PVN and the CeA induced by naloxone-precipitated withdrawal following chronic morphine treatment (McNally and Akil, 2002). The administration of CRF receptor antagonist (1, 3 or 5 µg of α-helical CRF, i.c.v.) in the lateral ventricle decreases the somatic signs of OW such as jumping, salivation, ptosis and decreases level of corticosterone. Furthermore, the administration of CRF receptor antagonist in CeA decreases the opiate somatic signs without any effect at the corticosterone level. Finally, the administration of CRF receptor antagonist in the BNST has no effect on the opiate somatic signs and corticosterone level (McNally and Akil, 2002).

Funada and his colleagues (1993 & 2001) administered mice with intermittent and increasing morphine doses (8 to 45 mg/kg over a period of 5 days) to induce dependence. Afterward, the injection of naloxone (3 mg/kg, i.p.) in these animals results in the emergence of some somatic withdrawal signs such as jumping and BW loss, 2 hr after morphine cessation (Funada et al., 2001; Funada et al., 1993). Most importantly, the injection of the CRF_1 receptor antagonist (CRA1000; 20 mg/kg, i.p.) 5 min prior naloxone attenuates these somatic signs (Funada et al., 2001). Similarly, pretreatment with CRF_1 receptor antagonist antalarmin or CP-154526 attenuates the BW loss and the irritability observed among morphine-dependent rats (Navarro-Zaragoza et al., 2010). In other studies, OW was induced by naloxone (20 mg/kg, s.c.) in male rats implanted with morphine pellet (75 mg, s.c.) during six days. The consequence of these manipulations is the emergence of somatic signs including: writhing, weight loss, lacrimation, salivation and irritability. Interestingly, these somatic signs are attenuated by the antagonism of CRF_1 receptor using CP-154,526 (20 mg/kg, i.p.) (Iredale et al., 2000). Thus, it is known that OW decreases the CRF_1 but not the CRF_2 receptor mRNA expression in CeA, Nacc and striatum (Iredale et al., 2000) which are brain regions implicated in the OW somatic signs (Maldonado et al., 1992). The down-regulation of CRF_1 receptor expression is hypothesized to represent a compensatory response which serves to attenuate OW somatic signs (Iredale et al., 2000). This hypothesis is also comforted by the experiments of Shaham and colleagues (1998) that reported a crucial role of the CRF and of CRF_1 receptor activation in the stress-induced

opiate-seeking behavior elicited by OW (Shaham et al., 1998; Shaham et al., 1997). Supporting this data, another study showed an important role of the CRF_1 receptor in the emergence of negative affect during OW (Stinus et al., 2005). Effectively, in male rats, dependence was instigated using morphine pellet subcutaneous implantation during 12 days. Subsequently, OW was precipitated by naloxone and rats were tested in CPA apparatus. Rapidly, rats showed aversion with an environment associated with naloxone-paired compartment which is dose dependently inhibited by the CRF_1 receptor antalarmin (Stinus et al., 2005). Similarly, the systemic administration of a the CRF_1 receptor antagonist and the intracerebral administration of a CRF_1/CRF_2 antagonist, attenuate CPA induced by precipitated OW (Heinrichs et al., 1995; Stinus et al., 2005).

The specific CRF_2 antagonist antisauvagin-30 (AS-30) (Ruhmann et al., 1998) was developed to enhance the knowledge about the exact implication of the CRF_2 receptor in the OW syndrome. In male rats, morphine dependence was instigated by daily intermittent and increasing morphine doses injections (twice per day during 5 days) and OW was precipitated by naloxone. Notably, the administration of the non-specific α-helical CRF (10 μg, i.c.v.) and the selective CRF_1 receptor antagonist CP-154,526 (30 mg/kg, i.p.) attenuate the emergence of somatic sings such as jumping, teeth chattering, writhing, wet dog shakes, lacrimation, irritability, piloerection and diarrhea 1hr after morphine treatment cessation (Lu et al., 2000). However, the administration of the CRF_2 antagonist AS-30 (10 μg, i.c.v.) does not affect somatic signs, which are developed after morphine treatment, suggesting a minimal role of the CRF_2 receptor during acute morphine withdrawal (Lu et al., 2000). In the same study, 28 days following morphine CPP procedure, CP-154,526 or α-helical CRF block the reinstatement of preference to drug-paired environments in rats after a single injection of morphine while the AS-30 has no effect in the reinstatement of CPP.

Norepinephrine is a neurotransmitter of the central nervous system which is likely involved in responses to stress and anxious behavior. The localization and the projection of cell bodies of the brain norepinephrine system include prefrontal cortex, septum, Nacc, medial forebrain bundle, hypothalamus, ventral and dorsal noradrenergic ascending bundle. Besides, several evidences showed an implication of norepinephrine in OW. It is known that precipitated OW increases norepinephrine release in the CeA and BNST (Watanabe et al., 2003). Also, the clonidine blocks the suppression of food-seeking behavior (Sparber and Meyer, 1978), somatic sings (Delfs et al., 2000) and place aversion elicited by OW (Schulteis et al., 1998). Because the noradrenergic and the CRF system have an important role in the response to stressors, several authors suggested a parallel adaptations and interactions of the two systems during the stressful conditions of OW (Harris and Aston-Jones, 2003). Indeed, hyperactivation of both CRF and norepinephrine systems during OW was reported (Milanes et al., 1998). Moreover, a

biochemical analysis of cerebral brain cortex in morphine-dependent mice, which displayed a somatic signs after administration of naloxone, showed an increase of norepinephrine ratio. Norepinephrine, originating in the noradrenergic pathways and projecting in the CeA and BNST, is implicated in the footshock-induced opiate-seeking behavior (Koob and Le Moal, 2006). As the non selective antagonist α-helical CRF, the $α_2$-noradrenergic agonists (inhibit norepinephrine release) eliminate footshock-induced opioids-seeking behavior (Shalev et al., 2010). In mice, the administration of the CRF_1 receptor antagonist CRA1000 (20 mg/kg, i.p.) attenuates the somatic signs and permits a recovery of normal basal norepinephrine ratio in the brain cortex (Funada et al., 2001). Others showed that in rats, that pretreatment with CRF_1 receptor antagonist CP-154526 or antalarmin attenuates the somatic signs of morphine withdrawal but does not block the increased noradrenergic activity in the PVN (Navarro-Zaragoza et al., 2010). However, the administration of the CRF_2 receptor antagonist AS-30 , in the same animals, decreases the norepinephrine turnover associated with an elevated mRNA CRF expression (Nunez et al., 2007) in the PVN and NST (Nvarro-Zaragoza et al., 2010).

The generation of CRF_1 (Smith et al., 1998) and CRF_2 (Bale et al., 2000) receptor deficient mice, provided a new interesting tool in the understanding of the role of the CRF system. The CRF_1 receptor deficient mice possess a functional CRF_2 receptor which can be activated by CRF and related peptide and vice versa. This characteristic permits the study of the role of CRF receptors which is difficult to explore with non specific CRF receptor antagonists. The CRF receptors deficient mice were generated using mouse strain 129 genomic DNA library. After the isolation of the genomic clone DNA of CRF receptors gene, a portion of gene was targeted and replaced with a neomycin resistance gene cassette. Southern analysis with an external probe revealed positive neomycin-resistant colonies. To generate chimaeric mice, the positive clones were injected into C57BL/6 blastocysts. Afterward, mice were crossed to produce heterozygous mutant mice on a mixed C57BL/6 x 129 genetic backgrounds which were maintaining in breeding via heterozygote x heterozygote crossing (Bale et al., 2000; Smith et al., 1998).

Altogether, studies cited previously reported that the CRF receptors have an important role in the anxiety-like behavior and stress-induced drug seeking behavior reinstatement. In our laboratory, the CRF_1 and CRF_2 receptor genetic deficient mice were used to dissecate the implication of the CRF system in the behavioral and molecular alterations linked to the spontaneous opiate withdrawal (SPO OW). In the context of human illicit opiate use, dependence is instigated by intermittent injections of increasing doses of drug and withdrawal emerges spontaneously. Similarly, in our laboratory animals, dependence was instigated using increasing and intermittent morphine doses and OW emerged preferably spontaneously.

The primary goal of our laboratory is to focus on the role of CRF_1 receptor components of the CRF system in the emergence of negative affective states and somatic signs motivating the

opiate-seeking behavior and relapse. Combining behavioral paradigm and biological assays, the early work sought to determine the implication of the CRF_1 receptor in the emergence of dysphoria and the alterations of neural biological substrates observed during OW (Contarino and Papaleo, 2005). In particular, a wild-type, heterozygous ($CRF_1^{+/-}$) and homozygous ($CRF_1^{-/-}$) mice were conditioned during 4 successive days in CPA apparatus in environment cues paired with the early phase of OW. Importantly, the total or partial genetic deletion of the CRF_1 receptor fully eliminates the aversion for environment cues paired with OW in CPA paradigm. In agreement with this data, a recent study, using CPA paradigm, reported attenuation of aversion for environmental cues paired with SPO OW in $CRF_1^{-/-}$ compared to wild-type mice (Garcia-Carmona et al., 2011). However in the latter study, genetic deletion of the $CRF_1^{-/-}$ does not completely eliminates place aversion as cited previously. Notably, intermittent injections of increasing morphine doses, from 10 to 60 mg/kg and from 20 to 100 mg/kg decrease (Garcia-Carmona et al., 2011) and eliminate (Contarino and Papaleo, 2005), respectively, the aversion in morphine-withdrawn $CRF_1^{-/-}$ compared to morphine-withdrawn wild-type mice. This data provides evidence that the CPA induced in $CRF_1^{-/-}$ genotype depends on the morphine regimen used to induce OW. Noteworthy that the morphine regimen doses used in our laboratory (20-100 mg/kg), to induce SPO OW, is an adequate regimen to study a larger behavioral difference between the wild-type and $CRF_1^{-/-}$ genotypes during OW.

In heroin-addicts, abnormal hypercortisolism was reported (Cami et al., 1992; Gerra et al., 2003). Indeed, high level of cortisol was observed during the third day of heroin withdrawal. This level decreases progressively until one month after heroine intake cessation and correlates positively with anxiety and depression (Shi et al., 2009). Thus, the $CRF_1^{+/-}$ mice showed a similar corticosterone level in comparison to the wild-type mice proving a central role of the CRF_1 receptor in OW induced CPA (Contarino and Papaleo, 2005). This data was in agreement with precedent studies showing an extrahypothalamic exclusive role of the CRF_1 receptor independent to the corticosterone in the reinstatement of drug-seeking behavior (Erb et al., 1998; Le et al., 2000; Shaham et al., 1997).

The CRF_1 receptor was localized in brain region involved in the cognitive process such as hippocampus and amygdala. In addition, several studies reported an important role of CRF_1 receptor in cognitive, learning, and memory processes and functions (Chen et al., 2000; Hogan et al., 2005; Ivy et al., 2010; Van Pett et al., 2000). The U50,488H is a κ-opioid receptor agonist known to induce CPA in mice (Funada et al., 1993). Using this agonist, wild-type, $CRF_1^{+/-}$ and $CRF_1^{-/-}$ mice showed place aversion response to environmental cues associated to the U50,488H, excluding a possible effect of genetic deletion on the associative learning processes underlying the acquisition and the expression of CPA (Contarino and Papaleo, 2005).

Importantly, the total or partial genetic deletion of the CRF_1 receptor also permits the recovery of normal basal DYN mRNA ratio in the NaccSH (Contarino and Papaleo, 2005), a brain region involved in the OW (Turchan et al., 1997). Indirect evidence concluded a contribution of the DYN /KOR of the Nacc in the dysphoria, associated with drug-withdrawal, by the decrease of dopamine release (Carlezon et al., 1998; Di Chiara and Imperato, 1988). In the same line, several studies in rodents also reported an increased DYN expression and increased levels of endogenous DYN peptide, in the Nacc, concomitant to the OW period (Nylander et al., 1995; Rattan et al., 1992; Turchan et al., 1997).

Thus, it is supposed that the disturbance of somatic signs associated with OW in $CRF_1^{-/-}$ mice may represent an argument which can explain the loss of dysphoria in CPA (Contarino and Papaleo, 2005). Nevertheless, $CRF_1^{-/-}$ mice showed greater expression of somatic signs than wild-type and $CRF_1^{+/-}$ mice 8h after the morphine treatment. However, the real impact of OW during CPA procedure was not explored. Even so, these primary observations revealed dissociation between negative affect and somatic signs and also suggested a minor impact of the somatic signs on the emergence of negative affective states elicited by OW. This latter finding does not exclude the deleterious effects of the various panels of somatic signs and symptoms in opiate intake and relapse. A possible contribution of the CRF system in this phenomenon is highly plausible. To answer this question, a second series of investigations were conducted to determine the role of the brain extrahypothalamic CRF/CRF_1 receptor circuitry in the expression of the somatic signs associated with stressful conditions of OW (Papaleo et al., 2007b).

It is reported that $CRF_1^{-/-}$ mice displayed abnormalities of the HPA axis and the HPA axis-related hormones responses to stressors (Smith et al., 1998; Timpl et al., 1998) which are identical to those observed in heroin addicts (Kostich et al., 1998). More precisely, $CRF_1^{-/-}$ mice displayed deficient levels of corticosterone and ACTH responses to stressors. These interesting particularities of the $CRF_1^{-/-}$ mice also permit the exploration of the supposed implication of HPA-axis in the expression of somatic signs, which are associated with stressful conditions of OW. Therefore, using the same protocol of morphine dependence induction previously described, the total genetic deletion of CRF_1 receptor exacerbates the somatic expression of OW (Papaleo et al., 2007b) confirming previous preliminary observations in this genetic mice model (Contarino and Papaleo, 2005). In addition, the $CRF_1^{-/-}$ mice exhibited alterations of CRF and DYN gene expression in the PVN and the striatum (Papaleo et al., 2007b). These brain regions and their related-peptides are involved in the behavioral and neuroendocrine responses to stress (Koob and Heinrichs, 1999; Sawchenko et al., 1993) and in stress-related OW (Houshyar et al., 2004; Lightman and Young, 1988; Zachariou et al., 2006). Interestingly, supplementation with corticosterone in $CRF_1^{-/-}$ mice restored a similar pattern of OW signs and restored also CRF PVN and striatal DYN gene expression alterations observed in wild-type opiate-withdrawn mice

(Papaleo et al., 2007b). Altogether, these original results showed a clear evidence of the contribution of a deficient HPA-axis functionality in the aggravation of somatic signs induced by biological alterations during OW (Papaleo et al., 2007b). To identify the role of extrahypothalamic CRF_1 receptor, pharmacological targeting of this receptor was established using antalarmin CRF_1 receptor antagonist. Thus, the behavioral alterations are HPA-axis independent because antalarmin injected in wild-type and $CRF_1^{+/-}$ withdrawn-mice, which displayed normal HPA-axis responses to stress, showed similar exacerbate somatic signs than $CRF^{-/-}$ withdrawn-mice (Papaleo et al., 2007b). Similarly, others showed that morphine-induced CPP was decreased by antalarmin without affecting the increase of plasma corticosterone levels (Grakalic et al., 2006). This latter data represents an additional evidence of no role of the HPA-axis in the behavioral alteration linked to OW. Nevertheless, as cited previously, the genetic deletion of CRF_1 receptor in mice induced an impaired HPA-axis functionality and serious molecular alterations in brain during OW (Papaleo et al., 2007b). Therefore, these latter data provide evidence of a strong limitation in treatment of OW by the targeting of the CRF_1 receptor.

The final experiments of our laboratory were focused on the specific role for the CRF_2 receptor pathway in the development of OW signs (Papaleo et al., 2008). Using the same SPO OW paradigm described above, but also pharmacological µ-opioid antagonism (naloxone), the $CRF_2^{-/-}$ mice displayed decreased somatic signs such as wet dog shake, jumping and paw tremor during OW, but also immediately after administration of opioid receptor antagonist. Recent work showed the same results in morphine-withdrawn rats after micro-infusion of the CRF_2 receptor antagonist AS-30 following the administration of naloxone (Navarro-Zaragoza et al., 2010).

It is reported that the $CRF_2^{-/-}$ mice showed no differences in structures of pituitary and adrenal gland and similar basal levels of ACTH and corticosterone compared to wild-type mice (Bale et al., 2000). In contrast to the $CRF_1^{-/-}$ mice, the $CRF_2^{-/-}$ mice showed an intact HPA-axis responsiveness during OW suggesting no role of the HPA-axis in the decreased somatic signs in this genotype (Papaleo et al., 2008). These data does not only indicate a crucial role of the CRF_2 receptor in the expression of somatic signs elicited by OW but also provides a new evidence of a dichotomous responses between the CRF receptors in the emergence of OW somatic signs. Indeed, these results are in line with the previous studies reporting an opposite role of these receptors in stress responsiveness (Bale et al., 2000; Contarino et al., 1999a; Contarino et al., 2000; Contarino et al., 1999b; Kishimoto et al., 2000; Smith et al., 1998; Timpl et al., 1998; Vetter et al., 2002). Although, many studies showed that the CRF_2 receptor has a minor role in alcohol and drug seeking and relapse (Blacktop et al., 2011; Schulz et al., 1996; Sharpe et al., 2005). However latter works in our laboratory shows an important role of this receptor in the somatic components of OW syndrome (Papaleo et al, 2008). Finally, these results are raising new attentions to the real implication of the CRF_2 receptor in drug dependence (**Table. 3**).

Table. 3 Behavioral and neuroendocrinological alterations associated with spontaneous opiate withdrawal among CRF$_1$ and CRF$_2$ receptor-deficient mice.

Abbreviations: **(+)** increased; **(-)** decreased; **(0)** no effect; **(A)** abolished; **ND** not determined.

	CRF$_1^{-/-}$	CRF$_2^{-/-}$
Components of negative affective states		
Dysphoria	(A)[a]	ND
Dysphoria (precipitated OW)	(-)[b]	ND
Somatic signs	(+)[c]	(-)[d]
Somatic signs (precipitated OW)	ND	(-)[d]
Anhedonia	ND	ND
Anxiety-like states	ND	ND
Motivational disorders	ND	ND
Stress-induced reinstatement	ND	ND
Neuroendocrinological alterations of OW		
Increased CRF in the NaccSH	ND	ND
Increased DYN in the NaccSH	(A)[a]	ND
c-fos in the PAG	ND	ND
Stress-coping abilities		
Increased CRF in the PVN	(-)[c]	ND
Increased DYN in the PVN	(+)[c]	ND
Increased TH in the LC	ND	ND
Corticosterone	(-)[c]	(0)[d]
Energy balance and lcomomotion		
Feeding	(0)[e]	ND
Body weight loss	(0)[c]	ND
Body weight loss (precipitated OW)	(-)[b]	ND
Locomotor activity	ND	ND

References: [a](Contarino and Papaleo, 2005), [b](Garcia-Carmona et al., 2011), [c](Papaleo et al., 2007b), [d](Papaleo et al., 2008), [e](Rouibi and Contarino, unpublished data)

OBJECTIVES

In the light of the aforementioned clinical studies, the cessation of opiate intake in opiate-dependent individuals is typified by the emergence of a plethora of physical and psychological signs and symptoms which motivate opiate seeking and relapse. Overall, the CRF system plays a key role in the stressful condition linked to the development of the OW syndrome. Therefore, preclinical studies reported that CRF_1 receptor antagonism reduces the stress-induced reinstatement of alcohol and drug-seeking behavior and ameliorates the negative affect associated with alcohol or drugs of abuse. Indeed, the genetic deletion of the CRF_1 receptor pathway eliminated the behavioral and brain correlates of dysphoria but exacerbated the somatic components of OW. In net contrast, genetic deletion of the CRF_2 receptor pathway decreased the somatic expression elicited by OW. Most importantly, the genetic inactivation of the CRF_1 receptor profoundly impairs HPA and brain stress-coping responses, suggesting that the CRF_1 receptor pathway might not be a suitable target for relieving OW distress. The role of the CRF_2 in the multiple components of the negative affective states such as dysphoria, anhedonia, motivational disorders, etc, is largely unexplored **(Table. 3)**. The aim of this work is to characterize the role of CRF_2 receptor in the emergence of the multiple components of the negative affective states developed during OW.

A. First of all, several evidences showed a dichotomy between CRF_1 and CRF_2 receptors on the stress responsiveness and on the emergence of OW somatic signs, which might presuppose an opposite role between CRF receptors in the emergence of negative affect during OW. Accordingly, it is known that the CRF_1 receptor-deficient mice possess a functional CRF_2 receptor. Inasmuch as the stimulation of the CRF_2 receptor by increased CRF activity in $CRF_1^{-/-}$ mice exhibited indirect evidence of the lack of dysphoria, we hypothesized that $CRF_2^{-/-}$ mice may exhibit an exaggerated development of negative affect elicited by OW. In the *chapter II*, we aimed to understand the role of the CRF_2 receptor in the behavioral and biological alterations linked to the dysphoria and anhedonia elicited by OW. A series of experiments was conducted in CRF_2 receptor-deficient mice, using behavioral and biological approaches to answer these questions.

B. Multiple data in both humans and animals showed motivational alterations to opiates and natural rewards during OW. It has been argued that the CRF system, by its action on the anxiety and the negative emotionality associated with dependence, increases the motivational effects of drug withdrawal. However, the contribution of the CRF system in the dramatic long-lasting motivational disorders showed during OW is largely unknown. The second objective of this work consisted to addressing the role of CRF_2 receptor during OW on the motivational properties of natural reward. However, another unresolved question in literature concerns the

sort of motivational alterations to natural reward during OW. For this purpose, at first, we developed a mouse model of HPF-driven operant behavior using a clinically relevant SPO OW paradigm previously validated in our laboratory (Article 1, *Chapter III*). Subsequently, using wild-type and $CRF_2^{-/-}$ female and male mice, we have adapted the animal model to identify the role of the CRF_2 receptor in the cognitive and motivational long-term sequelae of opiate treatment and withdrawal (Article 2, *Chapter III*).

C. Finally, as previously shown, clinical reports argued that stress is one of the critical factors leading individual-addicts to drug seeking and relapse during alcohol and drug withdrawal. Accordingly, preclinical studies mimicking the human stressful events showed alcohol and drug-seeking relapse in rats during abstinence. Because the CRF system is the major coordinator of behavioral and endocrine responses to stress, several studies investigated the role of CRF_1 receptor in the alcohol and drug-seeking relapse using a pharmacological antagonism. However the role of the CRF_2 receptor in the drug and/or food reinstatement seeking behavior is minimally explored. In addition, pharmacological antagonism of the CRF receptors largely used in these studies constitutes real limitations in the comprehension of the role of the CRF system in the alcohol and drug-seeking and relapse. In particular, there is no evidence in the scientific literature about the real specificity of the pharmacologic molecules employed to target the both two CRF receptors. Thus, in our knowledge no study investigated the role of the CRF receptors using genetic-deficient mice model which is more appropriate tool to dissecate the specific contribution of the CRF receptors in alcohol and drug-seeking and relapse. In *chapter IV* we explored the involvement of the CRF_2 receptor-deficiency in the stress-induced reinstatement of motivation for the palatable food during a prolonged OW.

Chapter II

Article 1 : La délétion génétique du récepteur CRF$_2$ élimine les effets du sevrage aux opiacés sans altérer l'adaptabilité au stress

RESUMÉ

La dépendance aux drogues opiacées est source de problèmes sanitaires et économiques majeurs dans le monde. Chez les personnes dépendantes, l'arrêt de la prise de substances opiacées se caractérise par l'émergence d'effets délétères physiques et psychologiques nommés syndrome de sevrage aux opiacés (SAO). Plusieurs évidences dans la littérature scientifique rapportent que les réponses comportementales et neuroendocriniennes au stress lors du syndrome de sevrage aux opiacés contribuent à la rechute et au maintien de la prise de drogue.

Le système CRF est un coordinateur essentiel des réponses adaptatives au stress par l'intermédiaire de ses deux récepteurs : le CRF$_1$ et le CRF$_2$. Des études menées sur des souris génétiquement invalidées aux récepteurs CRF$_1$ ont montré une abolition des états dysphoriques associés au SAO. Cependant, ces souris développaient une augmentation de l'intensité et de la durée de l'expression des signes somatiques. A l'opposé, les souris invalidées au récepteur CRF$_2$ (CRF$_2^{-/-}$) montraient une élimination des signes somatiques associés au SAO.

Afin de disséquer le rôle du récepteur CRF$_2$ nous avons utilisé des souris génétiquement invalidées à ce récepteur. En outre, nous avons exploré l'implication de ce dernier dans l'émergence de la dysphorie et de l'anhédonie constatées lors du SAO.

À l'encontre de notre hypothèse de départ, nos résultats montraient clairement une abolition des états dysphoriques et anhédoniques inféodés au sevrage spontané à la morphine chez des souris CRF$_2^{-/-}$. En utilisant le paradigme expérimental de conditionnement à un environnement aversif, nous avons pu mettre en évidence qu'un sevrage précipité à la naloxone ne provoquait pas l'émergence d'affects négatifs et de signes somatiques chez les souris CRF$_2^{-/-}$ contrairement aux souris de type sauvage. De surcroît, des analyses moléculaires des régions cérébrales impliquées dans le SAO chez les souris CRF$_2^{-/-}$, ne montraient pas d'augmentation de la DYN, du CRF ou de l'expression *c-fos* en comparaison avec les souris sauvages. Enfin, la délétion génétique du récepteur CRF$_2$ n'influençait pas l'activation de l'axe hypothalamo-hypothypsaire ainsi que l'expression du CRF et de la TH dans le PVN et le LC, respectivement.

En conclusion, ce travail montre que la délétion génétique du récepteur CRF$_2$ élimine complètement la dysphorie et l'anhédonie sans altérer les fonctions neuroendocriniennes et autonomiques essentielles à la réponse au stress. A l'heure actuelle, la concentration des efforts sur un antagonisme ciblé du récepteur CRF$_2$ semble être une approche prometteuse dans la recherche d'un traitement de la dépendance aux opiacés.

ORIGINAL ARTICLE

CRF$_2$ receptor-deficiency eliminates opiate withdrawal distress without impairing stress coping

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The opiate withdrawal syndrome is a severe stressor that powerfully triggers addictive drug intake. However, no treatment yet exists that effectively relieves opiate withdrawal distress and spares stress-coping abilities. The corticotropin-releasing factor (CRF) system mediates the stress response, but its role in opiate withdrawal distress and bodily strategies aimed to cope with is unknown. CRF-like signaling is transmitted by two receptor pathways, termed CRF$_1$ and CRF$_2$. Here, we report that CRF$_2$ receptor-deficient (CRF$_2^{-/-}$) mice lack the dysphoria-like and the anhedonia-like states of opiate withdrawal. Moreover, in CRF$_2^{-/-}$ mice opiate withdrawal does not increase the activity of brain dynorphin, CRF and periaqueductal gray circuitry, which are major substrates of opiate withdrawal distress. Nevertheless, CRF$_2$ receptor-deficiency does not impair brain, neuroendocrine and autonomic stress-coping responses to opiate withdrawal. The present findings point to the CRF$_2$ receptor pathway as a unique target to relieve opiate withdrawal distress without impairing stress-coping abilities.
Molecular Psychiatry advance online publication, 27 September 2011; doi:10.1038/mp.2011.119

Introduction

Opiate addiction is a chronic, relapsing disease with a major impact on public health (http://www.who.int/substance_abuse). Studies show an alarming rise in the recreational use of opiate drugs among adolescents, indicating that the incidence rate of opiate addiction may dramatically increase in the next years.[1] In opiate addicts, heroin 'highs' are inexorably followed by a severe opiate withdrawal syndrome composed of influenza-like somatic signs and negative affective symptoms, such as dysphoria, depressed mood and anhedonia.[2] Relief of opiate withdrawal signs and symptoms powerfully motivates compulsive drug-seeking and drug-taking behavior.[3,4] Moreover, opiate withdrawal serves as a severe stressor that strongly challenges stress-responsive systems,[5] thus further reducing the ability to abstain from addictive drug intake. Opiate addiction and withdrawal are currently treated mainly by substitutive opioid receptor agonist drugs, such as methadone and buprenorphine.[6] However, the latter drugs are very addictive and their discontinuation is

often followed by relapse to 'street' opiates.[6] Novel treatments for opiate addiction and withdrawal are thus urgently needed.

The corticotropin-releasing factor (CRF) system is a major coordinator of behavioral, neuroendocrine and autonomic responses to stressors.[7–10] The CRF system might also mediate the behavioral effects of ethanol or drug withdrawal. Indeed, functional antagonism of brain CRF neurotransmission attenuates stress-induced reinstatement of ethanol- or cocaine-seeking behavior,[11,12] decreases ethanol self-administration[13] and reverses anxiety-like behavior[14] induced by ethanol withdrawal in rats. Accordingly, cessation of ethanol or cocaine intake elevates CRF activity in the amygdala and in the bed nucleus of the stria terminalis,[15–18] brain regions implicated in the behavioral effects of substance withdrawal. In mammals, CRF-like signaling is transmitted by two receptor pathways, termed CRF$_1$ and CRF$_2$.[19] Genetically engineered mouse models have allowed the discovery of distinct, and often opposite, functions for the two known CRF receptor pathways. In particular, genetic disruption of the CRF$_1$ or the CRF$_2$ receptor pathway decreases or increases, respectively, anxiety-like and hypothalamic–pituitary–adrenal (HPA) axis responses to stressors.[20–24] CRF$_1$ and CRF$_2$ receptor pathways might also differentially contribute to the myriad of somatic opiate withdrawal signs. Indeed, CRF$_1$ or CRF$_2$ receptor-deficiency exacerbates or attenuates, respectively, the somatic signs of opiate

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withdrawal.[25,26] Moreover, CRF$_1$ receptor-deficiency eliminates the dysphoria-like states of opiate withdrawal[27] but profoundly impairs HPA axis[26,27] and brain[26] stress-coping responses, suggesting that the CRF$_1$ receptor pathway might not be a suitable target for relieving opiate withdrawal distress. CRF$_2$ receptor-deficiency increases HPA axis and negative affective-like responses to stressors other than opiate withdrawal.[20–22] However, the role for the CRF$_2$ receptor pathway in the negative affective symptoms and the stress response elicited by opiate withdrawal is unknown.

Thus, the present study aims to elucidate the role for the CRF$_2$ receptor pathway in critical components of opiate withdrawal distress, such as dysphoria and anhedonia, and the underlying brain mechanisms. Furthermore, the contribution of the CRF$_2$ receptor pathway to brain, neuroendocrine and autonomic responses aimed to cope with the stressful condition of opiate withdrawal is investigated. Noteworthy, in keeping with the clinical setting where opiate withdrawal signs and symptoms 'spontaneously' and gradually rise along with the drug removal from the body, behavioral, molecular and neuroendocrine studies are conducted in mice undergoing spontaneous opiate withdrawal (SPO OW). Furthermore, to compare our findings with the literature studies, the role for the CRF$_2$ receptor pathway in dysphoria-like behavior is also examined using a more classical opioid-receptor antagonist-precipitated opiate withdrawal paradigm.

Subjects and methods

Subjects
Group-housed, littermate female mice on a mixed C57BL/6J × 129 background that are wild-type or CRF$_2$ receptor-null mutant (CRF$_2^{-/-}$) are used throughout.[20] Mice are 4–9 months old at the time of the experiments and derived from mating CRF$_2^{+/-}$ mice. Wild-type and CRF$_2^{-/-}$ offspring of CRF$_2^{+/-}$ breeders are identified by PCR analysis of tail DNA. The mice are housed in a colony room maintained at $22 \pm 2\,°C$ on a 12-h light/dark cycle (lights on from 0800 until 2000 hours). Food and water are available *ad libitum*. All studies are conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and the European Communities Council Directive of 24 November 1986 (86/609/EEC), and are approved by the local Animal Care and Use Committee.

Opiate withdrawal-induced conditioned place aversion
The conditioned place aversion (CPA) apparatus consists of a rectangular Plexiglas box (length 42 cm, width 21 cm and height 21 cm) divided by a central partition into two chambers of equal size ($21 \times 21 \times 21$ cm). One compartment has black walls and a smooth Plexiglas floor, whereas the other compartment has vertical black and white striped (2 cm) walls and a slightly rough floor. During the test

sessions, an aperture (4×4 cm) in the central partition allows the mice to enter both sides of the apparatus, whereas during the conditioning trials the individual compartments are closed off from each other. Transparent Plexiglas lids allow observation of the animal's behavior on a video monitor situated in an adjacent room and connected to a camera placed above the apparatus.[27] Moreover, time spent in each of the two compartments of the CPA apparatus is quantified by an automated video-tracking system (ViewPoint, Champagne au Mont d'Or, France). Both the spontaneous and the naloxone-precipitated OW (NAL OW) CPA experiments last 14 days and consist of a preconditioning test, a conditioning and a post-conditioning phase. On day 1, each mouse is allowed to explore freely the entire CPA apparatus for 20 min and time spent in each of the two compartments is measured (preconditioning test). Within each genotype, mice are then divided in two groups with similar preconditioning time values in the preferred and the non-preferred compartment of the CPA apparatus. One group is assigned to receive saline and the other increasing morphine doses (20–100 mg kg^{-1}). In particular, starting on day 3, every 12 h (0800 hours; 2000 hours) the mice are treated with saline or morphine according to the following protocol: day 3: 20 mg kg^{-1}, day 4: 40 mg kg^{-1}, day 5: 60 mg kg^{-1}, day 6: 80 mg kg^{-1}, day 7: 100 mg kg^{-1} and day 8: 100 mg kg^{-1}, only one injection in the morning. Morphine is administered twice a day as, unlike constant drug delivery devices, it allows partial opiate withdrawal between drug injections, a condition encountered in the clinical setting. In the SPO OW experiment, conditioning trials take place on days 5–8, whereas morphine-treated mice are in an opiate withdrawal state. For this purpose, 8 h after the morning injection mice are confined for 30 min a day into their preferred compartment of the CPA apparatus, as determined during the preconditioning test.[27] In the NAL OW experiment, the mice are treated with saline or morphine, as described above. On days 5–8, all mice receive naloxone (0.23 mg kg^{-1}, subcutaneously (s.c.)) at 2 h after the morning saline or morphine injection and are immediately confined for 30 min a day into their preferred compartment of the CPA apparatus, as determined on the preconditioning test. During the conditioning trials, we quantify the number of jumps and wet dog shakes, and check for the presence of diarrhea. For each mouse, a global somatic opiate withdrawal score is also calculated as follows: number of jumps + number of wet dog shakes + 3 points for the diarrhea sign. Post-conditioning tests take place 6 days after the last conditioning trial (day 14), once somatic opiate withdrawal signs have largely dissipated in both genotypes.[25]

U-50488H-induced CPA
We use the same CPA apparatus and an experimental procedure similar to that described above. The only difference is that the mice are not exposed to

the twice daily treatment with saline or morphine. Moreover, during the four conditioning trials (days 5–8), immediately before being confined to their preferred compartment of the CPA apparatus, the mice are treated with saline or the kappa opioid-receptor (KOR) agonist compound U-50488H (National Institute on Drug Abuse, Bethesda, MD, USA; 5 mg kg^{-1}, s.c.). To evaluate the role for the CRF$_1$ receptor pathway in the U-50488H-induced CPA, three additional groups of CRF$_2^{-/-}$ mice are treated with saline or U-50488H (5 mg kg^{-1}, s.c.) immediately before the conditioning trials and with antalarmin (20 mg kg^{-1}, per os) or the appropriate vehicle 1 h before the conditioning trials. In the latter study, the mice undergo three, instead of four, conditioning trials (days 5–7).

Highly palatable food preference paradigm
The mice are individually housed 10 days before the beginning of the experiments. Standard chow food (SCF; 3.3 kcal g^{-1}, 72.5% carbohydrates, 8.2% fat, 19.3% protein, rodent diet, A04, Augy, France) and water are available *ad libitum*. Then, during three consecutive days several baseline measures are collected. In particular, body weight (BW) and home-cage SCF intake are measured every 12 h, at the beginning and at the end of the light phase of the 12-h light/dark housing cycle. Moreover, at 1600 1-h highly palatable food (HPF) preference tests are performed by offering a choice between a pre-weighed (~4 g) SCF pellet and three 1-g HPF pellets. For this purpose, we use a sucrose-enriched HPF (5-TUL, 3.44 kcal g^{-1}, 66.7% carbohydrate, 12.7% fat, 20.6% protein; Test-Diet, PMI Nutrition International, LLC, St Louis, MO, USA). Then, 1 h later, SCF and HPF pellets are removed from the home cage and intake of both diets calculated. Care is taken to look for spillage to measure as accurately as possible food intake. Within genotype, the mice are then assigned to two groups having similar home-cage SCF intake, percentage of HPF and total (SCF + HPF) intake, as measured during the third baseline day. One group is assigned to receive saline and the other increasing morphine doses, as described above. Saline and morphine treatments start 24 h after the last baseline measure. The mice are weighed immediately before each injection and BW changes calculated as percentage of BW recorded just before the first saline or morphine injection. Starting 8 h after the last saline or morphine injection, 1-h HPF preference tests are performed once daily up to 104 h following the last injection. Throughout the experiment, BW and home-cage SCF intake are measured, as described above. Moreover, caloric efficiency (CE), calculated as grams of BW gained per kilocalories ingested, is assessed during the three baseline days, the intermittent morphine injections (from the first injection to 24 h after the last injection) and 24–96 h after the last injection. For this purpose, the amount of SCF and HPF ingested during the 1-h HPF preference tests is also taken into account.

In situ hybridization experiments and plasma corticosterone assay
The mice are treated with saline or morphine and exposed to the CPA paradigm, as described above. The only difference is that at the end of the last 30-min conditioning trial, the mice are taken into another room, their brains rapidly removed, frozen in isopentane (−40 °C) and stored at −80 °C. Blood samples are collected from the trunk, centrifuged (4000 r.p.m., 15 min) and plasma samples stored at −20 °C until corticosterone assay. Plasma corticosterone levels are quantified by radioimmunoassay using a specific corticosterone antibody (ICN Pharmaceuticals, Orsay, France). The intra- and inter-assay coefficients of variation are approximately 3.5% and 8%, respectively. For *in situ* hybridization experiments, brains are cut in coronal sections (12 μm) using a cryostat and thaw mounted onto gelatin-coated slides as previously described.[28] The Paxinos and Franklin mouse brain atlas is used to identify the different brain regions examined.[29] *In situ* hybridization is performed with antisense ^{35}S-labeled complementary RNA probes designed to recognize CRF,[30] dynorphin (DYN),[31] tyrosine hydroxylase (TH)[28] and c-fos[28] mRNAs. All probes are prepared by *in vitro* transcription from 100 ng of linearized plasmid using [35S]UTP (>1000 Ci mmol^{-1}; Perkin-Elmer Life Sciences, Courtaboeuf, France) and appropriate RNA polymerases. After alkaline hydrolysis to obtain 0.25 kb complementary RNA fragments, the ^{35}S-labeled probes are purified on G50-Sephadex and precipitated in sodium acetate 3 M, pH 5.0 (0.1 volume), absolute ethanol (2.5 volumes). Sections are postfixed in 4% paraformaldehyde for 5 min at room temperature, rinsed twice in 4× sodium chloride—sodium citrate buffer (SSC) and placed into 0.1 M triethanolamine/4× SSC, pH 8.0, for 10 min at room temperature; 0.25% acetic anhydride is added for the last 5 min. After dehydration in graded alcohols, sections are hybridized overnight at 55 °C with 10^6 c.p.m. of antisense ^{35}S-labeled probes in 50 μl hybridization buffer (20 mM Tris–HCl, 1 mM EDTA, 300 mM NaCl, 50% formamide, 10% dextran sulfate and 1% Denhardt). The slides are rinsed in 4× SSC twice, treated with RNAse A for 15 min at 37 °C, washed in decreasing SSC concentrations, at room temperature and then at 65 °C with 0.1× SSC for 30 min twice. Slides are dehydrated and then exposed at room temperature to Biomax-MR X-ray film (Kodak, Eastman Kodak, Rochester, NY, USA) for 2–8 weeks. Quantification of mRNA expression is performed by densitometric analysis on X-ray film and data are expressed as mean of optical density × 1000 ± s.e.m.

Drugs
Morphine HCl (Francopia, Gentilly, France; 20–100 mg kg^{-1}, intraperitoneally), U-50488H (5 mg kg^{-1}, s.c.) and naloxone HCl (0.23 mg kg^{-1}, s.c.; Sigma-Aldrich, Lyon, France) are dissolved in physiological saline and injected in a volume of 10 ml kg^{-1}. Antalarmin HCl (20 mg kg^{-1}, per os; Sigma-Aldrich)

is dissolved in acidified saline (pH $\sim$ 2.5) and injected in a volume of $10 \, ml \, kg^{-1}$. Control mice are injected with the same volume of the appropriate vehicle.

Statistical analysis

Two-way analysis of variance (ANOVA) with genotype (wild type or CRF$_2^{-/-}$) and treatment (control, opiate withdrawal or U-50488H) as independent variables is used to examine the CPA scores, the *in situ* hybridization data (DYN, CRF, *c-fos* and TH mRNAs levels) and the plasma corticosterone levels. A one-way ANOVA is used to examine the CPA scores of the antalarmin/U-50488H experiment. Jumps, wet dog shakes and global somatic opiate withdrawal scores are examined using the nonparametric Mann–Whitney *U*-test. The 2×2 table χ^2-test is used to analyze the presence or absence of the diarrhea sign during the CPA conditioning trials. A three-way ANOVA with genotype and treatment (control or opiate withdrawal) as between-subject factors and repeated measures as a within-subject factor is used to analyze the percentage of HPF and the total amount of kilocalories ingested during the HPF preference tests. A three-way ANOVA with genotype and treatment (control or morphine) as between-subject factors and repeated measures as a within-subject factor is used to analyze the 12-h home-cage SCF intake and the percentage of BW changes induced by the stress of intermittent morphine injections and by its cessation. A two-way ANOVA with genotype and treatment (control or morphine) as independent variables is used to examine CE induced by the stress of intermittent morphine injections and by its cessation. The Student–Newman–Keuls *post hoc* test is used for individual group comparisons. The accepted value for significance is $P < 0.05$.

Results

Absence of dysphoria-like states of opiate withdrawal in CRF$_2$ receptor-deficient mice

In humans, the opiate withdrawal syndrome is composed of severe negative affective-like states, such as dysphoria and anhedonia, and influenza-like somatic signs.[2] To investigate the role for the CRF$_2$ receptor pathway in dysphoria-like states induced by opiate withdrawal, we use the CRF$_2^{-/-}$ mouse model,[20] a clinically relevant SPO OW CPA paradigm previously validated in our laboratory[27] and a more classical NAL OW CPA paradigm. To closely parallel drug intake patterns of opiate addicts, we use an intermittent morphine injection procedure, wherein drug doses are progressively increased. Moreover, in the SPO OW experiment, 30-min place-conditioning trials are started 8 h after morphine injection, that is, following body clearance of the opiate drug and during the maximal expression of somatic opiate withdrawal signs.[25,32] During the 20-min preconditioning test, time spent in the preferred compartment of the CPA apparatus is similar for wild-type and CRF$_2^{-/-}$ mice assigned to the control or the

opiate-withdrawn group (Supplementary Table 1). Post-conditioning tests reveal that the wild-type mice consistently avoid the environmental cues of the CPA apparatus previously paired with the SPO OW or the NAL OW (Figures 1a and b), indicating dysphoria-like states during the place conditioning trials. In contrast, in both the CPA experiments opiate-withdrawn CRF$_2^{-/-}$, mice do not differ from control wild-type and CRF$_2^{-/-}$ mice (Figures 1a and b). Noteworthy, despite the higher CPA scores observed in the wild-type mice during the NAL OW, as compared with the SPO OW experiment, CRF$_2^{-/-}$ mice do not show any sign of dysphoria-like behavior (compare Figures 1a and b). Thus, in contrast to the
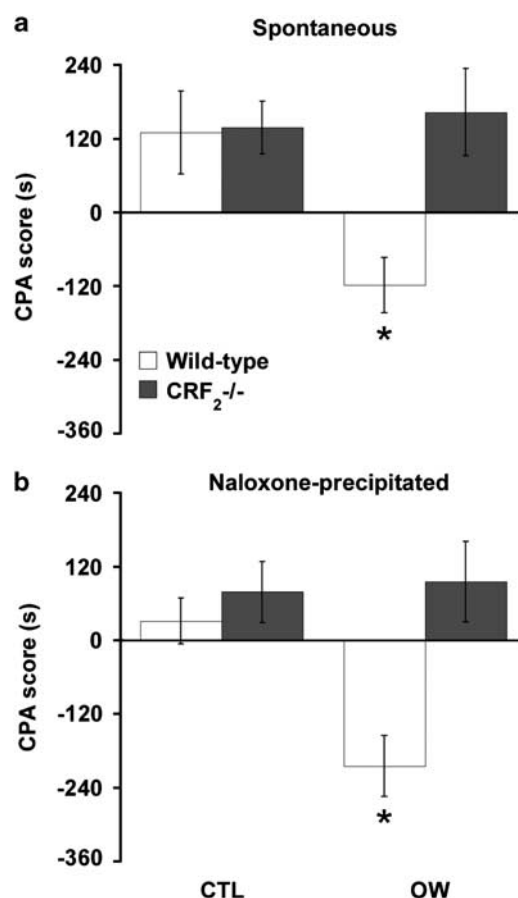
Figure 1 CRF$_2$ receptor-deficiency eliminates the dysphoria-like states of opiate withdrawal. Mean ($\pm$ s.e.m.) conditioned place aversion (CPA) scores (s) displayed by control (CTL) or opiate-withdrawn (OW) wild-type and CRF$_2^{-/-}$ mice during the (**a**) spontaneous (SPO) or the (**b**) naloxone (NAL)-precipitated OW experiment (SPO OW, genotype $\times$ opiate withdrawal interaction effect: F$_{1,35}$ = 5.6, $P < 0.05$; NAL OW, genotype $\times$ opiate withdrawal interaction effect: F$_{1,32}$ = 4.2, $P < 0.05$). *$P < 0.01$ versus OW CRF$_2^{-/-}$, CTL wild-type and CTL CRF$_2^{-/-}$ mice, Newman–Keuls *post hoc* test. For each mouse, a CPA score is calculated as the post- minus the preconditioning time spent in the conditioning-paired compartment of the CPA apparatus. N = 9–10 per group. Abbreviation: CRF, corticotropin-releasing factor.

wild-type mice, CRF$_2^{-/-}$ mice do not show any aversion for the environmental cues paired with opiate withdrawal, indicating the absence of dysphoria-like states. During both the SPO OW and the NAL OW experiments, the impact of CRF$_2$ receptor-deficiency upon major somatic signs of opiate withdrawal is also examined. Along with increasing morphine doses, during the place-conditioning trials of the SPO OW experiment the wild-type mice show a progressive increase in the severity of somatic opiate withdrawal signs, as assessed by the global score parameter (Supplementary Figure 1a). In contrast, opiate-withdrawn CRF$_2^{-/-}$ never differ from control wild-type and CRF$_2^{-/-}$ mice (Supplementary Figure 1a). In particular, opiate-withdrawn wild-type mice make more jumps and wet dog shakes than opiate-withdrawn CRF$_2^{-/-}$, control wild-type and CRF$_2^{-/-}$ mice (Supplementary Figures 1b and c). Noteworthy, none of the opiate-withdrawn CRF$_2^{-/-}$ mice displays the jump sign (Supplementary Figure 1b). Moreover, during the fourth place-conditioning trial, 5 out of 10 opiate-withdrawn wild-type mice show the diarrhea sign, whereas none of the opiate-withdrawn CRF$_2^{-/-}$ mice show this sign (Supplementary Table 2). On the other hand, no reliable somatic signs of opiate withdrawal are observed in the NAL OW experiment neither in the wild-type nor in the CRF$_2^{-/-}$ mice (data not shown), indicating a selective effect of the naloxone dose used (0.23 mg kg^{-1}, s.c.) upon affective-like states. We previously reported that CRF$_2$ receptor-deficiency attenuates the somatic expression of opiate withdrawal, as assessed 8–152 h after the last morphine injection.[25] Unlike our prior study, the present results provide novel evidence in favor of a role for the CRF$_2$ receptor pathway also in the progressive increase of somatic opiate withdrawal signs induced by repeated intermittent injections of morphine (Supplementary Figure 1). Thus, CRF$_2$ receptor-deficiency completely eliminates the dysphoria-like states and the somatic signs of opiate withdrawal occurring between successive injections of increasing morphine doses. These findings clearly indicate an essential role for the CRF$_2$ receptor pathway in critical negative affective-like and somatic components of opiate withdrawal distress.

Unaltered CPA abilities in CRF$_2$ receptor-deficient mice
CRF$_2^{-/-}$ mice show no conditioned aversions to the environmental cues associated with opiate withdrawal. Thus, it can be argued that in CRF$_2^{-/-}$ mice absence of opiate withdrawal-induced CPA might be due to developmental deficits in associative learning processes required for the acquisition and expression of conditioned behavior. To address this issue, an additional cohort of wild-type and CRF$_2^{-/-}$ mice is tested for CPA to the KOR agonist compound U-50488H.[33] During the 20-min preconditioning test, time spent in the preferred compartment of the CPA apparatus is similar for wild-type and CRF$_2^{-/-}$ mice assigned to be treated with saline or U-50488H (Supplementary Table 1). Post-conditioning tests

reveal that wild-type and CRF$_2^{-/-}$ mice display similar levels of CPA for the environmental cues previously paired with the KOR agonist U-50488H (Supplementary Figure 2a). The latter results clearly indicate that CRF$_2$ receptor-deficiency does not alter associative learning processes underlying the acquisition and the expression of CPA. These findings also indicate functional levels of the KOR pathway, the preferential target of endogenous DYN peptides,[34] in the CRF$_2^{-/-}$ mice. To assess the role for the CRF$_1$ receptor pathway in the U-50488H-induced CPA, another cohort of CRF$_2^{-/-}$ mice is pretreated with the CRF$_1$ receptor-preferring antagonist antalarmin 1 h before being conditioned with U-50488H. Analysis of time spent in the preferred compartment of the CPA apparatus during the preconditioning test reveals no group differences (F$_{2,13}$ = 1.3, P = nonsignificant, data not shown). Similarly to above, the KOR agonist U-50488H induces reliable CPA in the CRF$_2^{-/-}$ mice (Supplementary Figure 2b). However, pretreatment with antalarmin completely abolishes the U-50488H-induced CPA in the CRF$_2^{-/-}$ mice, indicating that dysphoria-like states induced by stimulation of the KOR pathway are mediated by the CRF$_1$ receptor pathway (Supplementary Figure 2b). Moreover, the latter results add further support to the notion of preserved CRF$_1$ receptor activity in the CRF$_2^{-/-}$ mice.[20,21]

Absence of anhedonia-like states of opiate withdrawal in CRF$_2$ receptor-deficient mice
A prominent negative affective-like state of opiate withdrawal is anhedonia, that is, an impaired capability to experience reward from natural pleasant activities, such as eating or sex.[2] Stress-induced decrease in the preference for HPF is thought to reflect anhedonia-like states.[35] Thus, we use a HPF preference test to assess the role for the CRF$_2$ receptor pathway in the anhedonia-like states of opiate withdrawal. In particular, the mice are injected with saline or morphine as in the opiate withdrawal CPA experiment mentioned above, and daily 1-h HPF preference tests carried out at 8–104 h following the last injection. Before the beginning of saline or morphine injections, wild-type and CRF$_2^{-/-}$ mice display similar levels of HPF preference (point B, Figure 2a). However, 8 h after the last morphine injection the opiate-withdrawn wild-type mice display a decrease in the percentage of HPF ingested, indicating the presence of an anhedonia-like state (Figure 2a). In contrast, opiate-withdrawn CRF$_2^{-/-}$ mice do not show any decrease in the percentage of HPF ingested and do not differ from control wild-type and CRF$_2^{-/-}$ mice (Figure 2a). The reduced HPF preference observed in opiate-withdrawn wild-type mice does not depend on energy intake. Indeed, 8 h after the last saline or morphine injection there is neither genotype nor opiate withdrawal effect in total kilocalories ingested (Figure 2b). However, we find a genotype-independent increase in total kilocalories ingested 32 and 56 h after the last morphine injection (Figure 2b), when no more reduction in HPF

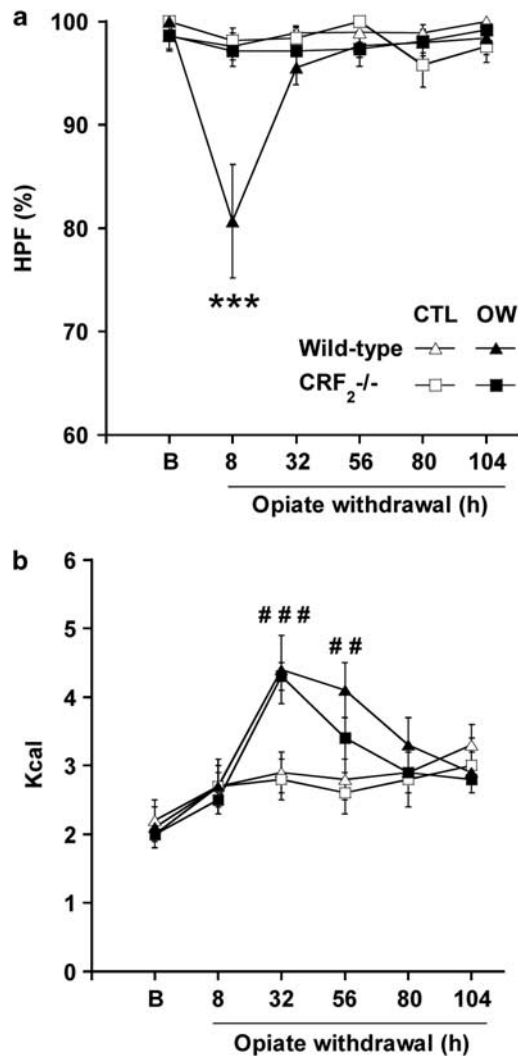**Figure 2** CRF$_2$ receptor-deficiency abolishes the anhedonia-like states of opiate withdrawal. (**a**) Mean (± s.e.m.) percentage of highly palatable food (HPF) ingested by control (CTL) or opiate-withdrawn (OW) wild-type and CRF$_2^{-/-}$ mice during the daily 1-h HPF preference tests carried out before (B, baseline) and 8–104 h after intermittent saline or morphine injections (genotype × opiate withdrawal × repeated measures interaction effect: F$_{5,150}$ = 4.3, $P < 0.005$; ***$P < 0.0001$ versus OW CRF$_2^{-/-}$, CTL wild-type and CTL CRF$_2^{-/-}$ mice, at the same time point, Newman–Keuls *post hoc* test). $N = 8$–9/group. (**b**) Mean (± s.e.m.) total kilocalories (kcal) ingested during the 1-h HPF preference tests (opiate withdrawal × repeated measures interaction effect: F$_{5,150}$ = 9.1, $P < 0.0001$; ##$P < 0.0005$, ###$P < 0.0001$ versus CTL mice at the same time point, Newman–Keuls *post hoc* test; genotype × opiate withdrawal × repeated measures interaction effect: F$_{5,150}$ = 0.3, $P =$ nonsignificant). $N = 8$–9 per group. Abbreviation: CRF, corticotropin-releasing factor.

preference is observed in the opiate-withdrawn wild-type mice. Noteworthy, the amount of kilocalories ingested during the 1-h HPF preference tests is relatively high, as compared with the kilocalories ingested daily (data not shown), a phenomenon due to the appetitive properties of the HPF used. Thus,

our studies demonstrate a fundamental role for the CRF$_2$ receptor pathway in the anhedonia-like states of opiate withdrawal. Moreover, our results show that CRF$_2$ receptor deficiency does not influence the increased HPF-driven energy intake.

CRF$_2$ receptor-deficiency abolishes the brain substrates of opiate withdrawal distress
Morphine withdrawal increases the expression of the opioid peptide DYN in the brain region of the nucleus accumbens shell (NaccSH).[27] Increased DYN/KOR activity in the nucleus accumbens may underlie the negative affective-like states of opiate withdrawal,[27] and also decrease the extracellular levels of dopamine,[36] a neurotransmitter mediating the hedonic properties of drugs of abuse[4] and palatable food.[37] Thus, to investigate the brain substrates underlying the CRF$_2$ receptor-mediated dysphoria-like and anhedonia-like states of opiate withdrawal, wild-type and CRF$_2^{-/-}$ mice are injected with saline or morphine as above, and NaccSH–DYN expression examined in brains collected 8.5 h after the last injection. Our results indicate that opiate withdrawal consistently increases NaccSH–DYN expression in the wild-type mice (Figures 3a and b). In contrast, opiate withdrawal does not affect NaccSH–DYN expression in the CRF$_2^{-/-}$ mice, which show DYN mRNA levels similar to control wild-type and CRF$_2^{-/-}$ mice (Figures 3a and b). We also examine the expression of the immediate-early gene *c-fos*, a marker of neuronal reactivity,[38] in the periaqueductal gray (PAG), a brainstem region implicated in opiate withdrawal distress.[39] Our analyses reveal that opiate withdrawal produces a 2.4-fold increase in PAG–*c-fos* expression in the wild-type mice (Figure 3c). However, opiate withdrawal does not produce any increase in PAG–*c-fos* expression in the CRF$_2^{-/-}$ mice, which display *c-fos* mRNA levels similar to control wild-type and CRF$_2^{-/-}$ mice (Figures 3c and d). Increased CRF activity in the central nucleus of the amygdala (CeA) might contribute to the behavioral effects of drug or ethanol withdrawal.[5] However, no prior studies have assessed the role for the CRF$_2$ receptor pathway in opiate withdrawal-induced CeA–CRF expression. Here, we demonstrate that opiate withdrawal reliably increases CeA–CRF expression in the wild-type mice (Figures 4a and b). However, opiate-withdrawn CRF$_2^{-/-}$ mice do not show any increase in CeA–CRF expression (Figures 4a and b), indicating a fundamental role for the CRF$_2$ receptor pathway in CeA–CRF responses to opiate withdrawal. Thus, our results clearly demonstrate that the activity of brain circuitry underlying negative affective-like and somatic components of opiate withdrawal distress totally depends on functional levels of the CRF$_2$ receptor pathway.

Preserved stress responses in opiate-withdrawn CRF$_2$ receptor-deficient mice
The opiate withdrawal syndrome is an extremely severe and challenging stressor. CRF and norepinephrine (NE) pathways originating from the
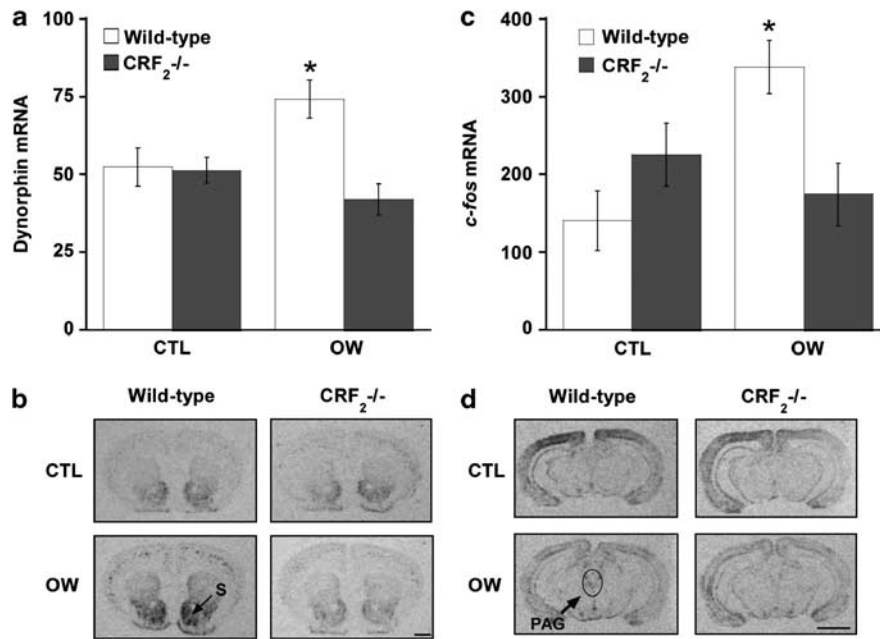
Figure 3 CRF$_2$ receptor-deficiency eliminates the brain substrates of opiate withdrawal distress. (**a**) Mean (±s.e.m.) dynorphin (DYN) mRNA levels in the nucleus accumbens shell (NaccSH; bregma interval: 1.10/0.86 mm) in control (CTL) or opiate-withdrawn (OW) wild-type and CRF$_2^{-/-}$ mice (genotype × opiate withdrawal interaction effect: F$_{1,28}$ = 8.3, $P < 0.01$; *$P < 0.05$ versus OW CRF$_2^{-/-}$, CTL wild-type and CTL CRF$_2^{-/-}$ mice, Newman–Keuls *post hoc* test). Results are expressed as optical density (OD; × 1000). $N = 7$–9 per group. (**b**) Representative images of brain sections illustrating DYN expression in the NaccSH (S) in CTL or OW wild-type and CRF$_2^{-/-}$ mice. Scale bar = 1 mm. (**c**) Mean (±s.e.m.) *c-fos* mRNA levels in the periaqueductal gray (PAG; bregma interval: −3.16/−3.40 mm) in CTL or OW wild-type and CRF$_2^{-/-}$ mice (genotype × opiate withdrawal interaction effect: F$_{1,36}$ = 10.5, $P < 0.005$; *$P < 0.05$ versus OW CRF$_2^{-/-}$, CTL wild-type and CTL CRF$_2^{-/-}$ mice, Newman–Keuls *post hoc* test). Results are expressed as OD (× 1000). $N = 10$/group. (**d**) Representative images of brain sections illustrating *c-fos* expression in the PAG in CTL or OW wild-type and CRF$_2^{-/-}$ mice. Scale bar = 2.5 mm. Abbreviation: CRF, corticotropin-releasing factor.

paraventricular nucleus of the hypothalamus (PVN) and the locus coeruleus (LC), respectively, orchestrate brain, neuroendocrine and autonomic stress responses.[7,8,10] Moreover, PVN–CRF pathways projecting to the median eminence of the hypothalamus–pituitary system coordinate stress-induced HPA axis activity.[9] Thus, we assess the impact of CRF$_2$ receptor-deficiency upon the opiate withdrawal-induced activity of PVN–CRF, HPA axis and LC–NE pathways. Noteworthy, analyses are carried out on the same brains used for the DYN, CRF and *c-fos* expression studies mentioned above. Our results demonstrate that, unlike the CeA–CRF expression, opiate withdrawal similarly increases PVN–CRF expression in wild-type and CRF$_2^{-/-}$ mice (Figures 4b and c). Accordingly, CRF$_2$ receptor-deficiency does not affect plasma corticosterone levels (Supplementary Figure 3), indicating preserved HPA axis and CRF$_1$ receptor activity[23,24] in opiate-withdrawn CRF$_2^{-/-}$ mice. However, despite the very elevated plasma corticosterone levels (47–65 µg 100 ml^{-1}), opiate-withdrawn mice do not differ from control mice, most probably due to a ceiling effect produced by the stressful 30-min confinement to the CPA apparatus. LC–NE activity is examined by assessing the expression of the TH enzyme, the rate-limiting NE synthesis

enzyme.[40] Similar to the PVN–CRF expression, we find that opiate withdrawal similarly increases LC–TH expression in wild-type and CRF$_2^{-/-}$ mice (Figures 5a and b). Thus, here we show increased PVN–CRF and LC–TH activity in mice (CRF$_2^{-/-}$) lacking the somatic signs and the negative affective-like states of opiate withdrawal. These results strongly suggest that activation of PVN–CRF and LC–NE circuitry does not contribute to the malaise of opiate withdrawal. Adaptive stress responses also include time-limited metabolic changes, such as decreased food intake and increased catabolism, which aim to maximize stress coping.[7,8] Thus, we investigate the impact of CRF$_2$ receptor deficiency upon SCF intake, BW and CE, an index of energy balance status, induced by the stress of intermittent morphine injections and the associated repeated cycles of 12-h opiate withdrawal. Furthermore, to examine the role for the CRF$_2$ receptor pathway in the post-stress recovery of metabolic functions, SCF intake, BW and CE are monitored up to 96 h after the last saline or morphine injection. Before the beginning of saline or morphine injections, wild-type and CRF$_2^{-/-}$ mice display similar daily SCF intake and BW (Supplementary Table 3). The intermittent morphine injections and their cessation induce a genotype-independent decrease and
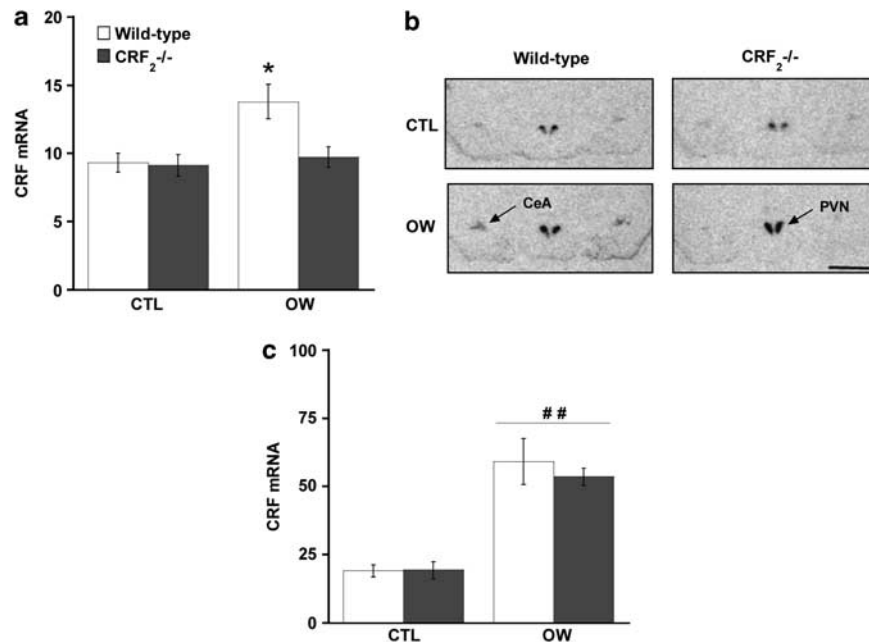
Figure 4 Dissociation of brain corticotropin-releasing factor (CRF) responses to opiate withdrawal in CRF$_2$ receptor-deficient mice. (**a**) Mean (± s.e.m.) CRF mRNA levels in the central nucleus of the amygdala (CeA; bregma interval: −0.82/−1.06 mm) in control (CTL) or opiate-withdrawn (OW) wild-type and CRF$_2^{-/-}$ mice (genotype × opiate withdrawal interaction effect: F$_{1,31}$ = 4.7, $P < 0.05$; *$P < 0.01$ versus OW CRF$_2^{-/-}$, CTL wild-type and CTL CRF$_2^{-/-}$ mice, Newman–Keuls *post hoc* test). Results are expressed as optical density (OD; × 1000). $N = 8$–10 per group. (**b**) Representative images of brain sections illustrating CRF expression in the CeA and in the paraventricular nucleus of the hypothalamus (PVN) in CTL or OW wild-type and CRF$_2^{-/-}$ mice. Scale bar = 2 mm. (**c**) Mean (± s.e.m.) CRF mRNA levels in the PVN (bregma interval: −0.82/−1.06 mm) in CTL or OW wild-type and CRF$_2^{-/-}$ mice (opiate withdrawal main effect: F$_{1,33}$ = 60.0, $P < 0.0001$; ##$P < 0.0005$ versus CTL mice, Newman–Keuls *post hoc* test; genotype × opiate withdrawal interaction effect: F$_{1,33}$ = 0.4, P = nonsignificant). Results are expressed as OD (× 1000). $N = 8$–10 per group. Noteworthy, in CRF$_2^{-/-}$ mice, opiate withdrawal increases the expression of CRF in the PVN but not in the CeA.

a rebound increase in SCF intake, respectively (Supplementary Figure 4a). Accordingly, CRF$_2$ receptor-deficiency does not affect BW loss and recovery induced by intermittent morphine injections and by their cessation, respectively (Supplementary Figure 4b). Finally, CRF$_2$ receptor-deficiency does not affect CE decrease and increase induced by intermittent morphine injections and by their cessation, respectively (Supplementary Figures 5b and c). Overall, our findings indicate that CRF$_2$ receptor-deficiency does not affect either the intensity or the duration of brain, neuroendocrine and autonomic stress-coping responses to opiate withdrawal.

Discussion

The main finding of the present study is that genetic disruption of the CRF$_2$ receptor pathway completely eliminates the negative affective-like and the somatic components of opiate withdrawal distress. Moreover, unlike wild-type mice, opiate-withdrawn CRF$_2$ receptor-deficient mice do not show any increase in DYN, CRF and *c-fos* expression in the NaccSH, the CeA and the PAG, respectively, that is, brain circuitry thought to underlie drug withdrawal distress.

Nevertheless, CRF$_2$ receptor-deficiency does not influence PVN–CRF/HPA axis, LC–TH and autonomic responses essential to cope with the stressful condition of opiate withdrawal.

Despite extensive research, the neural mechanisms underlying opiate withdrawal signs and symptoms remain largely unknown. Using clinically relevant mouse models of spontaneous drug withdrawal, here we report an essential role for the CRF$_2$ receptor pathway in the dysphoria-like and the anhedonia-like states of opiate withdrawal. To measure the dysphoric consequences of opiate withdrawal we use the CPA paradigm. In the latter behavioral test, avoidance of environmental cues paired with opiate withdrawal or other stressors is taken as a reliable measure of dysphoria-like states.[27,41] Our results demonstrate that, unlike the wild-type mice, CRF$_2^{-/-}$ mice do not avoid the environmental cues paired with opiate withdrawal, indicating the absence of dysphoria-like states. Furthermore, using a food-preference test designed to assess stress-induced hedonic changes,[35] we report that opiate-withdrawn CRF$_2^{-/-}$ mice do not show any reduction in HPF preference, indicating the absence of anhedonia-like states. The present results contrast with the increased anxiety-like responses to stressors other than opiate withdrawal previously
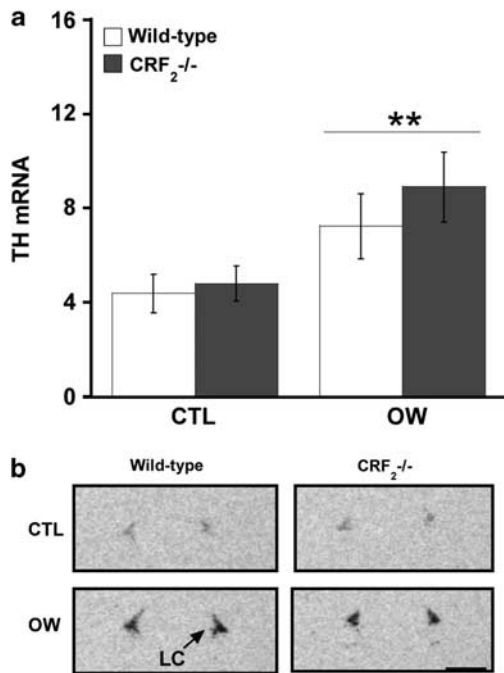
Figure 5 Preserved brain stress-coping responses in opiate-withdrawn CRF$_2$ receptor-deficient mice. (**a**) Mean (± s.e.m.) tyrosine hydroxylase (TH) mRNA levels in the locus coeruleus (LC; bregma interval: −5.40/−5.52 mm) in control (CTL) or opiate-withdrawn (OW) wild-type and CRF$_2^{-/-}$ mice (opiate withdrawal main effect: F$_{1,30}$ = 8.5, P < 0.01; **P < 0.01 versus CTL mice, Newman–Keuls *post-hoc* test; genotype × opiate withdrawal interaction effect: F$_{1,30}$ = 0.3, P = nonsignificant). Results are expressed as optical density (× 1000). N = 8–9 per group. (**b**) Representative images of brain sections illustrating TH expression in the LC in CTL or OW wild-type and CRF$_2^{-/-}$ mice. Scale bar = 1 mm. Abbreviation: CRF, corticotropin-releasing factor.

observed in CRF$_2$ receptor-deficient mice.[20,22] Thus, our findings put forward the original notion of a stressor-dependent role for the CRF$_2$ receptor pathway in negatively valenced affective-like states. In line with the behavioral findings, here we also show that CRF$_2$ receptor-deficiency eliminates the brain substrates of opiate withdrawal distress. In particular, we demonstrate that opiate withdrawal increases NaccSH–DYN expression in the wild-type mice. Increased DYN neurotransmission is suggested to underlie negative affective-like responses to stressors. For instance, conditioned aversions for places paired with foot-shock, intracerebroventricular injection of CRF or intraperitoneal injection of U-50488H are abolished by the systemic or nucleus accumbens administration of the KOR antagonist nor-binaltorphimine and by prodynorphin-deficiency.[41,42] Upregulated DYN/KOR system activity also decreases dopamine release within the nucleus accumbens,[36] a brain mechanism implicated in dysphoria-like states. Nucleus accumbens dopamine circuitry also mediate the hedonic properties of drugs of abuse[4] and natural

rewards such as palatable food.[37] Thus, the increased NaccSH–DYN expression may underlie the dysphoria-like and the anhedonia-like states observed herein in opiate-withdrawn wild-type mice. In contrast with the wild-type mice, opiate withdrawal does not influence the NaccSH–DYN expression in the CRF$_2^{-/-}$ mice, indicating a fundamental role for the CRF$_2$ receptor pathway in the brain mechanisms underlying the dysphoria-like and the anhedonia-like states of opiate withdrawal.

We also report that CRF$_2$ receptor-deficiency eliminates the opiate withdrawal-induced neuronal activity, as measured by *c-fos* expression, in the PAG. Systemic administration of naloxone elevates *c-fos* mRNA levels in the PAG in rats implanted with morphine pellets.[28] Moreover, injection of the hydrophilic poorly diffusing opioid receptor antagonist methylnaloxonium into the PAG precipitates somatic signs of opiate withdrawal and produces CPA in rats implanted with morphine pellets.[39] However, to our knowledge, no studies have yet assessed the role for the CRF system in PAG neuronal activity induced by opiate withdrawal. Here, we show that opiate withdrawal reliably increases PAG–*c-fos* expression in wild-type mice. Nevertheless, opiate withdrawal does not produce any increase in PAG–*c-fos* expression in CRF$_2^{-/-}$ mice, indicating that opiate withdrawal-induced activity of PAG is under the control of the CRF$_2$ receptor pathway. We also demonstrate that CRF$_2$ receptor-deficiency abolishes the opiate withdrawal-induced increase in CeA–CRF expression. CeA–CRF circuitry might mediate anxiety-like and substance-seeking behavior induced by cocaine or ethanol withdrawal. For instance, the administration of nonspecific CRF receptor antagonists into the CeA reverses the negative affective-like states and the enhanced ethanol self-administration induced by morphine or ethanol withdrawal in rats.[13,14,43] Accordingly, ethanol withdrawal enhances CRF release in the amygdala.[16] However, no prior studies have addressed the relative role for each of the two known CRF receptor pathways in opiate withdrawal-induced CeA–CRF expression. Here, we demonstrate that SPO OW increases CeA–CRF expression in wild-type mice. In contrast, CRF$_2$ receptor-deficiency completely eliminates the increased CRF mRNA levels induced by opiate withdrawal in the CeA, suggesting a presynaptic positive regulation of CRF synthesis and release by the CRF$_2$ receptor pathway. Another study demonstrates an essential role for CeA–CRF receptor pathways in gamma-aminobutyric acid release induced by ethanol dependence.[44] Thus, although further work is needed, the latter evidence points out to a presynaptic control of synthesis and release of neurotransmitters relevant to substance dependence and withdrawal by CRF receptor pathways. Thus, the lack of upregulated CeA–CRF expression observed herein in opiate-withdrawn CRF$_2^{-/-}$ mice may contribute to the absence of negative affective-like states, despite preserved CRF$_1$ receptor activity. Overall, the present results indicate

a cardinal role for the CRF₂ receptor pathway in the brain molecular changes thought to mediate the negative affective-like and the somatic components of opiate withdrawal distress. However, further work using more advanced genetic and/or pharmacological tools might be needed to confirm a direct link between the behavioral and the brain gene expression profiles displayed by opiate-withdrawn CRF₂ receptor-deficient mice in the present study. Moreover, as we use a genetic mouse model bearing a whole-body CRF₂ receptor-deficiency, the present study does not allow discerning the relative contribution of peripheral, versus central, CRF₂ receptor pathways to opiate withdrawal distress. Nevertheless, the findings of a recent rat study showing that activation of peripheral CRF₂ receptor pathways by urocortin 2 does not produce any sign of malaise, as assessed by the conditioned taste aversion paradigm,[45] suggest that negatively valenced affective-like states are mainly mediated by central CRF₂ receptor pathways.

The opiate withdrawal syndrome is an extremely stressful clinical condition. Effective coping with stressors requires functional stress-responsive systems. CRF and NE pathways originating from the PVN and the LC, respectively, widely project to the neuraxis and coordinate brain, neuroendocrine and autonomic responses essential to cope with stressors.[7–10] For instance, activation of PVN–CRF, HPA axis and LC–NE pathways facilitates arousal, vigilance and attention, stimulates the sympathetic nervous system and inhibits parasympathetic outflow, eating behavior and sleeping, thus favoring adaptation to intrinsic (that is, hypoglycemia and decreased blood pressure) or extrinsic (that is, environmental threat) stressors.[7,8,10] In the present study, we report that CRF₂ receptor-deficiency does not impair the increased PVN–CRF expression induced by the stress of opiate withdrawal. The unaltered plasma corticosterone levels observed in the CRF₂⁻/⁻ mice might contribute, at least in part, to the opiate withdrawal-induced PVN–CRF expression. Indeed, corticosterone deficiency produces an aberrant decrease in PVN–CRF responses to opiate withdrawal, which are 'normalized' by restoring nadir plasma corticosterone levels.[26] Furthermore, here we show that CRF₂ receptor-deficiency does not influence the opiate withdrawal-induced expression of LC–TH, a key coordinator of sympathetic nervous system responses to stressors.[10] We also report on the role for the CRF₂ receptor pathway in metabolic responses to intermittent injections of increasing morphine doses and to their cessation. Noteworthy, the intermittent morphine injections procedure used herein serves as a severe stressor, as it produces repeated cycles of 12-h opiate withdrawal. Our results demonstrate that, similar to wild-type mice, CRF₂⁻/⁻ mice show decreased food intake and BW during the 12-h opiate withdrawal periods occurring between successive morphine injections and display a negative energy balance status, as revealed by decreased

CE. The increased PVN–CRF and LC–TH expression may underlie the decreased food intake, the BW loss and the reduced CE displayed by opiate-withdrawn wild-type and CRF₂⁻/⁻ mice. Indeed, injection of CRF into the PVN powerfully decreases food intake in rats.[46] Furthermore, activation of PVN–CRF, HPA axis and LC–NE systems leads to primarily catabolic effects and mobilization of energy resources toward the brain, heart and skeletal muscles to cope with stressors. Most importantly, in the context of an adequate stress response, such a stress-related metabolic shift toward a generalized catabolic state promptly reverses upon retraction of the stressor. With regard to the latter point, here we show that cessation of the morphine injections is followed by a rapid BW recovery, increased food intake and a positive energy balance status, indicating self-restrained adequate stress-coping responses to opiate withdrawal. Noteworthy, the temporal dynamics of the latter responses is similar in wild-type and CRF₂⁻/⁻ mice, demonstrating that CRF₂ receptor-deficiency does not influence either the intensity or the duration of metabolic responses to the stress of opiate withdrawal. Thus, the present results of increased PVN–CRF and LC–TH expression in opiate-withdrawn CRF₂⁻/⁻ mice, together with a time-limited stressor-dependent catabolic state, strongly indicate that disruption of the CRF₂ receptor pathway spares the ability to cope with the extremely stressful condition of opiate withdrawal.

Studies suggest that brain CRF–NE feed-forward loops might be relevant to adaptive or maladaptive stress responses. For instance, both the CeA and the PVN are major sources of CRF neurons projecting to the LC.[10] Moreover, CRF mediates stress-induced LC–TH immunoreactivity,[47] increases the discharge rate of LC–NE neurons[48] and stimulates NE release in LC terminal regions.[48,49] On the other hand, stimulation of NE receptors increases CRF release in the amygdala and in the hypothalamus.[50] Here, we demonstrate that CRF₂ receptor-deficiency abolishes CeA–CRF but does not affect PVN–CRF and LC–TH responses to the stress of opiate withdrawal. These findings provide initial evidence of a CRF₂ receptor-dependent clear-cut dissociation in the activity of brain systems underlying key features of drug withdrawal and stress-coping abilities.

In conclusion, the present study demonstrates that CRF₂ receptor-deficiency completely abolishes major negative affective-like and somatic components of opiate withdrawal distress, yet sparing brain, neuroendocrine and autonomic responses essential for stress coping. Escape from opiate withdrawal distress is a powerful drive to addictive behavior. Thus, our findings bear important clinical implications for the treatment of opiate addiction and withdrawal: peripherally administered blood–brain barrier penetrating CRF₂ receptor antagonists might alleviate opiate withdrawal distress without altering stress-coping abilities, thus facilitating opiate abstinence in addicted individuals.

Conflict of interest

Acknowledgments

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Supplementary Information accompanies the paper on the Molecular Psychiatry website (http://www.nature.com/mp)

Supplementary Figure 1
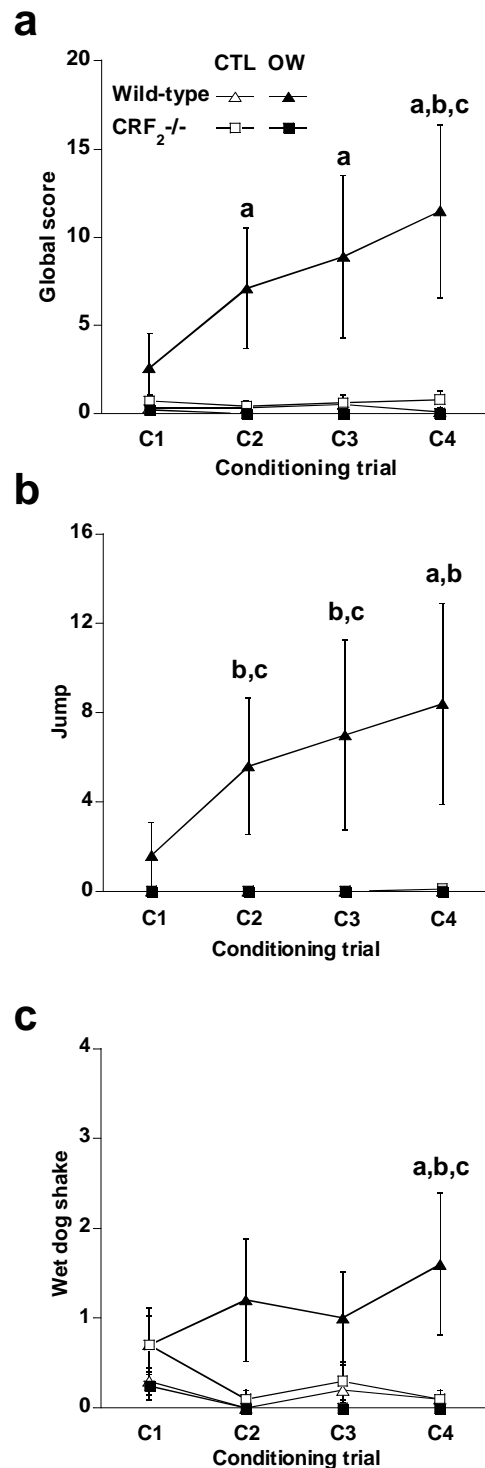
Supplementary Fig. 1 Absence of somatic opiate withdrawal signs in CRF_2 receptor-deficient mice. Mean (± s.e.m.) number of **(a)** global somatic opiate withdrawal scores, **(b)** jumps and **(c)** wet dog shakes displayed by control (CTL) or opiate-withdrawn (OW) wild-type and CRF_2-/- mice during the four 30-min place conditioning trials (C1 to C4) of the spontaneous opiate withdrawal (SPO OW) CPA experiment. [a]$P<0.05$, [b]$P<0.05$ and [c]$P<0.05$ versus OW CRF_2-/-, CTL wild-type and CTL CRF_2-/- mice, respectively, at the same time point. Pairwise comparisons, non-parametric Mann-Whitney U test. N= 9-10/group.

Supplementary Figure 2
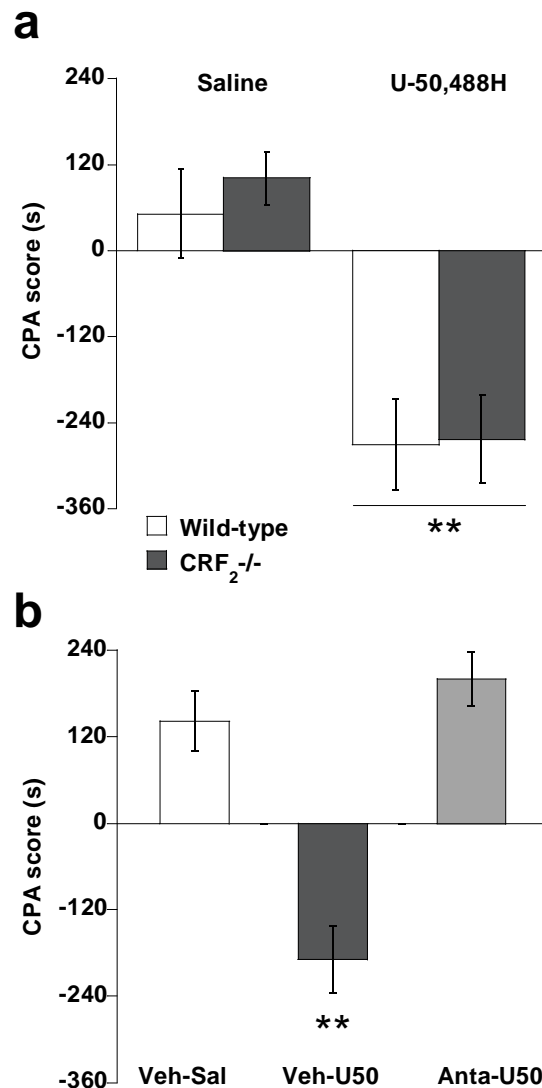
Supplementary Fig. 2 CRF$_2$ receptor-deficiency does not impair the ability to acquire and express conditioned place aversion (CPA). **(a)** Mean ($\pm$ s.e.m.) CPA scores (s) displayed by saline- or U-50,488H (5 mg/kg, s.c.)-treated wild-type and CRF$_2$-/- mice (U-50,488H main effect: $F_{1,22}$= 38.3, P<0.0001; **P<0.0005 versus saline-treated mice, Newman-Keuls post-hoc test). N= 5-8/group. **(b)** Mean ($\pm$ s.e.m.) CPA scores (s) displayed by CRF$_2$-/- mice treated with saline (Sal) or U-50,488H (5 mg/kg, s.c.; U50) immediately prior to the conditioning trials, and with antalarmin (20 mg/kg, *per os*; Anta) or the appropriate vehicle (Veh) one hour prior to the conditioning trials (group main effect: $F_{2,13}$=24.1, P<0.0001; **P<0.0005 versus Veh-Sal and Anta-U50, Newman-Keuls post-hoc test). N= 4-7/group. For each mouse, a CPA score is calculated as the post- minus the pre-conditioning time spent in the conditioning-paired compartment of the CPA apparatus.
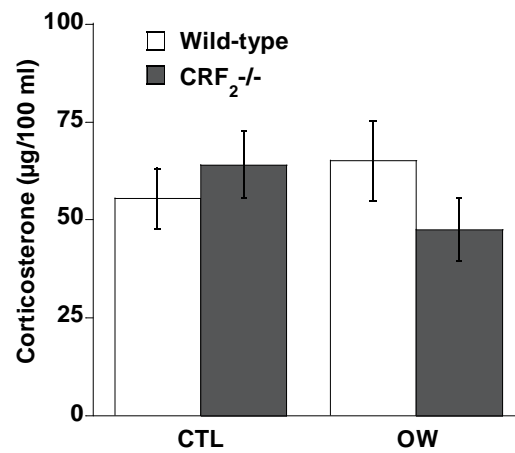
Supplementary Fig. 3 Preserved hypothalamus-pituitary-adrenal (HPA) axis activity in opiate-withdrawn CRF_2 receptor-deficient mice. Mean ($\pm$ s.e.m.) plasma corticosterone levels displayed by control (CTL) or opiate-withdrawn (OW) wild-type and CRF_2-/- mice, as measured immediately after the end of the fourth and last 30-min place conditioning trial of the spontaneous opiate withdrawal (SPO OW) conditioned place aversion (CPA) experiment (genotype effect: $F_{1,35}= 0.3$, P= n.s.; opiate withdrawal effect: $F_{1,35}= 0.1$, P= n.s.; genotype x opiate withdrawal effect: $F_{1,35}= 2.2$, P= n.s.). N= 9-10/group.

Supplementary Figure 4
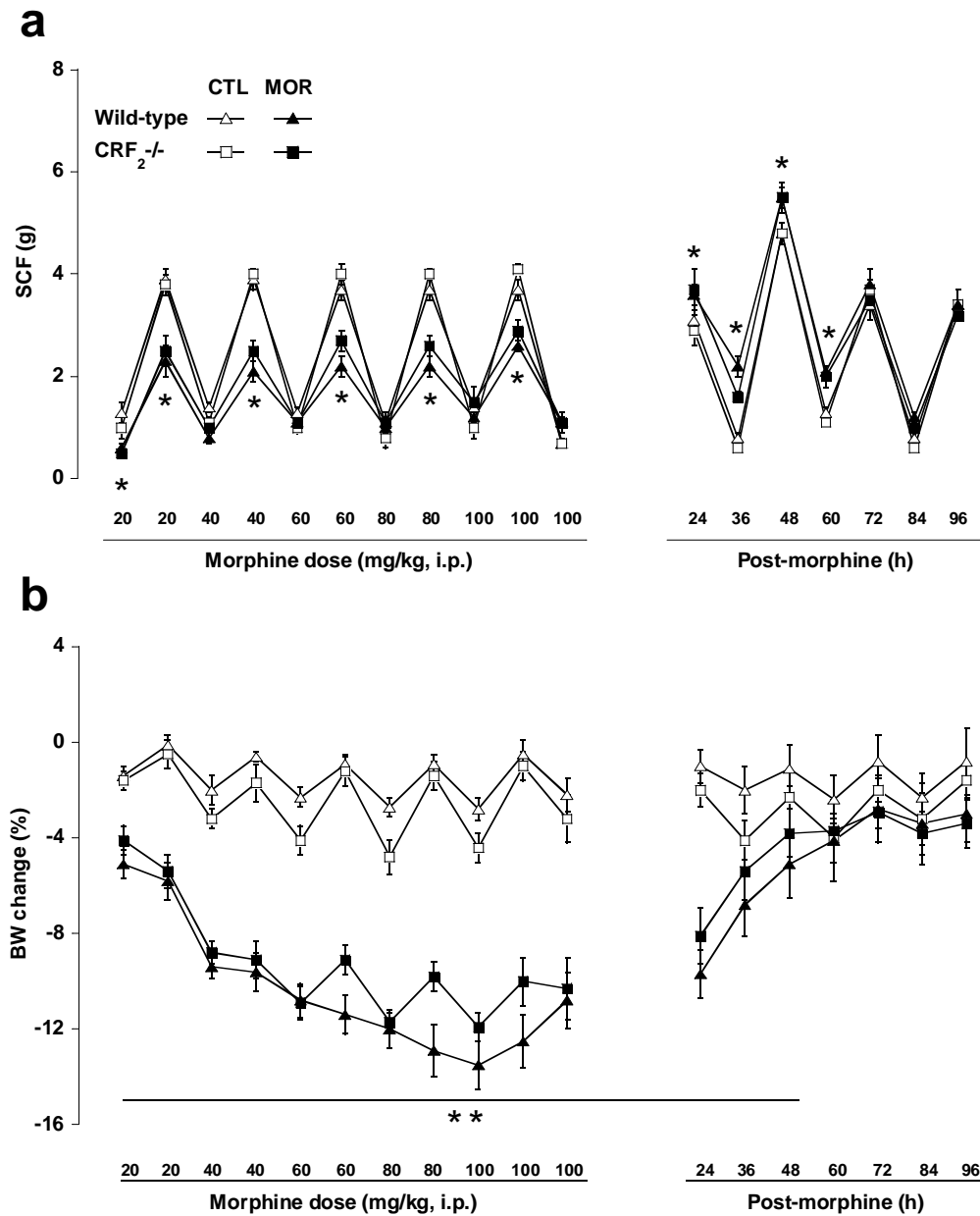
Supplementary Fig. 4 CRF_2 receptor-deficiency does not impair metabolic responses to the stress of intermittent morphine injections and to its cessation. Mean ($\pm$ s.e.m.) **(a)** 12-h home-cage standard chow food (SCF) intake (g) and **(b)** body weight (BW) changes, calculated as percentage of BW recorded just prior to the first injection, displayed by wild-type and CRF_2-/- mice during and 24 to 96 hours after intermittent saline (CTL) or morphine (MOR) injections. Noteworthy, the intermittent morphine injections procedure used herein serves as a severe stressor since it produces repeated cycles of 12-hour opiate withdrawal. The morphine injections induce a genotype-independent decrease in SCF intake (morphine x repeated measures interaction effect: $F_{10,300}= 25.7$, $P<0.0001$; *$P<0.005$ versus CTL mice at the same time point, Newman-Keuls post-hoc test; genotype x morphine x repeated measures interaction effect: $F_{10,300}= 0.5$, $P=$ n.s.). However, cessation of morphine injections produces a prompt genotype-independent increase in SCF intake that lasts at least 60 hours (morphine x repeated measures interaction effect: $F_{6,180}= 6.3$, $P<0.0001$; *$P<0.005$ versus CTL mice at the same time point, Newman-Keuls post-hoc test; genotype x morphine x repeated measures interaction effect: $F_{6,180}= 0.7$, $P=$ n.s.). The morphine injections also induce a genotype-independent BW loss which is fully recovered by 60 hours after their cessation (morphine x repeated measures interaction effect: $F_{17,510}= 30.5$, $P<0.0001$; **$P<0.001$ versus CTL mice at the same time point, Newman-Keuls post-hoc test; genotype x morphine x repeated measures interaction effect: $F_{17,510}= 0.7$, $P=$ n.s.). N= 8-9/group.

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Supplementary Figure 5
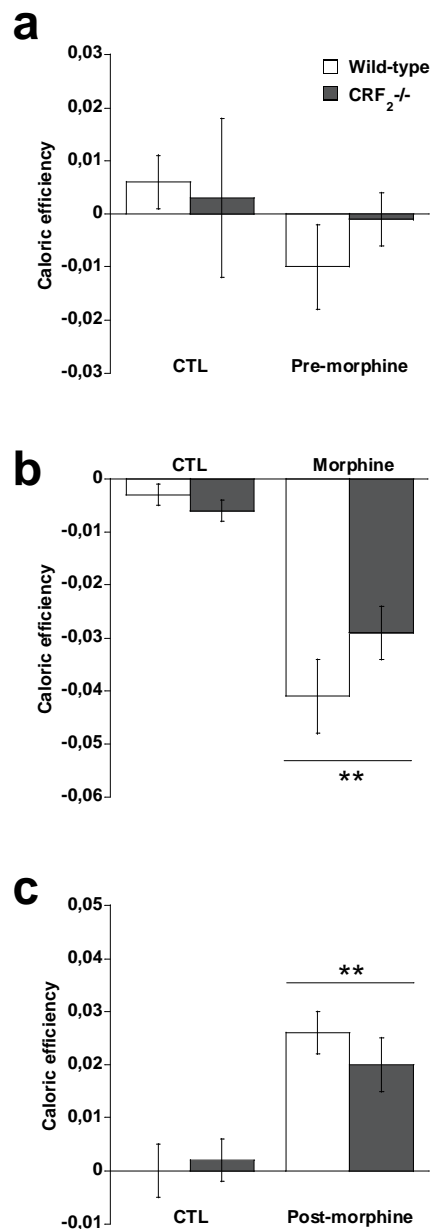
Supplementary Fig. 5 CRF$_2$ receptor-deficiency does not influence energy balance responses to the stress of intermittent morphine injections and to its cessation. Mean ($\pm$ s.e.m.) caloric efficiency (CE), calculated as grams of body weight gained/kilocalories ingested, displayed by wild-type and CRF$_2$-/- mice **(a)** prior to, **(b)** during and **(c)** 24 to 96 hours after intermittent saline (CTL) or morphine injections. The intermittent morphine injections procedure used herein serves as a severe stressor since it produces repeated cycles of 12-hour opiate withdrawal. Prior to saline or morphine injections, wild-type and CRF$_2$-/- mice display similar CE levels. The morphine injections induce a genotype-independent decrease in CE (morphine main effect: $F_{1,30}= 46.1$, $P<0.0001$, **$P<0.0005$ versus CTL mice, Newman-Keuls post-hoc test; genotype x morphine interaction effect: $F_{1,30}= 2.7$, $P=$ n.s.). In contrast, 24 to 96 hours after the last saline or morphine injection there is a genotype-independent increase in CE (morphine main effect: $F_{1,30}= 25.2$, $P<0.0001$, **$P<0.0005$ versus CTL mice, Newman-Keuls post-hoc test; genotype x morphine interaction effect: $F_{1,30}= 1.0$, $P=$ n.s.). N= 8-9/group.

Supplementary Table 1 CRF_2 receptor-deficiency does not affect unconditioned place preferences. Mean (± s.e.m.) seconds spent in the preferred compartment of the conditioned place aversion (CPA) apparatus by wild-type and CRF_2-/- mice during the 20-min pre-conditioning test of the spontaneous (SPO), the naloxone-precipitated (NAL) opiate withdrawal (OW) and the U-50,488H (U-50) CPA experiments. In the SPO OW and the NAL OW experiments, over a 6-day time period the mice are injected every 12 hours with saline (CTL) or increasing morphine doses (20-100 mg/kg, i.p.; MOR). In the SPO OW experiment, place conditioning trials are carried out eight hours after saline or morphine injection (n= 9-10/group). In the NAL OW experiment, place conditioning trials are carried out immediately after naloxone dosing (0.23 mg/kg, s.c.), two hours after saline or morphine injection (n= 6-12/group). In the U-50,488H CPA experiment, place conditioning trials are carried out immediately after saline (CTL) or U-50,488H (5 mg/kg, s.c.) dosing (n= 5-8/group).

		SPO OW	NAL OW	U-50,488H	
Genotype	*Group*	*Time (s)*	*Time (s)*	*Group*	*Time (s)*
Wild-type	CTL	731 ± 28	682 ± 24	CTL	759 ± 55
	MOR	737 ± 27	711 ± 23	U-50	836 ± 50
CRF_2-/-	CTL	742 ± 40	726 ± 23	CTL	760 ± 49
	MOR	747 ± 26	693 ± 24	U-50	808 ± 48

Supplementary Table 2 Absence of somatic opiate withdrawal signs in CRF_2 receptor-deficient mice. Number of control (CTL) or opiate-withdrawn (OW) wild-type and CRF_2-/- mice showing the diarrhea sign during the four conditioning trials of the spontaneous opiate withdrawal (SPO OW) conditioned place aversion (CPA) experiment. N= 9-10/group.

Genotype	Group	Conditioning trial			
		1st	2nd	3rd	4th
Wild-type	CTL	0	1	1	0
	OW	1	1	3	$5^{a,b}$
CRF_2-/-	CTL	0	1	1	2
	OW	0	0	0	0

[a]$P<0.05$ and [b]$P<0.01$ versus OW CRF_2-/- and CTL wild-type mice, respectively, during the fourth conditioning trial. Two x 2 table chi-square test.

Supplementary Table 3 CRF_2 receptor-deficiency does not affect daily food intake and body weight (BW). Mean (± s.e.m.) standard chow food (SCF) ingested during the 12-h light (SCF-L) or the 12-h dark (SCF-D) phase of the light/dark housing cycle and BW displayed by wild-type and CRF_2-/- mice prior to intermittent saline (CTL) or morphine (MOR; 20-100 mg/kg, i.p.) injections. SCF values represent the average of three days. BW values are those recorded immediately prior to the first saline or morphine injection. N= 8-9/group.

Genotype	Group	SCF-L (g)	SCF-D (g)	BW (g)
Wild-type	CTL	0.8 ± 0.1	4.1 ± 0.2	27 ± 0.9
	MOR	0.8 ± 0.1	4.1 ± 0.2	28 ± 0.5
CRF_2-/-	CTL	0.7 ± 0.1	3.9 ± 0.1	28 ± 1.1
	MOR	0.8 ± 0.1	4.2 ± 0.2	27 ± 1.0

Chapter III

Article 2 : Augmentation de la motivation à la nourriture chez des souris lors du sevrage aux opiacés

RESUMÉ

Chez les individus dépendants à la drogue, la motivation est dirigée exclusivement vers les activités liées à l'obtention de cette drogue au dépend des autres plaisirs de la vie. Plusieurs observations dans le champ clinique montrent clairement des désordres motivationnels vis-à-vis des récompenses naturelles chez les individus dépendants à la drogue. Cependant, à l'heure actuelle l'impact réel d'un sevrage aux opiacés sur la motivation reste peu identifié.

L'objectif de ce *chapitre III* était de mettre en place un modèle murin des troubles motivationnels liés au sevrage aux opiacés (SAO). A cet effet, des souris ont été entrainées dans une procédure de *« tâche opérante dirigée vers l'obtention d'une nourriture hautement palatable »*. Par la suite, une dépendance a été induite par des doses croissantes et intermittentes de morphine tout en maintenant le comportement opérant dirigé vers l'obtention de la nourriture.

Nos résultats montraient que le sevrage précoce, c'est-à-dire 8h après une dose de morphine, n'affectait pas la motivation envers la nourriture. Cependant, le sevrage tardif, c'est-à-dire 32 hr après la dernière dose de morphine, provoquait une augmentation drastique du comportement opérant dirigé vers la nourriture. Cette hyper motivation était observée tout au long des 12 jours de sevrage qui ont suivi la dernière administration de morphine. Par ailleurs, une procédure de renversement de la tâche opérante a été appliquée afin d'examiner d'éventuelles détériorations cognitives induites par le sevrage. Nos résultats montraient clairement que l'application d'une nouvelle tâche opérante n'affectait pas les capacités de réapprentissage des souris sevrées à la morphine. A l'opposé, le sevrage à la morphine améliorait de façon claire le réapprentissage à une nouvelle tâche opérante. Finalement, nos données montraient que l'hyper motivation à la nourriture est totalement indépendante des altérations de l'activité ambulatoire ou du poids corporel causées par le traitement à la morphine.

En conclusion ce modèle murin représente un outil nouveau pour l'étude des mécanismes neuronaux impliqués dans les désordres motivationnels observés durant le SAO.

ORIGINAL INVESTIGATION

Increased motivation to eat in opiate-withdrawn mice

Khalil Rouibi · Angelo Contarino

Abstract

Rationale In drug-dependent individuals, the primary excessive motivation is for drugs. Studies also indicate altered interest for "natural" rewarding activities associated with motivational disorders that may be relevant to drug dependence. However, to date, the impact of drug dependence and withdrawal upon motivation for "natural" rewards remains unclear.

Methods and objectives In the present study, we use a food-driven operant behavior paradigm to assess the impact of opiate intake and withdrawal upon the motivational properties of highly palatable food (HPF) in mice.

Results Our findings indicate that early (8-h) opiate withdrawal does not affect either the motivational or the discriminative properties of HPF intake. However, starting 32 h after the last morphine injection, opiate withdrawal increases operant behavior aimed at obtaining HPF. The increased HPF-driven behavior lasts at least 12 days following opiate withdrawal, indicating long-lasting effects upon motivation. Using a paradigm of reward contingency reversal, we also address the impact of opiate withdrawal upon cognitive functions. Our results indicate that opiate withdrawal does not affect the ability to learn a new operant rule to obtain HPF. Indeed, opiate withdrawal ameliorates the acquisition of the new HPF-driven operant task, most probably due to the persistent and long-lasting increased motivation. Finally, analysis of ambulatory activity and body weight (BW) changes reveal that motivational and cognitive effects are totally independent of caloric and/or motor effects of opiate dosing and withdrawal.

Conclusions These results clearly demonstrate that excessive opiate intake and withdrawal produces dramatic and long-lasting motivational disorders relevant to drug dependence.

Keywords Drug dependence · Opiate withdrawal · Motivation · Cognition · Mice

Abbreviations

ANOVA	Analysis of variance
B	Baseline
BP	Breakpoint
BW	Body weight
CRF	Corticotropin-releasing factor
CTL	Control
DI	Discrimination index
FR	Fixed ratio
HPF	Highly palatable food
i.p.	Intraperitoneally
OW	Opiate withdrawal
PR	Progressive ratio

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Introduction

Opiate dependence is a major health problem across the world (www.who.int/substance_abuse). Studies also

indicate that the incidence rate of opiate dependence may dramatically increase in the next years (Compton and Volkow 2006). In opiate-dependent individuals, heroin "highs" are followed by a severe opiate withdrawal syndrome composed of severe influenza-like somatic signs and symptoms and negative affective states, such as dysphoria, depressed mood, and anhedonia (APA 2000). The opiate withdrawal syndrome is central to opiate dependence (APA 2000; O'Brien 1996). Notably, escape from the extremely stressful opiate withdrawal signs and symptoms powerfully motivates drug-seeking and drug-taking behavior, thus strongly contributing to the perpetuation of opiate dependence (Schulteis and Koob 1996).

In drug-dependent individuals, the primary excessive motivation is for drugs (Koob and Le Moal 2001; Negus 2006; Robinson and Berridge 2003). Studies also indicate profound alterations in the motivation for "natural" rewards, such as food or sex. However, either clinical evidence or experimental studies provide discordant findings about the motivational disorders associated with drug dependence and withdrawal. For instance, human studies show decreased responsiveness to "natural" 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). In contrast, other studies report heightened preference for and/or consumption of liquid or solid sweets, elevated desire (craving) for sweet foods, and unaltered nutritional status in opiate-dependent individuals (Morabia et al. 1989; Weiss 1982). Severe motivational disorders are also observed in laboratory animals exposed to excessive opiate intake and withdrawal. In particular, morphine-withdrawn rats show lower levels of operant behavior for food than drug-naïve control rats (Harris and Aston-Jones 2003; Zhang et al. 2007). Moreover, "precipitation" of opiate withdrawal by relatively small amounts of the opioid receptor antagonist naloxone suppresses food-driven operant behavior in rats implanted with morphine pellets (Schulteis et al. 1994). On the other hand, a more recent study report increased operant behavior for food in rats undergoing early (~23-h) opiate withdrawal (Cooper et al. 2010). However, in the latter study, food-driven operant behavior may be due to the remarkable energy deficiency associated with intermittent morphine dosing and early opiate withdrawal phases.

The aim of the present study is to investigate the motivational effects of opiate dependence and withdrawal. For this purpose, highly palatable food (HPF)-driven operant behaviour is assessed in mice before, during, and following cessation of morphine dosing. HPF is used to facilitate the acquisition of food-driven operant behaviour since the mice are never food-deprived and tested during the light phase of the 12-h light/dark cycle, i.e., when food intake is relatively low. Notably, the motivational effects of opiate withdrawal are assessed up to 12 days after cessation of morphine

dosing, i.e., during prolonged opiate withdrawal phases. To avoid interference with the establishment of food-motivated behaviour, drug treatment is initiated after the acquisition of the operant task. Moreover, to mimic the clinical setting, the mice are treated with intermittent escalating doses of morphine and motivational changes assessed following body clearance of the drug, i.e., under spontaneous opiate withdrawal conditions. Finally, a paradigm of reward contingency reversal is also used to assess the effect of opiate withdrawal upon cognitive functions relevant to operant behaviour.

Materials and methods

Subjects

A total of 16 naïve female CD-1 mice (Charles River, L'Arbresle, France) is used. The mice are housed in groups of four and kept on a 12-h light/dark cycle (lights on from 8 a.m. to 8 p.m.) in a colony room maintained under standard laboratory conditions (relative humidity 50–60%, temperature $22\pm1°C$). Standard laboratory food (SAFE, Augy, France; 3.3 kcal/g; 72.5%, 19.3%, and 8.2% of kilocalorie from carbohydrates, protein, and fat, respectively) and water are available ad libitum. The mice are 14-week-old at the beginning of the experiment.

Experimental design

Starting 1 week prior to the beginning of the operant behaviour training, on alternate days, each mouse is handled for 1 min for a total of three times. Operant behaviour is assessed using 16 operant behaviour chambers. Each operant chamber (length 22 cm, width 14 cm, height 20 cm) is equipped with light sources for testing under dim light conditions 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 of the chamber, 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, results in food pellet delivery whereas nose-poking into the other hole, i.e., the inactive hole, has no consequences. Centred between the nose-poke holes is a feeding-trough for the delivery of food pellets. A food pellet delivery occurs when the photo-beam of the active nose-poke hole is interrupted for at least 500 ms. We use 20-mg HPF pellets (5-TUL; energetic value 3.44 kcal/g, 66.7%, 20.6%, and 12.7% of kilocalorie from carbohydrates, protein, and fat, respectively; PMI Nutrition International, LLC, St. Louis, MO, USA). The food pellets are delivered by an automated dispenser situated outside the operant chamber into a food trough situated 3 cm from the nose-poke holes and 2 cm from the grid floor. The food trough

is equipped with photo-beams that allow monitoring of food trough visits. An additional food pellet is not delivered until a food trough visit (removal of the previously delivered food pellet), thereby allowing resolution of food-directed behaviour at the unit of an individual food pellet. The wire grid floor of the cage allow the passage of uneaten food pellets to a sliding drawer, making storage impossible and allowing evaluation of food spillage. Each operant chamber is also equipped with two series of photo-beams that serve to record horizontal and vertical ambulatory activity. Starting on the day after the last handling session, at 4 p.m., the mice are daily confined to the operant behaviour chambers for a 2-h test. To avoid confounding factors linked to food restriction, the mice are always provided with food ad libitum. Half of the mice ($n=8$) are assigned the left and the other half to the right nose-poke hole as the active hole. A fixed ratio (FR)-1 reinforcement schedule is initially applied for three consecutive days, i.e., one nose-poke results in the delivery of one food pellet. Then, a FR-3 and a FR-6 reinforcement schedule are each applied for three consecutive days. During the FR-3 and the FR-6 schedules, three or six nose-pokes in the active nose-poke hole produce the delivery of one food pellet, respectively. The mice are then switched to a progressive ratio (PR)-2 reinforcement schedule for 21 days (3 baseline, 6 morphine treatment, and 12 opiate withdrawal days). During the last 6 opiate withdrawal days, a reversal operant behaviour exercise is applied, i.e., the active nose-poke hole become inactive, and vice versa. Under the PR-2 reinforcement schedule, the number of active nose-pokes required to obtain each successive food pellet is progressively increased, i.e., earning the first food pellet requires two active nose-pokes, the second four nose-pokes, the third six nose-pokes, etc. We also calculate the breakpoint (BP) as the last PR level achieved during each 2-h operant behaviour test. For example, to earn the fourth food pellet a mouse has to nose-poke $2+4+6+8$ times in the active hole, and thus is given a BP value of 8. The BP is a well-validated measure of the strength of the reinforcer and of the motivational state of the animal (Arnold and Roberts 1997; Hodos 1961). Learning criteria are as follows: (1) ingestion of at least ten food pellets/ test session and (2) a discrimination index (DI, active nose-pokes/total nose-pokes×100) of at least 75% during each of the first three PR-2 days (baseline). Then, the mice are assigned to two groups ($n=8$/group) having similar BP mean values, as averaged across the baseline days. One group is assigned to receive saline and the other escalating morphine doses (20–100 mg/kg, i.p.). In particular, starting on the fourth PR-2 day, every 12 h (8 a.m.; 8 p.m.), the mice are repeatedly treated with saline or morphine according to the following protocol: day 1, 20 mg/kg, day 2; 40 mg/kg, day 3, 60 mg/kg, day 4, 80 mg/kg, day 5, 100 mg/kg, day 6, 100 mg/kg, only one injection in the morning. Morphine is administered twice a day since, unlike constant drug delivery devices, it allows partial opiate withdrawal between drug injections, a condition

encountered in the clinical setting (Papaleo and Contarino 2006). The mice are weighed immediately before each injection and body weight (BW) changes calculated as percentage of the BW recorded just prior to the first injection. Finally, BW changes are monitored up to 120 h following cessation of morphine dosing. The normal estrus cycle of laboratory mice is 4–5 days in length, and it is divided into four phases (proestrus, estrus, metestrus, and diestrus). Since the different phases (acquisition of operant behavior, morphine administration, opiate withdrawal, etc.) of the experiments reported above last at least 6 days, the impact of a particular phase of the estrus cycle upon behavior is not monitored.

Drugs

Morphine HCl (Francopia, Gentilly, France) is dissolved in physiological saline and injected in a volume of 10 ml/kg. Control mice are injected with the same volume of saline.

Statistical analysis

The one-way analysis of variance (ANOVA) with repeated measure (daily operant behaviour tests) as a within-subject factor is used to analyse nose-pokes, food pellets ingested and DI observed during FR-1, FR-3, and FR-6 reinforcement schedules. The two-way ANOVA with treatment (saline, morphine) as a between-subject factor and repeated measure (daily operant behaviour tests) as a within-subject factor is used to analyze BP, food pellets ingested, DI, and ambulation (horizontal and vertical activity) observed during the interval between morphine injections and following cessation of drug dosing, i.e., during early (8-h) or more prolonged (up to 12 days) phases of opiate withdrawal, respectively. The two-way ANOVA with treatment (saline, morphine) as a between-subject factor and repeated measure as a within-subject factor is also used to analyze BW changes. The Student–Newman–Keuls post-hoc test is used for individual group comparisons. The accepted value for significance is $P<0.05$.

Results

CD-1 mice rapidly acquire food-driven operant behaviour

Analysis of active nose-pokes performed during the FR reinforcement schedules reveal a repeated measure effect ($F_{8,120}=24.54$, $P<0.0001$). The number of food-driven active nose-pokes progressively increases during the FR-3 ($P<0.05$, versus FR-1) and the FR-6 ($P<0.001$, versus FR-3) reinforcement schedule (Suppl. Fig. 1a). However, active nose-poke responding is stable across the 3 days of each FR reinforcement schedule ($P=0.33$). On the other hand, analysis of

inactive nose-pokes reveal no repeated measure effect ($F_{8,120}=0.71$, $P=0.72$; data not shown), indicating stability of non-rewarded behaviour. Examination of the DI parameter also reveal a repeated measure effect ($F_{8,120}=9.23$, $P<0.0001$). DI values are higher during the FR-3 ($P<0.05$) and the FR-6 ($P<0.01$) than during the FR-1 reinforcement schedule (Suppl. Fig. 1b). Analysis of food pellets ingested also shows a repeated measure effect ($F_{8,120}=24.18$, $P<0.0001$). Starting from the third FR-1 day, the mice eat more food pellets than during the first two operant tests ($P<0.01$, Suppl. Fig. 1c). In particular, ingestion of food pellets sharply increases during the three FR-1 days (Suppl. Fig. 1c). Thus, our results clearly indicate that CD-1 mice rapidly acquire and display consistent levels of food-motivated behaviour in operant paradigms.

Early opiate withdrawal does not affect food-driven operant behaviour

Following initiation of morphine dosing, the mice are daily tested while undergoing early (8-h) opiate withdrawal. Analysis of the BP parameter reveal no early drug withdrawal effect ($F_{1,14}=0.71$, $P=0.41$) and no early drug withdrawal×repeated measure interaction effect ($F_{5,70}=1.61$, $P=0.17$; Suppl. Fig. 2a). Similarly, examination of the DI parameter reveal no early drug withdrawal×repeated measure

interaction effect ($F_{5,70}=1.06$, $P=0.39$; Suppl. Fig. 2b). These results clearly indicate that early opiate withdrawal does not disrupt either the motivational or the discriminative properties of HPF intake. Analysis of horizontal activity performed during the operant behaviour tests reveal an early drug withdrawal×repeated measure interaction effect ($F_{5,70}=2,93$, $P<0.05$). Starting from the 60 mg/kg morphine dosing day, opiate withdrawal consistently decreases horizontal activity ($P<0.05$, versus control mice; Suppl. Fig. 2c). Examination of vertical activity also reveal an early drug withdrawal×repeated measure interaction effect ($F_{5,70}=3.82$, $P<0.005$). However, post hoc individual comparisons show no difference between control and opiate-withdrawn mice (Suppl. Fig. 2d).

Increased food-driven operant behaviour during prolonged opiate withdrawal

Analysis of the BP values observed up to 152 h following cessation of morphine dosing reveal a drug withdrawal×repeated measure interaction effect ($F_{6,84}=2.32$, $P<0.05$). Starting 32 h after the last morphine injection, opiate withdrawal consistently increases BP values ($P<0.001$, versus control mice; Fig. 1a). Examination of the DI parameter reveal no drug withdrawal effect ($F_{1,14}=0.03$, $P=0.87$) and no drug withdrawal×repeated measure interaction effect

Fig. 1 Prolonged opiate withdrawal dramatically increases food-driven operant behaviour. **a** Breakpoint (BP), **b** discrimination index (DI, active nose-pokes/total nose-pokes×100), **c** horizontal and **d** vertical activity displayed by control (CTL) or opiate-withdrawn (OW) mice 8–152 h after the last morphine injection. Values represent mean±SEM $N=8$/group. *$P<0.05$, **$P<0.001$, versus CTL mice. #$P<0.05$, versus 8-h opiate withdrawal
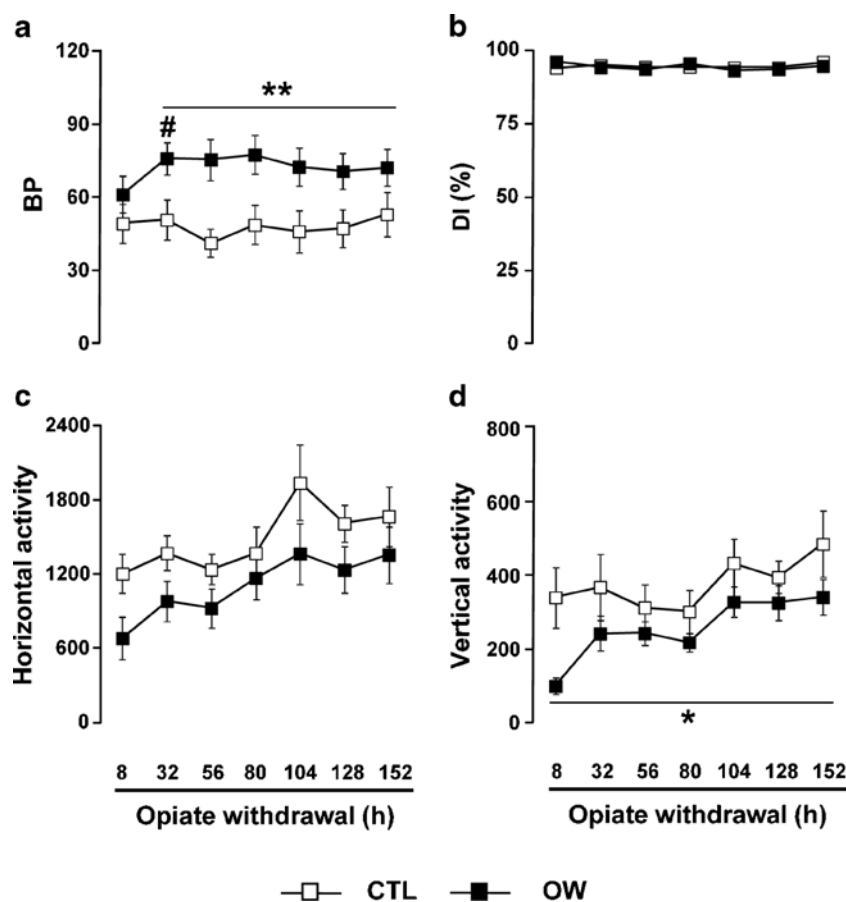
($F_{6,84}=0.66$, $P=0.68$), indicating that opiate withdrawal does not alter the ability to discriminate food-paired nose-poking (Fig. 1b). Analysis of horizontal activity reveal no drug withdrawal effect ($F_{1,14}=3.34$, $P=0.09$) and no drug withdrawal×repeated measure interaction effect ($F_{6,84}=0.42$, $P=0.86$; Fig. 1c). However, analysis of vertical activity reveal a drug withdrawal effect ($F_{1,14}=6.0$, $P<0.05$), indicating lower levels of activity in opiate-withdrawn mice ($P<0.05$, versus control mice; Fig. 1d). These results clearly show that opiate withdrawal sharply increases the motivational properties of palatable food intake. Moreover, they indicate that food-driven behaviour is independent of the ambulatory effects of opiate withdrawal.

Prolonged opiate withdrawal does not affect cognitive functions

Analysis of the BP values observed following reversal of the food-paired nose-poke hole (176–296 h after the last morphine injection) reveal a drug withdrawal effect ($F_{1,14}=11.82$, $P<0.005$), a repeated measures effect ($F_{5,70}=9.90$, $P<0.0001$) but no drug withdrawal×repeated measure interaction effect ($F_{5,70}=1.55$, $P=0.18$). Notably, opiate-withdrawn mice show higher BP levels than control mice ($P<0.005$; Fig. 2a). BP values are higher 200 than 176 h after the last saline or

morphine injection ($P<0.0005$, opiate withdrawal-independent repeated measure effect), indicating rapid reestablishment of pre-reversal food-driven motivation. However, following reversal of the active with the inactive nose-poke hole, only opiate-withdrawn mice show higher BP levels than 8–152 h after the last morphine injection (compare Figs. 1a and 2a). Likewise, examination of the DI parameter reveal a drug withdrawal effect ($F_{1,14}=6.94$, $P<0.05$), a repeated measures effect ($F_{5,70}=78.65$, $P<0.0001$) but no drug withdrawal×repeated measure interaction effect ($F_{5,70}=1.08$, $P=0.38$). Opiate-withdrawn mice show higher DI levels than control mice ($P<0.05$; Fig. 2b). Moreover, DI values are higher 200 than 176 h after the last saline or morphine injection ($P<0.0005$, opiate withdrawal-independent repeated measure effect), indicating rapid learning of the new operant rule. However, only in opiate-withdrawn DI values return to the pre-reversal levels (compare Figs. 1b and 2b). Finally, analysis of ambulatory activity reveal no drug withdrawal effect (horizontal activity, $F_{1,14}=2.08$, $P=0.17$; vertical activity, $F_{1,14}=3.96$, $P=0.07$) and no drug withdrawal×repeated measure interaction effect (horizontal activity, $F_{5,70}=0.64$, $P=0.67$; vertical activity, $F_{5,70}=0.58$, $P=0.72$; Fig. 2c, d). Notably, the stability of operant behavior across each experimental phase (acquisition of operant behavior, morphine administration, opiate withdrawal, etc.) reported above suggests that the

Fig. 2 Prolonged opiate withdrawal does not affect cognitive functions. **a** Breakpoint (BP), **b** discrimination index (DI, active nose-pokes/total nose-pokes×100), **c** horizontal and **d** vertical activity displayed by control (CTL) or opiate-withdrawn (OW) mice 176–296 h after the last morphine injection. During this experimental phase, the previously active nose-poke hole is inactive and vice versa (reversal of reward contingency). Values represent mean±SEM. $N=8$/group. *$P<0.05$, **$P<0.005$, versus CTL mice. BP and DI values are higher 200 than 176 h after the last saline or morphine injection ($P<0.0005$, opiate withdrawal-independent repeated measure effect), indicating a rapid reversal of food-motivated behaviour. However, DI levels return to pre-reversal values in opiate-withdrawn but not in CTL mice (compare Figs. 1b and 2b)
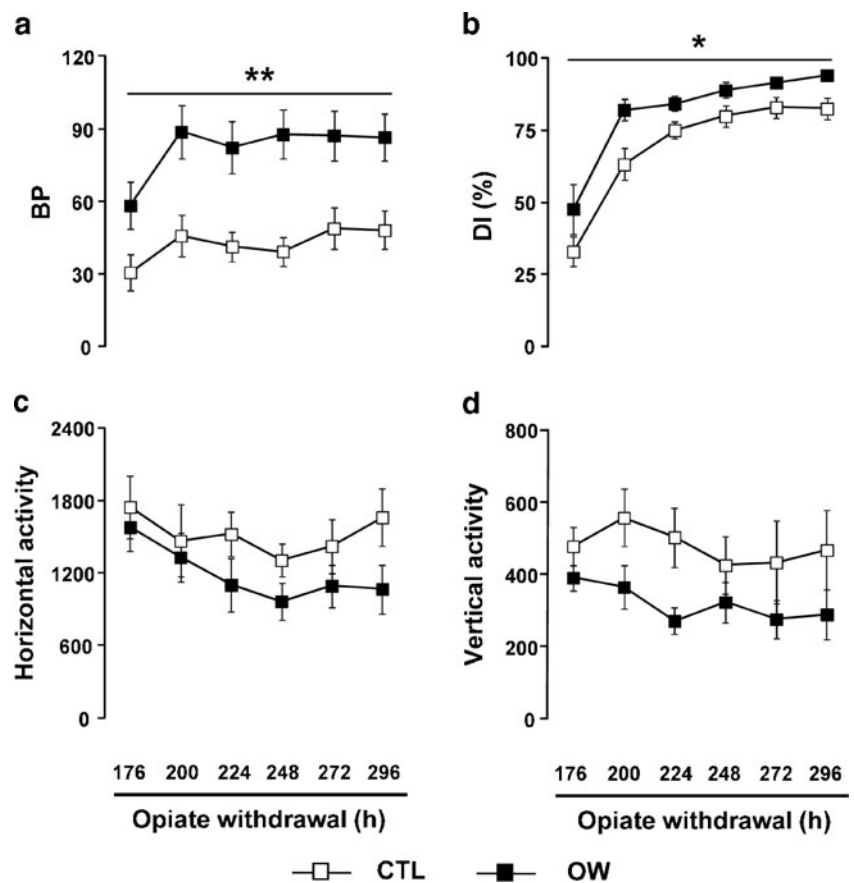
present results are not influenced by a particular phase of the estrus cycle.

Increased palatable food intake during prolonged opiate withdrawal

Starting on the first (PR)-2 day, all mice consumed all the HPF pellets earned up to the end of the experiments. Analysis of food pellets ingested during early (8-h) opiate withdrawal reveal no drug withdrawal effect ($F_{1,14}=0.71$, $P=0.41$) and no drug withdrawal × repeated measure interaction effect ($F_{5,70}=1.61$, $P=0.17$, Fig. 3). In contrast, analysis of food pellets ingested 8–152 h after the last morphine injection reveal a drug withdrawal effect ($F_{1,14}=5.61$, $P<0.05$) and a drug withdrawal × repeated measure interaction effect ($F_{6,84}=2.32$, $P<0.05$). Prolonged opiate withdrawal sharply increases the intake of HPF pellets, this effect being evident starting 32 h after morphine cessation ($P<0.001$, versus control mice; Fig. 3). Notably, opiate-withdrawn mice eat more food pellets 32 than 8 h after the last morphine injection ($P<0.05$; Fig. 3). Finally, analysis of food pellets ingested 176–296 h after the last drug injection (reversal phase) reveal a drug withdrawal effect ($F_{1,14}=11.57$, $P<0.005$), a repeated measures effect ($F_{5,70}=9.89$, $P<0.0001$) but no drug withdrawal × repeated measure interaction effect ($F_{5,70}=1.44$, $P=0.22$). Opiate-withdrawn mice ingest more food pellets than control mice up to the end of the experiment ($P<0.005$; Fig. 3). Overall, the mice also eat more food pellets 200 than 176 h after the last saline or morphine injection ($P<0.0005$, opiate withdrawal-independent repeated measure effect).

Rapid BW recovery following opiate withdrawal

Prior to the beginning of morphine dosing, control and opiate-withdrawn mice display similar BW (control=34.9±1.1, opiate-withdrawn=35.4±1.3; gram±SEM). Examination of BW changes induced by escalating morphine doses reveal an opiate withdrawal × repeated measure interaction effect ($F_{9,126}=2.81$, $P<0.005$). Opiate-withdrawn mice show a progressive BW loss that is evident starting from the 40 mg/kg morphine dosing day ($P<0.005$, versus control mice; Suppl. Fig. 3). However, following cessation of morphine dosing opiate-withdrawn mice display a rapid BW recovery. Indeed, no more difference in BW changes is observed between opiate-withdrawn and control mice by 24 h after the last saline or morphine injection (Suppl. Fig. 3).

Discussion

The present study demonstrates that opiate withdrawal increases the motivational properties of HPF in mice. Opiate withdrawal effects on motivation are relatively long-lasting since they last at least up to 12 days following cessation of opiate dosing. Prolonged opiate withdrawal does not affect cognitive functions necessary to learn a new operant rule to obtain HPF. Indeed, opiate withdrawal ameliorates the
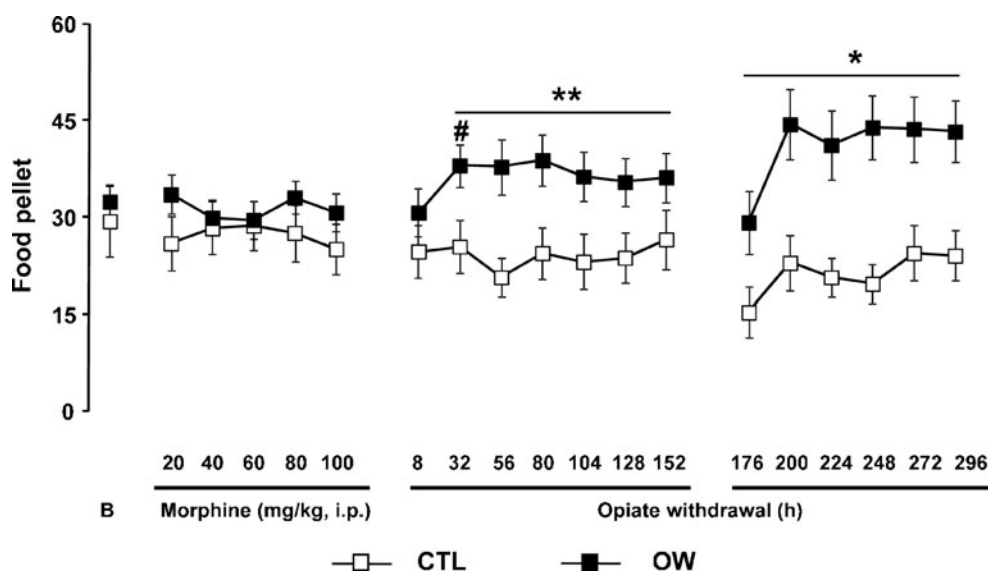
Fig. 3 Prolonged opiate withdrawal increases the intake of palatable food. Food pellets ingested by control (CTL) or opiate-withdrawn (OW) mice during baseline (B) or morphine (20–100 mg/kg, i.p.) dosing days and prolonged (8–296 h) opiate withdrawal, including the reversal operant behaviour phase (176–296 h). Values represent mean±SEM. N=8/group. $^{\#}P<0.05$ versus 8-h opiate withdrawal.

$^{*}P<0.005$, $^{**}P<0.001$, versus CTL mice. Similarly to the BP data illustrated in Fig. 2a, the mice ingest more food pellets 200 than 176 h after the last injection ($P<0.0005$, opiate withdrawal-independent repeated measure effect), suggesting a link between nose-poking and palatable food intake

acquisition of the new operant task, most probably due to the increased and long-lasting motivational drive for the HPF. Finally, the concurrent analysis of ambulatory activity and BW changes throughout the operant behaviour tests indicates that opiate withdrawal-induced increase in motivation is not due to motor and/or energy balance effects of opiate dosing and withdrawal.

Unlike prior reports (Cooper et al. 2010; Harris and Aston-Jones 2003), in the present study, mice are trained in a food-driven operant behaviour paradigm prior to being dosed with morphine. We find that CD-1 mice rapidly acquire a HPF-driven operant task, as indicated by the relatively elevated food-directed active nose-poke and DI (~100%) values. Then, the mice are treated with escalating doses of morphine and tested during the time interval between successive drug injections, i.e., during early (8-h) opiate withdrawal. Our results reveal that early phases of opiate withdrawal do not affect HPF-driven operant behaviour, as drug-treated and drug-naïve control mice display similar BP and DI levels. Moreover, early opiate withdrawal does not affect the cognitive abilities to discriminate food-driven from non-rewarded operant behaviour. The present findings differ from a prior study showing decreased remifentanil-driven operant behaviour during early (12-h) morphine withdrawal in rats (Cooper et al. 2008). However, in the latter study, increasing the remifentanil dose (the reward value) eliminates the difference between morphine-withdrawn and control rats, indicating that operant behaviour displayed during early opiate withdrawal phases largely depends on the reinforcing properties of the stimulus driving it. Thus, the HPF used in the present study may serve as a powerful reinforcing stimulus, as it can drive relatively elevated levels of operant behaviour, in spite of the significant motor-depressing effects of early opiate withdrawal phases, as indicated by the decreased horizontal activity.

Here, we also find that cessation of morphine dosing sharply increases the motivational properties of HPF intake. In particular, starting 32 h following the last morphine injection opiate-withdrawn mice show higher HPF-driven operant behaviour than drug-naïve control mice. Since the present study is carried out in group-housed mice, it is not possible to accurately measure home-cage "regular" food intake. However, it is unlikely that the operant behaviour results reported herein are due to a "general" increase in food intake. Indeed, we report that increased home-cage "regular" food intake following termination of the same morphine regimen used herein lasts less than 72 h in wild-type mice (Ingallinesi et al. 2011) whereas, in the same wild-type mice, increases in opiate withdrawal-induced motivation last much longer (Rouibi and Contarino, unpublished observations). Accordingly, in the present study, opiate withdrawal-induced motivational increases are relatively long-lasting since they are observed for at least 12 days

following the last morphine injection. Prior studies show increased food-driven operant behaviour in rodents withdrawn from psychostimulant drugs or nicotine (Krueger et al. 2009; Olausson et al. 2006). Nevertheless, the present findings are in contrast with previous rat studies showing that opiate withdrawal decreases food-driven operant behaviour (Harris and Aston-Jones 2003; Schulteis et al. 1994; Zhang et al. 2007). Methodological and/or species differences might underlie the discordances between the present and prior studies. Notably, prior studies use food pellets with a composition very similar to standard laboratory food as a reinforcer for operant behaviour and/or food restriction paradigms (Harris and Aston-Jones 2003; Schulteis et al. 1994). In the present study, the mice are never exposed to food restriction. Moreover, the food pellets (5-TUL diet) used herein in the operant behaviour task are highly palatable. Indeed, using a food-choice paradigm, we report that mice largely prefer the 5-TUL diet over standard laboratory food with a ratio preference of ~100% (Ingallinesi et al. 2011). Thus, in prior studies (Harris and Aston-Jones 2003; Schulteis et al. 1994), the relatively low reinforcing properties of the food used might fail to motivate operant behaviour in opiate-withdrawn rats. Alternatively, the food used is not able to attenuate the negative affective-like states of opiate withdrawal (see below for further discussion of the latter point). A more recent study shows that morphine withdrawal increases operant responding for standard food pellets in rats (Cooper et al. 2010). However, in the latter study, operant behaviour testing is carried out ~23 h after morphine dosing, while the rats are in a marked negative energy balance status. Indeed, unlike the control rats, the morphine-treated rats fail to gain BW throughout the study (Cooper et al. 2010). This makes it difficult to dissociate the motivational from the energy-driven properties of food intake in opiate-withdrawn rats. Indeed, it is possible that in the latter study, operant behaviour is largely driven by the strong food deprivation state induced by opiate withdrawal. Unlike the latter study, herein, the motivational effects of opiate withdrawal are observed up to 12 days after cessation of morphine dosing. In contrast, no BW difference is found between control and opiate-withdrawn mice by 24 h after the last morphine injection. Thus, our results indicate that the increased motivation for HPF induced by opiate withdrawal is largely independent of the energy balance status. Furthermore, unlike the Cooper et al. (2010) study cited above in which only a FR-1 reinforcement schedule is used; herein, food-driven operant behaviour is examined using three different FR schedules, i.e., FR-1, FR-2, and FR-3, and a PR-2 reinforcement schedule, allowing a more thorough investigation of the strength of the reinforcer and of the motivational state of the animal (Arnold and Roberts 1997; Hodos 1961). Moreover, unlike the Cooper et al. (2010) study, herein, a reward contingency reversal paradigm is also applied, further allowing the investigation of the motivational state of

opiate-withdrawn mice under more difficult operant conditions. As discussed below, testing in the reward contingency reversal paradigm also allows assessing the ability of opiate-withdrawn mice to learn a new operant behaviour rule.

Throughout the present study, we also monitor the ambulatory activity of control drug-naïve and opiate-withdrawn mice. Notably, both horizontal and vertical activity is monitored during operant behaviour testing. Our results show that early (8-h) or more prolonged (152-h) phases of opiate withdrawal decrease horizontal and vertical activity, respectively. However, despite the decreased ambulation, opiate-withdrawn mice never show lower levels of operant behaviour than control mice. In contrast, prolonged opiate withdrawal even increases food-driven operant behaviour. Prior studies examine the effect of opiate withdrawal upon ambulatory activity. In particular, spontaneous opiate withdrawal alter the circadian rhythm of ambulatory activity in rats (Stinus et al. 1998), whereas relatively low doses of naloxone reduce ambulation in rats implanted with morphine pellets (Schulteis et al. 1994). However, the latter studies do not examine the impact of ambulatory activity upon food-driven operant behaviour in opiate-withdrawn animals. Thus, the present results provide initial evidence of dissociation between the motivational and the ambulatory effects of opiate dosing and withdrawal.

Using a reward contingency reversal paradigm, in the present study, we also investigate the ability of opiate-withdrawn mice to learn a new operant rule to obtain HPF. Upon reversing the active with the inactive nose-poke hole, we find that opiate withdrawal ameliorates the re-acquisition (learning) of the new HPF-driven operant task. Indeed, during the reversal phase of the operant behaviour experiment, opiate-withdrawn mice show higher DI levels than control mice. Moreover, by the end of the experiment, only opiate-withdrawn mice display pre-reversal DI levels (see Fig. 2b). Reversal learning is shown to vary across mouse strains and is genetically linked to dopamine D2-like receptor expression in the ventral mesencephalon (Knapman et al. 2010; Laughlin et al. 2011). Interestingly, CD-1 mice show sporadic dopamine neuron abnormalities and lower D2-like receptor mRNA levels, as compared to C57BL/6J mice, in the substantia nigra pars compacta (SNpc) and the ventral tegmental area (VTA) (Prasad and Richfield 2008; Short et al. 2006). Thus, the decreased reversal performance reported herein in CD-1 mice might be due to dopamine neurotransmission deficits in brain areas relevant to motivated learning. Indeed, on the last reversal day, two out of eight control CD-1 mice show a DI lower than 75%, whereas all of the opiate-withdrawn mice show a DI higher than 85%. The present findings contrast with prior studies reporting severe deficits in attention, motivation, and learning in opiate- or cocaine-withdrawn animals (Dalley et al. 2005; Harris and Aston-Jones 2003; Krueger et al. 2009; Zhang et al. 2007). In particular, cocaine-withdrawn mice display severe learning deficits upon reversal of reward contingencies, indicating important alterations in cognitive flexibility (Krueger et al. 2009). Moreover, opiate-withdrawn rats show decreased food-driven operant behaviour and take more time to achieve a learning criterion in the operant task, as compared to control rats (Harris and Aston-Jones 2003). However, in the same study, the authors also show that opiate-withdrawn rats take longer than control rats to resume food-driven operant responding in a conditioned suppression paradigm (Harris and Aston-Jones 2003). Thus, it is possible that in the latter study, increased emotionality interfere, at least in part, with the expression of operant behaviour by opiate-withdrawn rats. Another major difference with the present study is that the rats are treated with morphine prior to being trained in the operant procedure (Harris and Aston-Jones 2003). In contrast, to avoid interference with learning the operant task, herein, mice are allowed to acquire the food-driven behaviour prior to being treated with morphine. Thus, our results clearly show that opiate withdrawal does not impair cognitive abilities but actually ameliorates the acquisition of a new rule in operant behaviour tasks. In this regard, it is possible that the opiate withdrawal-induced increased motivation for HPF greatly facilitates the acquisition and expression of the reversal operant behaviour. However, we cannot rule out that instrumental reversal learning tasks different from that used herein might unravel learning deficits in opiate-withdrawn mice.

The neurobiological substrates underlying the motivational effects of opiate withdrawal remain largely unknown. A prior study shows that rats allowed to ingest 20–30% sucrose solutions display a reliable decrease in the expression of major somatic signs of opiate withdrawal, such as escape attempts, diarrhea, and palpebral ptosis (Jain et al. 2004). Intake of HPF increases the activity of beta-endorphin pathways (Dum et al. 1983) and elevates plasma and cerebrospinal levels of this endogenous mu-opioid receptor ligand (Yamamoto et al. 2000). Furthermore, HPF intake increases dopamine release in the brain region of the nucleus accumbens (Bassareo and Di Chiara 1997; Mark et al. 1991; Rada et al. 2005). Thus, HPF intake may increase the activity of brain circuitry, which in turn might attenuate the negative affective-like states of opiate withdrawal. On the other hand, opiate withdrawal increases the activity of stress-responsive circuitry, which might mediate somatic and negative affective-like components of opiate withdrawal (Contarino and Papaleo 2005; Harris and Aston-Jones 2007; Koob 2008). Indeed, morphine-withdrawn animals display elevated brain corticotropin-releasing factor (CRF), plasma adrenocorticotropic hormone (ACTH), and corticosterone levels (Houshyar et al. 2003; Houshyar et al. 2004; McNally and Akil 2002; Papaleo et al. 2007). Moreover, disruption of CRF neurotransmission abolishes the somatic signs and the negative affective-like states of opiate withdrawal (Contarino and Papaleo 2005; Ingallinesi et al. 2011; Papaleo et al. 2008).

Interestingly, intake of highly palatable lard and sucrose decreases the hypothalamic expression of CRF and reduces plasma ACTH and corticosterone responses to a restraint stress (Pecoraro et al. 2004). Here, we find that increased levels of operant behaviour induced by opiate withdrawal are associated with elevated HPF intake (see Fig. 3). Thus, the operant behaviour observed herein might serve to increase HPF intake and thus modulate the activity of reward-linked and/or stress-responsive systems, which in turn may attenuate the malaise of opiate withdrawal.

Excessive intake of and/or withdrawal from drugs of abuse is thought to impair the brain reward system and thus contribute to the anhedonia-like symptoms commonly observed in drug-dependent individuals (Koob and Le Moal 2001). In particular, in the intracranial self-stimulation (ICSS) paradigm exposure to high amounts of drugs may increase the self-administration of electrical current, indicating heightened brain reward threshold (BRT). Notably, elevation in BRT is thought to reflect brain reward system deficiency and/or anhedonia-like states (Markou and Koob 1992). Accordingly, heightened BRT is observed in rats self-administering high amounts of heroin or cocaine (Ahmed et al. 2002; Kenny et al. 2006). Moreover, increased BRT is observed in naloxone-treated rats self-administering heroin (Kenny et al. 2006) or in rats withdrawn from continuous infusion of fentanyl (1.2 mg/kg/day) by a mini-pump device (Bruijnzeel et al. 2006). It is possible that in the present study, opiate withdrawal produces brain reward system deficiency and/or anhedonia-like states. However, several studies indicate that opiate withdrawal signs and symptoms, anhedonia and craving, and/or motivational disorders arise independently. Thus, it is unlikely that the motivational disorders observed herein in opiate-withdrawn mice are due to decreased brain reward function. Indeed, opiate withdrawal-induced elevations in BRT are relatively short-lasting since it largely dissipate by 72 h after cessation of opiate dosing (Bruijnzeel et al. 2006). Moreover, anhedonia-like effects of opiate withdrawal are observed 8 h but dissipate by 32 h after the last morphine injection in mice (Ingallinesi et al. 2011). In contrast, herein, opiate withdrawal-induced food-driven operant behaviour is long-lasting since it lasts at least up to 12 days following cessation of morphine dosing. Thus, since their intensity and temporal pattern do not appear to overlap, it is likely that motivational disorders are independent of brain reward system deficiency and/or anhedonia-like states associated with excessive drug intake and withdrawal.

In conclusion, using a clinically relevant mouse paradigm, the present study clearly demonstrates that opiate withdrawal dramatically increases the motivation to eat. In particular, opiate withdrawal-induced motivational disorders are long-lasting and independent of energy balance and ambulatory effects of drug intake and withdrawal. These findings may help to understand the neural mechanisms underlying motivational disorders associated with opiate dependence and withdrawal, which remain largely unknown.

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Suppl. Fig. 1
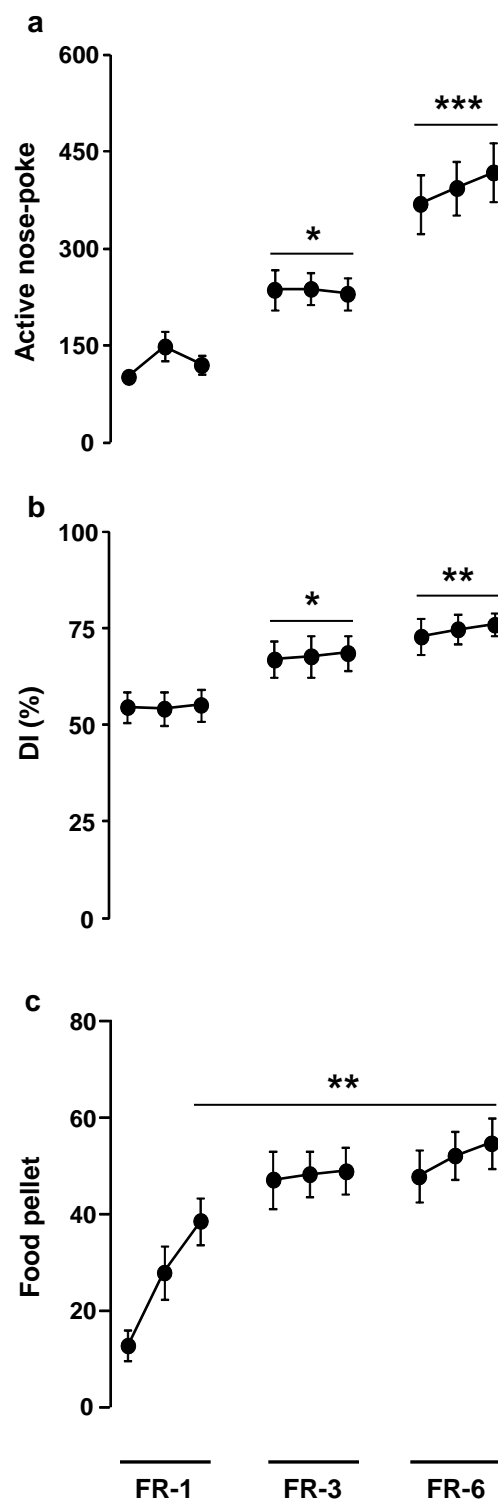
Suppl. Fig. 1 CD-1 mice rapidly acquire food-driven operant behaviour. **(a)** Active nose-pokes, **(b)** discrimination index (DI, active nose-pokes/total nose-pokes X 100) and **(c)** food pellets eaten during the nine daily sessions of the fixed ratio (FR) reinforcement schedules (FR-1, FR-3 and FR-6). Values represent mean±s.e.m. N=16. *P<0.05, **P<0.01, versus FR-1 days. ***P<0.001, versus FR-3 days.

Suppl. Fig. 2
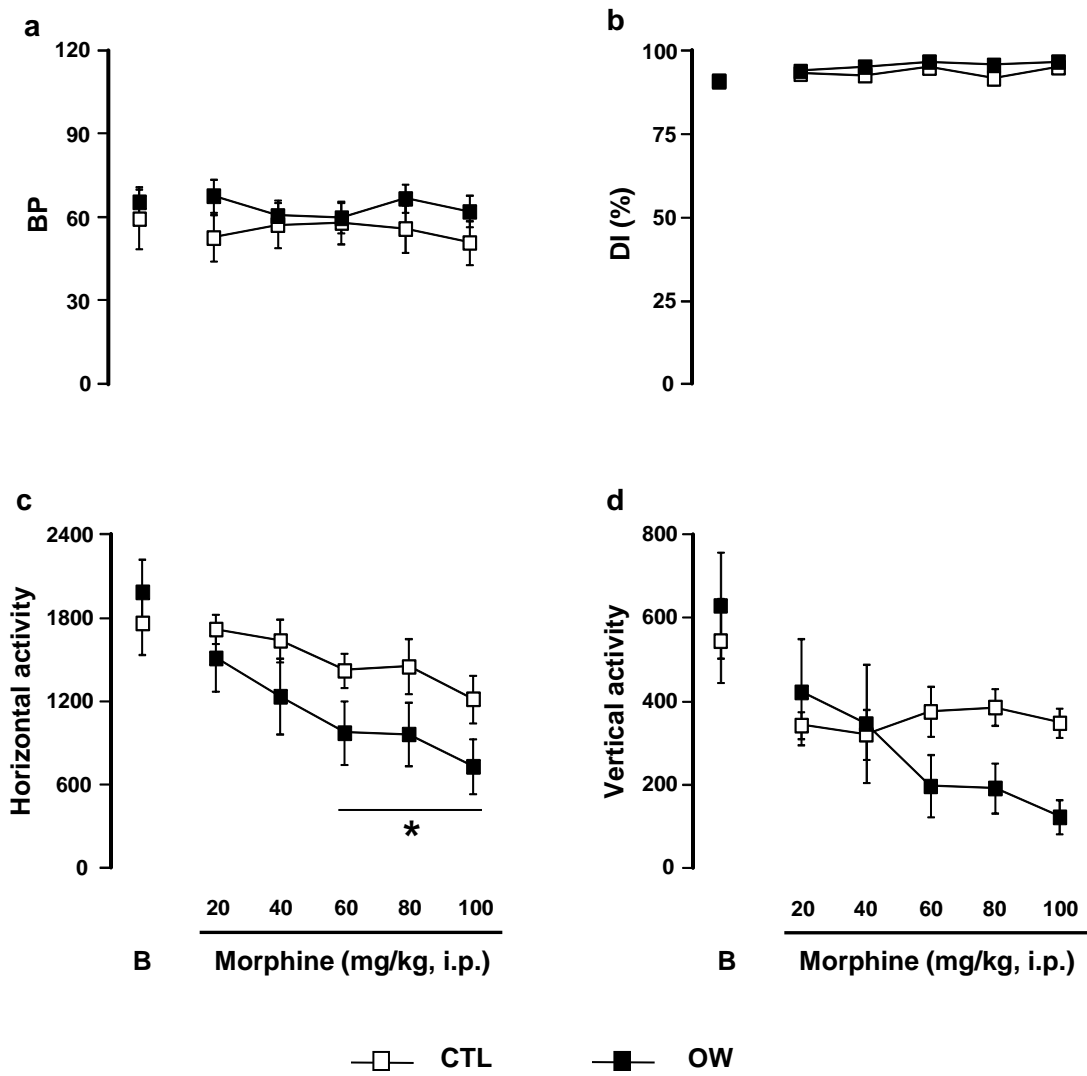
Suppl. Fig. 2 Early (8-hour) opiate withdrawal does not affect food-driven operant behaviour. **(a)** Breakpoint (BP), **(b)** discrimination index (DI, active nose-pokes/total nose-pokes X 100), **(c)** horizontal and **(d)** vertical activity displayed by control (CTL) or opiate-withdrawn (OW) mice during the baseline days (B) and the interval between successive morphine injections. Values represent mean±s.e.m. N=8/group. *P<0.05, versus CTL mice.

Suppl. Fig. 3
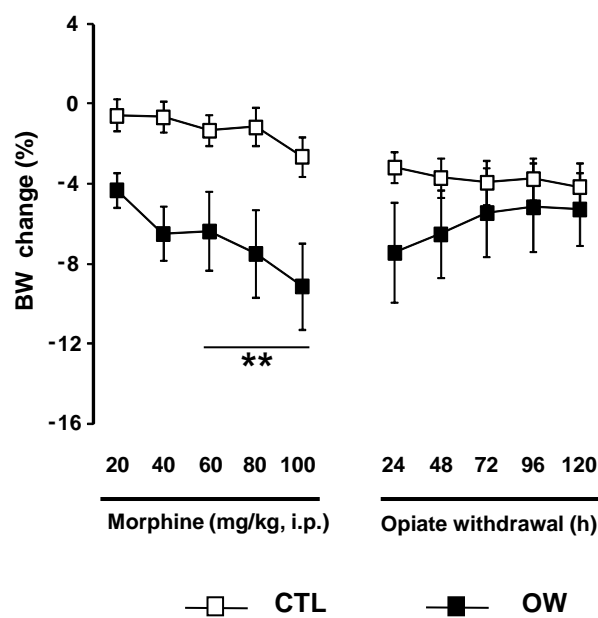
Suppl. Fig. 3 Body weight (BW) rapidly recovers following opiate withdrawal. BW changes, calculated as percentage of BW recorded just prior to the first saline or morphine injection, displayed by control (CTL) or opiate-withdrawn (OW) mice during the morphine (20-100 mg/kg, i.p.) dosing days and 24-120 hours following opiate withdrawal. BW is recorded 12 hr after the second injection of the morphine dose indicated. Values represent mean±s.e.m. N=8/group. *P<0.005, versus CTL mice. Notably, by 24 hr after cessation of morphine dosing no more opiate withdrawal effect is observed.

Article 3 : La délétion génétique du récepteur CRF$_2$ réduit les effets motivationnels du sevrage aux opiacés

RESUMÉ

Chez l'Homme, les désordres motivationnels qui surviennent suite à une discontinuité, une réduction ou à un arrêt brutal de la prise de drogues opiacées sont des éléments clefs déterminants dans les phénomènes de recherche et de prise de drogues. Dans l'état actuel des connaissances, les mécanismes neurobiologiques régissant les désordres motivationnels sont largement méconnus.

Par l'utilisation d'une approche comportementale de *ratio fixe* visant à obtenir de la nourriture hautement palatable, nous avons montré dans cette étude que la délétion génétique du récepteur CRF$_2$ diminuait le comportement opérant visant à obtenir de la nourriture hautement palatable chez des souris mâles mais pas femelles. Cependant l'exposition à un exercice de type *ratio progressif* montrait que la délétion génétique du récepteur CRF$_2$ n'altérait pas la motivation ou la prise de nourriture hautement palatable chez les souris mâles comme chez les souris femelles. Pendant et à la suite d'une procédure d'induction de dépendance par des doses croissantes de morphine (20-100 mg/kg, i.p.), des souris mâles et femelles de type sauvage augmentaient considérablement la tâche opérante visant à obtenir la nourriture indiquant des altérations motivationnelles importantes qui sont visibles longtemps (6 jours) après l'arrêt des injections de morphine. A l'inverse, les souris mâles et femelles invalidées génétiquement au récepteur CRF$_2$ réduisaient de façon marquée les altérations motivationnelles vis-à-vis de la nourriture hautement palatable. D'autre part, la délétion génétique du récepteur CRF$_2$ n'affectait pas les effets ambulatoires et les désordres de la balance énergétique suite à un traitement et à un sevrage à la morphine suggérant un rôle sélectif dans la motivation. Par l'application d'une procédure de renversement des conditions opérantes visant à obtenir la nourriture palatable, la délétion génétique du récepteur CRF$_2$, ainsi que le sevrage aux opiacés, n'affectaient pas l'aptitude des souris à apprendre une nouvelle tâche opérante.

Ces résultats montrent clairement une implication majeure du récepteur CRF$_2$ dans les désordres motivationnels induits par une prise et un sevrage aux opiacés suggérant de nouvelles stratégies pour un traitement efficace de la dépendance aux drogues opiacées.

The CRF$_2$ receptor pathway mediates the motivational disorders of opiate withdrawal

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Running title: CRF$_2$ receptor and motivation

ABSTRACT

Motivational disorders are key features of drug dependence and withdrawal, yet their neural mechanisms remain largely unknown. Here, we report that genetic disruption of the corticotropin-releasing factor receptor-2 pathway ($CRF_2^{-/-}$) does not impair the motivation for a highly palatable food (HPF) either in male or female mice, as assessed by a progressive ratio-2 (PR-2) operant behavior paradigm. However, unlike the drug-naïve mice CRF_2 receptor-deficiency effectively reduces the motivational effects of opiate injections and withdrawal. Indeed, treatment with and withdrawal from intermittent injections of escalating morphine doses (20-100 mg/kg, i.p.) dramatically increases the motivation for HPF either in male or female wild-type mice. Notably, in wild-type mice the opiate withdrawal (OW)-induced motivational increases last up to six days after the last morphine injection. In contrast, following OW either male or female $CRF_2^{-/-}$ mice show lower and shorter motivational increases than wild-type mice. Throughout, operant behavior parallels HPF intake, suggesting a link between the two phenomena. Moreover, wild-type and $CRF_2^{-/-}$ mice show similar ambulatory and body weight effects of intermittent morphine injections and withdrawal, ruling out a role for motor or energy balance influences upon the CRF_2 receptor-mediated motivation. Finally, following extinction of the OW-induced motivation, testing in a reversal reward contingency procedure reveals no effect of long-term OW or CRF_2 receptor-deficiency upon learning a new operant rule. The present results clearly demonstrate a gender-independent role for the CRF_2 receptor pathway in motivational disorders induced by intermittent morphine injections and withdrawal, suggesting new strategies for the treatment of opiate dependence.

INTRODUCTION

Opiate dependence is a major health problem across the world (www.who.int/substance_abuse). Studies show an alarming rise in the recreational use of opiate drugs among adolescents, indicating that the incidence rate of opiate dependence may dramatically increase in the next years (Compton and Volkow 2006). Severe motivational disorders are key features of opiate dependence and withdrawal that dramatically reduce the ability to overcome this devastating disease (APA 2000). In opiate-dependent subjects, the primary excessive motivation is for opiate drugs (Cooper et al 2008; Kenny et al 2006; Negus 2006). However, studies also indicate increased motivation for food or non-opiate drugs, especially during OW and/or abstinence periods. For instance, opiate-dependent individuals show heightened preference and desire (craving) for sweet food (Morabia et al 1989; Weiss 1982). Accordingly, opiate-withdrawn rats display increased motivation for food, as assessed by operant behavior paradigms (Cooper et al 2010). Moreover, OW increases cocaine-seeking and cocaine intake in either rats or rhesus monkeys (Gerak et al 2009; He and Grasing 2004). Thus, opiate dependence and withdrawal may increase behavior directed to and intake of non-opiate substances, suggesting profound and generalized alterations in motivational processes.

The corticotropin-releasing factor (CRF) system is a major coordinator of behavioral, neuroendocrine and autonomic responses to stressors (Koob 1999; Rivier et al 1982). The CRF system might also be implicated in substance dependence. Indeed, cessation of ethanol or cocaine intake elevates CRF activity in brain regions underlying negative affective-like states, such as the amygdala (Maj et al 2003; Merlo Pich et al 1995; Richter and Weiss 1999). Moreover, CRF receptor antagonists attenuate stress-induced reinstatement of ethanol- or cocaine-seeking behavior and decrease ethanol self-administration and anxiety-like behavior in ethanol- or cocaine-withdrawn rats (Basso et al 1999; Erb et al 1998; Funk et al 2006; Le et al 2000; Rassnick et al 1993). In mammals, CRF-like signaling is transmitted by two receptor pathways, termed CRF_1 and CRF_2 (Hauger et al 2003). The two known CRF receptor pathways may differentially contribute to the myriad of OW signs and symptoms. In particular, CRF_2 receptor-deficiency completely eliminates the somatic signs and the negative affective-like states of OW without impairing stress-coping abilities (Ingallinesi et al 2011; Papaleo et al 2008). However, to our knowledge the role for the CRF system in the motivational disorders associated with opiate dependence and withdrawal remains largely unknown.

To assess the role for the CRF_2 receptor pathway in the motivational effects of OW, in the present study we use male and female wild-type and $CRF_2^{-/-}$ mice (Bale et al 2000). In particular, motivation is assessed using a highly palatable food (HPF)-driven operant behavior paradigm recently established in our laboratory (Rouibi & Contarino, unpublished data). Notably, to mimic

the clinical setting the mice are injected twice a day with escalating doses of morphine and motivation assessed following body clearance of the drug, i.e., during "spontaneous" OW. Moreover, a reversal reward contingency paradigm is applied to evaluate the effect of CRF_2 receptor-deficiency and OW upon operant learning ability.

MATERIALS AND METHODS

Subjects

A total of 96, group-housed littermate female and male wild-type or CRF$_2$ receptor null mutant (CRF$_2^{-/-}$) mice are used throughout (Bale et al 2000). The mice derive from mating CRF$_2^{+/-}$ mice, are identified by PCR analysis of tail DNA and are 3–6 months old at the beginning of the experiments. They are housed in a colony room (22±2 °C, relative humidity: 50–60%) on a 12-h light/dark cycle (lights on at 0800 hours). Standard laboratory food (3.3 kcal/g; SAFE, Augy, France) and water are available *ad libitum*. All studies are 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.

Experimental design

Starting one week prior to the beginning of the behavioral experiments, on alternate days each mouse is handled for 1 min for a total of three times. Operant behavior is assessed using 24 apparatus. Each apparatus (length: 22 cm, width: 14 cm, height: 20 cm) is 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 of the apparatus, 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, results in food pellet delivery whereas nose-poking into the other hole, i.e., the inactive hole, has no consequences. Centered between the nose-poke holes is a feeding-trough for the delivery of food pellets. A food pellet delivery occurs when the photo-beam of the active nose-poke hole is interrupted for at least 500 msec. We use 20-mg HPF pellets (5-TUL, 3.4 kcal/g; PMI Nutrition International, LLC, St. Louis, MO, USA). The food pellets are delivered by an automated dispenser situated outside the apparatus into a food trough situated 3 cm from the nose-poke holes and 2 cm from the grid floor. The food trough is equipped with photo-beams that allow monitoring of food trough visits. An additional food pellet is 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 allow the passage of uneaten food pellets to a sliding drawer, making storage impossible and allowing evaluation of food spillage. Each apparatus is also equipped with 2 series of photo-beams that serve to record horizontal and vertical ambulatory activity. Starting on the day after the last handling session, at 1600 hours the mice are daily confined to the apparatus for a 2-hour test. Within genotype and gender, half of the mice are assigned the left and the other half the right nose-poke hole as the active hole. A fixed ratio (FR)-1 reinforcement schedule is initially applied for ten consecutive days, i.e., one nose-poke results in the delivery of

one food pellet. Then, a FR-3 and a FR-6 reinforcement schedule are each applied for three consecutive days. During the FR-3 and the FR-6 exercises, three or six active nose-pokes produce the delivery of one food pellet, respectively. The mice are then switched to a progressive ratio (PR)-2 reinforcement schedule for 30 days (3 baseline, 6 morphine treatment and 21 OW days). During the last 11 OW days, a reversal operant behavior exercise is applied, i.e., the active nose-poke hole become inactive, and vice versa. Under the PR-2 reinforcement schedule, the number of active nose-pokes required to obtain each successive food pellet is progressively increased by two. We also calculate the breakpoint (BP) as the last PR level achieved during each 2-hour test. For example, to earn the fourth food pellet a mouse has to nose-poke 2 + 4 + 6 + 8 times in the active hole, and thus is given a BP value of 8. The BP is a well-validated measure of the strength of the reinforcer and of the motivational state of the animal (Arnold and Roberts 1997; Hodos 1961). Learning criteria are applied: mice that ingest less than eight HPF pellets and/or show a discrimination index (DI, active nose-pokes ∕ total nose-pokes*100) lower than 75% during the third PR-2 day are excluded from the study. Then, within genotype and gender the mice are divided into two groups with similar BP mean values on the third PR-2 day, one assigned to receive physiological saline (10 ml/kg) and the other morphine HCl (Francopia, Gentilly, France;). In particular, starting on the fourth PR-2 day at 0800 hours and 2000 hours the mice are treated with saline (CTL) or morphine (OW), 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 at 0800 hours. The latter drug regimen allows partial opiate withdrawal between drug injections (Papaleo and Contarino 2006). The mice are weighed immediately before each injection and body weight (BW) changes calculated as percentage of the BW recorded just prior to the first injection. Following the termination of the morphine injections, BW changes are monitored every three days up to the end of the experiment.

Statistical analysis

The two-way analysis of variance (ANOVA) with genotype (wild-type or $CRF_2^{-/-}$) as a between-subject factor and repeated measure (daily operant tests) as a within-subject factor is used to analyze active nose-pokes, DI, horizontal and vertical activity recorded during the FR and the PR-2 experiments preceding drug dosing. The Student's t-test is used to examine the mean number of HPF pellets ingested during each day of the FR and the PR-2 experiments. A three-way ANOVA with genotype and treatment (saline or morphine) as between-subject factors and repeated measures (daily operant tests) as a within-subject factor is used to analyze BP, DI, HPF intake, horizontal activity, vertical activity and BW changes recorded on the third PR-2 day, during and following intermittent morphine injections. The Student-Newman-Keuls *post-hoc* test is used for individual group comparisons. The accepted value for significance is P<0.05.

RESULTS

Gender-dependent effect of CRF$_2$ receptor-deficiency on HPF-driven operant behavior

Analysis of active nose-pokes performed during the FR exercises shows a genotype effect in male ($F_{1,43}$= 4.15, P<0.05) but not in female ($F_{1,42}$= 0.58, P= n.s.) mice, a repeated measure effect (male: $F_{2,645}$= 30.59, P<0.0005; female: $F_{2,630}$= 10.67, P<0.0005) but no genotype X repeated measure interaction effect (male: $F_{15,645}$= 0.76, P= n.s.; female: $F_{15,630}$= 0.88, P= n.s.). CRF$_2$ receptor-deficiency decreases active nose-pokes in male but not in female mice (P<0.05, Figures S1A and S1B). However, increasing the FR exercise leads to a genotype-independent elevation in active nose poke in both genders (Figures S1A and S1B). Analysis of DI reveals no genotype effect (male: $F_{1,43}$= 1.55, P= n.s.; female: $F_{1,42}$= 0.49, P= n.s.), a repeated measure effect (male: $F_{15,645}$= 11.22, P<0.0005; female: $F_{15,630}$= 5.43, P<0.0005) but no genotype X repeated measure interaction effect (male: $F_{15,645}$= 1.57, P= n.s.; female: $F_{15,630}$= 0.63, P= n.s.). Increasing the FR exercise leads to a genotype-independent DI elevation in both genders (Figures S1C and S1D). Analysis of horizontal activity reveals no genotype effect (male: $F_{1,43}$= 0.23, P= n.s.; female: $F_{1,42}$= 1.73, P= n.s.) and no genotype X repeated measure interaction effect (male: $F_{15,645}$= 1.65, P= n.s.; female: $F_{15,630}$= 0.51, P= n.s.; Figures S1E and S1F). Analysis of vertical activity reveals a genotype X repeated measure interaction effect in male ($F_{15,645}$= 1.80, P<0.05) but not in female ($F_{15,630}$= 0.65, P= n.s.) mice. CRF$_2^{-/-}$ male but not female mice show lower vertical activity than wild-type mice during the 1st FR-1 day (P<0.05, Figures S1G and S1H). Finally, during the FR-1, the FR-3 and the FR-6 exercises male but not female CRF$_2^{-/-}$ mice ingest less HPF pellets than wild-type mice (P<0.05, Figures S1E and S1F).

CRF$_2$ receptor-deficiency does not affect the motivational properties of HPF

Analysis of active nose-pokes performed during the PR-2 exercise reveals no genotype effect (male: $F_{1,43}$= 1.04, P= n.s.; female: $F_{1,41}$= 0.27, P= n.s.) and no genotype X repeated measure interaction effect (male: $F_{2,86}$= 0.38, P= n.s.; female: $F_{2,82}$= 0.88, P= n.s.). Similarly, analysis of DI reveals no genotype effect (male: $F_{1,43}$= 0.59, P= n.s.; female: $F_{1,41}$= 0.09, P= n.s.) and no genotype X repeated measure interaction effect (male: $F_{2,86}$= 1.14, P= n.s.; female: $F_{2,82}$= 1.64, P= n.s.). Either male or female mice show relatively elevated genotype-independent levels of active nose-poke and DI (Figures 1A, 1B, 1C and 1D). Moreover, either gender shows no genotype difference in HPF intake (Figures 1E and 1F). Thus, in drug-naïve mice CRF$_2$ receptor-deficiency does not impair HPF-driven motivation.

CRF$_2$ receptor-deficiency reduces the motivational effects of opiate withdrawal

Analysis of BP observed prior to, during and after intermittent morphine injections reveals a genotype X OW X repeated measure interaction effect (male: $F_{16,656}$= 2.75, P<0.0005; female: $F_{16,624}$=1.72, P<0.05). Male OW wild-type mice show higher BP than CTL wild-type mice during the six days following the last morphine injection (P<0.05, Figure 2A). In contrast, male OW CRF$_2^{-/-}$ mice show higher BP values than CTL CRF$_2$-/- mice only during the first two days following the last morphine injection (P<0.005, Figure 2A). Moreover, male OW CRF$_2^{-/-}$ mice show lower BP values than OW wild-type mice up to eight days following the last morphine injection (P<0.05; Figure 2A). Female OW wild-type mice show higher BP than CTL wild-type mice from the 80 mg/kg morphine day to the sixth day following the last morphine injection (P<0.05; Figure 2B). In contrast, female OW CRF$_2^{-/-}$ mice differ from CTL CRF$_2^{-/-}$ mice only during the first day following the last drug injection (P<0.001, Figure 2B). Moreover, female OW CRF$_2^{-/-}$ mice show lower BP values than OW wild-type mice from the 80 mg/kg morphine day up to the fifth day following the last drug injection (P<0.05; Figure 2B). Analysis of HPF pellets ingested during the operant tests reveals CRF$_2$ receptor-deficiency effects similar to the BP findings reported above (Figures S3A and S3B). Finally, analysis of the DI reveals no OW effect (male: $F_{1,41}$= 3.31, P=n.s.; female: $F_{1,39}$= 1.30, P=n.s.) and no genotype X OW X repeated measure interaction effect (male: $F_{16,656}$= 0.57, P= n.s.; female: $F_{16,624}$= 0.82, P= n.s.). OW does not affect the DI in either wild-type or CRF$_2^{-/-}$ mice of both genders (data not shown). Thus, CRF$_2$ receptor-deficiency reduces the dramatic and long-lasting increases in motivation induced by OW without affecting discrimination performance.

CRF$_2$ receptor-deficiency or opiate withdrawal do not impair learning abilities

Analysis of DI following reversal of reward contingency reveals no OW effect (male: $F_{1,40}$= 1.11, P=n.s.; female: $F_{1,39}$= 1.25, P=n.s.), a repeated measure effect (male: $F_{11,440}$= 68.09, P<0.0005; female: $F_{11,429}$= 69.25, P<0.0005) and no genotype X OW X repeated measure interaction effect (male: $F_{11,440}$= 1.06, P=n.s.; female: $F_{11,429}$= 0.94, P= n.s.). In both genders, on the first reversal day DI is ~30% than that of the previous day but progressively increases from the second to the sixth reversal day (Figures S4A and S4B). Thus, neither CRF$_2$ receptor-deficiency nor OW affects learning a new operant behavior task.

CRF$_2$ receptor-deficiency does not affect opiate withdrawal-induced ambulation or body weight

Analysis of horizontal activity reveals an OW X repeated measure interaction effect (male: $F_{16,640}$= 9.81, P<0.0005; female: $F_{16,624}$= 8.08, P<0.0005) but no genotype X OW X repeated measure interaction effect (male: $F_{16,640}$= 0.43, P=n.s.; female: $F_{16,624}$= 1.12, P=n.s.). During the morphine days, OW reduces horizontal activity in both genotypes and genders (P<0.05, Figures 3A and 3B). Two days after the last morphine injection, OW female but not male mice show a genotype-independent increase in horizontal activity (P<0.05, versus CTL mice; Figures 3A and 3B). Analysis of vertical activity reveals an OW X repeated measure interaction effect (male: $F_{16,656}$= 7.86, P<0.0005; female: $F_{16,592}$= 7.98, P<0.0005) but no genotype X OW X repeated measure interaction effect (male: $F_{16,656}$= 1.02, P= n.s.; female: $F_{16,592}$= 1.18, P= n.s.). In both genders and genotypes, OW reduces vertical activity during the morphine days but increases it on the day after the last drug injection (P<0.05, versus CTL mice; Figures 3C and 3D). Just prior to the first injection, BW are as follows: male, CTL wild-type= 29.9±0.9, OW wild-type= 28.4±0.8, CTL CRF$_2^{-/-}$= 31.9±0.6, OW CRF$_2^{-/-}$= 31.8±0.5; female, CTL wild-type= 25.6±1.0, OW wild-type= 25.6±0.7, CTL CRF$_2^{-/-}$= 26.0±0.9, OW CRF$_2^{-/-}$= 26.9±0.6 (gram±s.e.m.). Analysis of BW changes reveals an OW X repeated measure interaction effect (male: $F_{12,492}$= 63.71, P<0.0005; female: $F_{12,468}$= 42.73, P<0.0005). In both genders, intermittent morphine injections induce a genotype-independent BW loss that is recovered by three days after the last morphine injection (P<0.0005, versus CTL mice; Figure S5A and S5B).

DISCUSSION

The present study provides initial evidence of a crucial role for the CRF_2 receptor pathway in the motivational disorders induced by OW. In particular, CRF_2 receptor-deficiency attenuates the dramatic and relatively long-lasting increases in motivation induced by intermittent morphine injections and withdrawal, in either male or female mice. In either genotype or gender motivation (operant behavior) parallels HPF intake but is independent of ambulatory or energy balance effects of intermittent morphine injections and withdrawal. Moreover, neither OW nor CRF_2 receptor-deficiency impairs the acquisition of a new operant behavior task, indicating preserved learning abilities.

To avoid possible interference with the establishment of food-driven behavior, in the present study the intermittent injections of escalating morphine doses is initiated after the acquisition of the operant task. We find that, in dug-naïve mice, CRF_2 receptor-deficiency affects HPF-driven operant behavior in a gender-dependent manner. Indeed, during the three FR reinforcement schedules used male but not female $CRF_2^{-/-}$ mice show lower active nose-pokes and HPF intake than wild-type mice. Nevertheless, increasing the FR exercise elevates active nose-poke and DI in either wild-type or $CRF_2^{-/-}$ mice of both genders, indicating that CRF_2 receptor-deficiency does not impair HPF-motivated behavioral adaptation. Accordingly, male CRF_2 receptor-deficient mice show preserved operant behavior for food pellets with a macronutrient composition similar to the home-cage chow (Tabarin et al 2007). Prior studies report gender-linked effects of a genetic mutation of the CRF system. For instance, male but not female $CRF_2^{-/-}$ mice show higher anxiety-like behavior than wild-type mice, although increased anxiety-like behavior in $CRF_2^{-/-}$ mice of both genders is also reported (Bale et al 2000; Kishimoto et al 2000). Moreover, female but not male urocortin 2-deficient mice show decreased immobility levels in the forced swim and in the tail suspension tests, as compared to wild-type mice (Chen et al 2006). Furthermore, in the latter study female but not male urocortin 2-deficient mice show increased hypothalamic expression of vasopressin and higher nocturnal plasma adrenocorticotropic hormone (ACTH) and corticosterone levels than wild-type mice, which are abolished by ovariectomy (Chen et al 2006). Thus, although further studies are needed to better understand the complex interaction between different elements of the CRF system and sexual hormones, the present study provides novel evidence of a gender-dependent role for the CRF_2 receptor pathway in behavior.

To assess the role for the CRF_2 receptor pathway in the motivational effects of OW, wild-type and $CRF_2^{-/-}$ mice are tested in a PR-2 reinforcement schedule prior to, during and after intermittent injections of escalating morphine doses. PR paradigms provide a reliable index of the motivational properties of a reinforcer and/or of the motivational state of the animal (Arnold

and Roberts 1997; Hodos 1961). Prior to drug exposure, either male or female wild-type or $CRF_2^{-/-}$ mice show similar levels of HPF-driven operant behavior, further strengthening the notion that CRF_2 receptor-deficiency does not impair motivation. These findings also suggest that disruption of the CRF_2 receptor pathway does not affect the hedonic valence of the HPF used. Moreover, since the mice are never food-deprived, the HPF-driven operant behavior observed herein is independent of energy needs. Intermittent morphine injections and withdrawal dramatically increase the motivation to eat and HPF intake in either male or female wild-type mice. Notably, OW-induced operant behavior and HPF intake display a similar time-course, gradually decreasing over the six days following termination of the morphine injections (see Figures 2 and S3). Notably, wild-type mice as those used in the present study show reliable somatic signs of OW that gradually decrease over 5-6 days following the last morphine injection (Papaleo et al 2008). Our prior and present findings suggest a link between motivation, HPF intake and distress induced by intermittent opiate injections and withdrawal. Studies show that termination of drug intake induces brain changes that may underlie somatic and negative affective-like states of drug dependence and withdrawal. In particular, OW increases CRF activity in the central nucleus of the amygdala (CeA) and in the paraventricular nucleus of the hypothalamus (PVN), increases dynorphin activity in the nucleus accumbens shell (NaccSH) and decreases extracellular dopamine levels in the nucleus accumbens (Acquas and Di Chiara 1992; Contarino and Papaleo 2005; Ingallinesi et al 2011; Maj et al 2003; McNally and Akil 2002; Papaleo et al 2007; Turchan et al 1997). Notably, morphine withdrawal increases CeA-CRF, PVN-CRF and NaccSH-dynorphin activity in wild-type mice as those used herein (Ingallinesi et al 2011). On the other hand, palatable food intake increases the activity of hypothalamic beta-endorphin pathways (Dum et al 1983) and dopamine release in the nucleus accumbens (Bassareo and Di Chiara 1997; Mark et al 1991; Rada et al 2005), decreases CRF activity in the hypothalamus as well as plasma ACTH and corticosterone responses to restraint stress (Pecoraro et al 2004). Thus, it is possible that the increased operant behavior observed herein in wild-type mice serves to heighten HPF intake, which in turn modulate the activity of reward- and/or stress-responsive systems to attenuate OW distress. Accordingly, sucrose intake decreases the expression of major somatic signs of OW, such as escape attempts, diarrhea and palpebral ptosis in rats (Jain et al 2004).

Here, we also show gender-dependent effects of OW in wild-type mice. In particular, during the period of intermittent morphine injections female but not male wild-type mice show higher levels of motivation than control wild-type mice. It is unlikely that the latter gender effect is due to differences in the pharmacokinetic properties of morphine. Indeed, no gender difference in blood and brain levels of morphine is found in animals treated with a relatively wide range of morphine doses (Cicero et al 2000; Cicero et al 1996; Cicero et al 1997; Craft et al 1996).

However, gender-linked differences are found in morphine-induced analgesia and in the somatic expression of OW (Cicero et al 1997; Cicero et al 2002; Papaleo and Contarino 2006). In particular, female but not male mice treated with a lower morphine regimen (10-50 mg/kg, i.p.) than that used herein display reliable somatic signs of OW (Papaleo and Contarino 2006). Thus, the increased motivation observed herein in female but not in male wild-type mice between successive morphine injections provides further support to the notion of a gender-linked vulnerability to OW.

In contrast with the wild-type mice, here we demonstrate that CRF_2 receptor-deficiency effectively reduces the motivational increases induced by intermittent morphine injections and withdrawal, in either male or female mice. Disruption of the CRF_2 receptor pathway completely abolishes the somatic signs and the negative affective-like states of OW (Ingallinesi et al 2011; Papaleo et al 2008). Accordingly, CRF_2 receptor-deficiency also eliminates the neural substrates of OW distress, such as the increased NaccSH-dynorphin and CeA-CRF activity (Ingallinesi et al 2011). Thus, the lack of drug withdrawal distress might, at least in part, underlie the relatively small motivational effect of intermittent morphine injections and withdrawal observed herein in CRF_2 receptor-deficient mice. However, several studies suggest dissociation between somatic, negative affective-like and motivational effects of OW. For instance, pharmacological agents that may modify the expression of somatic or negative affective-like components of OW, such as the α-2 noradrenergic agonist clonidine and the kappa-opioid antagonist 5'-guanidinonaltrindole, fail to influence OW-induced increase in heroin choice over food in rhesus monkeys (Negus and Rice 2009). Moreover, in rhesus monkeys somatic OW signs largely dissipate by 5-6 days whereas increased motivation for heroin is observed up to 9-10 weeks after termination of intermittent morphine injections (Gerak et al 2009). Accordingly, wild-type mice show the highest level of somatic OW (Papaleo et al 2008) and of HPF-driven operant behavior (present study) 8 and 32 hours after the last morphine injection, respectively. Furthermore, despite the relatively small motivational increase, $CRF_2^{-/-}$ mice never show somatic signs of OW (Ingallinesi et al 2011; Papaleo et al 2008). Thus, it is possible that the CRF_2 receptor pathway mediates the motivational disorders of OW independently of its role in somatic signs and negative affective-like states.

In conclusion, motivational disorders dramatically diminish the ability to overcome drug dependence and withdrawal by promoting drug-seeking and drug-taking behavior, yet their neural substrates are largely unknown. Here, we provide initial evidence of a key role for the CRF_2 receptor pathway in the motivational increase induced by intermittent morphine injections and withdrawal. This study reveals a key substrate of the motivational disorders that characterize opiate dependence, suggesting new strategies for the treatment of this devastating disease.

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FIGURE LEGENDS

Figure 1. CRF$_2$ receptor-deficiency does not impair HPF-driven motivation. (A, B) Active nose-pokes, **(C, D)** discrimination index (DI) and **(E, F)** mean daily number of highly palatable food (HPF) pellets ingested by wild-type or CRF$_2^{-/-}$ (left panels) male or (right panels) female mice under a progressive ratio (PR)-2 schedule of reinforcement during three consecutive days. Values represent mean±s.e.m. N= 10-12∕group.

Figure 2. CRF$_2$ receptor-deficiency reduces the motivational increases induced by opiate withdrawal. Breakpoint (BP) displayed by control (CTL) or opiate-withdrawn (OW) **(A)** male or **(B)** female wild-type or CRF$_2^{-/-}$ mice under a progressive ratio (PR)-2 schedule of reinforcement prior to (third PR-2 day, P), during and following termination of intermittent morphine injections. Values represent mean±s.e.m. N= 10-12∕group. [a]$P<0.05$, [b]$P<0.005$ and [c]$P<0.0005$ versus same genotype CTL mice. [d]$P<0.05$, [e]$P<0.005$ and [f]$P<0.0005$, OW CRF$_2$-/- versus OW wild-type mice.

Figure 3. CRF$_2$ receptor-deficiency does not affect the ambulatory effects of intermittent morphine injections and withdrawal. (A, B) Horizontal and **(C, D)** vertical activity displayed by control (CTL) or opiate-withdrawn (OW) wild-type or CRF$_2^{-/-}$ male (left panels) or female (right panels) mice during the daily 2-hour operant behavior tests carried out prior to (P), during and following termination of intermittent morphine injections. The values are expressed as percentage changes of the activity recorded the day preceding the onset of morphine injections (third PR-2 day, P). Values represent mean±s.e.m. N= 10-12∕group. [*]$P<0.05$, versus CTL mice.

Figure 1
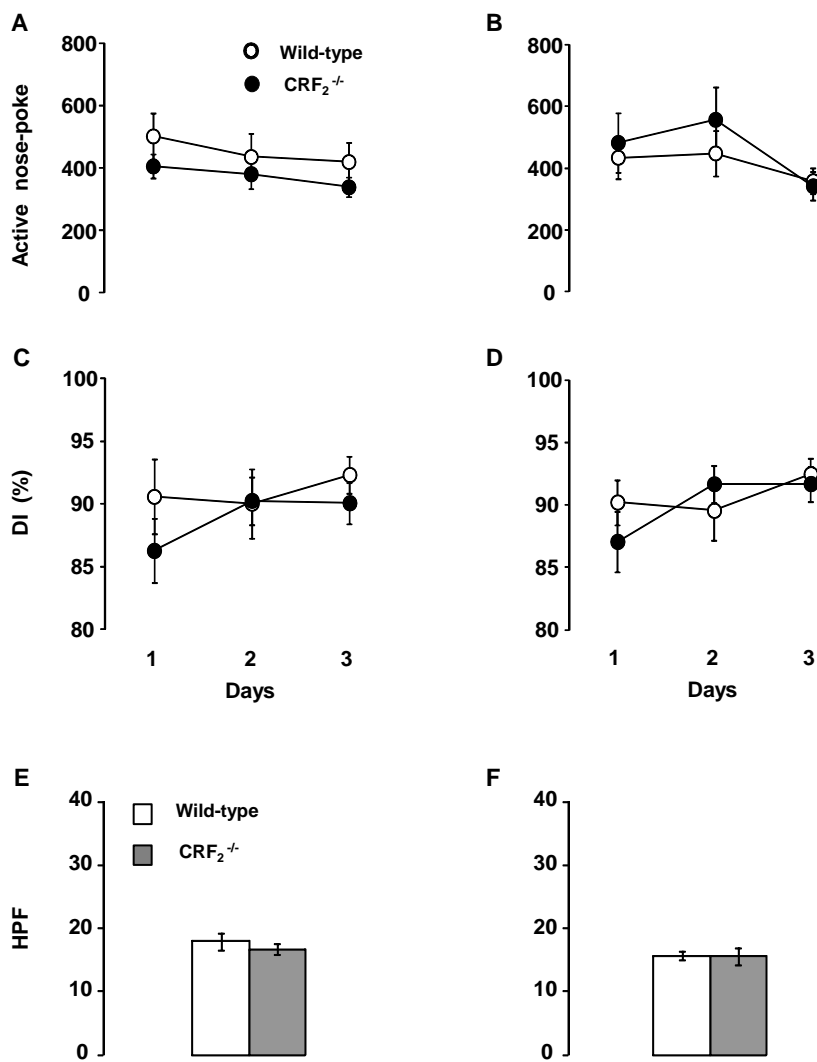
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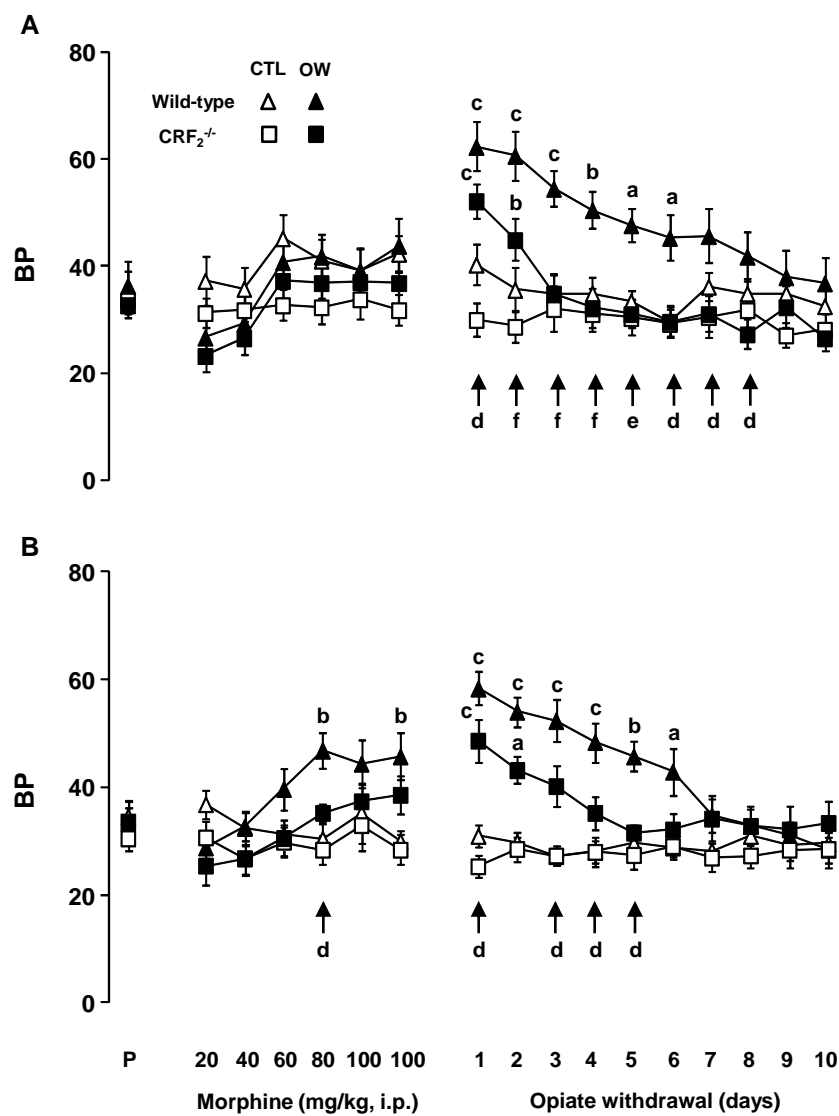
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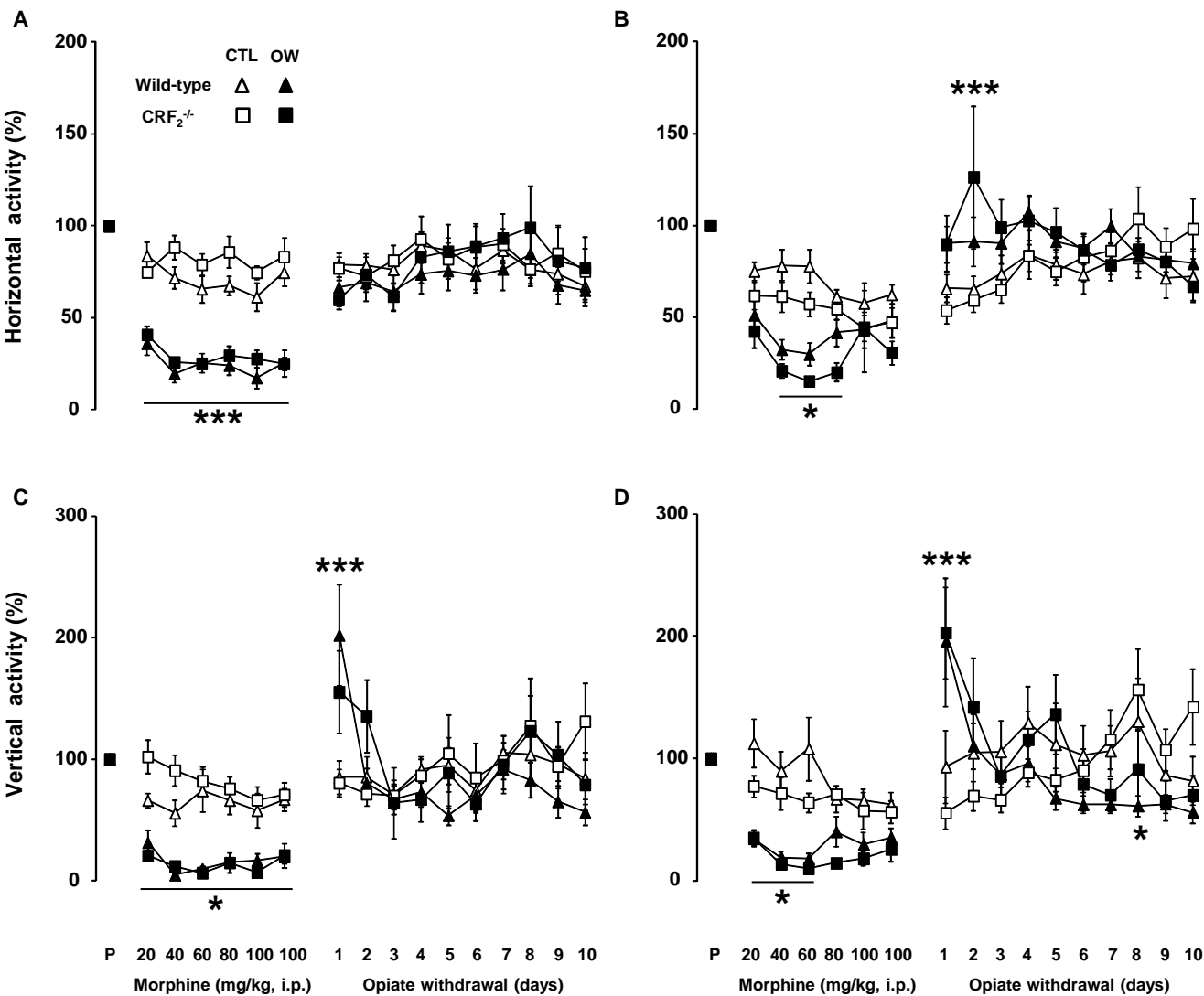
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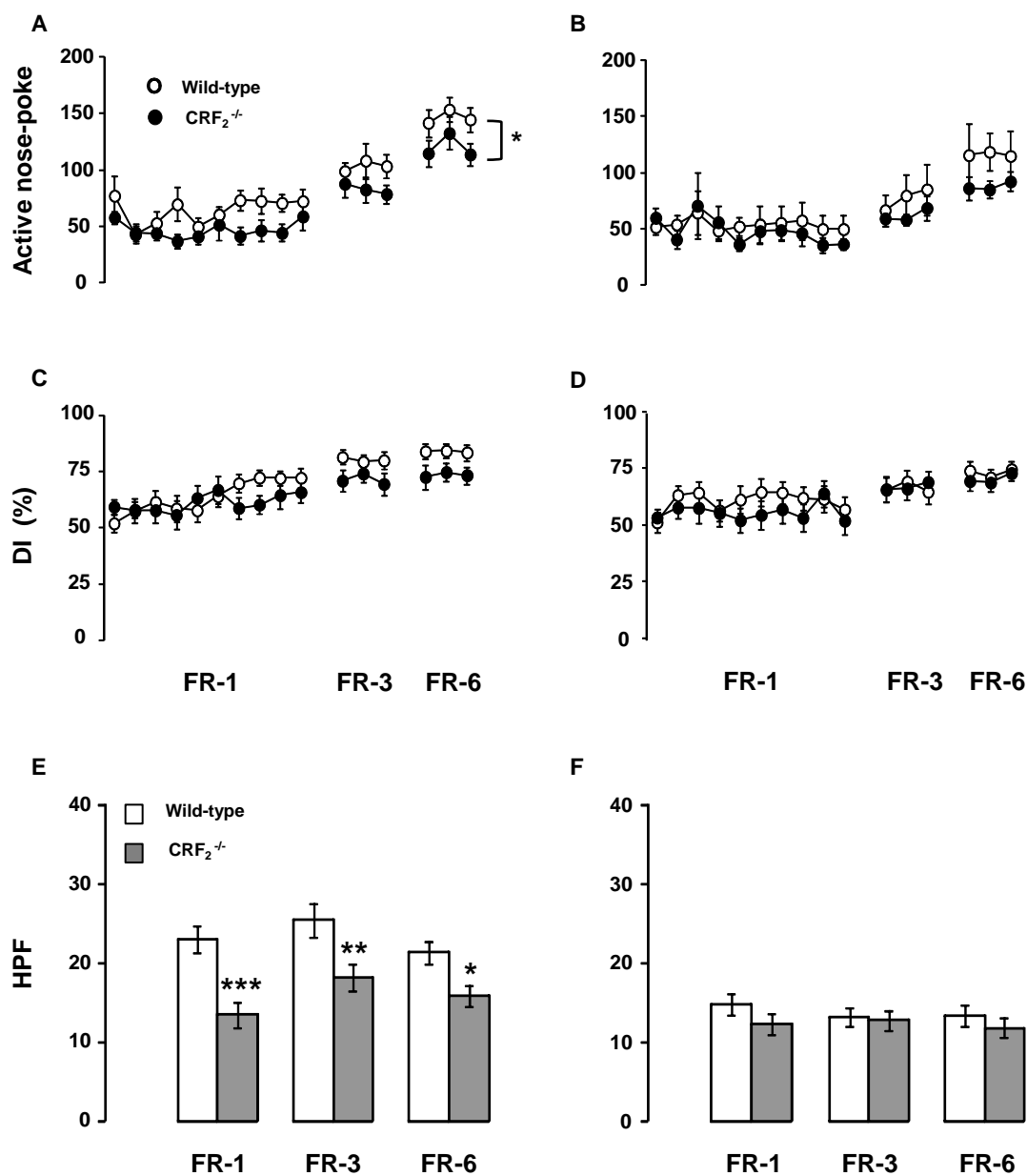
Figure S1. Gender-dependent effect of CRF_2 receptor-deficiency upon highly palatable food-driven operant behavior. (A,B) Active nose-pokes, **(C,D)** discrimination index (DI) and **(E,F)** mean daily number of highly palatable food (HPF) pellets ingested by wild-type or $CRF_2^{-/-}$ (left panels) male or (right panels) female mice during the FR-1 (10 days), the FR-3 (3 days) and the FR-6 (3 days) schedules of reinforcement. Values represent mean±s.e.m. N= 22-23/group. *P<0.05, **P<0.01, ***P<0.0005, versus wild-type mice.

Figure S2
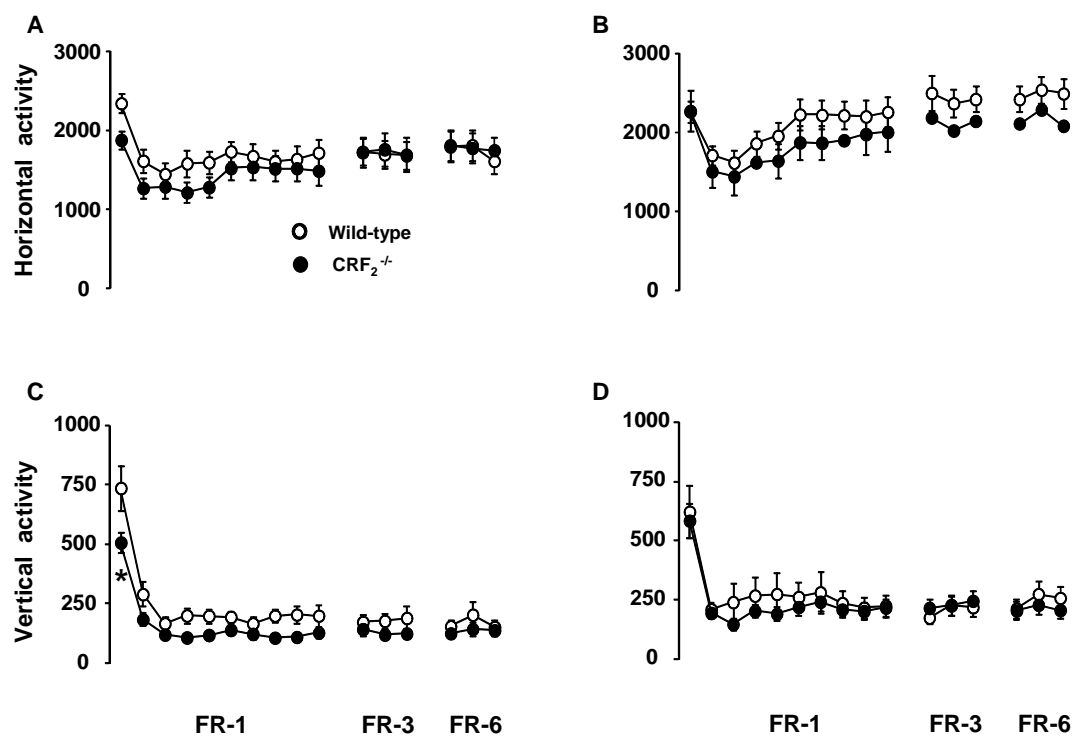
Figure S2. CRF$_2$ receptor-deficiency does not affect ambulation. (A,B) Horizontal and **(C,D)** vertical activity displayed by wild-type or CRF$_2^{-/-}$ (left panels) male or (right panels) female mice during the FR-1 (10 days), the FR-3 (3 days) and the FR-6 (3 days) schedules of reinforcement. Values represent mean±s.e.m. N= 22-23⁄group. *P<0.05, versus wild-type mice, at the same time point.

Figure S3
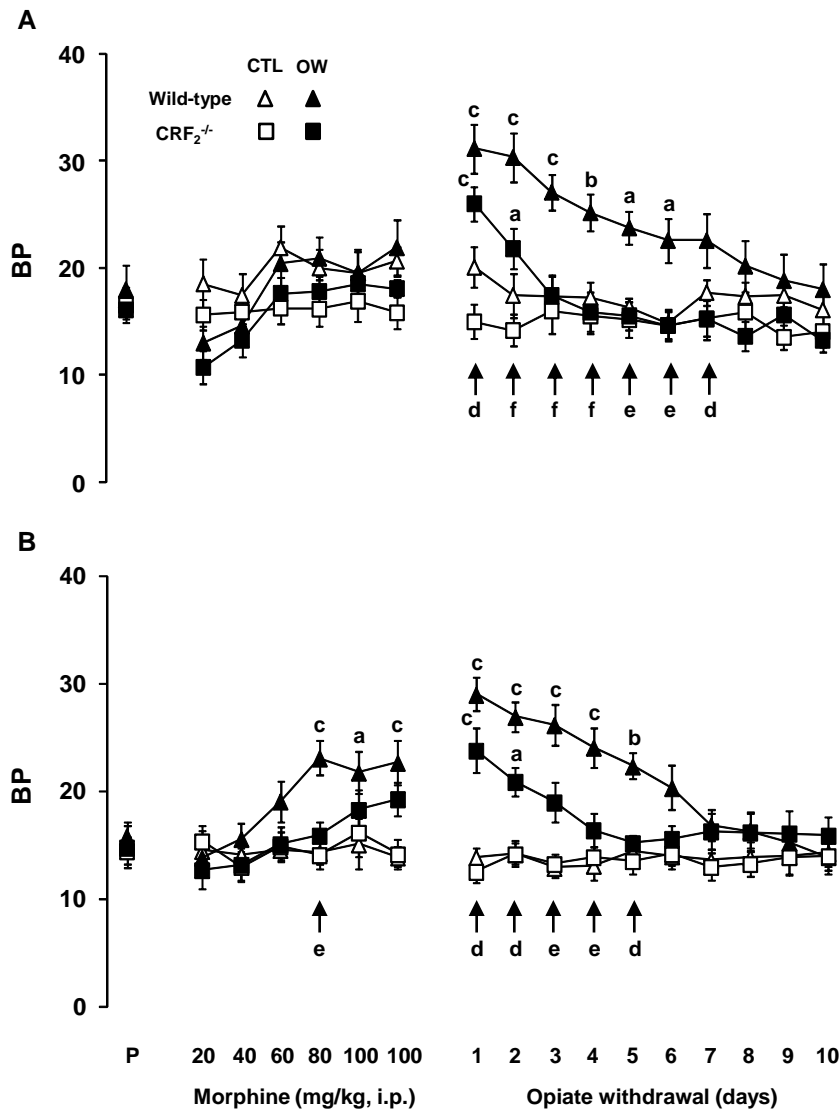
Figure S3. CRF$_2$ receptor-deficiency reduces the increase in highly palatable food intake induced by opiate withdrawal. Highly palatable food (HPF) pellets ingested by control (CTL) or opiate-withdrawn (OW) **(A)** male or **(B)** female wild-type or CRF$_2^{-/-}$ mice under a progressive ratio (PR)-2 schedule of reinforcement prior to (P, third PR-2 day), during and following intermittent morphine injections (opiate withdrawal). Values represent mean±s.e.m. N=10-12∕group. Statistical analysis reveals a genotype X opiate withdrawal X repeated measure interaction effect (male: $F_{16,656}$= 2.76, P<0.0005; female: $F_{16,624}$= 1.72, P<0.05). N= 10-12∕group. [a]P<0.05, [b]P<0.005 and [c]P<0.0005, versus same genotype CTL mice. [d]P<0.05, [e]P<0.005 and [f]P<0.0005, OW CRF$_2^{-/-}$ versus OW wild-type mice.

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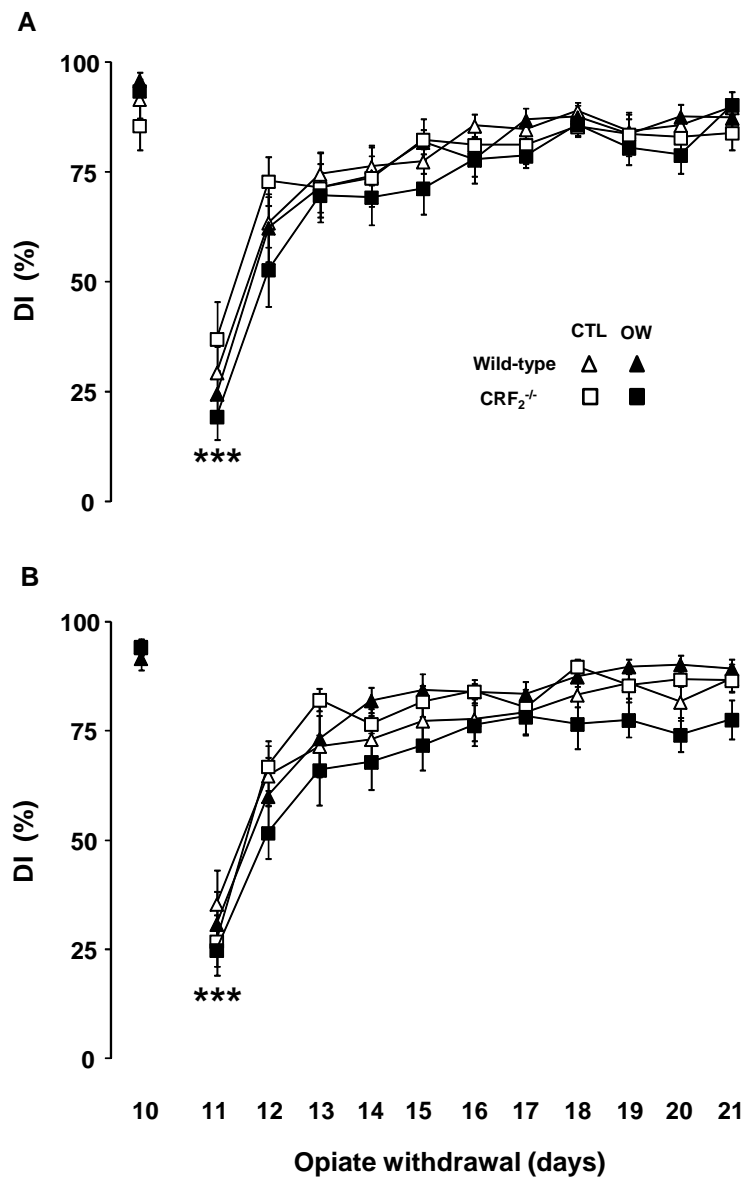
Figure S4

Figure S4. CRF$_2$ receptor-deficiency does not affect operant behavior learning during opiate withdrawal. Discrimination index (DI) displayed by **(A)** male or **(B)** female wild-type or CRF$_2^{-/-}$ mice under a progressive ratio (PR)-2 schedule of reinforcement during the 10th opiate withdrawal day and following reversal of highly palatable food (HPF) contingency. The reversal exercise is started on the 11th opiate withdrawal day. Values represent mean±s.e.m. N=10-12/group. *P<0.0001, versus the 10th and the 12th opiate withdrawal day.

Figure S5
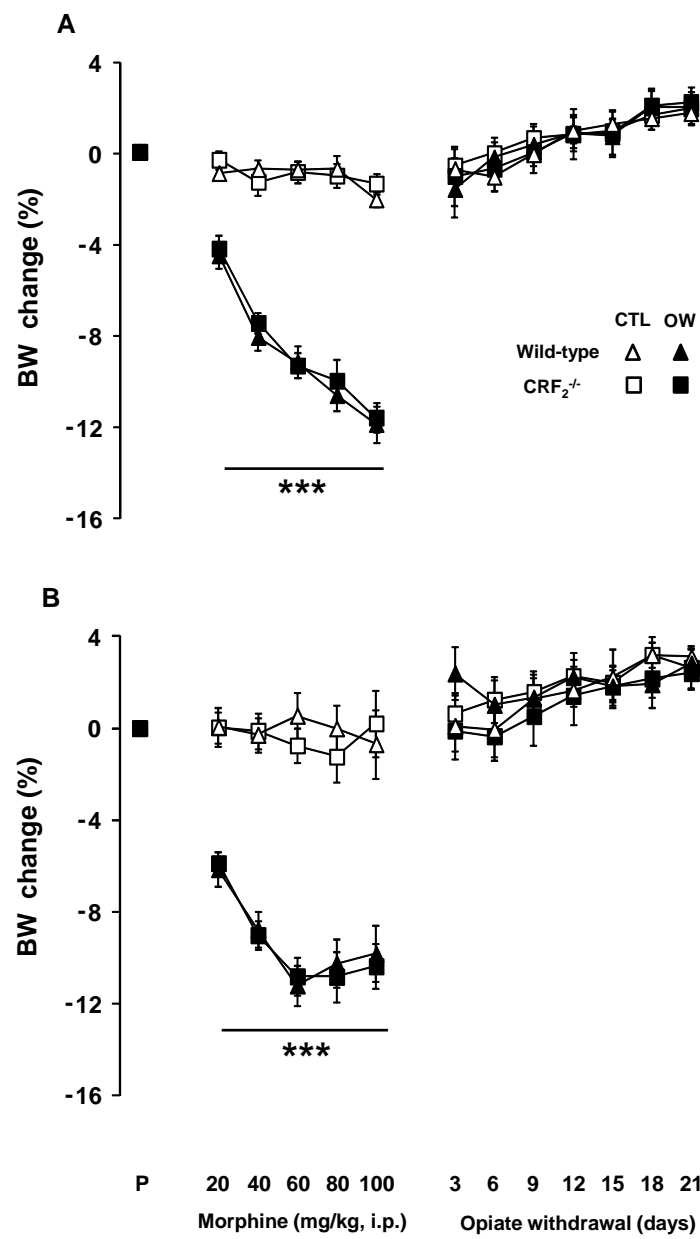
Figure S5. CRF$_2$ receptor-deficiency does not affect body weight changes induced by opiate injections and withdrawal. Body weight (BW) changes, calculated as percentage of BW recorded just prior to the first saline or morphine injection, displayed by control (CTL) or opiate-withdrawn (OW) **(A)** male or **(B)** female wild-type or CRF$_2^{-/-}$ mice just prior to (P), during and following intermittent morphine injections (opiate withdrawal). Represented are the BW changes recorded twelve hours after the second injection of the morphine dose indicated. Values represent mean±s.e.m. N=10-12/group. *P<0.0005, versus CTL mice.

Chapter IV

Etude 4 : La délétion génétique du récepteur CRF$_2$ élimine les effets du stress sur le rétablissement du comportement de recherche à la nourriture lors d'un sevrage aux opiacés chez les souris mâles mais pas femelles

RESUMÉ

L'implication du stress dans le phénomène de rechute à l'alcool et aux drogues, durant la période d'abstinence, a fait l'objet de plusieurs études chez l'Homme. Par exemple, chez les personnes dépendantes aux drogues opiacées, des évènements stressants ainsi que des désordres psychologiques tels que la dépression et l'anxiété sont considérés comme des facteurs à risque pouvant conduire à une rechute de prise de drogues opiacées pendant la période de sevrage.

L'objectif de ce *chapitre IV* était d'explorer le rôle du récepteur CRF$_2$ comme possible mécanisme contribuant au phénomène de rechute lors du sevrage aux opiacés (SAO) en mimant les évènements stressants observés chez l'humain dans un model murin.

Suite à une procédure de tâche opérante vis-à-vis d'une nourriture hautement palatable, des souris ont été soumises à une période d'extinction durant laquelle la nourriture a été enlevée. Nos résultats montraient que les souris mâles et femelles de type sauvage qui ont subit un traitement préalable aux opiacés, effectuées un niveau élevé de tâche opérante par rapport aux souris naïve à la drogue. Par ailleurs, les souris CRF$_2$$^{-/-}$ mâles et femelles sevrées aux opiacés ne montraient pas de différence avec les souris du même génotype et naïves à la drogue. Suite à l'application d'un stress avant une session de conditionnement à la tâche opérante, les souris de type sauvage sevrées à l'opiacé montraient un rétablissement du comportement opérant en comparaison aux souris de type sauvage, naïves à la drogue. De façon intéressante la délétion génétique du récepteur CRF$_2$ abolissait ce dernier effet chez les souris mâles mais pas chez les souris femelles. Par la suite, la mesure du comportement de type anxieux chez les souris sevrées à l'opiacé montrait des états de type anxieux indépendamment du sexe des souris suggérant que l'induction d'un comportement de recherche de nourriture suite à l'application d'un stress n'était pas causée par des états anxiogènes induits par le SAO.

Ces résultats montrent pour la première fois chez l'animal l'implication du récepteur CRF$_2$ ainsi que l'importance du facteur sexe dans le phénomène de rechute lors de SAO.

CRF$_2$ receptor deficiency eliminates stress-induced food seeking behavior in opiate-withdrawn male but not female mice

BACKGROUND AND OBJECTIVES

It is increasingly recognized that stress is one of the crucial factors predisposing and leading to alcohol and drug seeking and relapse during abstinence (Cooper et al., 1990; McFall et al., 1992; Sarnyai et al., 2001; Shiffman et al., 1985; Sinha, 2007; Sinha and Li, 2007). In the case of human opiate dependence, the atypical responsivity and the exposure to stressful events correlate well with opioid-relapse during abstinence (Ilgen et al., 2008; Kosten et al., 1986).

Along the same line, several preclinical studies have suggested that stress enhanced the acquisition and the maintenance of psychostimulant and opioid self-administration in rats (Bozarth et al., 1989; Goeders and Guerin, 1996a, b; Piazza and Le Moal, 1996; Shaham and Stewart, 1994). Furthermore, in rats previously trained to self administer drugs; the human stressful events were recreated through the use of a variety of stressor types during the drug-free period named extinction (Lu et al., 2003). In particular, footshock stress is one of these stressors largely employed to induce reinstatement of drug self-administration or to reactivate the preference for drug-paired environment in conditioned place preference (CPP) paradigm during extinction (Lu et al., 2003). For example, the footshock stress provokes the reinstatement of operant seeking behavior during extinction for alcohol (Le et al., 1998; Martin-Fardon et al., 2000) and the majority of drugs such as opioids (Ahmed et al., 2000; Shaham et al., 2000; Shaham and Stewart, 1995b), cocaine (Ahmed and Koob, 1997; Erb et al., 1996; Mantsch and Goeders, 1999) and nicotine (Buczek et al., 1999). Likewise, footshock stress reactivates cocaine and morphine-CPP in rats during extinction (Lu et al., 2001; Wang et al., 2000). Noteworthy, other stressors such as restraint stress, chemicals, food restriction, noise, social isolation, and maternal separation can be also used to provoke the reinstatement of alcohol and drug seeking behavior (Liu and Weiss, 2003). For example, it has been shown that the induction of a "stress-like" state, by food restriction (Shalev et al., 2000) or intracranial administration of the CRF peptide (Shaham et al., 1997) induced reinstatement for heroin-seeking behavior in rats.

Therefore, the OW is characterized by a multiple alterations of the behavioral and neurobiological homeostasis responses to stress which are in their turn determinants of opiate seeking and relapse (Koob, 2008). Thus, several studies have reported a fundamental contribution of the CRF system components in opiate seeking and withdrawal behavior (Koob, 2008; Sarnyai et al., 2001). In fact, it has been already shown that footshock stress induced reinstatement of heroin seeking behavior in rats previously extended (Shaham and Stewart,

1995b). Interestingly, enough is that this reinstatement is sharply attenuated by the CRF receptors α-helical CRF antagonist suggesting a major contribution of the CRF receptors in heroin relapse (Shaham et al., 1997). Subsequently, in the case of rats previously trained to self-administer drug during 3-4 hr daily session, the targeting of the CRF_1 receptor by the systemic administration of the selective CP-154,526 antagonist both attenuates and abolishes the footshock stress-induced reinstatement of respectively heroin and cocaine seeking (Shaham et al., 1998). Latter work, showed that the intra-ventral tegmental area (VTA) administration of the antisauvagin-30 (AS-30), which is considered as a specific antagonist for the CRF_2 receptor, completely blocks the footshock stress-induced reinstatement of cocaine seeking in rats previously trained to self-administer cocaine during 4hr daily session (Wang et al., 2007). These findings indicated the implication of the both CRF receptors in drug reinstatement. However, further experiments showed that the antagonism of CRF_1 receptor, by intra-VTA administration of the NBI-27914 or R121919, has no impact on the footshock stress-induced cocaine seeking reinstatement behavior (Wang et al., 2007); which stands as the exact opposite to the above study. Finally, a very recent study reported that footshock stress or intra-VTA administration of CRF induced reinstatement of cocaine seeking behavior in rats previously trained to self-administer cocaine during 6 hr daily session (Blacktop et al., 2011). In contrast to Wang study's (2007), the intra-VTA CRF-induced reinstatement was abolished by the administration of the CRF_1 receptors antagonists: antalarmin or CP-376395, but not the CRF_2 receptor antagonists: astressin-2B or AS-30 into the VTA. Similarly, the footshock stress-induced reinstatement was abolished as well by intra-VTA antalarmin but not astressin-2B (Blacktop et al., 2011). Altogether, these studies are constrained by differences in experimental procedures. In fact this might explain the lack of consensus in terms of the role of each CRF receptor in drug relapse. In particular, footshock stress-induced drug reinstatement is not only dependent on the time access to drug during daily session of self-administration (Blacktop et al., 2011; Mantsch et al., 2008) but probably also on the way of administration and the brain region targeted by the CRF receptor antagonists. In addition, what has to be mentioned is that the pharmacological specificity of the multiple antagonists targeting the CRF receptors may be controversial. Insofar as the specificity of CRF receptor antagonists was not well established, we further privileged the use of genetically engineered mice which are invalidated for the CRF_2 receptor. In addition, because the $CRF_2^{-/-}$ mice possess a functional level of CRF_1 receptor, this model provides a good tool to better explore the role of the different components of the CRF system.

It is well established that the mesolimbic systems mediate the motivational behavior to rewarding effects of drug and food (Lutter and Nestler, 2009; Wise and Bozarth, 1987). More specifically, animal studies have shown that palatable food and sex-related olfactory cues increased dopamine release in the nucleus accumbens (Nacc) (Mitchell and Gratton, 1992).

Similarly, dopaminergic input from the VTA increase dopamine activity onto neurons in the Nacc following drug intake (Lutter and Nestler, 2009). Consequently, animal models of drug dependence that have been employed to study the impact of stress in relapse can also be generalized to explore the role of stress on the reinstatement of natural reward seeking behavior such as palatable food. Some studies in humans and animals reported the ability of stress to induce reinstatement of seeking behavior for food. As matter of fact, the relapse to aberrant eating habits in humans is inexorably linked to various psychological factors such as anxiety, depression and mood disorders (Herman and Polivy, 1975; Parylak et al., 2011; Torres and Nowson, 2007). In rats, the induction of stress-like states by the anxiogenic-drug yohimbine reinstates highly palatable food (HPF) seeking after 10-16 daily extinction sessions (Ghitza et al., 2006; Nair et al., 2008; Richards et al., 2009). Interestingly, Ghitza and colleagues (2006) reported that the CRF_1 receptor antagonist antalarmin attenuates the yohimbine-induced reinstatement of food seeking. Similar results have been observed in the yohimbine induced reinstatement of alcohol seeking (Marinelli et al., 2007) suggesting a major contribution of the CRF system in food and alcohol seeking and relapse. Yet it seems that no study has investigated the role of the CRF system in stress induced food seeking behavior and relapse associated to the critical period of OW.

In the following studies, we would be then focusing on the involvement of the CRF_2 receptor in the stress-induced reinstatement of HPF-driven operant behavior during OW. Additionally, we would also examine anxiety-like behavior during OW. The findings of the present study might enhance the understanding of the physiopathological role for the CRF system in the behavioral effects of OW, thus contributing to the development of new therapies for opiates dependence.

MATERIALS AND METHODS

Subjects

Studies are conducted in a total of 88, group-housed, littermate female and male wild-type or CRF_2 receptor null mutant ($CRF_2^{-/-}$) mice that are previously used in a series of experimental studies (See materials of Article 3).

Experimental design

Operant training:

In this study we used the same experimental groups of mice previously trained in a series of behavioral sessions of progressive ratio (PR) reinforcement schedule (see experimental design described in Article 3). In particular, the mice were tested under PR-2 upon 11 daily sessions at the 11th day following the last injection of saline/morphine treatment. During this latter experimental phase, mice were subjected to a new operant task during which the active nose-poke hole became inactive and vice-versa. The objective of this procedure is to investigate the ability of opiate-withdrawn mice to learn a new operant rule to obtain HPF in a reward contingency reversal paradigm. Soon afterward, we tested mice in daily sessions of an extinction procedure during which the HPF was removed. In particular, extinction began at the 22th day following the last injection of saline/morphine treatment. Of note, during the extinction phase, the environmental cues associated to HPF delivery such as light and the noise of the pellet dispenser motor were identical to those for the previous phases. This experimental condition was chosen to obtain a long period of extinction which permits the observation of possible differences between the animal experimental groups. Indeed, previous works conducted in rats and pigeons showed resistances to extinguish when the context food reinforcers were available during extinction phase (Mace et al., 2010; Podlesnik and Shahan, 2009). To explore the role of the CRF_2 receptor in stress induced HPF seeking behavior during OW, we applied *elevated platform* (EPF) stress (**Photo. 4)** just before operant sessions during the extinction phase of experiments to reinstate HPF-driven operant behavior. More precisely, we exposed mice to stress in within-subject and counter balanced design (morphine treatment, sex, genotype and day of stress) at the 32th and 34th days of the extinction phase. Furthermore, all animal of each experimental group are stressed during an additional day (corresponding to the 39th last day of experiments). Mice were placed during 10 min on platform (10 X 10 cm) at 40 cm of higher on the floor and were tested immediately following the stress in operant chamber.

Anxiety-like studies

Mice were tested for anxiety-like behavior using the *Light/Dark* (LD) exploration test during OW days (37 day after the last injection of saline/morphine treatment). This test is based upon

the innate aversion of rodents to illuminated environment (Costall et al., 1989; Crawley, 1985). The apparatus consists of a rectangular Plexiglas box divided in two compartments. One of these boxes is darkened ($14.5 \times 27 \times 26.5$ cm; light intensity: 0 lux) covered with Plexiglas. The other compartment is illuminated by lamps fixed on the ceiling ($28.5 \times 27 \times 26.5$ cm; light intensity in the centre of the illuminated box was 200 lux). During test, an aperture (7.5 x 7.5 cm) in the central partition allowed the mice to enter in both sides of the apparatus. Mice were tested using a split-half balanced design (genotype and treatment), i.e., within each experimental group. On the test day, the mice were transferred at 08:00 a.m. from the colony room to a room adjacent to the testing room to 1hr habituation, and tests was performed between 9.00 a.m. and 13.00 p.m. for males and between 13.30 a.m. and 16.00 p.m. for females. To initiate the test session, mice were individually placed into the centre of the lit area facing back the entrance of the dark chamber. Each animal was tested in a 10 minute session, with test time starting after the first entry into the dark area (the animal was eliminated if it exceeded 6 min of his first entrance to the dark compartment). The LD apparatus was cleaned with plain water after each 10 min test. Behaviors scored were: a) the latency of the initial movement from the lit to the dark area, b) latency to re-enter the lit area, c) the time spent in the lit area, d) the number of transitions between the two compartments and e) the number of exploratory rearing in the lit area. A camera mounted above the apparatus allowed the observation and scoring of the animal's behavior on a video monitor placed in an adjacent room.

Statistical analysis

A three-way ANOVA with genotype and treatment (saline or morphine) as between subjects factors and repeated measures as a within-subject factor was used, for each sex, to analyze the nose-pokes and percentage of discrimination index (DI) observed throughout the operant conditioning associated with OW days. A two-way ANOVA with genotype and treatment as independent variables was used to examine anxiety-like behaviors (light-dark and dark-light latencies, time spent in the lit area, dark-light transitions, exploratory rearing) induced by the past morphine experience. The Student's–Newman–Keuls post-hoc test was used for individual group comparisons. The accepted value for significance was $P < 0.05$. Testing will be performed under blind conditions (the operator could not associate an animal to an experimental group).

RESULTS

CRF$_2$ receptor deficiency and prolonged opiate withdrawal do not affect the ability to maintain HPF-seeking behavior during new operant task:

Using a reward contingency reversal paradigm we reported previously in *chapter III* (Article 3) that the wild-type and the CRF$_2$$^{-/-}$ CTL and opiate withdrawn mice displayed no alterations of cognitive functions assessed by the DI. Analysis of the active nose-pokes performed by male mice following reversal of the food-paired nose-poke hole (11 days after the last morphine injection) revealed no repeated measure effect (F$_{11-444}$=1.35, P=ns) and no genotype x treatment x repeated measure effect (F$_{11-444}$=0.69, P=ns; **Fig. 1A**). Similarly, analysis of active nose-poke performed by female revealed no repeated measure effect (F$_{11-429}$=1.62, P=ns) and no genotype x treatment x repeated measure effect (F$_{11-429}$=0.73, P=ns; **Fig. 1B**). Importantly, these data revealed that both CRF$_2$ genetic deficiency and OW did not affect the ability of mice to engage a new operant behavior to obtain HPF during a new operant task.

Long-lasting resistance to food seeking behavior during extinction in wild-type opiate-withdrawn but not in CRF$_2$$^{-/-}$ opiate-withdrawn mice

Analysis of active nose-pokes performed by male mice during extinction revealed repeated measure effect (F$_{9-360}$=46.21, P<0.0005), genotype x treatment effect (F$_{1-40}$=4.81, P<0.05) and not genotype x treatment x repeated measure effect (F$_{9-360}$=0.97, P=ns). Post-hoc comparison of the repeated measure effect showed a complete extinction of operant behavior in the 5th day of extinction days **(Fig. 1A)**. Post hoc comparison of genotype x treatment effect showed a higher level of total active nose-pokes performed by wild-type opiate-withdrawn mice compared to others groups (P<0.005) indicating a higher global maintain of operant responding for HPF in wild-type opiate-withdrawn mice compared to CRF$_2$$^{-/-}$ opiate-withdrawn mice (see column representation of total active-nose poke in Fig. 1A). Analysis of active nose-pokes performed by female mice revealed repeated measure effect (F$_{9-351}$=43.42, P<0.0001) and treatment x repeated measure effect (F$_{9-351}$=1.89, P=0.05). Post-hoc comparison of repeated measure effect showed a complete extinction of operant behavior in the 6th day of extinction days **(Fig. 1B)**. Post hoc comparison of treatment x repeated measure effect revealed a higher level of active nose-pokes performed by opiate-withdrawn mice than CTL mice at 2nd and 3rd day of extinction phase (P<0.05). However, closer inspection of the statistical data revealed a higher tendency to nose-poking in wild-type opiate-withdrawn female mice compared to all other groups (see column representation of total active-nose poke in Fig. 1B).

Analysis of DI revealed no genotype x treatment x repeated measure effect in male (F$_{9-360}$=0.76, P=ns) and female mice (F$_{9-351}$=0.68, P=ns). This results indicate that the discriminative

properties of HPF-driven operant behavior is not altered in wild-type and $CRF_2^{-/-}$ mice during extinction (Data not shown).

Reinstatement of food-driven operant behavior after acute stress is blocked in opiate-withdrawn $CRF_2^{-/-}$ male but not female mice

Analysis of active nose-pokes performed by male mice after acute stress revealed genotype x treatment x repeated measure effect (F_{2-78}=3.12, P<0.05). Post-hoc comparison of this effect indicate clearly a reinstatement of operant food seeking behavior in wild-type opiate-withdrawn mice compared to wild-type CTL mice after each stress (P<0.0005). However, the $CRF_2^{-/-}$ opiate-withdrawn mice do not exhibited reinstatement of operant food seeking behavior in stress 1 as well as stress 2 (P=n.s; **Fig. 3A**). On the other hand, the analysis of active nose-pokes performed by female mice after acute stress revealed a treatment x repeated measure effect (F_{2-78}=7.86, P<0.001). Post-hoc comparison of this effect indicate clearly a reinstatement of operant food seeking behavior in wild-type as well as $CRF_2^{-/-}$ opiate-withdrawn mice compared to CTL mice after each stress (P<0.0005; **Fig. 3B**).

Analysis of DI revealed no genotype x treatment effect x repeated measure effect in male (F_{2-78}=0.27, P=ns) as well as in female mice (F_{2-78}=0.19, P=ns) indicating that genetic deletion or/and OW condition did not alter the ability to discriminate food-paired nose-poking during stress-induced HPF-seeking behavior (data not shown).

Overall, the opiate-withdrawn mice showed a strong vulnerability to reinstate food operant seeking behavior which is abolished by the genetic deletion of CRF_2 receptor in male mice. These results provide a novel evidence of a gender-dependant genotype effect in the relapse to food reward during the critical period of OW.

Genotype-independent opiate withdrawal-induced anxiety-like behavior

Exposure to opiate treatment profoundly increases anxiety-like behavior several weeks after last morphine injection in wild-type and $CRF_2^{-/-}$ male and female mice. In particular, a decrease of LD latency and increases in the DL latency, displayed by opiate-withdrawn wild-type and $CRF_2^{-/-}$ male and female mice is considered as markers of anxiety-like behavior. Similarly, a decrease of time spent in the lit compartment and number of transition and rearing performed by opiate-withdrawn wild-type and $CRF_2^{-/-}$ male and female mice reflect an increase of anxiety-like behavior.

More precisely, analysis of time spent before the first passage in the dark compartment (Light-dark latency) performed by male and female mice revealed treatment effect (Male: F_{1-39}=9.09, P<0.005; Females: F_{1-39}=12.98, P<0.001). Post-hoc comparison of light-dark latency showed a rapid passage of opiate-withdrawn mice to the dark compartment compared to CTL

mice (P<0.001; **Fig. 4A and B**). Analysis of the time spend before the first passage to the lit compartment (Dark-light latency) performed by male and female mice revealed treatment effect (Male: $F_{1-39}=8.32$, P<0.01; Female: $F_{1-39}=7.28$, P<0.05). Post-hoc comparison of dark-light latency revealed a slow passage of opiate-withdrawn mice to the lit compartment compared to CTL mice P<0.05; **Fig. 4C and D**). Analysis of the time spend by male and female mice in the lit compartment during test revealed treatment effect (Male: $F_{2-39}=9.54$, P<0.005; Female: $F_{1-39}=10.74$, P<0.005). Post-hoc of this latter parameter comparison showed less time spent by opiate-withdrawn mice in the lit compartment compared to CTL mice (P<0.005; **Fig. 4E and F**). Subsequently, analysis of transitions performed by male and female mice between the lit and the dark compartments during test revealed treatment effect in male mice ($F_{1-39}=12.35$, P<0.005) but not in female mice ($F_{1-39}=3.82$, P=ns; **Fig. H**). In particular, post-hoc comparison showed less transition performed by opiate-withdrawn male mice than CTL mice (P<0.005; **Fig. 4G**). Finally, analysis of rearing performed by male and female mice in the light compartment during LD test revealed treatment effect (Male: $F_{1-39}=10.54$, P<0.005; Female: $F_{1-39}=8.72$, P<0.01). Post-hoc comparison showed less rearing performed by opiate-withdrawn male and female mice than CTL mice (P<0.01; **Fig. 4I and J**).

DISCUSSION

The current study used an operant behavior paradigm to assess the long-term effect of past opiate experience on the reinstatement of food seeking behavior following unpredictable stress. In first, the prolonged OW and CRF_2 genetic deletion do not affect food-seeking operant behavior following application of reward contingency reversal paradigm. Along the same line with data previously reported in *chapter III*, the OW induced long-term changes in motivation for HPF following cessation of opiate dosing. In particular, opiate-withdrawn wild-type mice, but not $CRF_2^{-/-}$ mice, displayed a higher operant behavior responding during extinction phase indicating an increased motivation for HPF. In addition, the present results extend and corroborate previous findings by demonstrating that the motivational alterations induced by OW is least mediated by the activation of the CRF_2 receptor. Using an atypical stress procedure, we consistently found that prior opiate treatment induced HPF-reinstatement in wild-type male and female mice. The major finding of the present work showed that the genetic deletion of the CRF_2 receptor prevented the stress-induced HPF-reinstatement in male but not in female mice. These latter results showed novel evidence supporting the role of the CRF system upon gender-linked opiate withdrawal-induced hyper-motivation. Finally, CRF_2 receptor-deficiency did not contribute to anxiety-like responses elicited by long-term OW.

Using a reward contingency reversal paradigm, we investigate the ability of wild-type and $CRF_2^{-/-}$ mice to maintain HPF operant seeking behavior during OW. We argued previously that the powerful reinforcing hedonic effect of the HPF, leads the mice to improved rapidly their DI during the first daily sessions of the reversing the active with the inactive nose-poke hole (reversal phase). Supporting this latter notion, here we reported that the level of active nose-pokes performed by mice was unchanged before and during reward contingency reversal paradigm (compare baseline and the first day of the reversal phase in figure 1A and B). This behavior has as consequence to conserve a nearly similar level of HPF intake in mice between baseline conditions and the first day of the reversal phase. In particular, the mean number of pellets ingested by the mice during the baseline day and the first day session of the reversal phase is ~ 15±1.4 and 12± 2.2 pellets, respectively. Altogether, these data showed that despite new conditions in operant rule, all mice have re-acquired and maintained the HPF-driven operant behavior independently with genotype, gender or opiate treatment.

Afterward, we tested mice in daily sessions of extinction during which the HPF was removed. In first, we reported that male and female mice have completely decreased the responses rates of active nose-pokes at the 5th and 6th days of extinction phase, respectively. Remarkably, this extinction is rapid despite the continuous presence of environmental stimulus (light and noise) in operant chamber that was previously associated to the HPF delivery

following the active nose-poking. This agree with previous studies conducted in rats shown a rapid extinction which does not exceeded 6 daily session in the presence of environmental cues previously associated with palatable food-driven operant behavior (Ghitza et al., 2006; Nair et al., 2008; Nair et al., 2006). Secondly, our data showed a higher level of total active nose-pokes performed by wild-type opiate-withdrawn male and female mice compared to CTL groups during extinction phase. This data provides initial evidence of an effect of the OW on long-lasting motivational alterations following cessation of opiate dosing. We reported previously that morphine withdrawal increases HPF-drive operant behavior approximately 1 week following opiate regimen (Article 3). However, the development of motivational alterations is temporally concomitant to a marked negative-energy balance status which makes difficult to dissociate between the motivation and the energy-driven properties of food intake under "food restriction like-states" elicited by OW. Because mice are group-housed during experiments, we did not measure the regular home cage food intake, which represents a limitation in the interpretation of our results. However, herein we reported that the motivational effects of OW are observed during extinction sessions when HPF was removed. In addition, during the previous reversal phase, mice displayed no motivational alterations suggesting that motivational and energy balance alterations are not causal-linked. Finally, long-lasting resistance to food seeking behavior extinction was abolished by the genetic deletion of the CRF_2 receptor.

Most recently, evidence showed that a hyperactivity of the CRF_2 receptor is implicated in the motivational disorders elicited by OW (Article 3). The main finding of this study provides new support toward fundamental gender-related role of the CRF_2 receptor in the drug seeking and relapse. In particular, EPF stress reinstate HPF-driven operant seeking behavior in wild-type opiate-withdrawn female and male mice. However, the genetic deletion of the CRF_2 receptor prevented the HPF reinstatement in male but not in female mice. In agreement with our results, several studies showed that footshock stress failed to reinstate food seeking behavior in drug-free mice (Martin-Garcia et al., 2011) and rats (Ahmed and Koob, 1997; Buczek et al., 1999). However, others showed reinstatement of brain stimulation reward induced by footshock stress (Howarth and Deutsch, 1962; Shaham et al., 2000) suggesting differential physiological and neurological adaptations controlling the stress induced brain reward stimulation and food seeking behavior (Shalev et al, 2000). As far as we know, this is the first study which reported reinstatement of food-seeking behavior with other stressor than the yohimbine (Nair et al., 2009; Nair et al., 2006). Nevertheless, our data showed clearly that past opiate experience is a necessary condition to reinstate food seeking behavior following stressful episode. Noteworthy, our data suggested also that the stress-induced reinstatement behavior might be generalized to other animal species, non-drug reinforcers and stressors under the particular condition of the OW.

Studies on the neuronal mechanisms underlying drug priming-induced heroin and cocaine reinstatement have identified the mesocorticolimbic DA system as a major region implicated in the motivational reinforcing effect of drugs (Shaham et al., 2003). In particular, a series of experiments using D_2-like receptor agonists showed a similar effect of heroin or cocaine on reinstatement of drug seeking behavior (Khroyan et al., 2000; Self and Nestler, 1998; Spealman et al., 1999). In opposite, the systemic injections of D_1- and D_2-like receptor antagonists or opioid receptor antagonists have been found to block reinstatement of drug seeking behavior provoked by priming injections of heroin (Shaham et al., 1996; Shaham and Stewart, 1996). Latter woks showed that lesions of the VTA or the Nacc Shell attenuate reactivation of morphine-CPP (Wang et al., 2002). One possible explanation of the reinstatement of seeking behavior in wild-type opiate-withdrawn mice is that past opiate experience resulted in a profound long-term neuroadaptations in the mesocorticolimbic DA system.

Furthermore, clinical reports showed that stress influence eating pattern in human and might contribute to the development of eating disorders (Torres and Nowson, 2007). Similarly, previous works showed that consumption of comfort food induced dopamine release in the Nacc (Bassareo and Di Chiara, 1997; Rada et al., 2005) and reduced neuro-physiological responses to stress in animals (Dallman et al., 2003; Dallman et al., 2005; Pecoraro et al., 2004). For example, Pecoraro and colleagues (2004) showed attenuation of hypothalamic CRF expression and reduction of plasma ACTH and corticosterone responses to restraint stress following HPF intake. On the other hand, OW increases CRF, plasma adrenocorticotropic hormone (ACTH) and corticosterone levels in rodents (Houshyar et al., 2003; Houshyar et al., 2004; McNally and Akil, 2002; Papaleo et al., 2007a). Based on several behavioral and biological observations, it is well reported that HPF decreases somatic and negative affective components of OW (Contarino and Papaleo, 2005; Ingallinesi et al., 2011; Jain et al., 2004; Papaleo et al., 2008). Altogether, increase level of an operant seeking behavior previously associated to the hedonic properties of HPF in wild-type mice could protects against the behavioral and neurological sequelae of unavoidable OW stressful conditions.

Although, CRF_2 receptor deficiency eliminates the stress induced HPF seeking in male but not female opiate-withdrawn mice. Thus, stress typically evokes neuronal and physiological responses characterized by the activation of the CRF system. The CRF_2 antagonism by intra-VTA administration AS-30 completely blocks the footshock stress-induced reinstatement of cocaine seeking in rats previously trained to self-administer (Wang et al., 2007). However, others reported a crucial contribution of the CRF_1 receptor, but not CRF_2 in cocaine seeking behavior (Blacktop et al., 2011). Of note, these studies used CRF_2 antagonism with molecules such as AS-30 or astressin-2B which the specificity toward the CRF_2 receptor is not well established. However, it is highly plausible that other various factors (in the present and the cited studies)

such as animal species, reinforcers, stressors, gender, environment, etc, could impact differently on drug and food seeking behavior making difficult the knowledge of the role of the CRF system.

Interestingly, the genetic deletion of the CRF_2 receptor does not protect female mice toward the stress-induced reinstatement suggesting complex contribution of this receptor in drug relapse in female. In human literature, compared to men, women are more vulnerable to stress-related psychiatric disorders such as depression and anxiety (Kessler et al., 1993; Young et al., 2007). Using electrophysiological study, some authors showed that the major components of brain stress system such as CRF system and neuroepinephrine system in the locus coereleus (LC) are more sensitive to stressors in female than male rats (Curtis et al., 2006). Of note, the CRF hyper-secretion and neuronal hyper-sensitivity of the LC is a marker of the development of psychiatric disorders (O'Donnell et al., 2004). Thus, Bangasser and colleagues suggested that sex differences in these systems contribute to the higher compromised stress adaptations and vulnerability to psychiatric disorders observed in female. In particular, sexual dimorphism in CRF_1 receptor signaling, trafficking and internalization was well determined in rats (Bangasser et al., 2010). More precisely, compromised internalization and increased CRF signaling in female compared to male results in an enhancement of post-synaptic CRF function. In addition, immune-fluorescence studies showed that LC dendrites are longer and complex than those of male, suggesting heightened emotional arousal response in female (Bangasser et al., 2011).

Using light-dark test, in the present study we also investigate the anxiety-like states during OW. Compared to CTL group, opiate withdrawn male and female mice exhibited significant differences in anxiety-like behavior. In these experiments the anxiogenic effect of OW in not mediated by the CRF_2 receptor because no differences were observed between genotypes. These results suggested that stress-induced HPF reinstatement of seeking behavior is not linked to anxiety behavior. In fact the $CRF_2^{-/-}$ opiate withdrawn male mice which do not exhibited reinstatement of food seeking behavior shown similar anxiety-like behavior than wild-type opiate-withdrawn mice.

Throughout the present study, we demonstrate that OW induced long-term changes in motivation for HPF following cessation of opiate dosing. We showed also that prior opiate treatment facilitates stress-induced HPF reinstatement in wild-type male and female mice. This effect is abolished by the genetic deletion of the CRF_2 receptor male but not in female mice. Finally, food seeking relapse during OW is not related to anxiogenic-like effect of protracted abstinence. These findings underscore the importance of considering gender dimorphism in CRF receptor function in developing reliable treatment of opiate dependence.

Photo. 4 Elevated platform stress. Mice were placed during 10 min on platform (10 X 10 cm) at 40 cm of higher on the floor and were tested immediately following the stress in operant chamber.

Figure 1
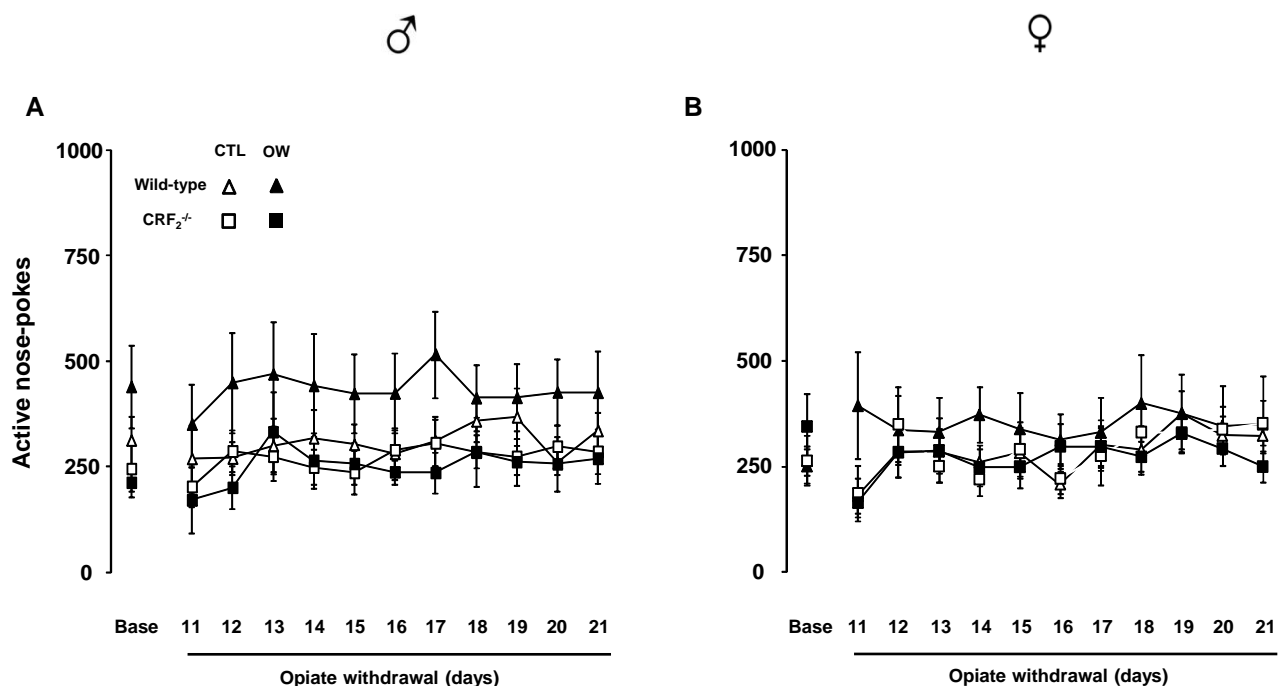
Fig. 1 CRF$_2$ receptor deficiency and prolonged opiate withdrawal do not affect the ability to maintain HPF-seeking behavior during new operant task. Active nose-pokes displayed by control (white symbols) or opiate-withdrawn (black symbols) wild-type (triangle symbol) and CRF$_2^{-/-}$ (square symbol) male **(A)** and female **(B)** mice 11-21 days after the last morphine injection. During this experimental phase, the previously active nose-poke hole represented here by the baseline day (base) is inactive, and vice versa (reversal of reward contingency). Values represent mean±s.e.m. N=10-12⁄group.

Figure 2
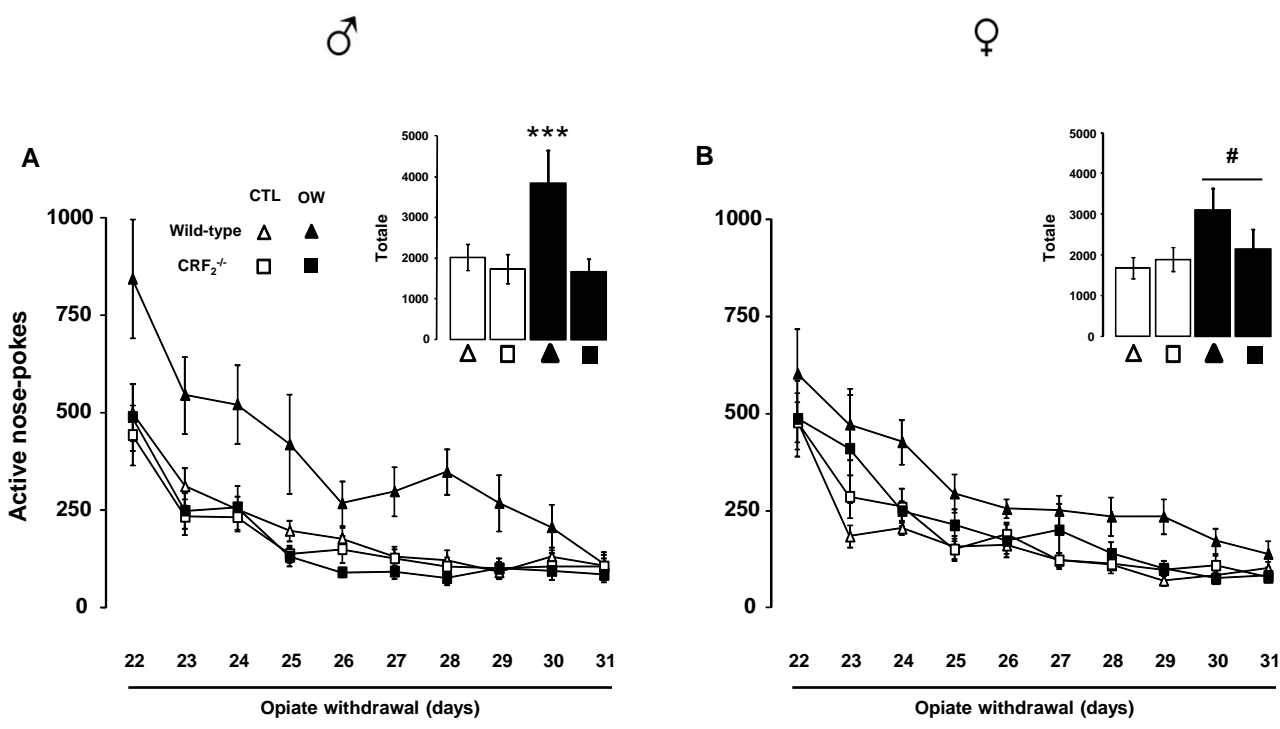
Fig. 2 Long-lasting resistance to food seeking behavior during extinction in wild-type opiate-withdrawn but not in CRF$_2^{-/-}$ opiate-withdrawn mice. Active nose-pokes displayed by control (white symbols) or opiate-withdrawn (black symbols) wild-type (triangle symbol) and CRF$_2^{-/-}$ (square symbol) male **(A)** and female **(B)** mice at 22 to 31 days after the last morphine injection. During this experimental phase, the HPF was removed. ***P<0.005, versus all other groups, #P<0.05, versus CTL mice. Values represent mean±s.e.m. N=10-12⁄group.

Figure 3
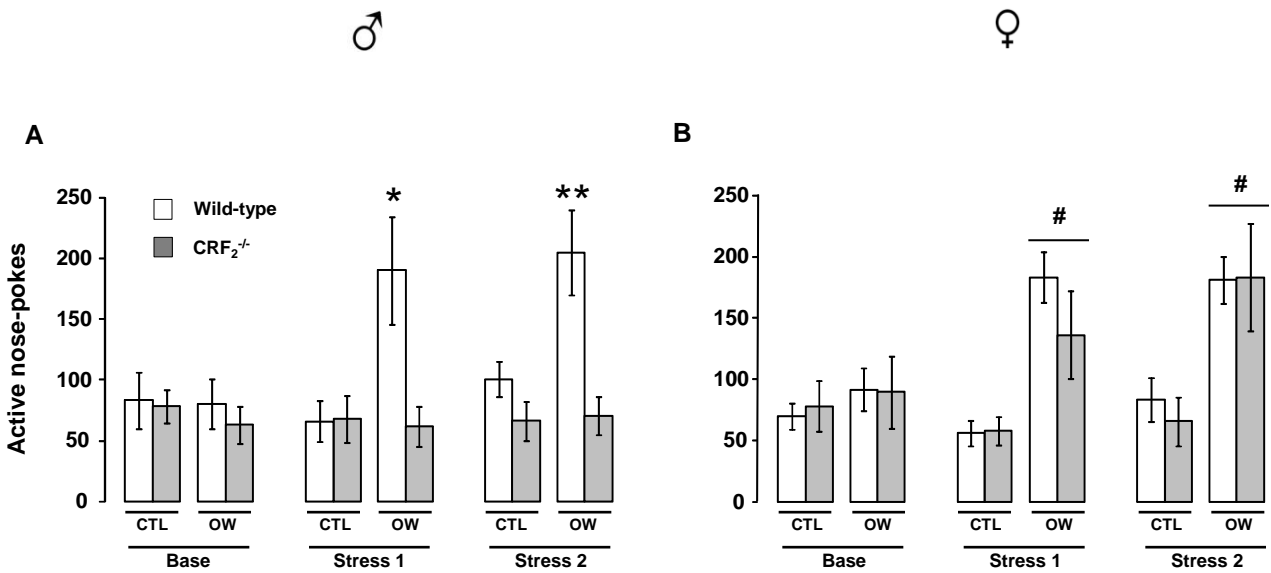
Fig. 3 Reinstatement of food-driven operant behavior after acute stress is blocked in opiate-withdrawn CRF$_2^{-/-}$ male but not female mice. Active nose-pokes displayed by control (CTL) and opiate-withdrawn (OW) wild-type (white column) and CRF$_2^{-/-}$ (grey column) male **(A)** and female **(B)** mice during baseline conditions (Base), stress 1 (32th and 34th day after last injection of morphine) and stress 2 (39th day after last injection of morphine). *P<0.001, **P<0.0005, versus all other groups, #P<0.0005, versus CTL mice. Values represent mean±s.e.m. N=10-12/group.
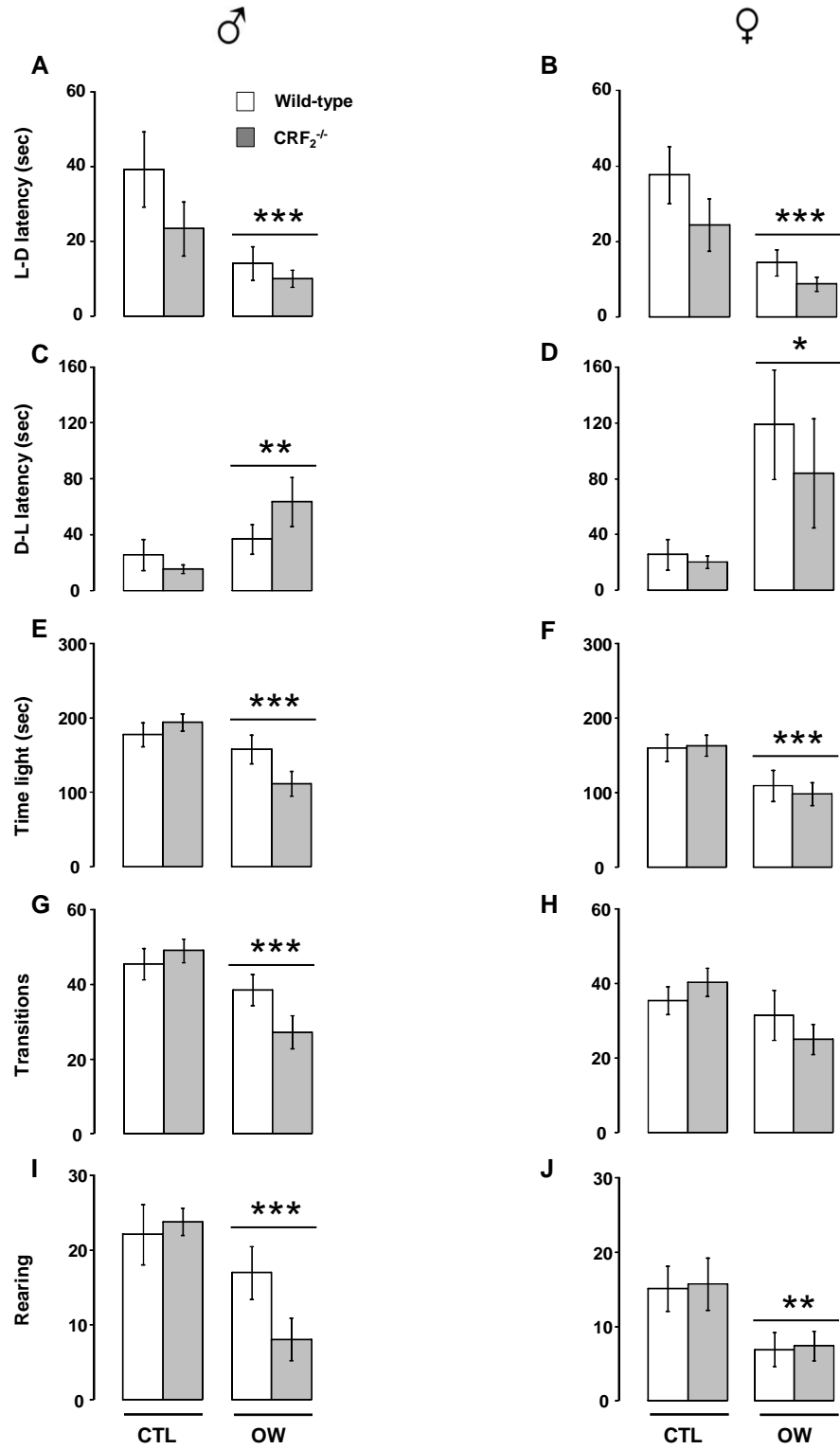
Fig. 4 Genotype-independent opiate withdrawal-induced anxiety-like behavior. Time spent before the first entrance to the dark compartment (L-D latency) in control (CTL) and opiate-withdrawn (OW) wild-type and CRF$_2$$^{-/-}$ male **(A)** and female **(B)** mice. Time spent before the first entrance to the lit compartment (D-L latency) in control (CTL) and opiate-withdrawn (OW) wild-type and CRF$_2$$^{-/-}$ male **(C)** and female **(D)** mice. Total time spent in the lit compartment by CTL and OW wild-type and CRF$_2$$^{-/-}$ male **(E)** and female **(F)** mice. Number of transitions between compartment displayed by CTL and OW wild-type and CRF$_2$$^{-/-}$ male **(G)** and female **(H)** mice. Number of rearing in the lit compartment displayed by CTL and OW wild-type and CRF$_2$$^{-/-}$ male **(I)** and female **(J)** mice. The wild-type and CRF$_2$$^{-/-}$ genotypes were represented by white and grey column, respectively. *P<0.05, **P<0.01, ***P<0.005versus CTL mice. Values represent mean±s.e.m. N=10-12/group.

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Chapter V

DISCUSSION

Stress is one of the major commonly reported factors leading to opiate dependence and relapse. Through its two receptors: CRF_1 and CRF_2, the CRF system is a major instigator of the behavioral and neuroendocrine responses to stress. Because OW is a stressful condition, it is not surprising that several authors hypothesize that CRF system could play a fundamental role in the critical problem of opiate intake and relapse during OW. Accordingly, intensive researches, specially this latter decade, contributed to the characterization of the implications of the CRF_1 receptors in opiate dependence. However, main questions about the role of the CRF_2 receptor in the emergence of the multiple components of the negative affective states of the OW syndrome are still unsolved. The aim of the present thesis is to answer the following research questions (Compare the contents of **table 3 and 4**):

A. What is the role of the CRF_2 receptor pathways in the critical components of OW distress, such a dysphoria and anhedonia, and the underlying brain mechanisms?

In *chapter II*, we reported that the genetic deletion of the CRF_2 receptor in mice completely eliminates dysphoria- and anhedonia-like states provoked by OW. Moreover, unlike wild-type mice, opiate-withdrawn $CRF_2^{-/-}$ mice do not show increase in DYN, CRF and *c-fos* expression in the NaccSH, the CeA and the PAG, respectively, that is brain circuitry thought to underlie drug withdrawal distress. Furthermore, opiate-withdrawn $CRF_2^{-/-}$ mice do not show impairment of the PVN-CRF/HPA-axis, LC-TH and autonomic responses that is essential to counteract the stressful conditions of OW.

B. What is the contribution of the CRF_2 receptor in the motivational disorders of OW?

To answer to this question, initially work consisted to develop a mouse model of HPF seeking behavior using a clinically relevant SPO OW paradigm. In *chapter III* we reported that OW increases the operant behavior several days after morphine dosing, indicating excessive motivation for HPF. Moreover, OW does not affect cognitive function assessed in paradigm of reward contingency reversal. Based on these latter results, a second study was conducted in the CRF_2 receptor-deficient mice to identify the role of the CRF_2 receptor pathways in the cognitive and the motivational long-term sequelae of opiate treatment and withdrawal. For this purpose, we tested wild-type and $CRF_2^{-/-}$ female and male mice in HPF-driven operant behavior paradigm. Along the same line with data above-mentioned, we found that OW consistently increased HPF motivation in wild-type mice. Moreover, the opiate withdrawal-induced motivation is associated with increased HPF intake but is independent of ambulatory or energy balance effects of intermittent morphine injections and their cessation. In contrast, $CRF_2^{-/-}$ mice showed a very minor alteration of HPF motivation during OW. Finally, the genetic deletion of the CRF_2 receptor does not affect the ability to learn a new operant rule to obtain HPF during OW.

C. Another unresolved question concerns the role of the CRF_2 receptor pathways in the stress-induced drug relapse during OW:

To answer to this question, we used experimental groups of mice tested in the *chapter III* in additional series of experiments. In particular, we applied stress procedure following daily extinction sessions. In line with our prior data, we found in *chapter IV* that the CRF_2 receptor deficiency eliminates long-lasting resistance to food seeking behavior during extinction. Nevertheless, CRF_2 receptor-deficiency abolished stress induced-reinstatement of HPF seeking behavior in opiate-withdrawn male but not in female mice. Moreover, CRF_2 receptor-deficiency did not affect anxiogenic-like responses induced by past opiate experience. Therefore, to a large extent, these findings outline a fundamental gender-dependent critical role for the CRF_2 in stress-induced food seeking relapse during OW.

Table. 4 Identification of the role of the CRF$_2$ receptor in the behavioral and neuroendocrinological alterations elicited by spontaneous opiate withdrawal.

Abbreviations: (+) increased; **(-)** decreased; **(0)** no effect; **(A)** abolished; **(A*)** abolished only in male; **ND** not determined. **NB:** the contribution of this thesis work is represented **in red**.

	CRF$_1$$^{-/-}$	CRF$_2$$^{-/-}$
Components of negative affective states		
Dysphoria	(A)[a]	(A)
Dysphoria (precipitated OW)	(-)[b]	(A)
Somatic signs	(+)[c]	(-)[d]
Somatic signs (precipitated OW)	ND	(-)[d]
Anhedonia	ND	(A)
Anxiety-like states	ND	(0)
Motivational disorders	ND	(-)
Stress-induced reinstatement	ND	(A*)
Neuroendocrinological alterations of OW		
Increased CRF in the NaccSH	ND	(A)
Increased DYN in the NaccSH	(A)[a]	(A)
c-fos in the PAG	ND	(A)
Stress-coping abilities		
Increased CRF in the PVN	(-)[c]	(0)
Increased DYN in the PVN	(+)[c]	(0)
Increased TH in the LC	ND	(0)
Corticosterone	(-)[c]	(0)[d]
Energy balance and lcomomotion		
Feeding	(0)[e]	(0)
Body weight loss	(0)[c]	(0)
Body weight loss (precipitated OW)	(-)[b]	ND
Locomotor activity	ND	(0)

References: [a](Contarino and Papaleo, 2005), [b](Garcia-Carmona et al., 2011), [c](Papaleo et al., 2007b), [d](Papaleo et al., 2008), [e](Rouibi and Contarino, unpublished data)

The role of the CRF$_2$ receptor pathway in the dysphoria elicited by opiate withdrawal:

It is well validated that the CRF$_1$ receptor increases the dysphoria provoked by OW. Based upon the opposite actions of the CRF$_1$ and CRF$_2$ receptors on the stress responsiveness and on the emergence of OW somatic signs, we hypothesized an opposite role for the two CRF receptor pathways in opiate dependence. More precisely, we expected that hyper-activation of the CRF$_2$ receptor may be involved in the attenuation of the dysphoria elicited by OW. Surprisingly, the main finding of the experiments reported in the *chapter II* showed clearly that the disruption of the CRF$_2$ pathways completely eliminate the dysphoric linked to OW. This finding is the exact opposite of our hypothesis. More precisely, we used CPA paradigm to assess the dysphoria during SPO OW or following μ-opioid antagonist administration. Thus, unlike the wild-type mice, CRF$_2^{-/-}$ mice does not avoid environmental cues repeatedly paired with OW. Furthermore, we reported here that the genetic deletion of the CRF$_2$ receptor pathways decreases dramatically somatic signs of OW during place conditioning trials. This latter finding agree with previous work showing an elimination of the OW somatic signs by CRF$_2$ genetic deletion (Papaleo et al., 2008) or pharmacological blockade (Navarro-Zaragoza et al., 2010). Thus, based on the literature addressing the role of the CRF$_1$ receptor on the emergence of negative affective states and somatic signs, the latter result suggest that CRF$_1$ and CRF$_2$ receptors-mediated opiate withdrawal effects involve separate brain circuitry.

The role of the CRF$_2$ receptor pathway in anhedonia-like states elicited by opiate withdrawal:

Therefore, in the *chapter II* we reported an essential role for the CRF$_2$ receptor pathway in anhedonia-like behavior induced by SPO OW. In fact, upon opiate discontinuation wild-type mice displayed a marked decrease in the preference for HPF. The latter findings are in agreement with a previous demonstration showing attenuated preference for sweet saccharin solutions in morphine-withdrawn rats (Lieblich et al., 1991). Similarly, heroin-dependent individuals displayed decreased responsiveness to "natural" rewarding stimuli which is attributing to anhedonia (Lubman et al, 2009).

Furthermore, we reported that no more reduction in HPF preference was observed in the opiate-withdrawn mice from 32 to 104 hr after the last morphine injection. This suggests that the anhedonia-like state is a brief phenomenon which is manifested during the early hours of OW but not during long-term OW. Supporting this latter notion, a clinical study reported a higher anhedonia scores in opiates-addicts during the early OW which inversely correlated with OW detoxification period (Janiri et al., 2005).

In net contrast, we found that CRF$_2^{-/-}$ mice did not show any decrease in the percentage of HPF as compared to drug-naïve control mice. Also, similarly to control drug-naïve mice, during SPO OW CRF$_2^{-/-}$ mice displayed HPF preferences that were indistinguishable from those

detected prior to morphine treatment (baseline). Our result provides new insights of the role of the brain stress systems components in the hedonic allostatic adaptations of the reward system which may be involved in the development of the anhedonia like-states during opiates dependence (Koob and Le Moal, 2001).

As in opiate dependence, the brain stress system is implicated in the anhedonia-like states liked to psychiatric disorders such as depression and anxiety (Mitchell, 1998; Schedlowski et al., 1995). Supporting this, very recent work reported an implication of the substance P and its receptor neurokinin pathways (NK_1) in animal model of depression-related behaviors. In particular, genetic deletion of the *tac1* gen (coding the substance P) eliminates anhedonia-like states assessed by the saccharine preference paradigm (Frisch et al., 2010). Others reported that the genetic deletion or blockade of the NK_1 receptor, by the selective antagonist RP67580, induces a decrease in the somatic expression of naloxone-precipitated morphine abstinence in rodents (Maldonado and Koob, 1993; Murtra et al., 2000). Taken together these results suggested an interconnection between the CRF/CRF_2 and substance P/NK_1 pathways in the anhedonia and somatic signs related to drug dependence and psychiatric disorder.

Thus, excessive opiate exposure and withdrawal produce important BW loss and decreases food intake, both in humans and rodents (Boghossian et al., 2001; Houshyar et al., 2003; Papaleo and Contarino, 2006; Thornhill et al., 1976). Despite the marked effect upon HPF preferences, here we also report that CRF_2 receptor-deficiency did not affect BW loss and recovery induced by treatment and discontinuation, respectively. Throughout the entire HPF preference experiment, we also monitored home-cage ingestive behavior. In line with the BW loss and recovery results, morphine treatment and discontinuation induced a genotype-independent decrease in home-cage regular chow intake and a rebound hyperphagia, respectively. Finally, morphine treatment or discontinuation decreased or increased caloric efficiency, respectively. Clearly, our results indicate that CRF_2 receptor-deficiency does not influence energy balance alterations induced by OW

CRF_2 receptor-deficiency eliminates the brain substrates of opiate withdrawal distress:

Using hybridization in situ analysis, we also reported that CRF_2 receptor-deficiency abolishes the increased activity of DYN, CRF and periaqueductal gray circuitry, which are major brain substrates of OW distress. Drug withdrawal has been associated to increased DYN expression in the NaccSH (Turchan et al., 1997). Accordingly, CRF_1 receptor-deficient mice lacked both the negative affective-like states and the increased DYN expression in the nucleus NaccSH induced by OW (Contarino and Papaleo, 2005). Here we reported that OW consistently increases NaccSH–DYN expression in the wild-type mice but not in the $CRF_2^{-/-}$, indicating an interesting interaction between CRF and opioid system in dysphoric consequences of OW.

Increased activation of the DYN/kappa opioid receptor system in the Nacc has been suggested to decrease dopamine neurotransmission (Di Chiara et imperato, 1988), a brain circuitry underlying the rewarding effects of drugs of abuse and palatable food (Avena et al., 2008; Koob, 2008). Thus, our results provide a new evidence of the impact of increased DYN neurotransmission in the NaccSH on the emergence of the negative affect and the decreased HPF preference observed in the wild-type mice. Interestingly, wild-type mice displayed increased neuronal activity in the PAG during OW assessed by *c-fos* expression. Several data indicate an implication of this brain region in the somatic and negative affective states of OW (Frenois et al., 2005). In *chapter II* we report that the CRF_2 receptor-deficiency eliminates the opiate withdrawal-induced neuronal activity in PAG indicating clearly a crucial role of CRF_2 receptor in this brain region. Subsequently, we found that CRF_2 receptor-deficiency abolishes the opiate withdrawal-induced increase in CeA-CRF expression. It is well documented that the increase level of CRF in the CeA is an important factor involved in anxiety-like behavior and drug dependence. Similarly, here we reported also that wild-type mice displayed an increase of CRF- CeA during OW. Taken together, our results clearly demonstrate a key role of the CRF_2 receptor in the brain molecular changes related to the motivational aspects of opioids.

CRF_2 receptor deficiency does not affect the brain, neuroendocrine and autonomic stress-coping responses to opiate withdrawal:

We reported previously that the genetic deletion of CRF_1 receptor eliminates the dysphoria-like states of OW but profoundly impairs HPA axis and provokes serious molecular alterations in brain (Contarino et Papaelo, 2005; Papaleo et al, 2007). This latter finding represents a limitation in treatment of OW syndrome by the targeting of the CRF_1 receptor. Here we report that CRF_2 receptor-deficiency does not impair the increased PVN–CRF expression induced by the stress of OW. In addition, unaltered corticosterone level was observed in $CRF_2^{-/-}$ mice (Papaleo et al., 2008) and after blockade of CRF_2 receptor by AS-30 antagonist (Navarro-Zaragoza et al., 2010). Noteworthy, several evidences showed that corticosterone is implicated in the withdrawal-induced PVN–CRF expression (Papaleo et al., 2007b). Finally, we found that CRF_2 receptor-deficiency does not affect the OW-induced expression of LC–TH, a fundamental coordinator of sympathetic nervous system responses to stressors (Valentino and Van Bockstaele, 2008). Overall, in contrast to the CRF_1 receptor, the CRF_2 receptor could be a privileged target for relieving OW syndrome without altering stress-coping abilities.

Motivational disorders provoked by opiate withdrawal:

In *chapter III* (Article 2) we aimed to investigate the impact of OW on the motivational properties to natural reward. Using a clinically relevant paradigm of intermittent escalating doses

of morphine, we demonstrated that OW increases the motivational properties of HPF in CD1 mice starting from 32 hours and last at least up to twelve days following morphine treatment. Also, we found that prolonged OW does not disturb cognitive functions necessary to learn a new operant rule to obtain HPF. It should be mentioned that we found a similar pattern of increased motivation for HPF during OW in several strain of mice. In particular, compared to control drug-naïve mice, the C57BL morphine-withdrawn mice increased food-driven operant behavior and their HPF-pellet intake during FR paradigm (data not shown).

The second main finding of this work is that prolonged OW ameliorates the acquisition of a new rule in operant behavior tasks. This result is in opposite to human and animal studies. In medical field, deleterious cognitive disorders such as alterations of learning processes, attention and memory function were observed among opiate-dependant patients (Ornstein et al., 2000; Rogers and Robbins, 2001). Using food-driven operant behavior and self-administration of heroin paradigm, a deficit in learning operant task and attention were observed, respectively, in morphine-dependant rats (Dalley et al., 2005; Harris and Aston-Jones, 2003). One possible explanation of our data is that the opiate withdrawal-induced increased motivation for HPF which greatly facilitates the acquisition and expression of a new operant task.

CRF$_2$ receptor-deficiency reduces the motivational effects of opiate withdrawal:

Using the same clinically relevant paradigm of SPO OW employed previously in Article 2, we aimed to identify the role of the CRF$_2$ receptor in motivational alteration elicited by OW. We find that intermittent OW increased the HPF motivation during the operant behavior independently with gender in wild-type mice. This latter result is in agreement with our previous observation in the CD1 opiate-withdrawn female mice despite some differences in the temporality of the apparition of the motivational alteration toward HPF attributed probably to experimental condition such as mice strain or period of exposition to HPF. Most importantly, here we reported that CRF$_2$ receptor-deficiency reduces both HPF-driven operant behavior and HPF intake linked to intermittent morphine administration and withdrawal in either male or female mice, providing initial strong evidence of a critical role for the CRF$_2$ receptor pathway in the motivational disorders induced by drug intake and withdrawal. This main result consolidates the hypothesis of a fundamental contribution of the CRF$_2$ receptor in the behavioral and molecular alterations elicited by OW.

CRF$_2$ receptor deficiency eliminates stress-induced food seeking behavior in male but not female mice during opiate withdrawal:

Throughout the present study, we demonstrate that OW induced long-term changes in motivation for HPF following cessation of opiate dosing. In particular, compared to opiate-

withdrawn $CRF_2^{-/-}$ mice, opiate-withdrawn wild-type mice showed long-term motivational alterations during extinction confirming previous finding reported in Article 3. We argued also that this latter finding provide a dissociation between motivation and energy balance alteration long-term after opiate dosing. We reported also that prior opiate treatment facilitates stress-induced HPF reinstatement in wild-type male and female mice. Most importantly, this effect is abolished by the genetic deletion of the CRF_2 receptor male but not in female mice. These latter findings demonstrate the fundamental implication of the CRF system as a neuroadaptative system recruited during opiate dependence to produce changes in motivational systems in male mice. Koob and le Moal hypothesized that this allostatic states of dependence contribute to the vulnerability to drug relapse cycle (Koob and Le Moal, 2001). However, herein we reported that CRF_2 receptor deficiency does not affect stress-induced HPF seeking behavior in female mice suggesting others gender-linked neuroendocrine adaptation involved in relapse. To explore the role of anxiogenic-like states on stress-induced HPF reinstatement, we tested mice in LD paradigm. We expected that anxiety developed during OW might contribute to the allostatic state hypothesized to drive opiate dependence. Surprisingly, our data revealed a gender-independent increases of anxiety-like sates in wild-type and $CRF_2^{-/-}$ opiate-withdrawn mice compared to CTL suggesting that HPF reinstatement in not causal-linked to anxiogenic effects of OW.

Finally, these findings underscore the importance of considering gender dimorphism in CRF receptor function in developing reliable treatment of opiate dependence.

Perspective:

Overall, the findings of this thesis provided initial evidence of a critical but complex role for the CRF system in the physiopathology of opiate dependence. Importantly, our results pointed to the CRF_2 receptor pathway as a promising target for the management of opiate addiction and withdrawal. Thus, future work will focus upon the involvement of the CRF_2 receptor pathway in other major features of drug dependence, notably the reward and cognitive alterations during OW. In addition, studies will focus on the role for the stress-responsive CRF/CRF_2 receptor system in the activity of brain circuitry implicated in the motivational disorders linked to opiate dependence and withdrawal.

A. Implication of the CRF_2 receptor pathway in the reward and cognitive alterations elicited by opiate intake and withdrawal:

Based on clinical and preclinical studies, it is reported that the increase of opiate-taking reflect a motivational changes of the rewarding properties of opiates induced by prolonged exposition to opioids (Ahmed and Koob, 2004; Koob and Le Moal, 2001). Supporting this, previous animal studies showed escalation of opioid self-administration when drug is available in long access conditions (Ahmed et al., 2000; Kenny et al., 2006). In particular, rats trained to

self-administer heroin increased progressively heroin intake to counteract the alteration of brain reward system (Kenny et al., 2006). Furthermore, deleterious cognitive disorders such as alterations of learning processes, attention and memory function were observed among opiate-dependant patients (Rogers and Robbins, 2001). Using food-driven operant behavior and self-administration of heroin paradigm, a deficit in learning operant task and attention were observed, respectively, in morphine-dependant rats (Dalley et al., 2005; Harris and Aston-Jones, 2003). These cited studies reinforce the hypothesis that cognitive disorders contribute to the pattern of impulsive opiates seeking and intake. The CRF system might play a role in the regulation of cognitive process such as learning and memory. Overall, an optimal level activation of the CRF system might enhance cognitive process, while a hyper/down activation might damage it (Heinrichs and Richard, 1999). Because the CRF system is implicated, through its two receptors, in the emergence of the somatic signs and negative affective states of OW, we suppose that the CRF system might contribute to the deregulation of reward set-point and cognitive function observed. Some initial evidence in the present work showed that the inactivation of the CRF_2 receptor does not impair operant behavior in mice. Future investigation in our laboratory will focuses about the contribution of the CRF system in the motivational rewarding effect of opioids and cognitive alteration during opiate withdrawal

B. Implication of the CRF_2 receptor pathway in the neural substrates changes underlying the motivational disorders induced reinstatement of food-driven operant behavior:

In situ hybridization experiments will be used in a future study aiming to evaluate gene expression for DYN, TH and CRF in brain regions implicated in the behavioral alterations induced by excessive opiate intake and withdrawal. These analyses will be carried out on the mouse brains already collected at the end of the experiments of exposure to the test of EPF-induced reinstatement of food-driven operant behavior. The DYN expression will be examined in the Nacc, a brain region implicated in drug dependence. In fact, the κ-opioid system is hypothesized to play a role in the anxiety-like behavior, negative emotional states and reward deregulation (Wee and Koob, 2010). For example, κ-agonist produces in rodent place aversion (Zhang et al., 2005), depression-like behavior (Carlezon et al., 2006) and brain reward deficit (Dinieri et al., 2009). Furthermore, the functional antagonism or the genetic deletion of the DYN gen, exert antidepressant effect and reduce negative emotional states, respectively (Carr et al., 2010). In addition, the DYN is involved strongly in the morphine self-administration (Glick et al., 1995; Kuzmin et al., 1997) somatic and negative affective signs of OW (Contarino and Papaleo, 2005; Papaleo et al., 2007b). Finally, DYN pathways exert a crucial control upon dopamine neurotransmission in the Nacc (Di Chiara and Imperato, 1988), a neural substrate of the rewarding and motivational properties of drugs of abuse and palatable food (Koob and Le Moal, 2001). We will also examine the expression of TH, the key enzyme of noradrenaline and

dopamine synthesis (Markey and Sze, 1984), in the VTA. Indeed, VTA originating dopamine neurons projecting to the Nacc have been proposed to underlie reward-driven motivation (Salamone et al., 1994). Finally we will also examine CRF transmission in the CeA. Increased CeA-CRF activity might indeed mediate the deleterious effects of stress during drug dependence and withdrawal (Koob, 2008).

C. Interaction of the CRF_2 receptor pathway with other brain stress system in the opiates dependence:

There are several evidences suggesting interconnection between CRF system and other brain stress systems such as the substance P/NK pathways and the noradrenergic system in the critical problem of opiate dependence. For example, recent reports showed interconnection between the CRF and noradrenergic systems in the expression of the somatic signs of OW (Garcia-Carmona et al., 2011; Navarro-Zaragoza et al., 2011). Other researches focus on the substance P/NK pathways are another component of brain stress system which is implicated in the risk of psychiatric disorders and drug dependence. Interestingly, several data revealed a very similar pattern between the CRF_2 and NK receptor pathway in mediating the motivational aspects of opioids. For example, $NK_1^{-/-}$ mice displayed a similar profile than $CRF_2^{-/-}$ by reducing naloxone-induced place aversion following morphine treatment (Murtra et al., 2000). Similarly, the NK_1 antagonist RP67580 (Maldonado and Koob, 1993) or the genetic deletion of the NK_1 receptor (Murtra et al., 2000) decreases somatic signs of OW. In addition, $CRF_2^{-/-}$ and $NK_1^{-/-}$ mice displayed no anhedonia in model of opiate dependence (*Chapter II*) and depression (Frisch et al., 2010), respectively. Together, these reports suggested relationship between the CRF and substance P/NK receptor systems and possible common pathways in the modulation of the motivational aspects of opioids. In this regard, future investigations about the interconnection between these receptors may enhance the understanding of the multiple mechanisms implicated in the opiate dependence.

In conclusion, our findings might open the way to new research-based pre-clinical and clinical trials and thus contribute to the development of novel and effective pharmacological agents targeting the CRF_2 receptor for the management of opiate dependence.

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Annex

LIST OF PUBLICATIONS

FULL PAPERS

1. Ingallinesi M*****, **Rouibi K***, Le Moine C, Papaleo F, Contarino A.
CRF$_2$ receptor-deficiency eliminates opiate withdrawal distress without impairing stress-coping.
Mol Psychiatry. Publication, 27 September 2011; doi:10.1038/mp.2011.119
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2. Rouibi K & Contarino A.
Increased motivation to eat in opiate-withdrawn mice
Psychopharmacology. Publication, 30 December 2011; doi: 10.1007/s00213-011-2612-x

3. Rouibi K & Contarino A.
CRF$_2$ receptor-deficiency reduces the motivational effects of opiate withdrawal
Submitted for publication.

ORAL PRESENTATIONS

1. Rouibi K. & Contarino A. Role of the CRF system in motivational disorders induced by opiate withdrawal. Forth Symposium in "Nutrition & Neurosciences", Institut de Neurosciences de Bordeaux, France, 30 mars 2011.

2. Rouibi K. Role of the CRF system in opiate withdrawal-induced motivational disorders. Young Scientiste Symposium, Institut européen de chimie et biologie. Pessac, France, 19-20 mai 2011.

POSTER PRESENTATIONS

1. Rouibi K. & Contarino A. Role of the CRF system in motivational disorders induced by opiate withdrawal. 11ème Journée Scientifique de l'Ecole Doctorale Sciences de le Vie et de la Santé, Université de Bordeaux, 7 avril 2011, Arcachon, France.

2. Rouibi K, Tancredi M, Contarino A. Implication of the CRF system in opiate withdrawal-induced anhedonia. Aquitaine Conferences in Neurosciences. Insights into the Neurobiology of Addiction. 12-15 October 2010, Arcachon, France.

3. Rouibi K, Tancredi M, Contarino A. Implication of the CRF system in opiate withdrawal-induced anhedonia. 10ème Journée Scientifique de l'Ecole Doctorale Sciences de le Vie et de la Santé, Université de Bordeaux, 28 avril 2010, Arcachon, France.

4. Rouibi K, Contarino A. Evidence of a link between stress-induced eating and emotionality. 3rd Mediterranean Conference of Neuroscience, 13-16 December 2009, Alexandria, Egypt.

5. Rouibi K, Tabarin A, Contarino A. A mouse model of stress-induced aberrant eating associated with altered emotionality. 9ème Colloque de la Société Française des Neurosciences, 26-29 Mai 2009, Bordeaux, France.

6. Rouibi K, Tabarin A, Contarino A. A mouse model of stress-induced aberrant eating associated with altered emotionality. 9ème Journée Scientifique de l'Ecole Doctorale Sciences de la Vie et de la Santé, Université de Bordeaux, 8 avril 2009, Arcachon, France.

7. Rouibi K, Tabarin A, Contarino A. Addiction à la nourriture: études préliminaires chez la souris. 25ème Congrès de la Société Française d'Endocrinologie, 1-4 Octobre 2008, Lille, France.