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Cellular and molecular mechanisms involved in morphine dependence-induced behavioral alterations

Mecanismos celulares y moleculares implicados en los cambios comportamentales inducidos durante la dependencia a morfina

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"Between stimulus and response there is a space.

In that space is our power to choose our response.

In our response lie our growth and our freedom"

(Viktor E. Frankl)

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INTRODUCTION

Chronic exposure to drugs of abuse produces molecular or neurochemical adaptive changes in the brain, and as a consequence there are alterations in the individual behavior which characterize the addicted stage.

To identify either these drug-induced adaptive changes in the brain or the behavioral alterations (also drug-induced), is important to understand how addiction is developed, which from a clinical perspective, is the time-point where pharmacotherapy may be most effectively employed.

1. NEUROBIOLOGY OF ADDICTION

Drug addiction is a chronically relapsing disorder characterized by (a) compulsion to seek and take the drug, (b) loss of control in limiting intake, and (c) emergence of a negative emotional state (e.g., dysphoria, anxiety, irritability…) which reflect a motivational withdrawal syndrome when access to the drug is prevented (3).

Addiction is described by a series of events initiated by the acute rewarding effects of drugs followed by a transition into chronic drug use. This idea of positive reinforcement by drugs of abuse has been widely seen as a primary factor behind drug dependence (4). Additionally, long-term drug use often results in aversive psychological and physiological effects if intake is withheld, thus turning it into a continued consumption as a means to avoid the aversive consequences of drug withdrawal (that is, negative reinforcement) (5;6).

A psychiatric-motivational framework that provides sources of both positive and negative reinforcement for drug taking is the conceptualization that drug addiction has aspects of both impulsive control disorders and compulsive control disorders. Impulsive control disorders are characterized by an increasing sense of tension or arousal before committing an act when pleasure, gratification or relief are expected. Impulsive control disorders are largely associated with positive reinforcement mechanisms. In contrast, compulsive control disorders are characterized by anxiety and stress before committing compulsive repetitive behavior, and relief from the stress by performing the compulsive behavior.

Compulsive disorders are largely associated with negative reinforcement mechanisms and automaticity (3).

Collapsing the cycles of impulsivity and compulsivity yields a composite addiction cycle of three stages: binge/intoxication, withdrawal/negative effect, and preoccupation/anticipation (Figure 1), in which impulsiveness often dominates at the early stages and impulsiveness combined with compulsiveness dominates at the later stages. As an individual moves from impulsiveness to compulsiveness, a shift occurs from positive reinforcement driving the motivated behavior to negative reinforcement and automaticity (7).

These three stages are conceptualized as interacting with each other, becoming more intense, and ultimately leading to the pathological state known as addiction (8). The transition from occasional drug use to addiction involves neuroplasticity and may begin with initial drug use in vulnerable individuals or individuals at particularly vulnerable developmental periods (e.g., adolescence; (9)). In the last few years, several publications have suggested that this vulnerability develops persistent alterations in the behavior of the individual, which are due to significant changes in the gene expression (10;11).

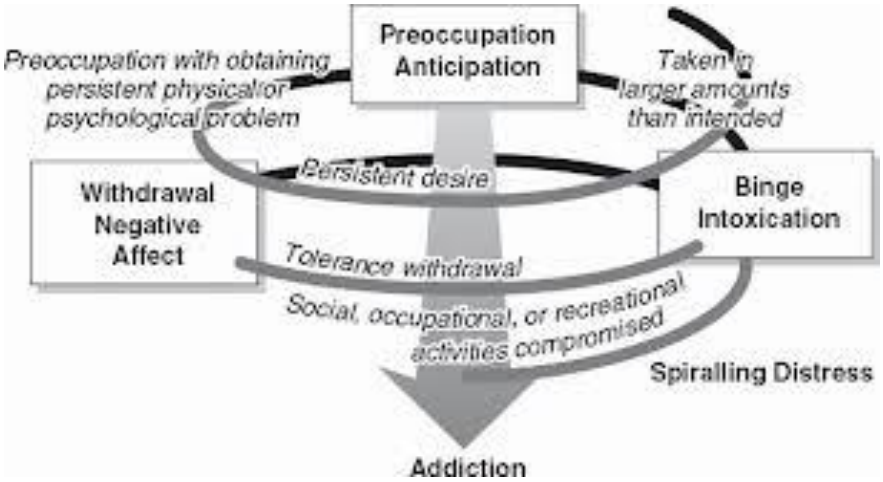

Figure 1. Diagram describing the spiraling distress/addiction cycle from a psychiatric/addiction perspective. The figure shows the three major components of the addiction cycle with the different criteria for substance dependence (8).

1.1. Neurocircuitry of Addiction

Addiction involves plasticity in specific brain pathways. Depending on the stage of addiction the individual is at, the activity in the brain pathways varies. Evidence converges to show that addictive substances cause synaptic adaptation, constituting a first permissive step in a cascade of cellular events that ultimately lead to circuit remodeling and changes in behavior, which may eventually be diagnosed as addiction (12;13)(Figure 2).

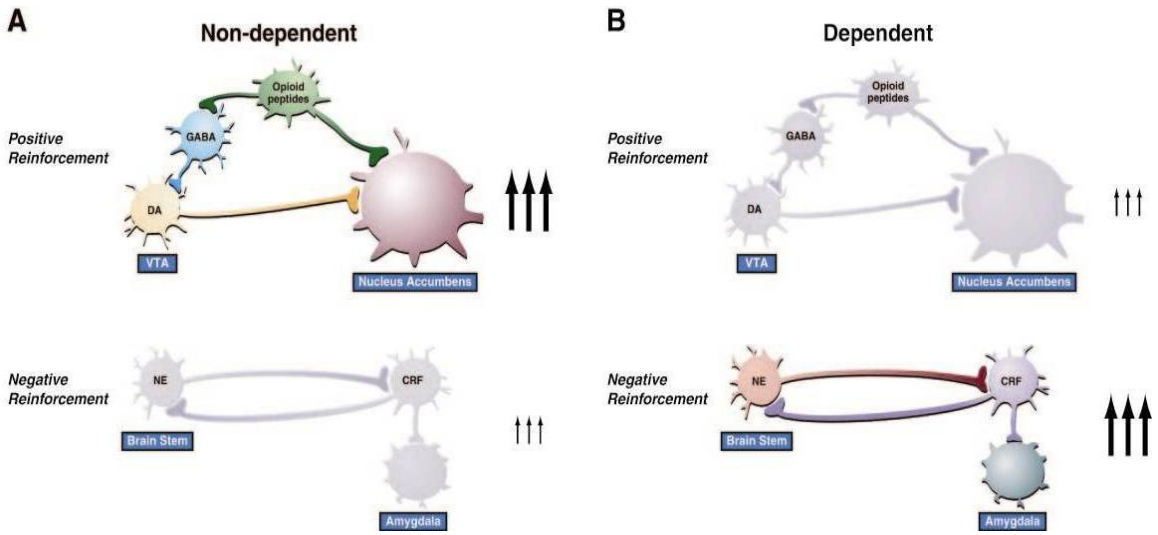

Figure 2. Neurocircuitry associated with acute positive reinforcing effects of drugs of abuse and negative reinforcement of dependence. Changes in the transition from non-dependent to dependent drug taking. Reproduced fromKoob et al. (2008) (14).

Neuroimaging studies in humans add credence to this hypothesis. Credible evidence also implicates dopaminergic, serotonergic, opioid, endocannabinoid, γ-aminobutiric acid-ergic (GABAergic) and glutamatergic mechanisms in addiction. Critically, drug addiction progresses from occasional recreational use to impulsive use and in consequence to habitual compulsive use. This correlates with a progression from reward-driven to habit-driven drug-seeking behavior. This behavioral progression correlates with a neuroanatomical progression from ventral striatal (nucleus accumbens; NAc) to dorsal striatal, with the characteristic lost of control over drug-seeking behavior, and inducing craving and/or relapse. The three classical sets of craving and relapse triggers

are: (a) re-exposure to addictive drugs, (b) stress, and (c) re-exposure to environmental cues (people, places, things) previously associated with drug-taking behavior. Drug-triggered relapse involves the NAc and the neurotransmitter dopamine (DA). Stress-triggered relapse involves (a) the central nucleus of the amygdala (CeA), the bed nucleus of the stria terminalis (BNST), and the neurotransmitter corticotropin-releasing factor (CRF), and (b) the noradrenergic nuclei of the brain stem and the neurotransmitter noradrenaline (NA)(15).

Thus, other neurotransmitter systems have been involved in emotional dysregulation of the motivational effects of drug withdrawal, such as dynorphin (dyn), orexins (Ox), arginine-vasopressin (AVP) or endocannabinoids (9). Knowledge of neuroanatomy, neurophysiology, neurochemistry and neuropharmacology of addictive drug action in the brain is currently producing a variety of pharmacotherapeutic treatment strategies for drug addiction, some of which appear promising (15).

1.1.1. Dopaminergic Reward System

Drugs of abuse have diverse pharmacologic profiles and can produce various behavioral effects, although all of them share a common characteristic, an increased mesocorticolimbic dopaminergic activity after intake through different levels of interaction (16;17).

Addictive substances, in general, evoke an activation of the reward circuitry with an increment of DA release in the NAc. A number of studies relate this DA release to the positive reinforcements of drugs of abuse, triggering the addictive cycle (18;19). Even though DA has been largely considered the main neurotransmitter involved in drug addiction, recently it has been proposed that other neurotransmitters, such as NA, glutamate, endocannabinoids, GABA, endogenous opioid peptides and orexins (OX), to take part in the different stages of addiction (Figure 3) (9;20).

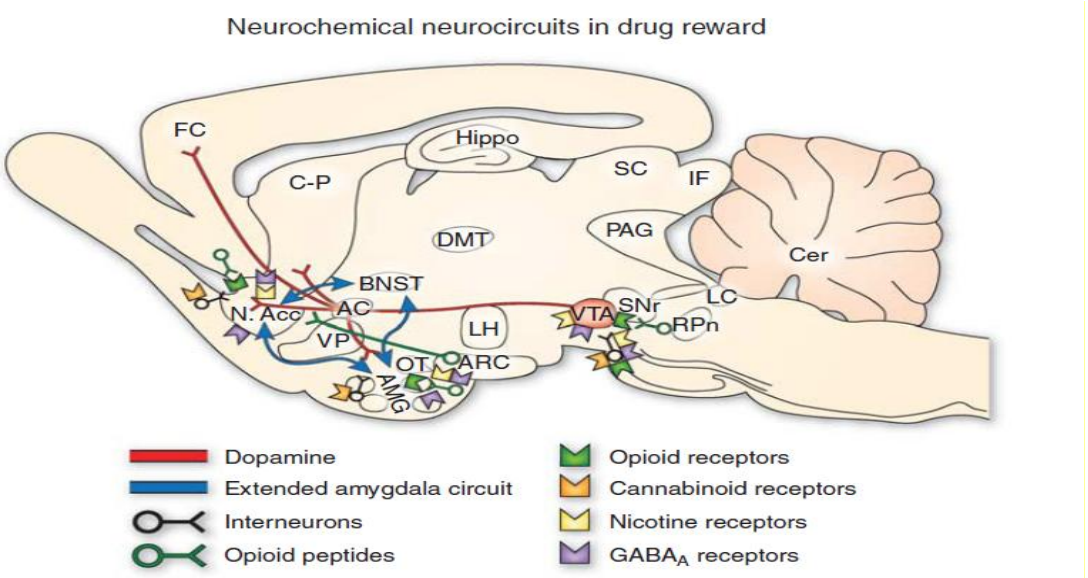

Figure 3. Neural circuits involved in addiction. AMG, amygdala; FC, frontal cortex; Hippo, hippocampus; LC, locus coeruleus; LH, lateral hypothalamus;; VTA, ventral tegmental area. Reproduced from (21).

Dopaminergic neurons located in the ventral tegmental area (VTA) project to limbic structures (mesolimbic pathway) such as the amygdala (AMG), ventral pallidum, hippocampus (Hippo) and NAc, and to cortical areas (mesocortical pathway) including the prefrontal cortex (PFC), the orbitofrontal cortex and the anterior cingulated area (22). NAc and ventral pallidum have been postulated to be the areas responsible for initial reinforcing drug effects, while the AMG and Hippo are involved in conditioned learning during addiction. On the other hand, PFC is responsible for the regulation of emotional behavior, cognitive control and the executive function (23).

The augmented DA release in the NAc elicited by addictive substances has been related to drug self-administration (16;17;24). In agreement, previous evidence showed severe attenuation in cocaine and heroin self-administration after damaging the NAc, VTA and ventral pallidum (25). Moreover, *in vivo* studies using microdialysis showed DA increased levels in the shell of the NAc during morphine (26) and alcohol (27) self-administration, corroborating the essential role of this neurotransmitter in positive reinforcement. In addition, it is known by neuroimaging studies in humans using a positron emission tomography (PET),

that the increased in DA release induced by drugs of abuse, is linked to a subjective euphoric sensation (28;29).

Dopaminergic receptors are distributed throughout several areas of the central nervous system (CNS) related to addiction such NAc, PFC and AMG. Five types of dopaminergic receptors have been identified, all of them coupled to G proteins, and classified in two families: D_1 receptors (subtypes D_1 and D_5) mostly located postsynaptically, and coupled to G_s proteins; and receptors D_2 (subtypes D_2, D_3, D_4) mostly located presynaptically, and coupled to G_i proteins. Interaction of DA with D_1 type receptors initiates the cyclic adenosine monophosphate/protein kinase/ cAMP response element binding (cAMP/PKA/CREB), and in consequence, begins the expression of genes with an important role in addiction, like c-Fos or pro-dyn. In contrast, when DA activates D_2 type receptors, cAMP synthesis is inhibited, K^+ channels open and calcium income to the cell is reduced through specific voltage dependent channels (30). Several imaging studies (PET) with agonist and antagonist substances of D_2 receptors have revealed that chronic administration of ethanol, cocaine, nicotine, methamphetamine or heroin, induced reductions of D_2 receptor density in the ventral striatum that persisted long after detoxification (30;31). Furthermore, an important role for presynaptic D_3 receptors in drug dependence has been hypothesized. Selective antagonist administration has revealed that D_3 receptors may play an important role in drug-induced reward, drug-taking, and cue-, drug, and stress-induced reinstatement of drug-seeking behavior (32).

The rewarding effects of opiates are mainly due to their actions on μ-opioid receptors. These receptors are localized in GABAergic interneurons, which synapse with dopaminergic cell bodies in the VTA. Supporting this hypothesis, the activation of morphine opioid receptors (MOR) in the VTA results in increasing levels of DA in the NAc and cortex (33). Recent studies have showed data indicating that activation of μ- and δ-opioid receptors in PFC enhances DA D_1-like receptor signaling. This previously unrecognized synergistic interaction may selectively affect DA D_1 transmission at specific postsynaptic sites where the receptors are co-localized, and may play a role in prefrontal DA D_1 regulation of opioid addiction (34). Moreover, chronic exposure to drugs of abuse induces upregulation of the κ-opioid receptors/dyn system. Such endogenous activation

of κ-opioid receptors tone by dyn is thought to underlie aversion, dysphoria/anhedonia, and depression-like or anxiety-like neuropsychiatric states. This counter regulatory action by the κ-opioid receptors/dyn system may therefore partially mediate in the negative reinforcing aspects of withdrawal from drugs of abuse and may exacerbate the chronic relapsing nature of addictive diseases (11).

1.1.2. Brain Stress System

Stress and addiction are closely related. Exposure to stressors may exacerbate drug intake, facilitating initial drug exposure and therefore increasing the drug taking behavior and relapse. It has been stated that drug withdrawal triggers the same physiological events as those observed during a stressful situation. Previous evidence has shown that either stressors or addictive substances activate the same brain regions, including the dopaminergic mesolimbic and mesocortical system that release DA in such regions (35-37).

Within-system neuroadaptations to chronic drug exposure include decreases in the functionality of the neurotransmitters communicating within the neurocircuitry involved in the acute reinforcing effects of drugs of abuse. One prominent hypothesis is that DA systems are compromised in such crucial phases of the addictive cycle, as withdrawal, and lead to decreased motivation for nondrug-related stimuli and increased sensitivity to the abused drug (38). A decreasing activity of the mesolimbic DA system and a decrease in serotonergic neurotransmission in the NAc occur during acute drug withdrawal from all major drugs of abuse in animal studies (39;40;40).

A second component of the withdrawal/negative affect stage is a between-system neuroadaptation, in which different neurochemical systems involved in stress modulation also may be engaged within the neurocircuitry of the brain stress and aversive systems in an attempt to overcome the chronic presence of the perturbing drug, to restore normal function despite the presence of the drug. Both the hypothalamic stress system, composed of the hypothalamic–pituitary–adrenal (HPA) axis, and the extrahypothalamic brain stress system, also called extended amygdala, are activated during withdrawal from chronic administration

of all major drugs with potential abuse. This has a common response of elevated adrenocorticotropic hormone (ACTH), corticosterone, and amygdaloidal CRF during acute withdrawal (14;41). Acute withdrawal from all drugs of abuse also produces an aversive or anxiety-like state in which CRF and other stress-related systems (including noradrenergic pathways) have key roles (3).

1.1.2.1. Extended Amygdala

The neuroanatomical entity termed the extended amygdala (42) represents a common anatomical substrate, where brain arousal–stress systems with hedonic processing systems are integrated in order to produce the negative emotional states that promote negative reinforcement mechanisms, associated with the development of addiction (3).

The extended amygdala is composed of the CeA, BNST, and a transition zone in the medial (shell) subregion of the NAc. Each of these regions has cytoarchitectural and circuitry similarities (42). The extended amygdala receives numerous afferents from limbic structures defining the specific brain areas that interface classical limbic (emotional) structures with the output of extrapyramidal motor system (43)(Figure 4). Additionally, this neurocircuitry has been long hypothesized to have a key role not only in fear conditioning (44), but also in the emotional component of pain processing (45).

Within the main components of the extended amygdala, are described the key elements of the brain stress system associated with negative addiction reinforcements: CRF, NA, Ox and dyn (46).

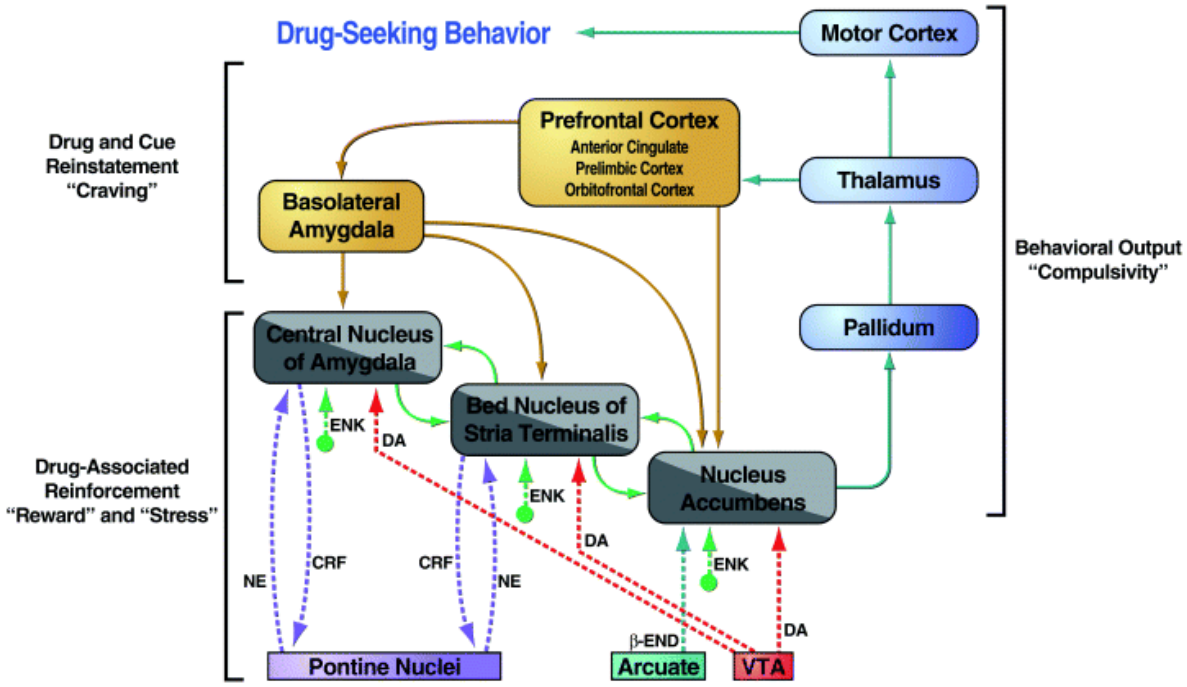

Figure 4. The three major neurocircuits that underlie addiction. A drug-reinforcement circuit is comprised of the extended amygdala including the CeA, the BNST, and the transition zone in the shell of the NAc. In the extended amygdala, there are not only multiple reciprocal CRF connections between CeA and BNST, but also afferent CRF projections from other amygdaloidal and extra-amygdaloidal structures, and noradrenergic inputs from the brainstem. Reproduced from (47).

CRF, a central neuropeptide to the stress response, may be a key solving the relapse cycle. CRF modulates anxiety observed in both acute and protracted abstinence from multiple drugs of abuse and thus presents an ideal target for the development of medication (48).

CRF was first characterized as the central activator of the endocrine stress response. In this way, exposure to a stressor triggers the synthesis of CRF at two levels, the paraventricular nucleus (PVN) of the hypothalamus, which is the apex of the HPA axis, and in the extended amygdala. CRF from extrahypothalamic sources was demonstrated to play a key role in the expression of behavioral responses to stressors (49).

CRF-immunoreactive perikarya can be found in various brain regions, with particularly strong expression in the extended amygdala and lateral septum (50)., all of which are activated and implicated in the expression of behavioral responses to stressors (51-53). CRF itself has been shown to be central involving these nuclei in behavioral stress responses, independently from HPA axis activation (54).

1.1.2.2. HPA Axis

The HPA axis is a complex neuroendocrine system involved in several physiological functions, either central or peripherial, most of them related to the adaptation to stressful situations, like drug addiction (55). This axis is composed of three important structures: PVN, the anterior lobe of the pituitary gland and the adrenal gland (56).

Focusing on the PVN, which is the apex of the HPA axis, two regions are differentiated, the magnocellular region and the parvocellular region (Figure 5). Main neuropeptides from the magnocellular region are AVP and oxytocine (OT), which project into the neurohypophysis and deliver these neurotransmitters to the peripheral circulation. The peripherial actions of AVP are maintaining water homeostasis and blood pressure (57;58). In the parvocellular region, where most hypothalamic CRF neurons are localized, AVP is also released. AVP projections from the parvocellular PVN, connect with the external zone of the median eminence besides CRF (59), and regulate the HPA axis in this way.

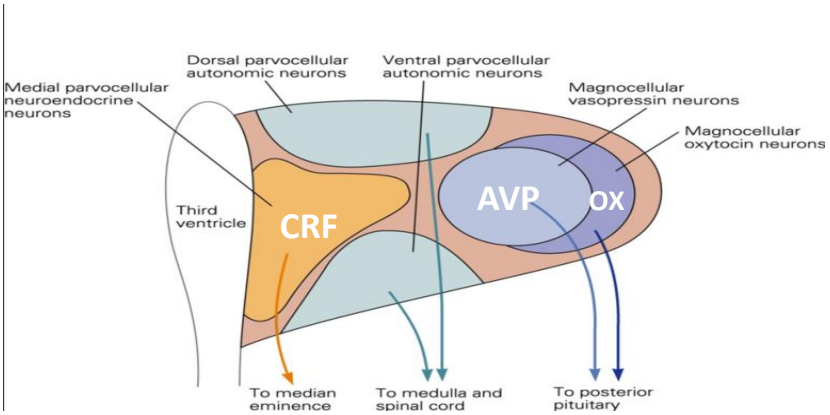

Figure 5. PVN subdivisions and neurotransmitters connections. Reproduced from http://www.ib.cnea.gov.ar/~redneu/2013/BOOKS/Principles of Neural Science - Kandel/gateway.ut.ovid.com/gw2/ovidweb.cgisidnjhkoalgmeho00dbookimagebookdb_7c_2fc

It has been shown in non-stressed rats that 50% of CRF neurosecretory cells co-express AVP (60). The ability of both neuropeptides (CRF and AVP) to stimulate the secretion of ACTH has been demonstrated in humans (61-63) and various other species, including rats (59;64) and mice (65). In all cases, a marked synergism between CRF and AVP has been observed (59;63;66;67). The impact of AVP on ACTH secretion is often regarded as ancillary to CRF, as far as AVP alone is a weak ACTH secretagogue, but acts synergistically with CRF to facilitate ACTH release in both humans (61-63) and rodents (59;64).

It has been shown that the corticotrophs AVP/vasopressin receptor-1b (V1b) and the CRF/ corticotropin releasing factor receptor-1 (CRF_1-R) signaling pathways converge to increase ACTH secretion (68;69). In particular, it has been suggested that AVP plays a role in stimulating the primary nuclear transcripts induced by CRF in pituitary corticotrophs (59). Studies in recent years suggest that the CRF–AVP synergism may also be conditioned by changes in the physical state of corticotrophs V1b and CRF_1 receptors (69;70).

Synthesis and release of ACTH is a physiological event evoked by activation of CRF receptors (stimulated by AVP release). There are two types of CRF receptors: CRF_1-R and corticotropin releasing factor receptor-2 (CRF_2-R) (71;72); both are 7-transmembrane G-protein-coupled receptors that principally function by interacting with the stimulatory G-protein (G_s), resulting in elevated Adenyl Cyclase and cAMP levels, although the receptors may also couple to other G-proteins (48).

ACTH in turn, stimulates glucocorticoids (GC) (cortisol in humans, corticosterone in rodents) synthesis and secretion from the adrenal gland into blood circulation. Moreover, circulating GC makes a negative feedback modulation on the HPA axis (73). This modulation is triggered by the activation of mineralocorticoid receptors (MR) and glucocorticoid receptors (GR), which clearly differs either in anatomical distribution or in pharmacological properties (74). Circulating GC not only inhibit CRF release from the PVN, but also inhibit ACTH release from the anterior lobe of the pituitary gland (75) (Figure 6).

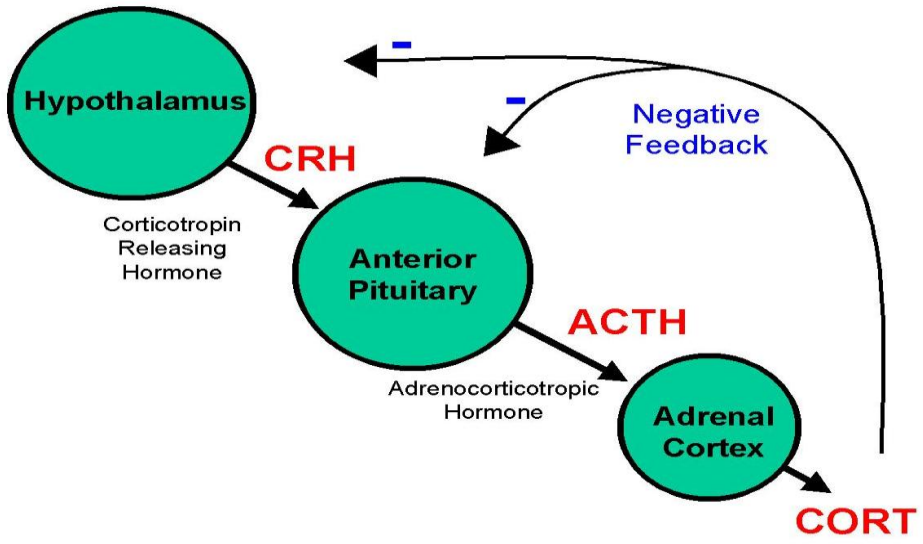

Figure 6. Simplified scheme of the HPA axis. Reproduced from (1).

From the perspective of addiction, progressive changes in the HPA axis take place during the transition from acute to chronic administration of drugs of abuse. Acute administration of most drugs of abuse in animals activates the HPA axis and may first facilitate the activation of the brain motivational circuitry, developing drug reward and, in consequence, progression in the acquisition of drug-seeking behavior (76). These acute changes are deregulated by repeated administration of cocaine, opioids, nicotine and alcohol (41;77;78).

Chronic exposure to opiates results in the development of tolerance to the HPA axis endocrine effects and physical dependence, characterized by increasing ACTH secretion, and therefore an increased release of GC after the administration of an opioid antagonist (79-81). Thus, previous research of our group has shown an enhancement in CRF heteronuclear RNA in the PVN during morphine withdrawal (82).

Apart from these neuroendocrine events, morphine dependence induce long term effects through changes in genetic expression (83;84). Previous studies in our laboratory have described that morphine withdrawal induces neural expression of c-Fos in the parvocellular division of the PVN (85). Furthermore, recent research demonstrates that chronic overexpression of CRF from the CeA produced HPA axis hyperactivity and behavioral anxiety associated with gene

expression changes in the PVN (86). An aversive stage during withdrawal has also been described, which is reverted administrating CRF antagonists (87).

Nonetheless, the intracellular signaling pathways in charge of the activation of the HPA axis are still unknown. Previous studies showed an enhancement of PKA and protein kinase C (PKC) levels in the PVN during morphine withdrawal (88;89). This PKA up-regulation could mean a first step towards the stimulation of the transcription of different genes, such as c-Fos or CRF.

1.1.2.3. Noradrenergic Pathways

Noradrenergic systems that innervate the extended amygdala are involved in negative symptomatology, and therefore, in the increasing self-administration linked to dependence to drugs of abuse.

Evidence for the NA involvement in motivation-reward has emerged from studies of self-administration, conditioned place preference (CPP), reinstatement, and locomotor activation. The noradrenergic system regulates the activity of the ascending DA pathways, via locus coeruleus (LC) (90). They meet in the VTA, where noradrenergic neurons modulate the DA cell firing pattern, via excitatory postsynaptic α_1-adrenoreceptors (91). The LC noradrenergic system also regulates the mesencephalic dopaminergic system indirectly, via the PFC. DA release in the PFC is regulated by local noradrenergic nerve terminals, and electrical stimulation of the LC neurons increases both extracellular DA and NA in the PFC (92)(Figure 7). When NA release is blocked, DA release is similarly attenuated. If the NA blockade becomes chronic, the DA system gradually compensates by upregulating the high-affinity state of postsynaptic receptors (i.e., increasing their density) (93). This process result in hypersensitivity to psychostimulants and to any other substance (or behavior) that increases intrasynaptic DA levels (30).

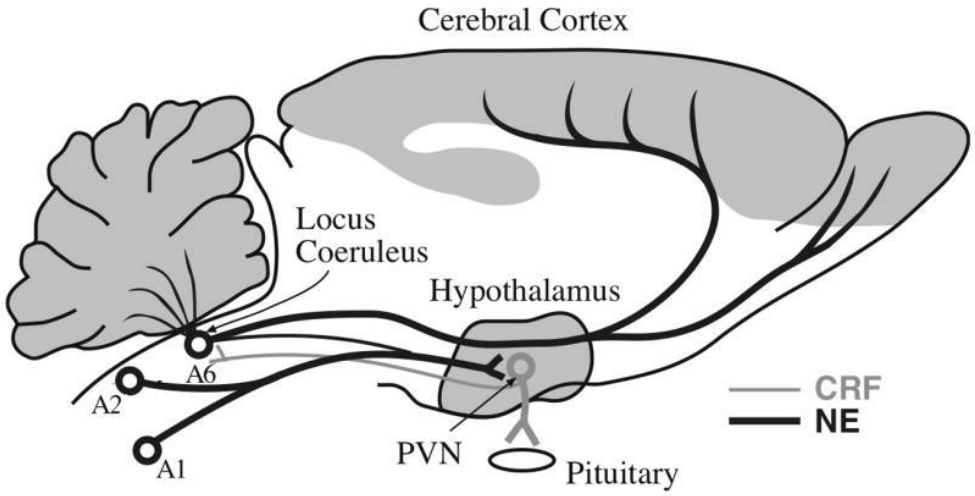

Figure 7. Simplified diagram indicating the major ascending noradrenergic projection systems: the dorsal LC system, the ventral A_1/A_2 (ventrolateral medial area/nucleus of solitary tract; VLM/NTS) system and their interactions within PVN CRF-containing neurons. Reproduced from Dunn et al. (94).

On the other hand, an interaction between the CRF and noradrenergic systems in the CNS it has been postulated. There is pharmacological, physiological and anatomic evidence of a CRF-NA interaction in the LC after exposure to a stressor. Thus, CRF neurons would activate the NA cell firing rate, and noradrenergic neurons would stimulate CRF release (95;96). NA also augments CRF release in other nuclei such as PVN, BNST and CeA (97) , and this CRF from these nuclei would induce the release of NA by the brainstem noradrenergic areas. Therefore, there is a highly marked interaction between the stress brain system and the noradrenergic circuitry of CNS, which has been defined as a feed-forward system.

Additionally, CeA CRF neurons project into the LC, increasing the firing rate of LC neurons, resulting in an increased NA release that modulates VTA DA neurons activity. Besides, CRF released by PVN neurons is associated with the increasing activity of noradrenergic neurons from the NTS, which also connect to BNST, CeA, VTA and NAc shell (98). Noradrenergic afferents from the NTS to the extended amygdala and PVN mediate not only in the development of the motivational effects of opiate withdrawal, but also in the stress-induced reinstatement of drug seeking behavior (87;99).

Noradrenergic function plays a main role in the reinstatement of drug seeking behavior induced by stress, which takes place during addiction (100). Administration of α_2-adrenergic agonists attenuates stress-induced reinstatement of compulsive drug seeking behavior for alcohol, cocaine and heroin (30).

Previous studies from our laboratory concluded that administration of α_1 and α_2 adrenoceptor antagonists, prazosin and yohimbine respectively, strongly blocked the characteristic increased NA turnover in the PVN, the enhancement of corticosterone plasma levels, and c-Fos neuronal expression in CRF cells from the PVN during morphine withdrawal. Studies made with propanolol also showed an inhibition of c-Fos expression in the PVN after naloxone administration to morphine dependent animals. All this evidence suggest that, during morphine withdrawal, the HPA axis is activated through the modulation of noradrenergic connections from the NTS to the PVN (85;101;102).

Finally, our laboratory has also shown an increased enzymatic activity of tyrosine hydroxylase (TH; the limiting enzyme in catecholamine's synthesis) and TH phosphorylation (activation) in the NTS, during morphine withdrawal (82;103). Further research relates NA to CRF, since an augment of noradrenergic activity in the PFC has been observed as a consequence of risen CRF intracerebroventricular (i.c.v.) administration (104). Additionally, co-expression of CRF and TH in LC (105), and CRF1-R expression in the catecholaminergic neurons from the LC, VTA and NTS (105;106) have been revealed. Such systems may have a strong functional significance in the response of an organism to environmental challenge, turning a system vulnerable to pathology and therefore, a relevant pharmacological target in stress system deregulation (107).

2. OREXINERGIC SYSTEM

Ox-A and Ox-B (also known as hypocretin 1 and 2) are neuropeptides derived from a prepro-orexin molecule made exclusively in hypothalamic neurons (108;109). Since the discoveries by de Lecea et al. and Sakurai et al., considerable work has characterized this neurotransmitter system. Orexin neurons are exclusively localized to the perifornical area (PFA), the lateral (LLH) and dorso-medial hypothalamus (DMH) in the rat brain (110-112), and this distribution has been confirmed in human tissue (113) (Figure 8). Sakurai et al. (1998) characterized two receptors for the orexin system, termed OxR1 and OxR2 (also denoted HcrtR1 and Hcrt2). OxR1 binds orexin A with 30 nM affinity but has much lower affinity for orexin B, whereas OxR2 binds both orexin peptides with similar high affinity. Further, OxR1 is coupled exclusively to Gq subclass of G proteins, and OxR2 is coupled to both Gq and Gi/o proteins (114).

The orexin neurons give rise to a highly divergent system of fiber projections that spans the entire neuraxis, including enervations in the cerebral cortex, Hippo, thalamus, midbrain, and spinal cord (110;115). Likewise, the two orexin receptors are widely distributed throughout the CNS but are regionally selective and largely non-overlapping (116-119) (Figure 8). Great interest was focused on this system shortly after its discovery, when two groups virtually simultaneously reported that dysfunction in the orexin system is strongly associated with narcoleptic symptoms in animals (120). Subsequent work in humans verified that narcoleptic patients (particularly those with cataplexy) have little orexin in their cerebrospinal fluid (CSF) and lack most or all orexin neurons (121;122). With these compelling findings, the prevailing view of orexin function started focusing on arousal and maintenance of the waking state. Supporting this view, findings showed that the major targets of orexin projections are classic brain arousal nuclei such as the LC (110;115). On the other hand, it has been also demonstrated how typical orexin application strongly activates cells in this brain area (123-127). However, a potential role for orexins in reward processing was evident from one of the first publications of their discovery.

Sakurai et al. (109) reported that administration of Ox-A or Ox-B into the lateral ventricle produced feeding in rats, which prompted him to name the new

peptides “orexins”, meaning appetite. The first report of a possible role for orexins in the effects of addictive drugs appeared in 2003 and showed that orexin neurons play a role in opiate withdrawal (128). Subsequent studies examined a possible role in conditioned responses to stimuli associated with drug rewards. This reward-associated function of the orexin system may be separate from its role in the maintenance of the waking state, and mediated by a separate population of (laterally located) orexin neurons. Thus, it has been proposed that the DMH and PFA orexin neurons are involved in arousal, whereas LLH orexin cells are related to reward and reinforcement (129-131).

2.1. Orexins and Addiction

Numerous studies have shown that orexins play important roles in drug seeking in animal models of addiction. However, the exact nature of these roles has remained elusive. The orexin receptors (118;119;132) and orexinergic projections from the LH (112;133) are found throughout the brain, including regions known for their involvement in drug reward and addiction.

2.1.1. Orexin and the Mesocorticolimbic Reward Pathway

Early studies showed broad projections of orexin neurons include the midbrain DA neurons of the VTA (110;134;135). Studies of drug reward and drug-induced neuroadaptations have focused on orexins’ effects on the VTA and the mesocorticolimbic target regions, such as the NAc and AMG as well as the medial prefrontal cortex (mPFC) (136-138). The hypothalamic orexin system is positioned so that it can interact with the mesocorticolimbic pathway via many brain regions, but most work has focused on the VTA (Figure 8).

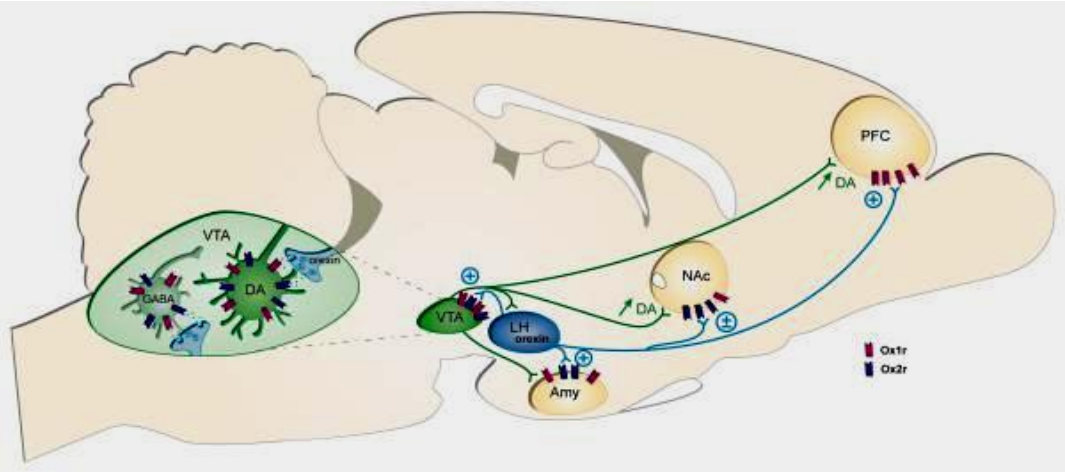

Figure 8. Schematic representation of the orexin system and its interactions with the mesocorticolimbic reward pathway. Reproduced from (139).

Orexin projections from the LH are localized in the VTA (110;134;135;140) and orexin terminals contact TH positive cells (135;140). Both orexin receptors have been found to be present in an increased density in the VTA (132;141) on both DA-containing (127;141) and GABA-containing neurons (127). Orexins excite the VTA and activates both DA (127;140) and non-DA cells (127) via a direct postsynaptic effect. Intra-VTA application of orexin evoked an increased c-Fos expression in DA neurons located specifically in the caudomedial portion of the VTA (142). This application also increases DA at the level of the NAc shell, but not in the NAc core or the mPFC (141;143). Intra-VTA orexin also results in an enhancement of N-methyl-d-aspartate (NMDA) receptor-mediated excitatory postsynaptic currents (144) indicating a role for orexins in long-term neural plasticity.

Although both OxR1 and OxR2 have been reported to be expressed in the NAc (145), OxR1 are at low levels (118) and orexin actions have been attributed to OxR2 binding (145). Unlike the excitatory effects of orexin in the VTA, activation of these receptors in the NAc has been reported to have inhibitory results (145). However, a more recent finding reports that orexin application depolarizes NAc shell neurons via OxR2 (146). Different experimental methodologies may explain these conflicting findings or perhaps OxR2 has both excitatory and inhibitory postsynaptic effects. Further investigation is necessary to clarify orexinergic

effects in the NAc. Orexinergic action in the AMG is excitatory, and mediated by OxR2 (147); however both orexin receptors have been reported in the AMG (118). Orexin is also excitatory in the mPFC via activation of the OxR1 (148) and the OxR1 is the only receptor type reported in the mPFC (118;119).

2.1.2. Drug action on Orexin System

Drugs of abuse have different effects on the orexin system and these effects are drug specific. Moreover, regional variations within the LH have been reported in response to some drugs (Figure 9).

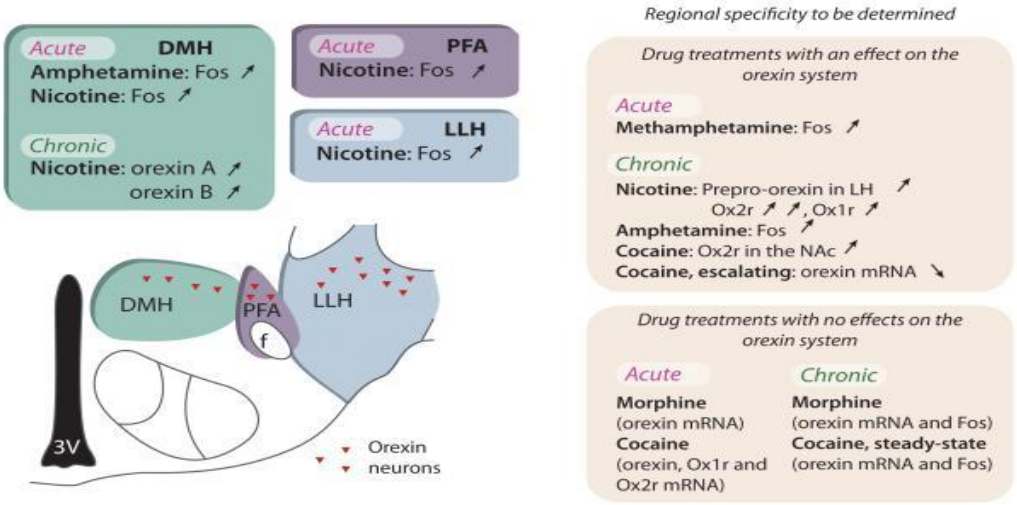

Figure 9. Effects of acute and chronic drug administration on LH orexin cells. Reproduced from (139).

For instance, c-Fos expression is increased in orexin neurons following acute administration of methamphetamine (149), amphetamine (135), and nicotine (150;151).

In addition to the effects of acute drugs, chronic drugs of abuse also influence the orexin system via changes in orexin neural activation and mRNA levels of orexin or its receptors. Chronic administration of nicotine is associated with increased expression of prepro-orexin mRNA in the LH (152). Chronic amphetamine exposure results in increased c-Fos expression in orexin neurons

(153). In contrast, chronic morphine treatment (128;154;155) and steady-state chronic cocaine (156) fail to affect orexin mRNA levels or c-Fos expression in LH orexin neurons. It should also be recognized that negative results on gene expression analysis, could depend on the time chosen for the analysis.

2.2. Orexin role in drug dependence and addiction

2.2.1. Behavioral actions of orexin

Drug-induced hyperlocomotion and behavioral sensitization is argued to represent a way of drug-induced neural plasticity (157) that may contribute to dependence and addictive processes. To assess orexin involvement in drug dependence, manipulation of the orexin system via a blockade of OxR1s by the administration of SB-334867 has been proven to be a useful strategy. SB-334867 is a selective OxR1 antagonist with 50-fold selectivity for OxR1 over OxR2 (158). In addition, orexin -/- mice (OKO) which lack the gene for prepro-orexin, have been useful in research where the orexinergic role in drug hyperlocomotion and sensitization has been limited. However, the limited and conflicting findings suggest that more investigation into the role of orexin in locomotor responses to drugs of abuse is needed.

The role of orexin in drug reward, using self-administration paradigms, has been reported. SB-334867 administration results in decreased self-administration of nicotine (159) and alcohol (160) and intra-LH Ox-A increases alcohol intake (161). However, these results are not seen with all drugs of abuse, as with i.c.v. administration of Ox-A (162) or treatment with SB-334867 (130) which fails to affect the response during active cocaine self-administration.

The conditioned place preference (CPP) test has become a widely-used paradigm for the measure of reward-related behavior. Here, an animal is placed in an enclosed environment, which is divided into two or more distinct compartments, each containing distinctive environmental cues that differentiate it from the other(s). After the animal is allowed to move freely among the compartments, it is confined to one enclosure and is presented with a reward

stimulus. The animal forms an association between the stimulus and the environmental cues during this time. After the pairing, when allowed to roam freely among the compartments, the animal will spend more time in the reward-paired chamber if the drug was rewarding.

It has been shown that animals which exhibit a preference for an environment previously paired with food, morphine, or cocaine show increased c-Fos expression in orexin neurons (163). Moreover, the amount of c-Fos expression in orexin neurons in the LLH, is positively correlated to the amount of time that the animals spend in the drug paired chamber (163). The conditioning phase of a morphine CPP paradigm is also associated with increased stimulation of orexin neurons specifically in the LLH (164). Conversely, cocaine CPP has been associated with decreased orexin mRNA levels specifically in the LLH (156). Interestingly, administration of SB-334867 reduces the expression of morphine place preference (163), but not of cocaine place preference. Narita et al. (2006) have also recently found that OKO and wild type (WT) mice prefer a morphine-paired environment equally; whereas, as it has been previously reported, OKO mice fail to develop such a preference (141). Further investigation is needed to clarify this discrepancy.

Previous reports suggest a role for orexin in the formation of a place preference to a morphine-paired environment. Bilateral excitotoxic lesions of the LH block the acquisition of morphine CPP. Unilateral lesions of the LH and intra-VTA administration of SB-334867 on the contralateral side also block the development of morphine CPP (164). Together, these data suggest that orexin activity in the VTA is essential for the development of morphine place preference. A role for the VTA in the expression of morphine place preference has also been reported as intra-VTA SB-334867, which suppresses the expression of morphine place preference (165).

2.2.2. Drug Withdrawal

Withdrawal from drugs of abuse can also alter the orexinergic system, and the first study connecting orexin to drugs of abuse demonstrated the involvement of orexin in morphine withdrawal (128).

Naloxone- or naltrexone-precipitated morphine withdrawal leads to the induction of CRF activity in orexin neurons (128) and c-Fos expression in orexin cells (128;154). Interestingly, increases in c-Fos expression were restricted to the DMH and PFA, and were not seen in the LLH (154). Spontaneous morphine withdrawal increases orexin mRNA in the LH (155). Activation of orexin neurons in response to withdrawal appears to be opiate specific and is not seen following spontaneous cocaine withdrawal (156).

Manipulations of the orexin system can affect behavioral responses seen during withdrawal. For instance, naltrexone-precipitated morphine withdrawal is attenuated in OKO mice (128). Similarly, SB-334867 treatment prior to naloxone-precipitated withdrawal attenuated somatic withdrawal symptoms (154). The degree of somatic withdrawal symptoms is correlated with c-Fos expression in the NAc shell, and blockade of OxR1 indirectly attenuates NAc cellular activation (154). Interestingly, the VTA does not show changes in c-Fos expression in the same animals, suggesting a possible non-VTA mechanism by which orexin alters NAc neuronal activity.

2.2.3. Orexin and stress response

Based on evidence gathered using the CPP test, it becomes evident that orexin signaling, particularly in the VTA, is important for the processing of drug-associated stimuli. However, some evidence suggests that orexin involvement in the reinstatement of extinguished drug seeking may be mediated by a stressing component. Following orexin administration, corticosterone levels (166-168) and plasma ACTH (168) are augmented.

Furthermore, orexin mRNA levels and c-Fos expression in orexin neurons are increased following stressors, such as immobilization, foot-shock, and cold

stress (114;166). It has been reported that i.c.v. Ox-A induced reinstatement of an extinguished cocaine-seeking response, is abolished when orexin is co-administered together with CRF antagonist (162). In addition, foot-shock stress-induced reinstatement of cocaine self-administration is abolished by SB-334867 administration (162). These data suggest that drug seeking is induced by activation of the stress pathway. Interestingly, intra-VTA CRF antagonist does not block Ox-A induced reinstatement of cocaine seeking, and foot-shock stress induced cocaine reinstatement is not blocked by intra VTA SB-334867 (169), suggesting that, at least at the level of the VTA, orexin and CRF have independent actions, and that blockade of stress induced reinstatement by SB-334867 is not VTA mediated.

The distinction between stress mediated and reward mediated effects of orexins has been previously suggested. c-Fos activation in animals that prefer a place previously paired with food, cocaine, or morphine would be specific to the LLH (163) whereas c-Fos activation in animals undergoing a stressful morphine withdrawal would be specific to the DMH and PFA (154). As previously mentioned, orexin action in the VTA has been implicated in CPP reward processing (164), but in response to withdrawal, the VTA fails to be affected by orexin (154). Thus, distinct orexin neuron populations and neural circuits may mediate orexin role in stress or reward.

2.2.4. Link between CRF and orexin

Many similarities exist between the actions of CRF and orexin in the VTA. Both CRF and Ox-A increase firing and potentiate NMDA receptor-mediated synaptic transmission of DA neurons in the VTA (127;144;170-173). Either neuropeptide, when administered to the VTA, promote DA release in target regions of the VTA (141;142;174;175). Additionally, either intra-VTA administration of CRF or Ox-A can induce reinstatement to cocaine seeking in rats as well as cause increased local DA and glutamate release (169;174).

The studies described above suggest that there may be direct interactions between CRF and orexin systems in the VTA. However, while both orexin and CRF can reinstate cocaine seeking through actions in the VTA (169), the

mechanisms by which they induce reinstatement to extinguished cocaine seeking may be independent. For example, intra-VTA Ox-A-induced reinstatement can be blocked by a locally infused OxR1 antagonist, but not by a CRF_1-R antagonist (169). Likewise, an intra-VTA administered OxR1 antagonist did not block CRF dependent foot shock-induced reinstatement or associated increases in local glutamate or DA release (169). Furthermore, while reinstatement of cocaine seeking by intra-VTA CRF is completely glutamate-dependent, reinstatement by intra-VTA Ox-A is not, suggesting a separate mechanism (169). Therefore, even though administration of SB-334867 blocks foot shock-induced reinstatement to cocaine (162), these effects are not likely mediated by the VTA and separate mechanisms may exist for the ability of Ox-A or CRF to induce reinstatement to extinguished cocaine seeking.

Harris et al. (2005; 2007) provided evidence in favor of a functional dichotomy of the orexin system by showing that orexin neurons in the LLH, but not those in the PFA or DMH, are strongly involved in reward-related behaviors. Retrogradely labeled LLH orexin neurons from the VTA show higher c-Fos activation with a drug-associated environment (164). Moreover, foot shock activates orexin neurons in PFA and DMH, but not those in the LLH (163). Recent anatomical and functional studies provide evidence for projections from CRF neurons to orexin neurons and vice versa (129;176). Taken together, these studies suggest that PFA and DMH orexin neurons, as opposed to LLH orexin neurons, respond to stressful events and might activate, or be activated by, CRF systems (Figure 10).

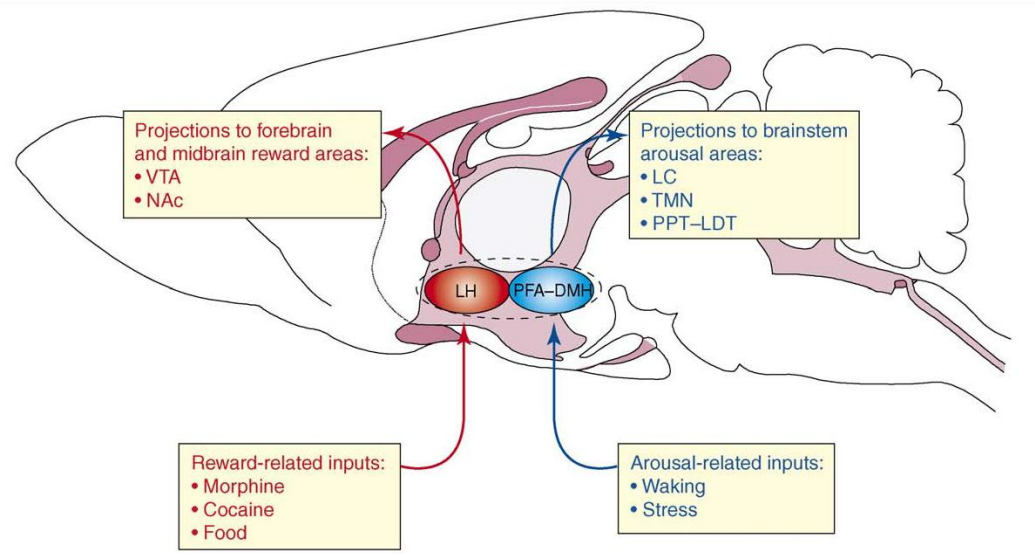

Figure 10. Schematic sagital section through the rat brain illustrating orexin neurons located in the LLH or in the PFA or DMH having distinct functional attributes. Reproduced from (129).

Clearly, both CRF and Ox-A play important roles in the development of addiction-associated behaviors and reinstatement of drug use. Although both neuropeptides mediate effects in the VTA, the mechanisms of these effects on reinstatement may be independent. This avenue of research is particularly important because a deeper understanding of this functional interaction between CRF and orexin systems would provide a very interesting opportunity to design better therapies to counteract pathological stress-related behaviors. For example, a combination therapy of two antagonists might offer strong efficacy against stress-induced relapse compared with a single-target approach.

It has been proposed that the PFA-DMH orexin system drives relapse through activation of stress systems (perhaps involving CRF or NA), whereas the LLH orexin system drives relapse through activation of brain circuits associated with reward learning. Future studies need to be conducted, in order to test these and other theories concerning the mechanism by which orexin plays a role in addiction. The orexin system, with its roles in both stress activation and reward-based learning and memory, could provide an important target for future pharmacotherapies designed to prevent drug relapse.

AIMS

Although DA has been extensively implicated in the initial reinforcing effects of drugs during self-administration and CPP, NA and other systems may play a larger role than previously suspected (177).

Recently, the role of NA in addiction has also received interest stemming from its importance in stress-induced reinstatement, drug-induced locomotion and opioid CPP (93). Furthermore, it has also been described an activation of the brain stress system after acute administration of drug of abuse (6). CRF is the main neuropeptide regulating the stress system activity, which is widely distributed throughout the brain and plays a key role in coordinating behavioural and autonomic responses to stress (178). The VTA receives CRF innervations from multiple sources (179;180), and CRF also projects to noradrenergic brainstem nuclei and LH (129;176). Additionally, recent investigations revealed participation of the orexinergic system in brain stress system (181). It has been reported that Ox-A activates PVN neurons (182) and that i.c.v. Ox-A increases plasma levels of GC and ACTH (183). However, it remains to be determined whether Ox-A modulates the activity of the extrahypothalamic CRF neurons.

Thus, the aims of the present work were:

1. Given the importance of catecholaminergic pathways in reward, the first aim was to investigate the possible changes in TH expression and phosphorylation during morphine-induced CPP in the NAc (shell and core), VTA, and NTS neurons. To this end, we first evaluated the changes in TH phosphorylation at serine 40 and 31 (pSer40 and pSer31) in addition to total levels of TH. We also examined changes in the NAc content of DA and its metabolite dihydroxyphenylacetic acid (DOPAC) and NA and its metabolite 3-methoxy-4-hydroxyphenyl-ethylene glycol (MHPG) during morphine-induced CPP to determine DA and NA turnover, respectively.

2. Because of the relevance of the CRF inputs to the neurocircuitry involved in the rewarding effects of drugs of abuse, our following goal was to evaluated the role of CRF_1-R in (i) the somatic and behavioural states produced by morphine administration; (ii) the activation (as shown by c-

Fos expression) of different brain nuclei involved in drug reward in morphine-paired mice; (iii) the responses of both the mesolimbic dopaminergic system and its noradrenergic innervations to morphine-induced CPP; and (iv) the activity of the HPA axis elicited by morphine-associated stimuli.

3. Our study was also designed to evaluate changes in the orexinergic system during naloxone-precipitated morphine withdrawal by the quantification of preproorexin mRNA levels and Ox-A neuron activation in the hypothalamus. We addressed the role of orexinergic inputs in the development of somatic signs of the opiate abstinence syndrome. For that, we blocked the orexinergic system, by OxR1 (SB334867) and OxR2 (TCSOX229) antagonist administration, and measured the somatic symptoms produced during morphine withdrawal. In addition, c-Fos expression in the hypothalamic and extrahypothalamic brain stress systems was evaluated after OxR1 blockade.

4. The role of the OxR1 and OxR2 was also evaluated in the morphine withdrawal-induced activation of the HPA axis. To this end, we measured the activity (by means of c-Fos expression) of CRF neurons in the parvocellular region of the PVN and the plasma corticosterone levels in morphine-withdrawn rats pretreated with SB334867 and TCSOX229.

MATERIALS AND METHODS

1. ANIMALS

All surgical and experimental procedures were performed in accordance with the European Communities Council Directive of 24 November 1986 (86/609/ EEC), and approved by the local Committees for animal research (REGA ES300305440012, Murcia and Institutional Animal Care and Use Committee of the Institute of Experimental Medicine, Budapest).

Adult male Swiss mice (25-35 g at the beginning of the experiments) were housed in cages sized 36x15x20 cm, and in groups of seven animals per cage. Male Wistar rats (220–240 g, at the beginning of the experiment; Harlan, Barcelona, Spain) were housed 2–3 per cage (45x24x20). Temperature (22±2ºC) and humidity (50±10%) were controlled in both cases. Food and water were available *ad libitum*, (Harlan Teklad standard rodent chow; Harlan Interfauna Ibérica, Barcelona, Spain). Animals were adapted to standard 12-h light-dark cycle (lights on: 08:00–20:00 h) for 7 days before the beginning of the experiments. Animals were conditioned and tested during the light phase of the cycle. They were handled daily during the first week after arrival to minimize stress. The study was approved by the University of Murcia bioethics committee (RD 1201/2005) and Ministerio de Ciencia y Tecnología (SAF2009-07178), Spain.

2. DRUG TREATMENT AND EXPERIMENTAL PROCEDURE

The experimental groups and treatment regimens used in the present study are shown in Figure 11.

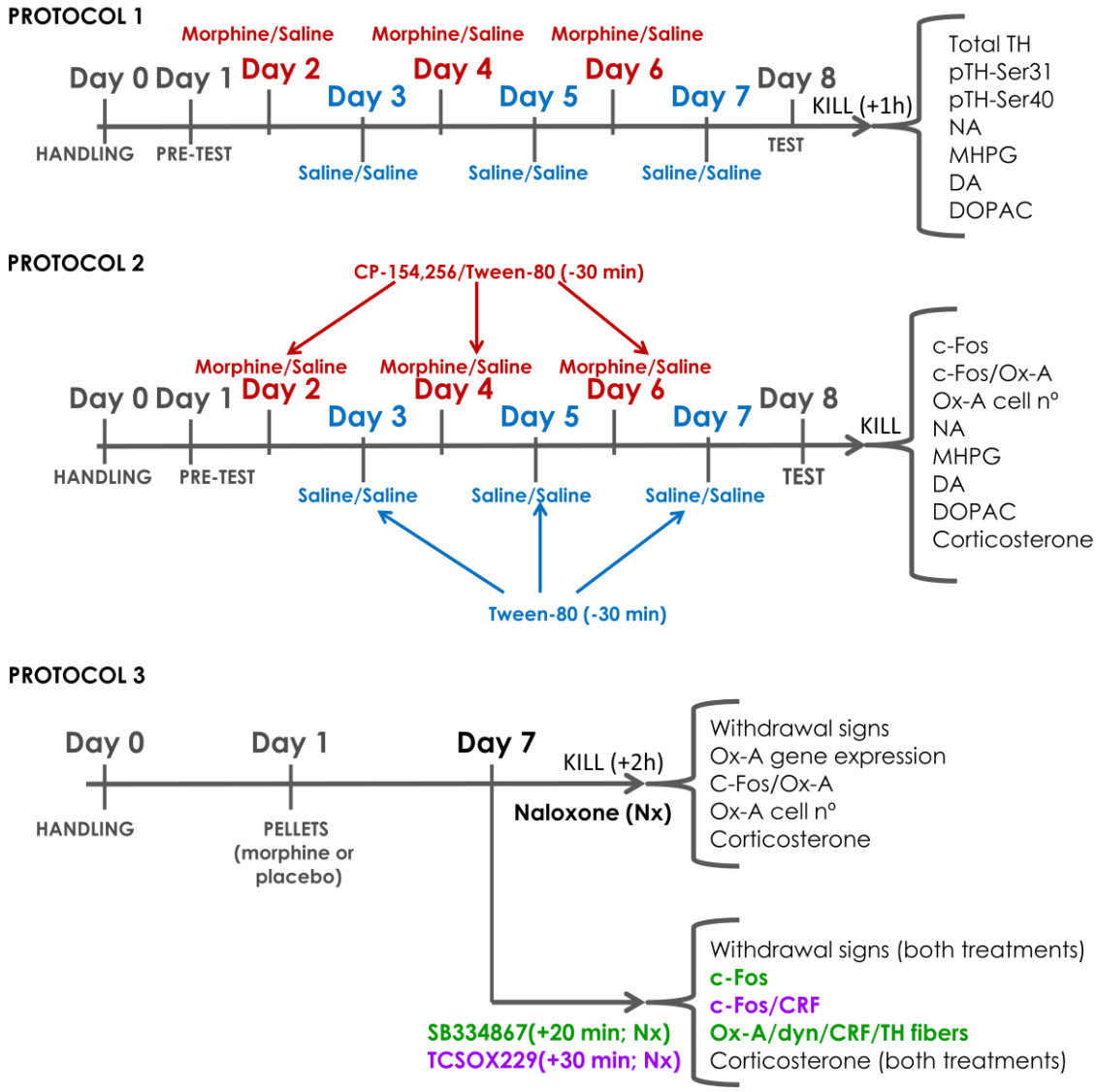

Figure 11. Protocols used during the different experiments. Protocols 1 and 2 were applied into mice. Protocol 3 was applied into rats.

2.1. Apparatus of place preference

The place conditioning apparatus is based on that used by (2) with some modifications and consisted in two rectangular polycarbonate compartments (20×18×25 cm) spaced at 4 cm from each other, both accessible from a rectangular polyvinyl chloride exterior area (20×7×25 cm). In order to distinguish the three compartments, visual and sensory texture cues were used; one compartment was gray-striped wall with black smooth floor and the other was

black-spotted wall with gray rough floor. The neutral area, providing access to the compartments, had transparent wall and floor. One compartment of the place preference apparatus was randomly chosen to be paired to drug administration and the other to saline. The time spent in each chamber was recorded for each mouse by a computer program (CPP Win 2.0. Panlab, Barcelona, Spain).

2.2. Place preference paradigm

The rewarding effects of morphine were evaluated using the CPP paradigm (184) (Figure 12).

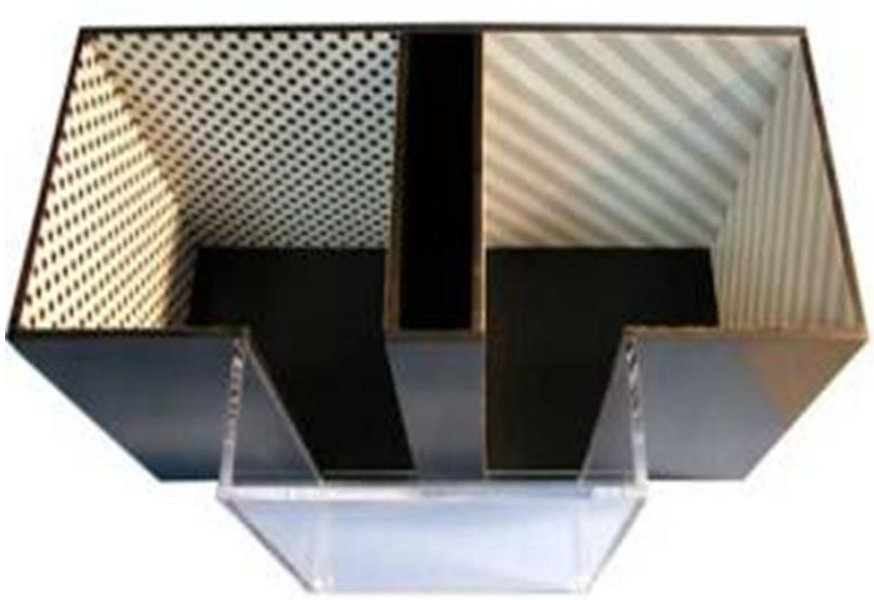

Figure 12. Conditioned place preference box. (2)

The CPP schedule consisted of three phases:

Preconditioning phase. During this phase, mice were placed in the middle of the neutral area and their localization was recorded for the following 15 min. After the session, animals were randomized to be paired to drug (morphine-paired group) or saline (saline-paired group) administration and to be assigned to a compartment. Animals that spent less that 6 min in either white or black

compartment were considered not to be neutral in preference for either side, and were excluded from further studies (less than 5% of mice).

Conditioning phase. During this phase, mice were treated for six consecutive days. To evaluate the rewarding effects of morphine, mice received an injection of morphine hydrochloride (0.5, 1, 4, 6, and 8 mg/kg, s.c.; Alcaliber Laboratories, Madrid, Spain) on days 1, 3 and 5, and an injection of saline solution (0,9%; 10 ml/kg, s.c.) on days 2, 4 and 6. Doors matching the walls of the compartment allowed confinement of the mice for 20 min immediately after morphine or saline injections. Control animals received saline every single day.

Testing phase. It was conducted exactly as the preconditioning phase (free access to both compartments for 15 min, 24 h after the final conditioning session).

A score was calculated for each mouse as the difference between post-conditioning and the pre-conditioning time spent in the drug-paired compartment, in order to measure this parameter.

Furthermore, we tested if blockade of CRF_1-R would affect the morphine rewarding effects. For that, another group of mice were treated with the same protocol described above, plus an intraperitoneal (i.p.) injection of CP-154,526 , [N-butyl-N-ethyl-2,5-dimethyl-7-(2,4,6-trimethylphenyl)-pyrrolo-[3,2-e]-pyrimidin-4-amine] (a selective CRF_1-R antagonist; 30 mg/kg, i.p., provided by Pfizer, New York, USA) dissolved in 10% of Twen-80 (Sigma-Aldrich, St Louis, MO, USA). Peripherally administered CP-154,526 crosses the blood brain barrier and reaches maximal brain concentration after 20 min (185). Therefore, the antagonist (CP-154,526) was administered 30 min before receiving the morphine hydrochloride dose (6 mg/kg, s.c.) on days 1, 3 and 5; and the vehicle (Tween-80) was also administered 30 min before the dose of saline (10 ml/kg, s.c.), on days 2, 4 and 6. Control animals received saline 30 min after the vehicle (Tween-80) every single day.

After the post-conditioning phase mice were decapitated at different times (between 10:00-12:00 h to avoid circadian variations in plasma levels of the hormones), the brains were rapidly removed, and stored immediately at -80°C until use for Western blot analysis (total TH, pSer31 TH and pSer40 TH), or High Performance Liquid Chromatography (HPLC) (DA, DOPAC, NA and MHPG). After

24 hours, brains were placed on an ice-cooled plate for dissection of the bilateral NAc. To isolate this area, the remaining rostral brain section was scalped bladed as described in Heffner et al. (186), with a triangle centred over each anterior commissure, according to Franklin and Paxinos atlas (187).

2.3. Measurement of the withdrawal syndrome in rats

Rats were made dependent on morphine by subcutaneous (s.c.) implantation of two 75 mg slow-release morphine pellets (provided by the Ministerio de Sanidad, Madrid, España) for 6 days under light ether anesthesia. Control rats received placebo pellets, the excipient without morphine. This procedure has been shown to produce constant plasma morphine concentration starting few hours after pellets implantation and morphine dependence, as revealed by expression of full withdrawal syndrome after acute injection of opioid antagonists [34]. Dependence on morphine (as measured by withdrawal response) is achieved 24 h after implantation of pellets and remained constant for 15 days [35]. Six days after the implantation of morphine or placebo pellets, precipitated withdrawal was induced by s.c. injection of naloxone (1 mg/kg; in a volume of 1 ml/kg; Sigma-Aldrich).

Experiments were carried out in a quiet room. The observer was blind to the drug combination used. Rats were individually placed into transparent plastic cages right after the naloxone injection, and observed continuously for the occurrence of somatic signs of opiate withdrawal up to 30 min (188). Subsequently, previously identified behavioral characteristics of the rat opiate withdrawal (189) were evaluated including: wet-dog shakes, jumping, paw tremor, teeth chattering, mastication, ptosis, piloerection, sniffing, writhing, tremor and diarrhea. The number of wet-dog shakes, jumping, sniffing, and paw tremor was counted as the number of events occurring during the total test time period (graded signs). Teeth chattering, body tremor, mastication, ptosis, piloerection and diarrhea were scored 1 for appearance or 0 for non-appearance within each 5 min time. To obtain a comprehensive index of the severity of somatic opioid withdrawal including all the signs examined, a global withdrawal score was calculated for each animal by giving each individual sign a relative weight as

previously reported by Maldonado (190): jumping x 0,8; wet dog shakes x 1; paw tremor x 0,35; sniffing x 0,5; writhing x 0,5; ptosis x 1,5; teeth chattering x 1,5; body tremor x 1,5; diarrhea x 1,5; mastication x 1,5 and piloerection x 1,5.

Body weight loss was calculated as the difference between the weight determined immediately before naloxone injection and a second determination 2 h later. The weight gain of the rats was checked during treatment to ensure that the morphine was liberated correctly from the pellets because it is known that chronic morphine treatment induces a decrease in body weight gain due to lower caloric intake (103;191).

In order to investigate the effect of the OxR1 and OxR2 blockade on the physical symptoms of morphine withdrawal, rats were pretreated with the respective antagonists. The selective OxR1 antagonist, SB334867; Tocris, Bristol, UK) which was suspended in 2% dimethylsulfoxide (DMSO) and 10% 2-hydroxypropyl-bcyclodextrin; Sigma), at the dose of 20 mg/kg i.p.; was administered 20 min before the saline or naloxone injection. Another set of animals was pretreated with the selective OxR2 antagonist TCSOX29 (Tocris dissolved in saline, 10 mg/kg i.p., 30 min prior saline or naloxone injection. Doses of these antagonists were chosen according to previous studies (154;160;192). Vehicle was delivered at the same volume as the SB334867 or TCSOX29 solutions.

3. ELECTROPHORESIS AND WESTERN BLOTTING

Western blot analysis was used for total TH determination and for TH pSer31 and pSer40 analysis. NAc samples were placed in homogenization buffer (82), homogenized and sonicated 30 seconds prior centrifuge at 6000x g for 5 minutes at 4ºC. Equal quantities of total protein were separated by 10% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) electrophoresis and transferred to polyvinylidene difluoride membranes. Blots were incubated in the following primary antibodies: polyclonal anti-total TH (against phosphorylated and non-phosphorylated TH; 1:1000; AB152, Chemicon International, Temecula, CA, USA), polyclonal anti-pSer40 TH (1:500; AB5935, Chemicon International), and polyclonal anti-pSer31 TH (1:300; AB5423, Chemicon International) in Tris-

buffered saline Tween-20 (TBST) with bovine serum albumin overnight at 25°C. After extensive washings with TBST, the membrane was incubated 1 h at room temperature, with peroxidase labeled secondary antibody (anti-rabbit sc-2004; 1:2500; Vector Laboratories, Burlingame, CA, USA). Quantification of immunoreactivity was carried out by densitometry. The integrated optical density of the bands was normalized to the background values. Relative variations between bands of the experimental samples and the control samples were calculated in the same image. Antibodies were stripped from the blots by incubation with stripping buffer (glycine 25mM and sodium dodecyl sulfate 1%, pH 2) for 1 h at 37°C. Blots were subsequently reblocked and probed with 1:1000 anti-β-actin (Cell Signalling, Beverly, MA, USA) as loading control. Each level is corrected for individual β-actin levels.

4. HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) ANALYSIS

Tissues isolated for HPLC were frozen in liquid nitrogen, weighted, placed in perchloric acid (0.1 M), homogenized and centrifuged 10000x g at 4°C for 20 minutes. Supernatants were used for analysis. HPLC samples were twice filtered through 0.22 μm GV filter (Millipore Corporation, Bedford, MA, USA). NA and its metabolite in the central nervous system MHPG and DA and its metabolite DOPAC were determined by HPLC with electrochemical detection (Milanés et al. 1998; Fuertes et al. 2000; Laorden et al. 2000). The mobile phase consisted of a 95% (v/v) mixture of water and methanol with sodium acetate (50 mM), citric acid (20 mM), L-octylsodium sulfonate (3.75 mM), di-n-butylamine (1 mM), and ethylenediaminetetraacetic acid (EDTA) (0.135 mM), adjusted to pH 4.3. The flow rate was 0.9 ml/min, and chromatographic data were analyzed with Millennium 2010 Chromatography Manager Equipment (Millipore Corporation). Data were calculated by using an external standard calibration. All the reagents (NA bitartrate, MHPG hemipiperazinium salt, DA HCl, and DOPAC; used as HPLC standards) were purchased from Sigma Chemical.

5. QUANTIFICATION OF REAL TIME PCR (Q-PCR)

For q-PCR, animals were decapitated at different time-points (15, 30, 60 and 120 min). The brains were rapidly removed from the skull, placed to an RNasa free rubber surface and the cerebellum was removed. The boundaries of the hypothalamic block, were at the optic chiasm in rostral-, at the mammillary bodies in caudal-, and at the hypothalamic sulcus in the lateral directions. Immediately after the extraction of the hypothalamic block, samples were frozen on dry ice and stored at -70ºC until assay.

In order to determine orexin mRNA expression either during addiction or withdrawal, q-PCR was applied on the hypothalamic tissue.

5.1. Primer design

Primers used for the comparative CT (threshold cycle) experiments were designed by the Primer Express 3.0 program. Primer sequences were the following:

Orexin-A:

Forward: TCCTTCAGGCCAACGGTAAC

Reverse: GGCAGGGATATGGCTCTAGCT

GAPDH:

Forward: ACAGCCGCATCTTCTTGTGC

Reverse: GCCTCACCCCATTTGATGTT

5.2. Quantification of the q-PCR

Total RNA was isolated from hypothalamic samples with QIAGEN RNeasy MiniKit (Qiagen, Valencia, CA, USA) according the manufacturer's instruction. To eliminate genomic DNA contamination DNase I treatment was used (100 ml Rnase free DNase I (1 uDNase I, Fermentas) solution was added). Sample quality control and the quantitative analysis were carried out by NanoDrop (Thermo Scientific). Amplification was not detected in the RT-minus controls. cDNA

synthesis was performed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). The designed primers (Invitrogen) were used in the real-time PCR reaction with Power SYBR Green PCR master mix (Applied Biosystems) on ABI StepOne instrument. The gene expression was analyzed by ABI StepOne2.0 program. The amplicon was tested by Melt Curve Analysis on ABI StepOne instrument. Experiments were normalized to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression.

6. SAMPLE PROCESSING FOR IMMUNOHISTOCHEMISTRY

Mice and rats were anesthetized and perfused at different time points. Anesthesia was induced by a subletal dose of sodium pentobarbital. Perfusion through the ascending aorta was performed with saline (0.9%), followed by ice-cold fixative (4% paraformaldehyde in 0.1 m/l borate buffer). Brains were post - fixed in the fixative for 3 h, and then placed in PBS (0.1 M phosphate buffered saline, pH 7.4), containing 30% sucrose overnight. Series of 30 µm frontal sections were cut on freezing microtome, according to the atlas of Franklin and Paxinos (187) for mice and Paxinos and Watson (193) for rats. Sections were collected in a cryoprotecting solution and stored at -20ºC until processing.

7. IMMUNOHISTOCHEMICAL DETECTION AND QUANTIFICATION OF TOTAL AND PHOSPHORYLATED TH, AND c-Fos

Analysis of total TH, TH pSer40, TH pSer31 and c-Fos were made on freefloating sections (30 µm). After blocking the samples with H_2O_2 and normal goat serum (Sigma), tissue sections were incubated in the primary antibody anti-total TH (1:6000; AB152, Chemicon International), polyclonal anti-phospho-Ser40 TH (1:400; AB5935, Chemicon International), polyclonal anti-phospho-Ser31 TH (1:400; AB5423, Chemicon International) and anti-c-Fos (1:10000, Santa Cruz Biotechnology, Santa Cruz, CA, USA), overnight at room temperature. This was followed by the application of a biotinylated anti-rabbit IgG (1:500, Vector Laboratories). Antigens were visualized by a conventional avidin–biotin immunoperoxidase protocol using reagents from the Vectastain ABC Elite Kit

(Vector Laboratories) and 3,3'-diaminobenzidine (DAB, Sigma Chemical) as chromogen for total TH, TH pSer40 and TH pSer31; and DAB followed by nickel intensification for c-Fos. Sections were mounted onto chrome-alum gelatin-coated slides, dehydrated, and cover-slipped.

Immunoreactivity images of brain sections were captured using a video camera (DFC 290; Leica, Madrid, Spain) on a light microscope (DM4000B; Leica). The number of total TH, TH pSer40, TH pSer31 and c-Fos immunoreactive neurons was counted by a computer-assisted image analysis system (QWIN; Leica) using an identical rectangular frame. Anatomical regions were delineated from the captured images of the tissue using the mouse brain atlas (187) and the rat brain atlas (193). Eight to ten sections from each mouse and rat were counted bilaterally at distinct rostrocaudal levels.

The number of neurons of total TH, TH pSer40, TH pSer31, and c-Fos cytosolic and nuclear profiles within the confines of cell groups of interest were counted bilaterally in complete series of sections and estimates were corrected for double-counting errors. To avoid observer bias, all sections were quantified by a blinded investigator. Total counts for different brain regions are expressed as mean ± SEM.

8. DOUBLE-LABELING IMMUNOHISTOCHEMICAL DETECTION AND QUANTIFICATION OF c-Fos IMMUNOREACTIVE NUCLEI FOR OxA-POSITIVE NEURONS, CRF-POSITIVE.

For double labeling, tissue sections from each mouse or rat, in each treatment group samples were processed for cFos immunoreactivity. Briefly, c-Fos immunostaining was performed as described above. Following the c-Fos staining, sections were rinsed in PBS. Then were treated with bovine albumin serum or goat serum and incubated with rabbit polyclonal anti-OxA antibody (1:2000, Santa Cruz), or rabbit polyclonal anti-CRF antibody (1:1000, a generous gift from Wylie Vale, The Salk Institute, La Jolla, CA, USA). This, was followed by application of a biotinylated anti-goat or anti-rabbit IgG (1:500, Vector Laboratories), and then with the avidin–biotin complex (Vector Laboratories).

Visualization of the antigen–antibody reaction sites was performed using DAB (Sigma). Sections were mounted onto chrome-alumn gelatin coated slides, dehydrated through graded alcohols, cleared in xylene and cover slipped with dibutylphtalate (DPX).

Images were captured by means of DM4000B Leica microscope (Leica) equipped with a video camera (DFC290; Leica). The LH was further subdivided into its three sub-populations: DMH, PFA and LLH of the LH, and we measured the number of Ox-A neurons in the three subpopulations. These parameters were measured in either mice or rats. After labeling the area of interest by a computer assisted image analysis system (QWIN) using identical rectangular frame first, single labeled Ox-A neurons were manually counted (X20 magnification) in LH. Then, c-Fos/Ox-A double stained neurons were identified as profiles with blue/dark nuclear staining for c-Fos and brown cytoplasmic deposits for Ox-A. Sections from each animal were counted bilaterally at distinct rostro-caudal levels of the LH, and averaged to obtain a single value for each animal. c-Fos-positive/ CRF (PVN, BNST, and CeA) double-stained neurons were quantified in the same way.

9. DOUBLE-LABELING IMMUNOHISTOCHEMISTRY OF Ox-A FIBERS AND dyn- TH- OR CRF-POSITIVE NEURONS

For double labeling, tissue sections from each placebo-treated rats were processed for Ox-A fibers immunoreactivity using DAB nickel intensification and then pro-dyn, CRF or TH were revealed using DAB chromogen only. Briefly, Ox-A fibers immunostaining were performed as described above, using DAB followed by nickel intensification. Following the Ox-A fibers staining, sections were rinsed in PBS, treated with 3% bovine serum albumin and then incubated with the guinea-pig anti-dyn antibody (1:2000, Neuromics, Edina, MN, USA), rabbit anti-CRF antibody or anti-TH antibody (1:6000, Chemicon). The same immunohistochemistry procedures described above were followed. The dyn - and CRF- and TH antibody– peroxidase complex was developed in DAB. The sections were mounted onto chrome-alumn gelatin coated slides and coverslipped.

10. RADIOIMMUNOASSAY

Trunk blood was collected into ice-cooled tubes containing 5% EDTA and was centrifuged (500x g; 4ºC; 15 min). Plasma was separated, divided into two aliquots and stored at -80ºC until assayed for corticosterone. Plasma levels were quantified using specific corticosterone antibodies for mice and rats (^{125}I-CORT RIA; MP Biomedicals, Orangeburg, NY, USA). The sensitivity of the assay was 7.7 ng/mL

11. STATISTICAL ANALYSIS

Data are presented as mean ± standard error of the mean (SEM). Data were analyzed using two-way or one-way (when required) analysis of variance (ANOVA) followed by a post hoc Newman–Keuls test or by a Dunnet's test. The Newman Keuls *post hoc* test was used for individual group comparisons. Dunett's test was used in order to compare the conditioning scores in control and experimental groups. Differences with a $P < 0.05$ were considered significant.

RESULTS

1. ENHANCED TYROSINE HYDROXYLASE PHOSPHORYLATION IN THE NUCLEUS ACCUMBENS AND NUCLEUS TRACTUS SOLITARIUS-A2 CELL GROUP AFTER MORPHINE-CONDITIONED PLACE PREFERENCE

As described previously, the aim of the present study was to investigate the TH phosphorylation on the CPP mice conditioned by morphine in the NAc, VTA, and NTS neurons. To this end, we first evaluated the changes on TH phosphorylation at Ser40 and Ser31 in addition to total levels of TH. We also examined changes in NAc content of DA, its metabolite DOPAC, and NA and its metabolite MHPG in mice conditioned by morphine, to determine DA and NA turnover respectively.

Before performing immunodetection assays, the weight of the animals was checked. Mice treated with morphine showed a significantly lower body weight gain than animals receiving saline injection (Figure 13).

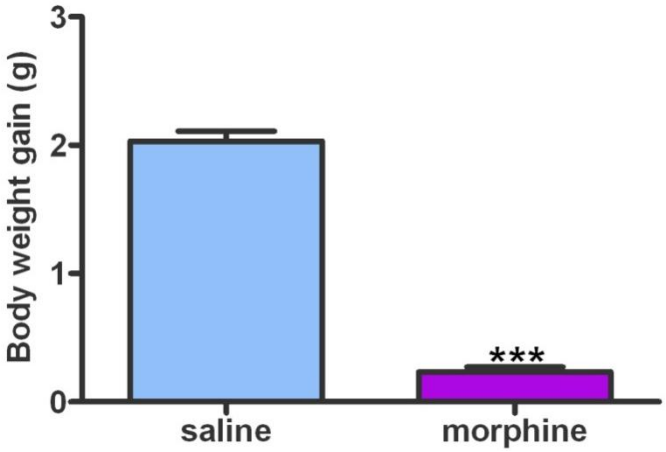

Figure 13. Effect of a repeated saline or morphine injection on the mice body weight gain. ***p<0.001 versus the saline control group.

1.1. Morphine dose–response on conditioned place preference paradigm

The time spent in each chamber during the place preference test for mice conditioned with the different doses of morphine is presented in figure 14. A comparison of the differences in time spent on the drug-paired side revealed a

main effect of drug treatment (p=0.001). Post hoc analysis revealed that mice given morphine had significantly higher difference scores compared to mice in the saline-paired group (p<0.001). Morphine at the dose of 6 mg/kg (s.c.) produced maximum preference; therefore, this dose was chosen for subsequent experiments.

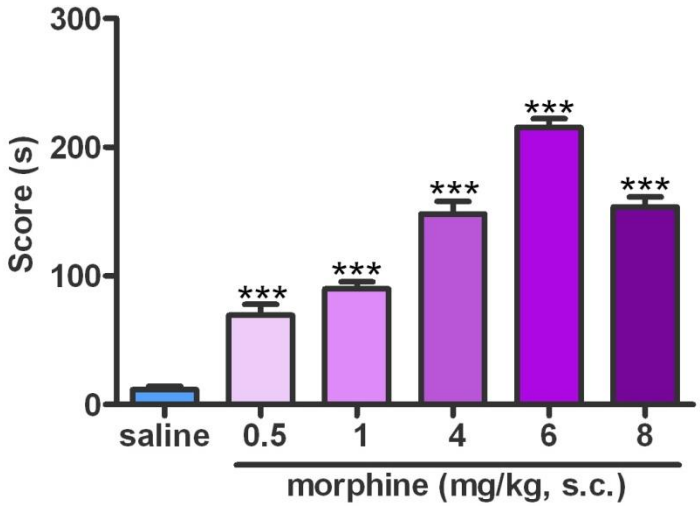

Figure 14. Conditioned place preference induced by different doses of morphine (0.5, 1, 4, 6, or 8 mg/kg, s.c) or saline (10 ml/kg, s.c.) during conditioning phase. Data are expressed as mean ± SEM (n=10–11). ***p<0.001 versus the saline control group

The 60% of the animals were receiving morphine in the preferred side while the 40% were receiving the drugs in the non-preferred side. The 53% of the animals that were receiving the drug in the preferred side showed a preference for the drug-paired side while the 60% of the animals that were receiving the drug in the non-preferred side showed a preference for the drug-paired side. There were no statistical differences between both groups.

1.2. Contents of DA, NA, and their metabolites in the NAc

The tissue contents of DA, NA, and their metabolites in the NAc were tested on the CPP mice conditioned by morphine. The results depicted in figure 15 showed an increase of the contents of DOPAC (p<0.01) and MHPG (p<0.01) and in the ratio DOPAC/DA (p<0.001) and MHPG/NA (p<0.001) after morphine-

induced CPP compared to the saline-paired group. There were no significant differences in the content of DA or NA between morphine- and saline- paired groups.

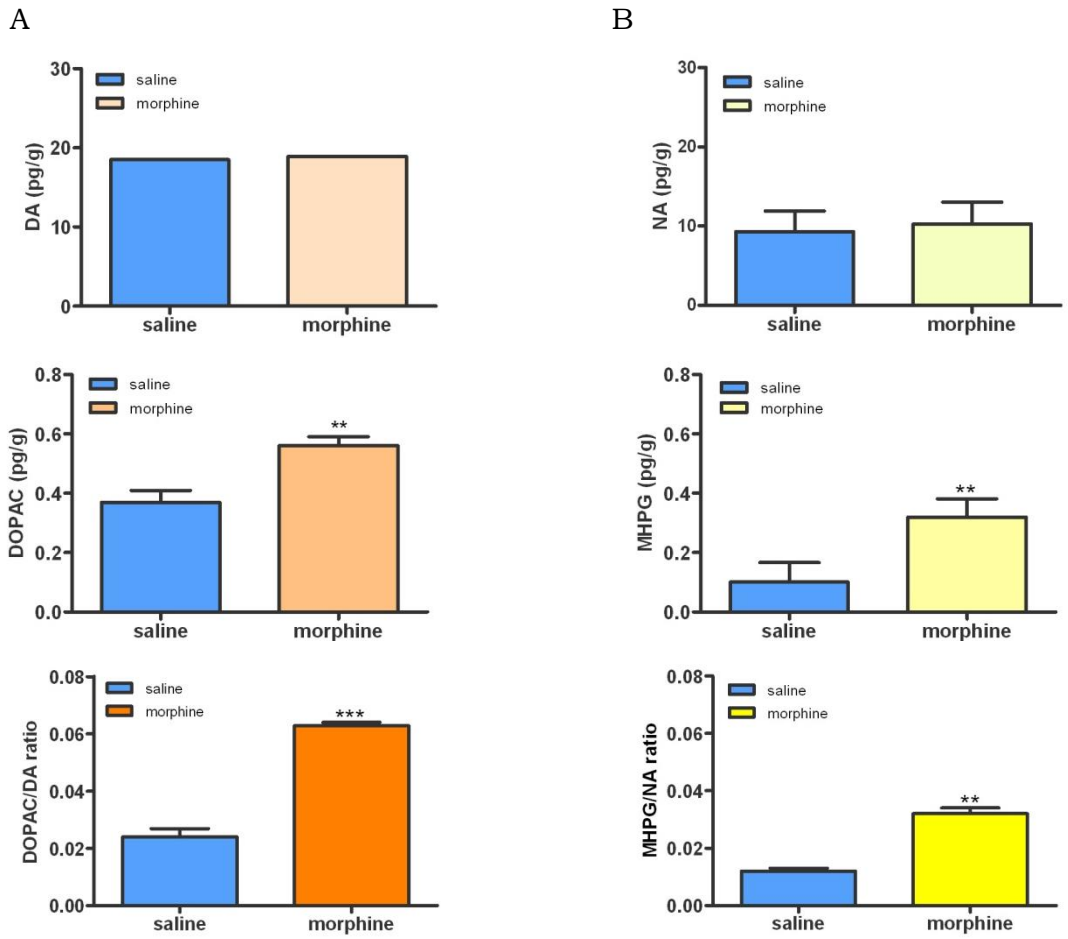

Figure 15. DA, DOPAC, DOPAC/ DA ratio (A) and NA, MHPG, and MHPG/NA ratio (B) 60 min after CPP test in the NAc from morphine-paired mice to morphine. Data are the means ± SEM (n=6–9). **p<0.01, ***p<0.001 versus the saline control group.

1.3. TH pSer40 and TH pSer31 in the NAc

The examination of total TH protein levels in the NAc of mice from saline-paired and morphine-paired groups revealed no differences in total TH expression between the two groups (Figure 16). We also tested the TH pSer40 and TH pSer 31 on the mice conditioned to morphine. TH pSer40 levels were significantly higher (p<0.01) in the morphine-paired group compared with saline-paired mice.

No significant difference in TH pSer31 levels in the NAc was observed between the two groups (Figure 16).

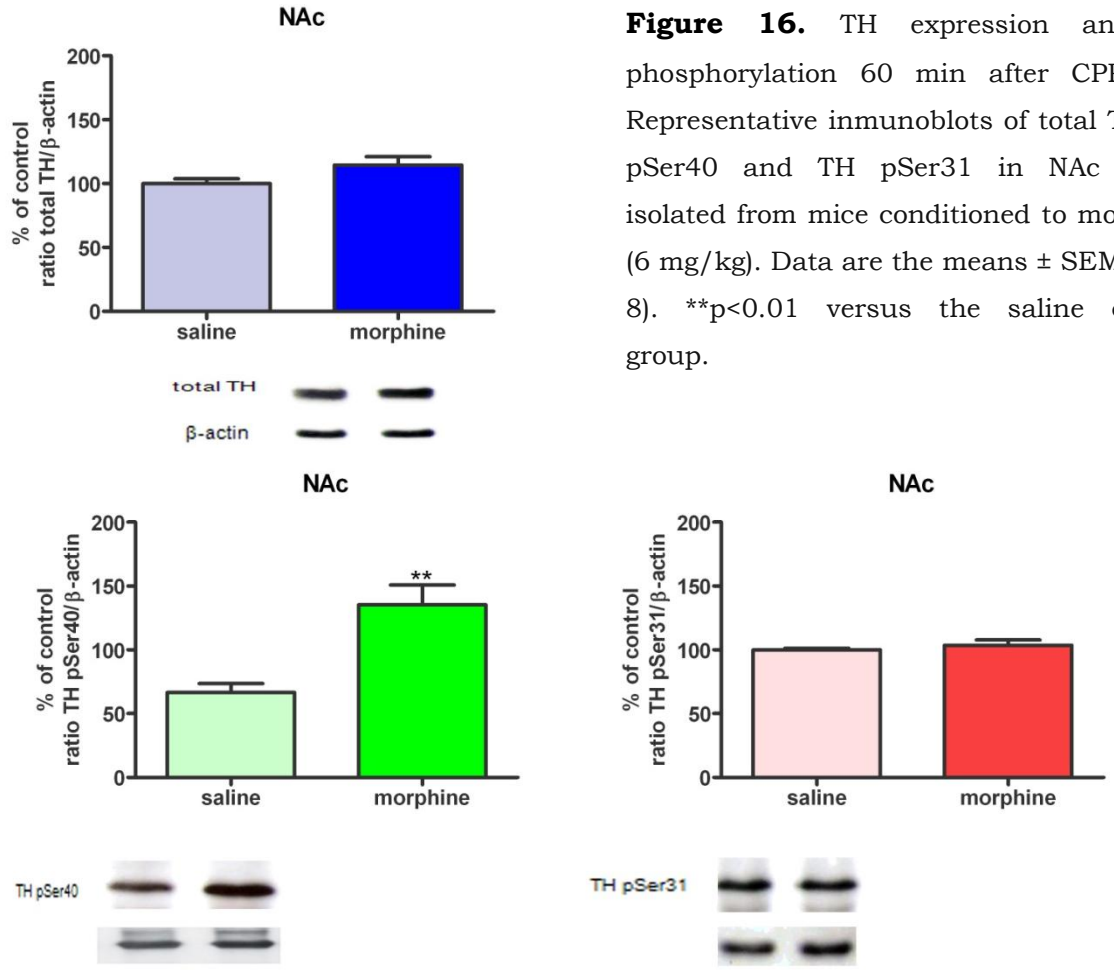

Figure 16. TH expression and TH phosphorylation 60 min after CPP test. Representative inmunoblots of total TH, TH pSer40 and TH pSer31 in NAc tissue isolated from mice conditioned to morphine (6 mg/kg). Data are the means ± SEM (n=5–8). **p<0.01 versus the saline control group.

1.4. TH pSer40 and at Ser31 in the VTA and NTS

The expression of total TH levels and the phosphorylation of TH in the VTA and NTS were examined after the CPP test. As shown in figure 17, there were no significant differences in the total TH levels between morphine- and saline-paired groups in the VTA. Analysis of phosphorylated forms of the above protein (Figure 17) showed no changes in pSer40 or pSer31, indicating that the amount of total protein and the activation (phosphorylation) were not changed after morphine-

induced CPP. However, in the same conditions, we have observed an increased on TH phosphorylation in the NTS.

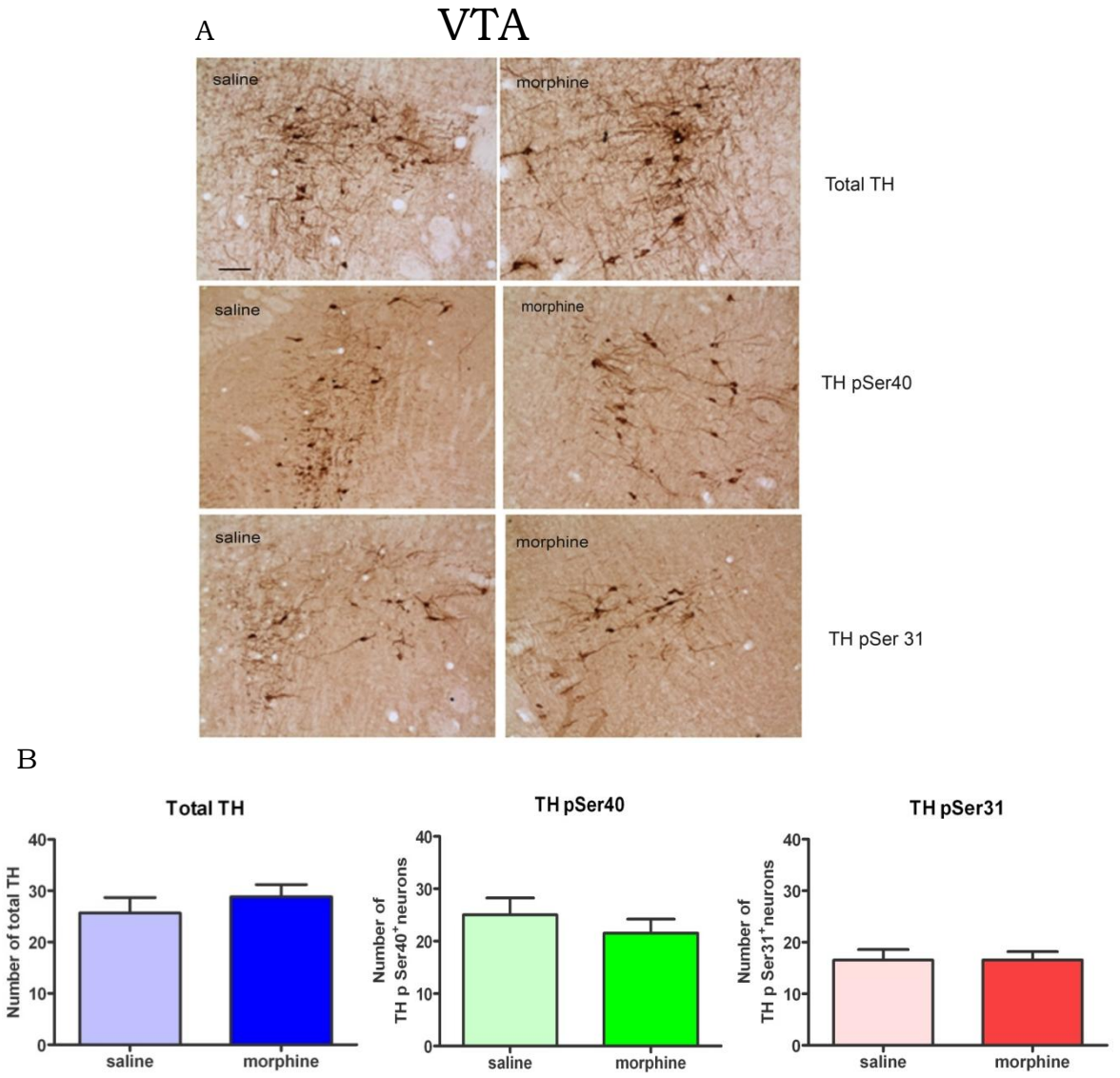

Figure 17. VTA TH expression and TH phosphorylation 60 min after CPP test. **A)** Photographs represent immunohistochemical detection of total TH, phosphorylated Ser40-TH and Ser31 TH in the VTA neurons from mice conditioned by morphine (6 mg/kg). Scale bar 100 µm. **B)** Quantitative analysis of the number of total TH, pSer40-TH, and pSer31 TH immunopositive neurons in the VTA. Data are the means ± SEM (n=5–8). *p<0.05, **p<0.01 versus saline group.

As shown in figure 18 there were quite few cells exhibiting pSer 40 or pSer31 TH staining in the saline-paired group. By contrast, higher levels of pSer40 ($p<0.01$) or pSer31 TH ($p<0.05$) immunoreactivity were observed in the NTS cell group on the mice conditioned to morphine. Quantitative analysis revealed that morphine-induced CPP produced a significant elevation in the number of pSer40 and pSer31 TH-positive neurons without any changes in total TH levels (Figure 18).

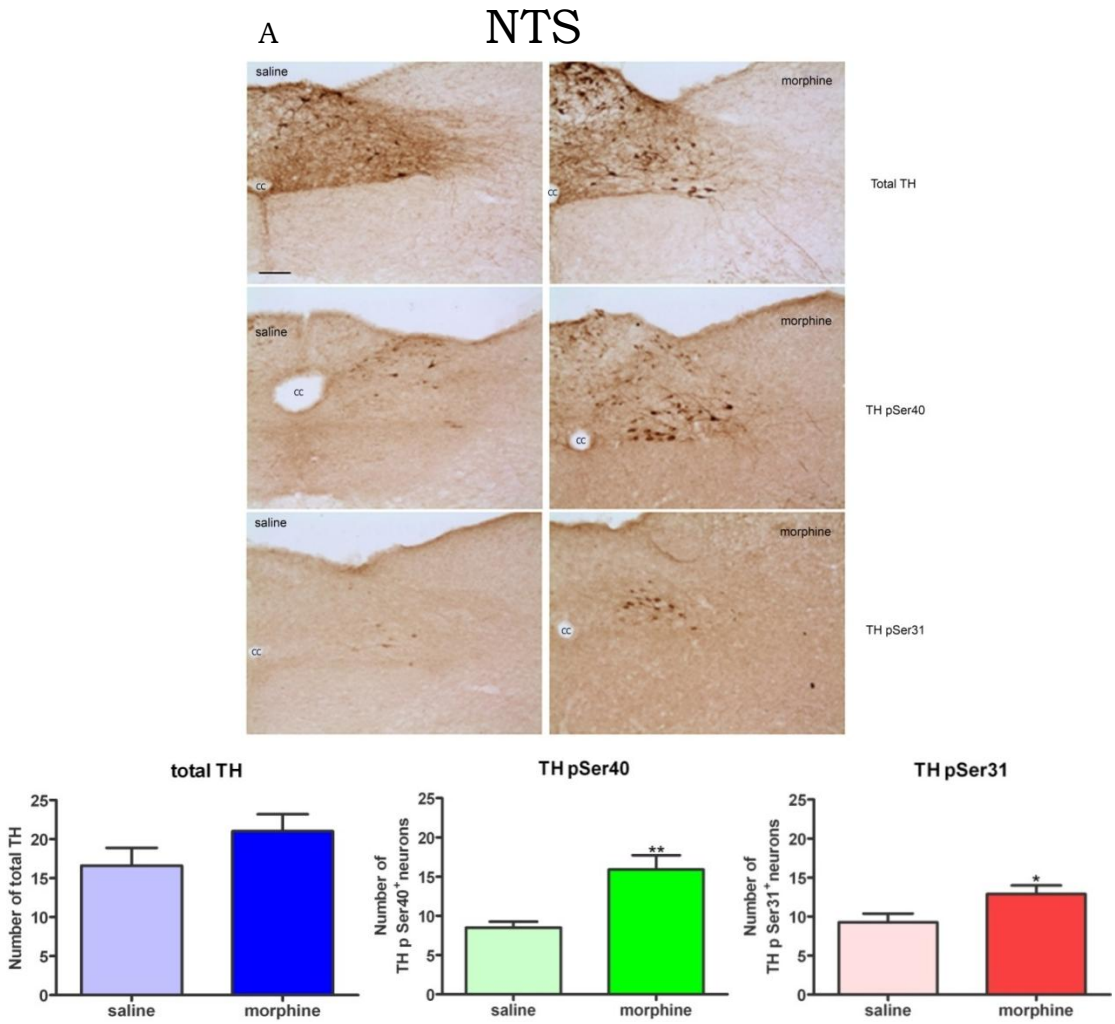

Figure 18. TH expression and TH phosphorylation 60 min after CPP test. **A)** Photographs represent immunohistochemical detection of total TH, phosphorylated Ser40-TH and Ser31 TH in the NTS neurons from mice conditioned by morphine (6 mg/kg). cc canal central. Scale bar 100 µm. **B)** Quantitative analysis of the number of total TH, Ser40-TH, and Ser31 TH immune-positive neurons in the NTS. Data are the means ± SEM (n=5–8). *p<0.05, **p<0.01 versus saline group.

2. CORTICOTROPIN-RELEASING FACTOR 1 RECEPTOR MEDIATES THE ACTIVITY OF THE REWARD SYSTEM EVOKED BY MORPHINE-INDUCED CONDITIONED PLACE PREFERENCE

Given the relevance of the CRF inputs to the neurocircuitry involved in the rewarding effects of drugs of abuse, in the present work we evaluated the role of CRF_1-R in (i) mediating somatic and behavioural states produced by morphine administration; (ii) the activation (as shown by c-Fos expression) of different brain nuclei involved in drug reward in morphine-paired mice; (iii) the responses of the mesocorticolimbic dopaminergic system and its noradrenergic innervations to morphine-induced CPP; and (iv) the activity of the HPA axis elicited by morphine associated stimuli.

2.1 Effect of CP-154,526 in body weight gain during morphine treatment

In agreement with previous results, Newman-Keuls *post hoc* test showed that morphine injection significantly ($P < 0.001$) decreased the body weight gain (Figure 19) when compared with the saline-injected group in animals pretreated with vehicle. The CRF_1-R antagonist administration to the saline-treated animals induced a significant ($P < 0.001$) decrease in the body weight gain in comparison to those administered with tween-80 (10%).

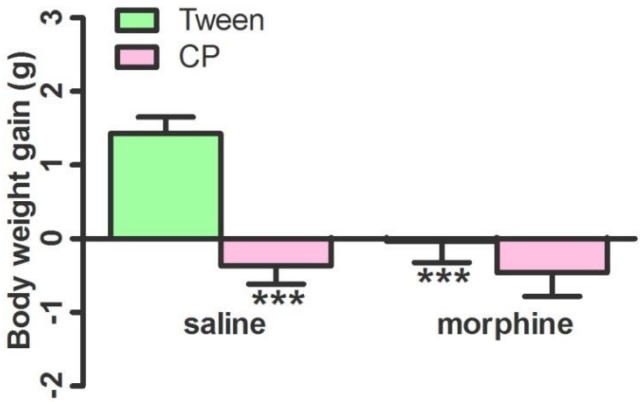

Figure 19. Body weight gain in saline- or morphine-treated mice 30 min after vehicle or CP-154,526 administration. Data shown are means ± SEM. ***$P < 0.001$ versus Tween-80 + saline.

2.2. CRF1R antagonism blocked morphine-induced CPP

Figure 20 shows that vehicle-given mice treated with morphine displayed significantly higher scores ($P < 0.001$) than those treated with saline. In addition, CP-154,526 administration significantly prevented ($P < 0.001$) the increased scores that were seen in the morphine-paired mice group.

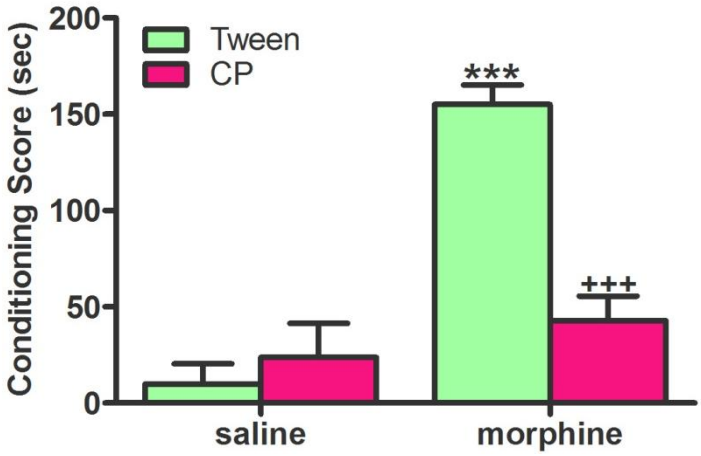

Figure 20. Conditioned place preference induced by morphine after vehicle or CP-154,526 administration. Data shown are means ± SEM. ***$P < 0.001$ versus Tween-80 + saline; +++$P < 0.001$ versus Tween-80 + morphine.

2.3. CP-154,526 attenuated morphine-induced CPP-evoked activation of the reward system

In the VTA, Newman-Keuls *post hoc* test revealed that there was a significant increase ($P < 0.001$) in c-Fos expression in vehicle-treated morphine-paired mice as compared to vehicle-treated saline-paired animals. We also observed that the CRF_1-R antagonist significantly ($P < 0.001$) blocked the activation of the VTA in morphine-paired animals (Figure 21F).

As depicts in Figure 21L, vehicle-injected morphine-paired mice displayed a significant ($P < 0.01$) augment in c-Fos expression in the NAc compared with vehicle-injected saline-paired animals. Additionally, CP-154,526 pretreated morphine-conditioned animals showed a significant ($P < 0.001$) decrease in c-Fos expression as compared to morphine-conditioned mice pretreated with vehicle and to CP-154,526 pretreated saline-paired animals.

2.4. CRF_1-R antagonism had no effect in the morphine-induced CPP-evoked increase in DA turnover in the NAc

In agreement with our previous studies (Figure 13), present results showed that morphine-induced CPP evoked a significant ($P < 0.05$) increase in DA turnover in the NAc of vehicle-treated mice compared with the vehicle-injected saline-paired animals. Additionally, DOPAC/DA ratio in saline-paired mice was significantly ($P < 0.01$) elevated over control levels in animals receiving CP-154,526 (Figure 21M).

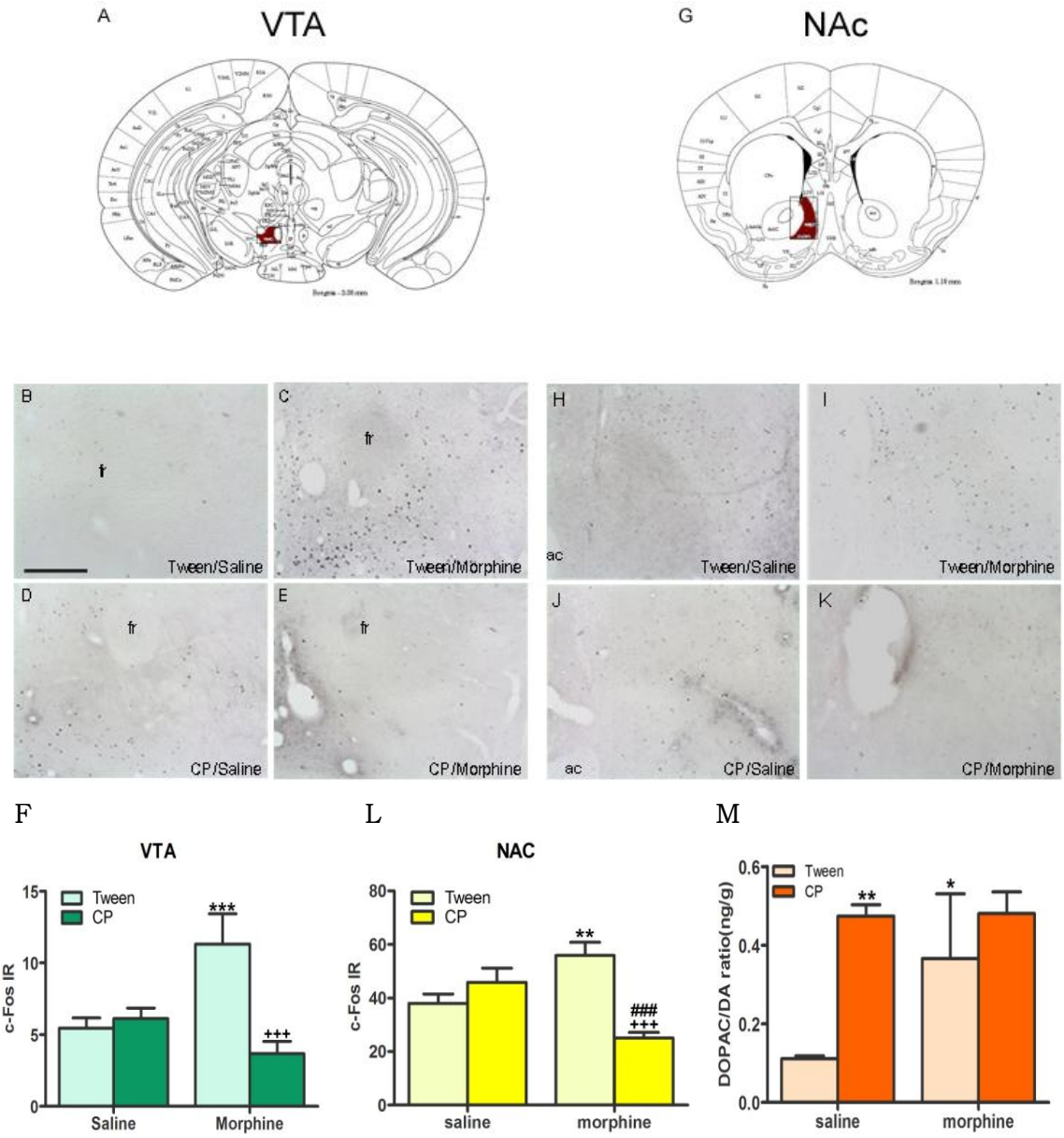

Figure 21. CRF_1-R antagonist administration blocked the enhancement in c-Fos expression in the VTA and NAc, but had no effect in the increased dopaminergic neurotransmission in the NAc evoked by morphine-induced CPP. (A, G) Schematic anatomic representation of the VTA and NAc adapted from Franklin and Paxinos stereotaxic atlas (2008). (B-E, H-K) Representative photographs illustrating c-Fos expression in the VTA (B-E) and NAc (H-K) of animals treated with tween-80 + saline (B, H), CP-154,526 + saline (C, I), tween-80 + morphine (D, J) and CP-154,526 (E, K). ac, anterior comissure. Scale bar, 100 µm. (F, L) Quantification of c-Fos-IR in the VTA (F) and NAc shell (L). (M) Evaluation of DA turnover in the NAc. Bars represent mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001 versus tween-80 + saline; ++P < 0.01, +++P < 0.001 versus tween-80 + morphine; ###P < 0.001 versus CP-154,526 + saline.

2.5. Effect of CRF_1-R antagonism in the activation of noradrenergic nuclei innervating the brain reward system during morphine-induced CPP

Figure 22F showed that there was a significant ($P < 0.001$) augment in c-Fos expression in the LC of morphine-paired mice compared with control animals, both pretreated with vehicle. In contrast, CP-154,526 administration significantly ($P < 0.001$) blocked the morphine-induced CPP-evoked increased in c-Fos expression in the LC. For the NTS, morphine-paired animals receiving vehicle or CP-154,526 displayed a significant ($P < 0.001$) higher c-Fos expression compared with those saline-paired mice, as revealed in figure 22L.

2.6. CRF_1-R antagonism blocked the morphine-induced CPP-evoked increase in MHPG/NA ratio in the NAc

As figure 22M depicts, there was a significant ($P < 0.001$) increase in NA turnover in vehicle-injected morphine-paired mice compared to the vehicle-injected control group. In addition, CP-154,526 pretreatment significantly ($P < 0.01$) antagonised the morphine-induced CPP-evoked increase in MHPG/NA ratio.

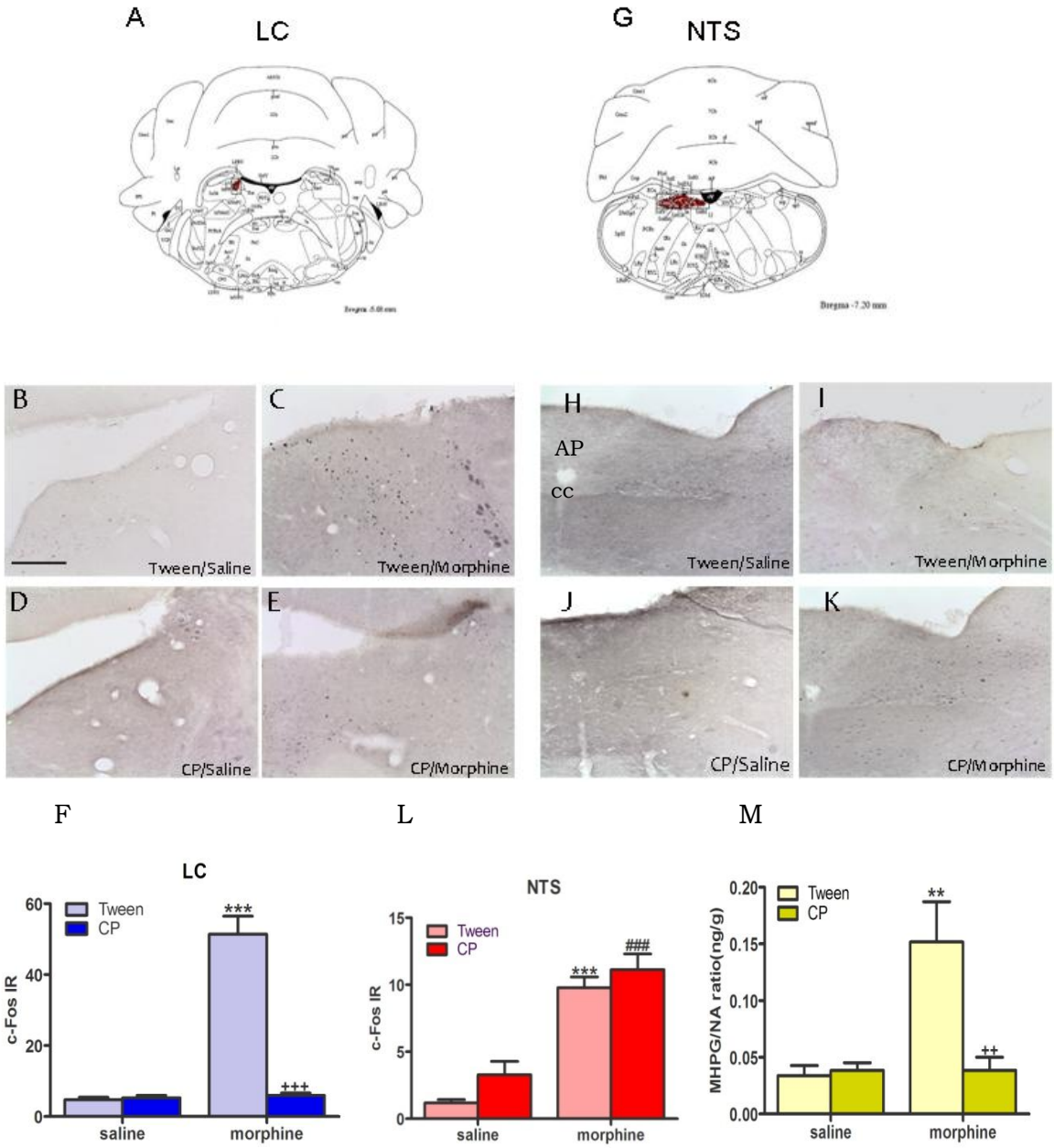

Figure 22. CRF_1-R antagonist administration blocked the enhancement in c-Fos expression in the LC and the increased noradrenergic neurotransmission in the NAc evoked by morphine-induced CPP. (A, G) Schematic anatomic representation of the LC and NTS adapted from (187). (B-E, H-K) Representative photographs illustrating c-Fos expression in the LC (B-E) and NTS (H-K) of animals treated with tween-80 + saline (B, H), CP-154,526 + saline (C, I), tween-80 + morphine (D, J) and CP-154,526 (E, K). AP, area postrema ; cc, canal central. Scale bar, 100 µm. (F, L) Quantification of c-Fos-IR in LC and NTS. (M) Evaluation of NA turnover in the NAc. Bars represent mean ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$ versus tween-80 + saline; $^{++}P < 0.01$ versus tween-80 + morphine; $^{###}P < 0.001$ versus CP-154,526 + saline.

2.7. Effect of CRF_1-R antagonism in c-Fos expression in the LH during morphine-induced CPP

CRF_1-R antagonist administration significantly ($P < 0.05$) diminishes c-Fos expression in the DMH of morphine-paired animals (Figure 23F). In the PFA, CP-154,526 injection significantly ($P < 0.001$) decreases c-Fos-IR in both saline and morphine receiving-mice (Figure 23G). Additionally, *post hoc* test showed that morphine-induced CPP significantly ($P < 0.001$) increases c-Fos expression in the LLH and that this enhancement was antagonised ($P < 0.001$) by CP-154,526 administration (Figure 23H).

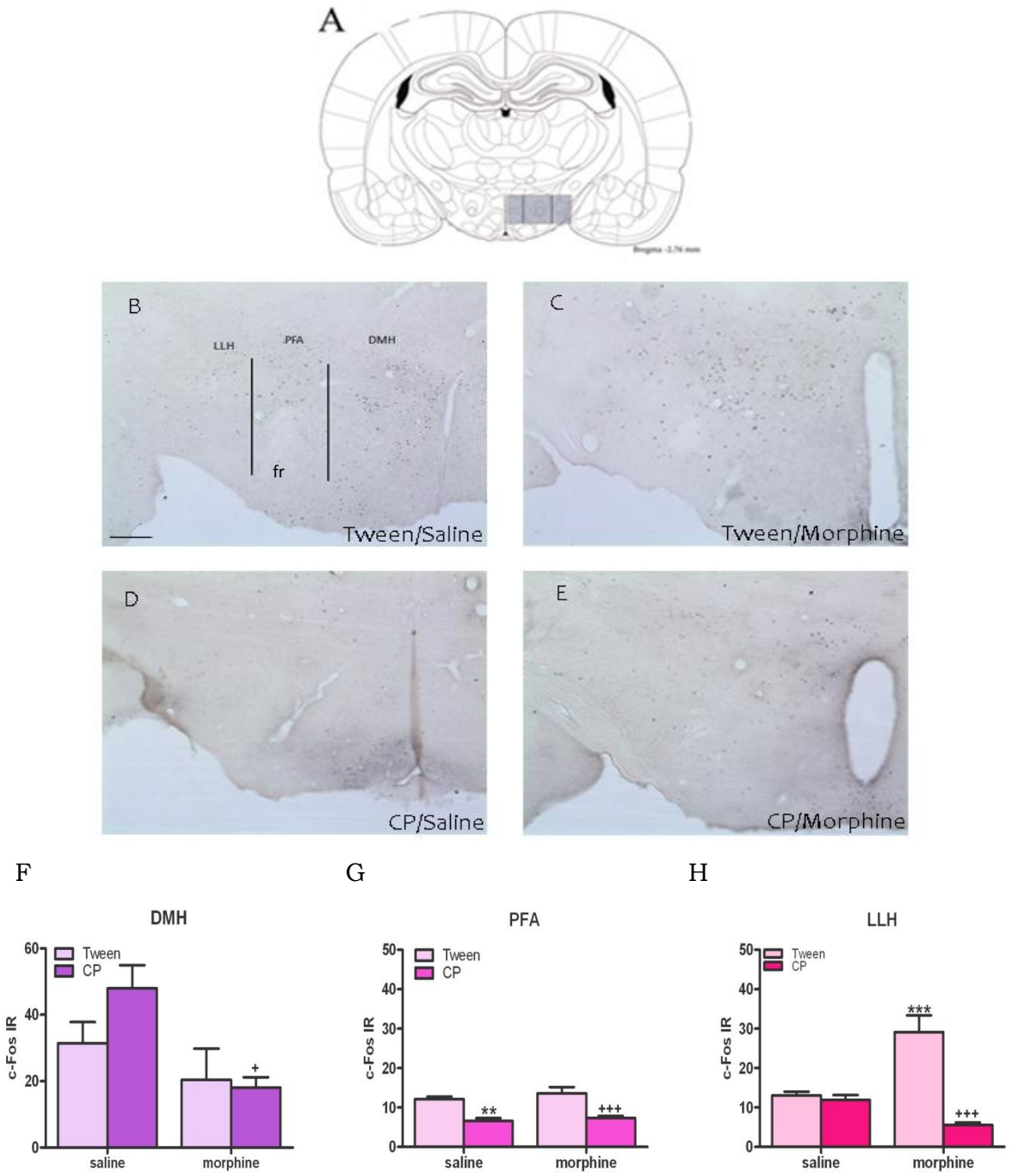

Figure 23. Morphine-induced CPP activates exclusively the lateral part of the LH. This activation was prevented by CP-154,526 administration. (A) Schematic anatomic representation of LH subdivisions adapted from (187). (B-E) Representative photographs illustrating regional c-Fos expression in the LH of animals treated with tween-80 + saline (B), tween-80 + morphine (C), CP-154,526 + saline (D) and CP-154,526 + morphine (E). fr, fornix. Scale bar, 100 µm. (F-H) Mean ± SEM in the three regions of the LH. $^{**}P < 0.01$, $^{***}P < 0.001$ versus tween-80 + saline; $^{+}P < 0.05$, $^{+++}P < 0.001$ versus tween-80 + morphine.

2.8. Morphine-induced CPP and CRF_1-R antagonism do not modify the number of Ox-A neurons in the LH

As showed in Table 1, administration of CP-154,526 significantly augmented the number of Ox-A positive neurons in the PFA of saline-paired animals.

Table 1. Effects of CRF_1-R antagonism on the number of Ox-A positive neurons in the three subdivisions of the LH during morphine-induced CPP.

Treatment	DMH	PFA	LLH
Tween + saline	12,85 ± 1,727	15,50 ± 1,354	14,68 ± 1,466
Tween + morphine	16,66 ± 2,005	18,71 ± 1,308*	19,31 ± 1,637
CP154,526 + saline	22,88 ± 4,286	22,31 ± 2,289	19,32 ± 1,325
CP154,526 + morphine	22,76 ± 2,584	23,30 ± 1,962	22,74 ± 1,232

Each value represents the mean ± SEM. n = 4-5 per group. Newman–Keuls' *post hoc* comparison test revealed a significant increase in the number of Ox-A positive neurons in the PFA of saline-paired animals pretreated with CP154,526 (*$P < 0.05$ vs tween + saline).

2.9. Effect of CRF_1-R antagonism in the activation of orexinergic neurons in the LH during morphine-induced CPP

There was an activation (as showed by c-Fos expression) of the orexinergic neurons only in the LLH of morphine-paired mice compared with saline-paired animals receiving vehicle ($P < 0.05$), that was antagonised ($P < 0.001$) by CP-154,526 (Figure 24H). CRF_1-R administration significantly ($P < 0.001$) decreased as well the number of c-Fos-positive and Ox-A positive neurons in the LLH of saline-treated mice compared with animals receiving vehicle. In addition, administration of CP-154,526 significantly decreased the number of c-Fos/Ox-A double labelled neurons in the PFA of both controls ($P < 0.001$) and morphine-paired ($P < 0.05$) animals.

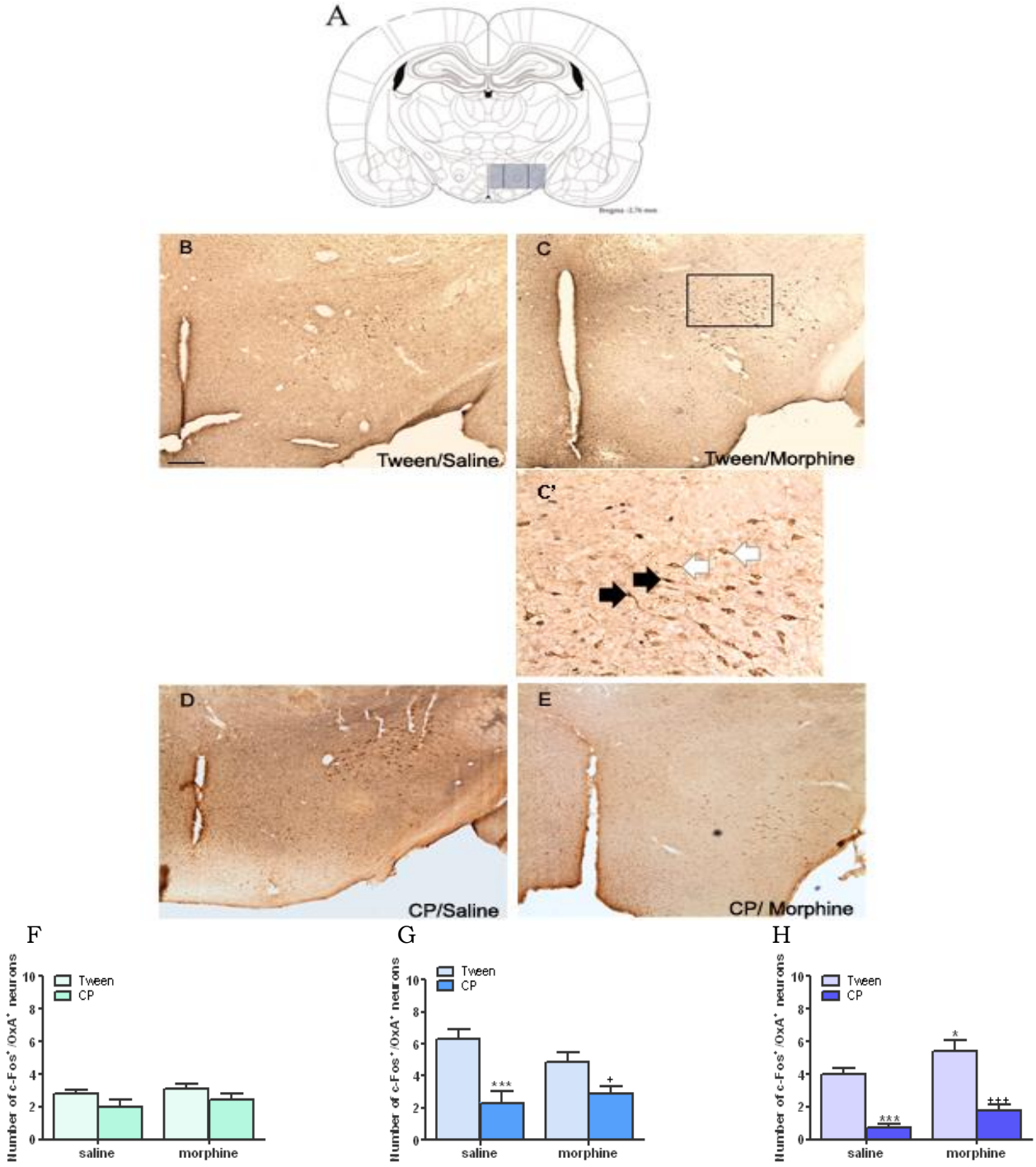

Figure 24. Morphine-induced CPP activates the orexinergic neurons from the lateral part of the LH. This activation was prevented by CP-154,526 administration. (A) Schematic anatomic representation of LH subdivisions adapted from (187). (B-E) Representative photographs showing double-label immunohistochemistry for c-Fos and OXA in the LH in animals treated with tween-80 + saline (B), CP-154,526 + saline (C), tween-80 + morphine (D) and CP-154,526 (E).

2.10. CRF_1-R blockade does not antagonise morphine-induced CPP-evoked HPA axis activation

As shown in figure 24, plasma corticosterone levels increased significantly ($P < 0.01$) in vehicle pretreated morphine-paired mice. Corticosterone levels in CP-154,526-injected morphine-treated animals were significantly ($P < 0.01$) higher than those observed in the control group also administered CP-154,526. In addition, morphine-paired mice receiving CP-154,526 showed significant ($P < 0.001$) higher plasma corticosterone concentrations compared with those receiving vehicle instead of CP-154,526.

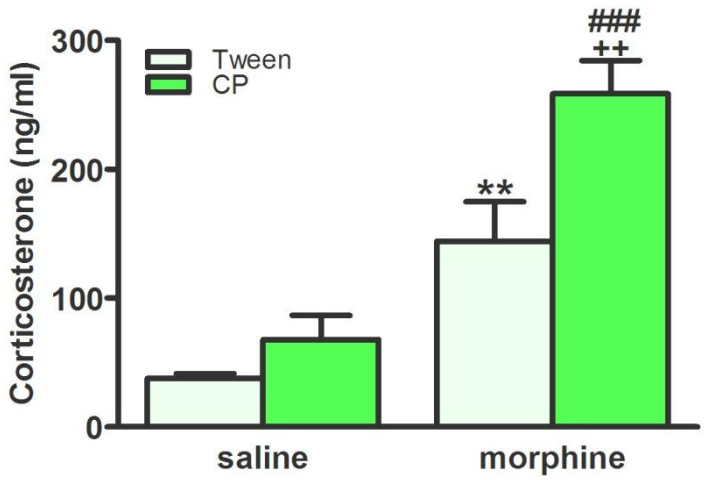

Figure 25. CP-154,526 augments the corticosterone secretion response to morphine induced CPP. Data represent the mean ± SEM of corticosterone concentration. $^{**}P < 0.01$ versus tween-80 + saline; $^{++}P < 0.01$ tween-80 + morphine; $^{###}P < 0,001$ versus CP-154,526 + saline.

3. HYPOTHALAMIC OREXIN A NEURONS ARE INVOLVED IN THE RESPONSE OF THE BRAIN STRESS SYSTEM TO MORPHINE WITHDRAWAL

Recent investigations revealed participation of the orexinergic system in brain stress system (194). The present study was designed to evaluate changes in the orexinergic system during naloxone-precipitated morphine withdrawal. We addressed the role of orexinergic inputs in the development of somatic signs associated to the opiate abstinence syndrome, and the brain stress systems responses to morphine withdrawal.

It has been reported that Ox-A activates PVN neurons (195) and that intracerebroventricular (i.c.v.) Ox-A increases plasma levels of glucocorticoids and ACTH (88). However, it remains to be determined whether Ox-A modulates the activity of the extrahypothalamic CRF neurons. Further aims of this work were to confirm the orexin projections to the main nuclei of the brain stress system, such as NAc shell, BNST, PVN, CeA, and NTS; and study the pharmacological blockade effect of the orexinergic system, by OxR1 antagonist (SB334867) administration, on the somatic symptoms produced during morphine withdrawal. In addition, c-Fos expression in the brain stress system and GC release during morphine withdrawal were measured in morphine-dependent SB334867-treated rats.

3.1. OxR1 antagonist SB334867 attenuates somatic expression of naloxone-precipitated morphine withdrawal

Six days after the implantation of morphine or placebo pellets, rats were challenged with naloxone (1 mg/kg s.c.) and immediately tested for the occurrence of somatic signs of opiate withdrawal. The following somatic signs were significantly present in morphine-treated groups when compared with placebo treated groups: wet dog shakes, sniffing, writhing, body tremor, ptosis, diarrhea, piloerection, teeth chattering, paw tremor, mastication, and body weight loss. The analysis of the global withdrawal score confirmed these differences between morphine- and placebo-treated rats. Results are shown in Table 2. When OxR1 receptors were blocked, comparisons between morphine (n= 9) groups

showed that wet dog shakes, sniffing, writhing, body tremor, ptosis, diarrhea, piloerection, and mastication were significantly decreased in morphine-dependent rats receiving SB334867 before naloxone (Table 2, Figure 26A–K). The analysis of the global withdrawal score confirmed that SB334867 significantly reduced somatic expression of withdrawal in morphine-treated rats (Table 2, Figure 26L). Thus, the blockade of OxR1 overall decreased the expression of naloxone precipitated somatic signs of opiate withdrawal, reducing global scores of morphine-dependent SB334867-treated rats. No changes were seen in the placebo-treated group receiving SB334867 (n= 6) and the control group injected with vehicle.

	Two-Way ANOVA					
	Chronic	treatment	Pretreatment		Interaction	
Signs	$F_{1,27}$	p<	$F_{1,26}$	p<	$F_{1,26}$	p<
Wet Dog Shakes	61.39	0.0001	4.19	ns	4.19	ns
Paw Tremor	10.62	0.0032	1.07	ns	1.07	ns
Sniffing	22.32	0.0001	15.94	0.0005	15.94	0.0005
Writhing	21.35	0.0001	4.20	ns	4.20	ns
Tremor	122.75	0.0001	16.47	0.0004	16.47	0.0004
Ptosis	101.57	0.0001	43.45	0.0001	43.45	0.0001
Mastication	70.96	0.0001	1.70	ns	1.70	ns
Teeth Chatering	16.08	0.0005	1.10	ns	1.10	ns
Piloerection	120.27	0.0001	3.79	ns	3.79	ns
Diarrhea	70.86	0.0001	21.63	0.0001	21.63	0.0001
Weight Loss	28.42	0.0001	0.18	ns	0.18	ns
Global Score	124.27	0.0001	8.66	0.0066	8.66	0.0066

Table 2. SB334867 attenuates the somatic expression of naloxone-precipitated morphine withdrawal. Two-way ANOVA with chronic treatment (morphine vs. placebo) and pretreatment before naloxone (SB334867 vs. vehicle) as between-subjects factors. When significant interactions in pretreatment or between these two factors were observed, a subsequent post hoc test was applied.

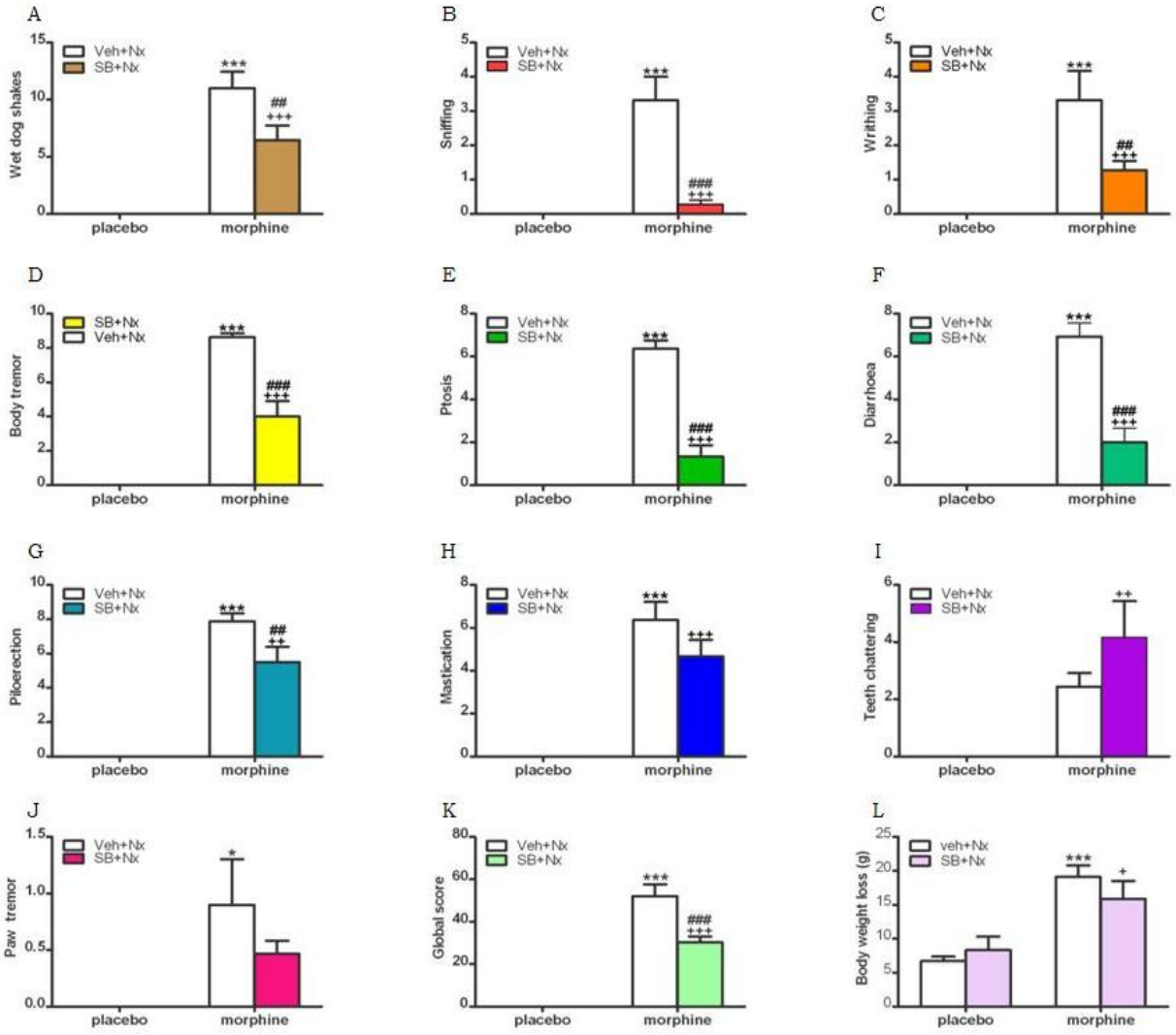

Figure 26. Attenuation of the severity of somatic signs of naloxone-precipitated morphine withdrawal up to 30 min after the naloxone injection by SB-334867 pretreatment. The following variables were counting: (A) wet-dog shakes; (B) sniffing; (C) writhing; (D) body tremor; (E) ptosis; (F) diarrhoea; (G) piloerection; (H) mastication; (I) teeth chattering; (J) paw tremor; (K) body weight loss. Somatic signs of withdrawal were observed during 30 min immediately after naloxone injection (1 mg/kg s.c.) A global withdrawal score (L) was calculated for each animal as described in Methods. Data are expressed as mean 6 SEM. *p<0.05; ***p<0.001, versus placebo + vehicle (veh) + naloxone (nx); ++p<0.01; +++p<0.001 versus similar groups receiving vehicle instead of SB-334867.

3.2. OxR2 antagonist TCSOX229 attenuates somatic expression of naloxone-precipitated morphine withdrawal

Six days after the implantation of morphine or placebo pellets, rats were challenged with naloxone (1 mg/kg s.c.) and immediately tested for the occurrence of somatic signs of opiate withdrawal. The following somatic signs were significantly present in morphine-treated groups (n= 6) when compared with placebo-treated groups (n =6): paw tremor ($p < 0.01$), sniffing ($p < 0.001$), writhing (p,0.001), body tremor ($p < 0.001$), ptosis ($p < 0.001$), diarrhea ($p < 0.001$), mastication ($p < 0.001$), piloerection ($p < 0.001$), teeth chattering ($p < 0.01$), and body weight loss ($p < 0.001$). The analysis of the global withdrawal score confirmed these differences between morphine- and placebo-treated rats ($p < 0.001$). When OxR2 receptors were blocked, comparisons between morphine (n= 7) groups showed that sniffing ($p < 0.001$), writhing ($p < 0.05$), body tremor ($p < 0.001$), ptosis ($p < 0.05$), diarrhea ($p < 0.01$), piloerection ($p < 0.001$), and body weight loss ($p < 0.01$) were significantly decreased in morphine-dependent rats receiving TCSOX229 before naloxone (Table 3, Figure 27A–K). However, wet dog shakes ($p < 0.001$) was significantly increased in morphine-dependent rats receiving TCSOX229. The analysis of the global withdrawal score confirmed that TCSOX229 significantly reduced somatic expression of withdrawal in morphine-treated rats ($p < 0.001$; Table 3, Figure 27L).

Thus, the blockade of OxR2 overall decreased the expression of naloxone-precipitated somatic signs of opiate withdrawal, reducing global scores of morphine-dependent TCSOX229-treated rats. No changes were seen in the placebo-treated group receiving TCSOX229 (n= 7) and the control group injected with vehicle.

	Two-Way ANOVA					
	Chronic	treatment	Pretreatment		Interaction	
Signs	$F_{1,22}$	p<	$F_{1,22}$	p<	$F_{1,22}$	p<
Wet Dog Shakes	10,97	0,0033	4,762	0,0406	4,762	0,0406
Paw Tremor	19,01	0,0003	1,791	0,1958	1,791	0,1958
Sniffing	51,65	0,0001	9,426	0,0058	9,426	0,0058
Writhing	24,57	0,0001	3,515	0,0742	3,515	0,0742
Tremor	196,3	0,0001	112,5	0,0001	112,5	0,0001
Ptosis	990,9	0,0001	2,327	0,1414	2,327	0,1414
Mastication	53,88	0,0001	0,1574	0,6954	0,1574	0,6954
Teeth Chatering	44,17	0,0001	1,191	0,2870	1,191	0,2870
Piloerection	80,08	0,0001	7,582	0,0116	7,582	0,0116
Diarrhea	246,9	0,0001	4,844	0,0385	4,844	0,0385
Weight Loss	210,6	0,0001	1,148	0,2975	13,22	0,0018
Score	347,0	0,0001	11,82	0,0024	11,82	0,0024

Table 3. TCSOX229 attenuates the somatic expression of naloxone-precipitated morphine withdrawal. Two-way ANOVA with chronic treatment (morphine vs. placebo) and pretreatment before naloxone (TCSOX229 vs. vehicle) as between-subjects factors. When significant interactions in pretreatment or between these two factors were observed, a subsequent post hoc test was applied.

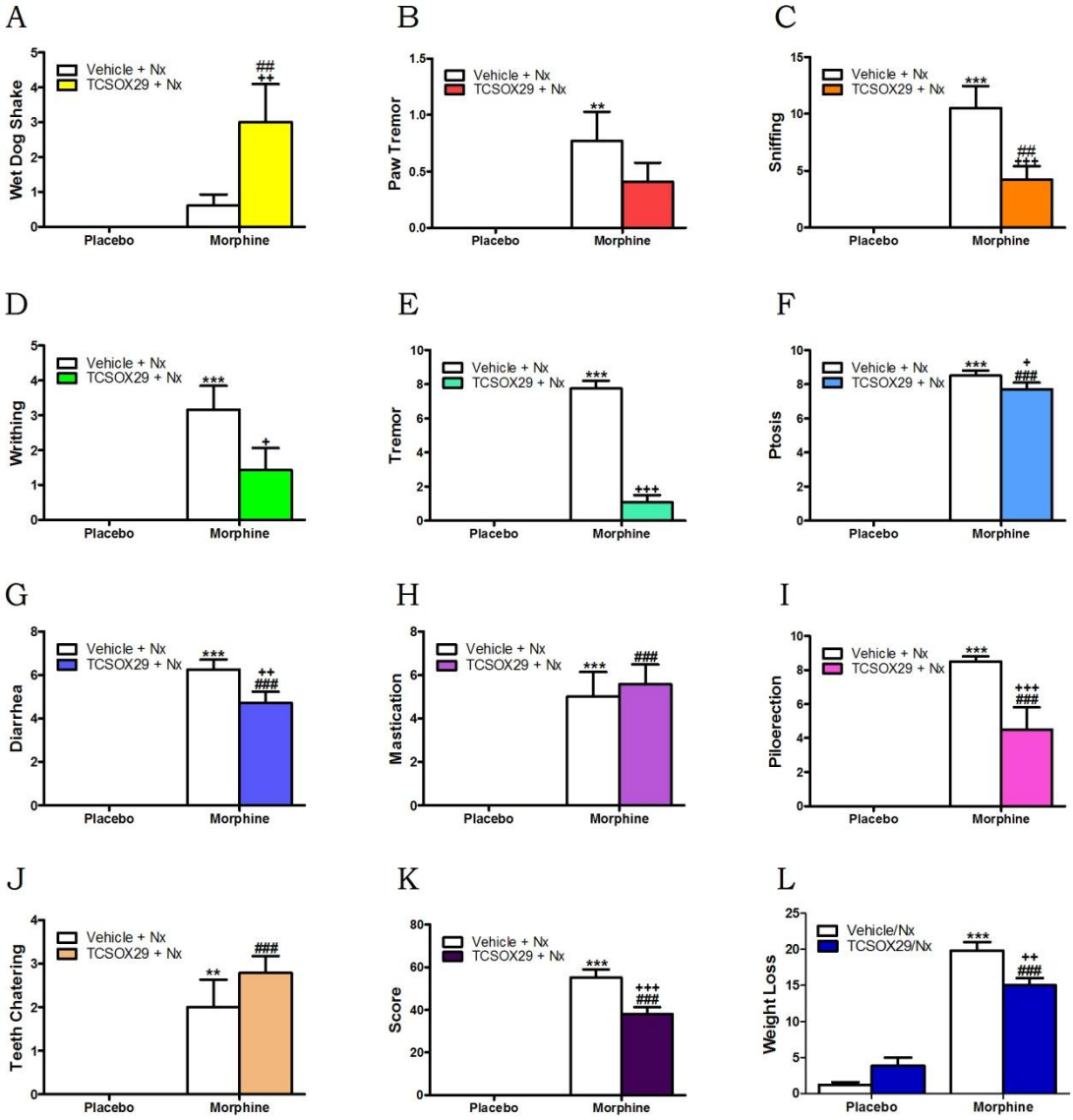

Figure 27. Attenuation of the severity of somatic signs of naloxone-precipitated morphine withdrawal up to 30 min after the naloxone injection by TCSOX229 pretreatment. The following variables were counting: (A) wet-dog shakes; (B) paw tremor; (C) sniffing; (D) writhing; (E) tremor; (F) ptosis; (G) diarrhoea; (H) mastication; (I) piloerection; (J) teeth chattering; (K) body weight loss. Somatic signs of withdrawal were observed during 30 min immediately after naloxone injection (1 mg/kg s.c.) A global withdrawal score (L) was calculated for each animal as described in Methods. Data are expressed as mean 6 SEM. *p<0.05; ***p<0.001, versus placebo + vehicle (veh) + naloxone (nx); ++p<0.01; +++p<0.001 versus similar groups receiving vehicle instead of TCSOX229

3.3. Naloxone-precipitated morphine withdrawal induces orexin gene expression

Next, we assessed if activation of orexinergic neurons in response to chronic morphine treatment and precipitated withdrawal results in induction of orexin gene expression. Time course of changes in OX-A mRNA levels was followed in the hypothalamic samples by quantitative RT-PCR (Figure 28). When compared to placebo-implanted controls no change in mRNA levels was detected in response to chronic morphine or to naloxone in morphine-naïve animals.

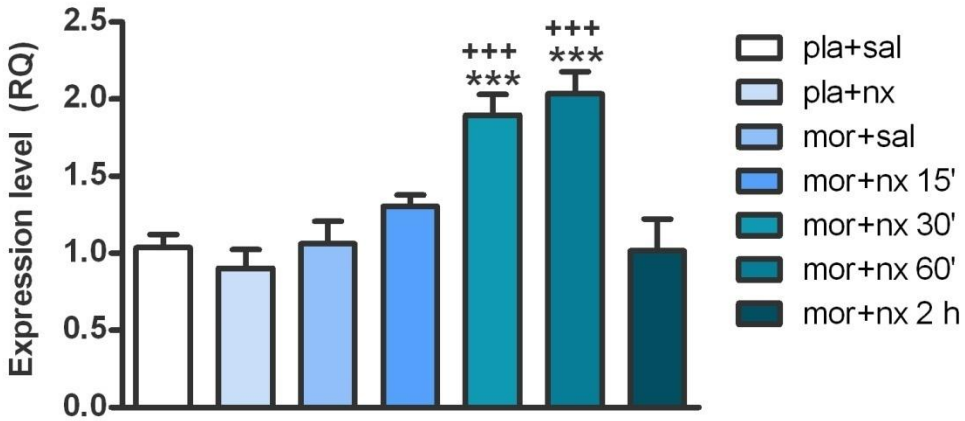

Figure 28. Morphine withdrawal induced Ox-A gene expression in the hypothalamus. Rats were sacrificed at different time-points (15, 30, 60 and 120 min) after saline or naloxone injection. Mean 6 SEM RQ (relative quantification of the comparative CT experiments) expression values were obtained by real-time q-PCR measurement, where the expression of Ox-A gene in morphine withdrawn rats is expressed relative to the placebo implanted controls receiving naloxone. ***p<0.001 versus placebo + naloxone; +++p <0.001 versus morphine + saline.

Neuman Keuls' post hoc test showed that at 30 and 60 min after naloxone injection there was a significant increase mRNA expression compared with placebo groups receiving naloxone (p,0.001; p,0.001, respectively) and with morphine-dependent animals receiving saline instead naloxone (p,0.001; p,0.001, respectively).

3.4. Chronic morphine treatment and naloxone-precipitated morphine withdrawal do not modify the number of Ox-A neurons in the LH

To evaluate if chronic morphine and naloxone-precipitated morphine withdrawal differentially affects the number of Ox-A neurons in the LH, the cell counts of Ox-A immunoreactive profiles were taken in three sub-population of the LH: DMH, PFA and LLH. The two-way ANOVA performed on the number of Ox-A-positive neurons in placebo and chronic morphine treated rats, with or without naloxone injection, indicated that as depicts in figure 29, chronic morphine or naloxone injection did not significantly alter the number of Ox-A neurons in the LH.

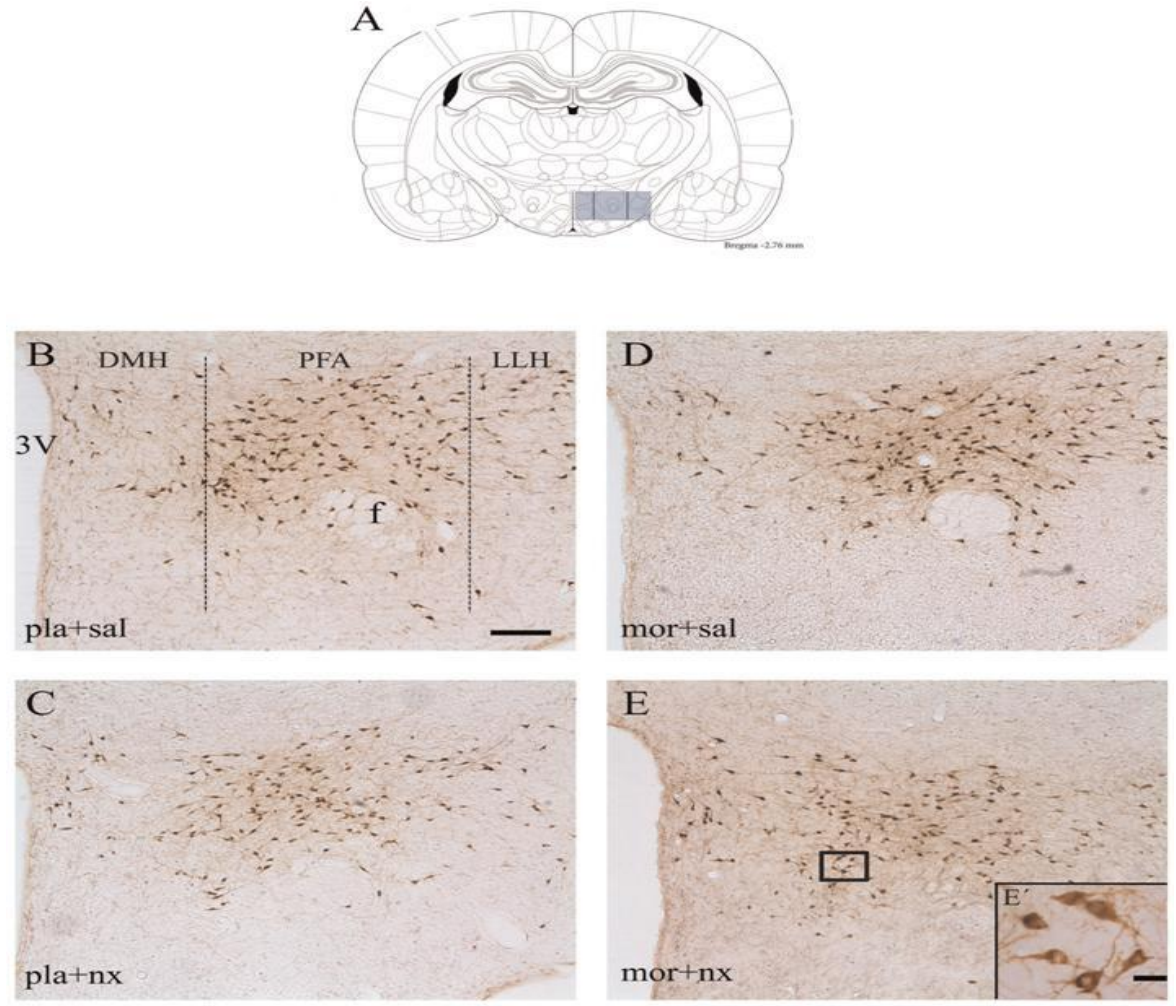

Figure 29. Chronic morphine and morphine withdrawal do not modify the number of Ox-A neurons in the DMH, PFA and LLH regions of the lateral hypothalamus. Placebo and morphine-dependent groups were sacrificed 120 min after saline or naloxone administration. (A) Schematic anatomic representation of LH subdivisions adapted from (193). (B–E, E9) Representative photographs illustrating regional orexin A cell expression in the LH. (F) Mean 6 SEM in the three regions of the LH. 3V, third ventricle. Scale bars, 200 mm (B–E) and 20 mm (E9).

3.5. Naloxone-precipitated morphine withdrawal activates Ox-A neurons

To reveal if Ox-A neurons are activated during naloxone-precipitated morphine withdrawal, c-Fos and Ox-A immunostaining was co-localized and quantified in sections of the lateral hypothalamic area (Figure 30A–D).

Post hoc analysis revealed that morphine withdrawal increased ($p < 0.001$) the number of Ox-A-containing neurons expressing c-Fos in all three subpopulation of the LH compared with placebo controls receiving naloxone and morphine-treated rats injected with saline instead of naloxone (Figure 30E). Administration of the opioid antagonist to control rats did not induce any modification, compared with control groups receiving saline.

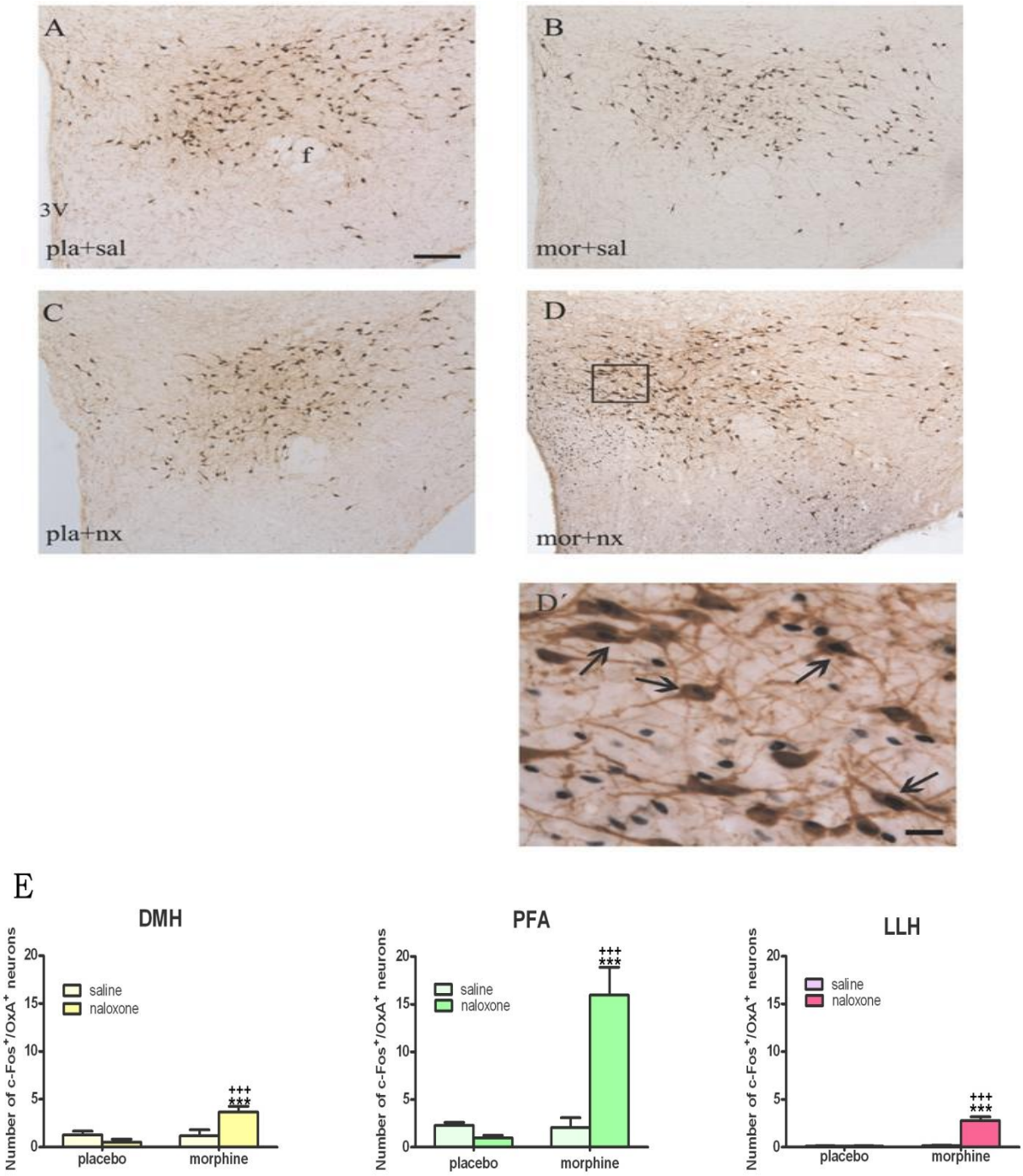

Figure 30. Naloxone-induced morphine withdrawal results in activation of orexin A cells in the DMH, PFA and in the LLH. (A–D) Representative photographs showing double-label immunohistochemistry for c-Fos and Ox-A in the LH. (D´) High magnification image showing c-Fos-positive (black)/Ox-A-positive (brown) neurons. Arrows indicate c-Fos in Ox-A-positive neurons. (E) Regional expression of c-Fos in Ox-A positive cells. Bars represent the mean 6 SEM. +++p<0.001 versus morphine + saline; ***p<0.001 versus placebo + naloxone.

3.6. OxR1 antagonism attenuates naloxone-precipitated morphine withdrawal-evoked activation of extrahypothalamic and hypothalamic brain stress systems

We assessed the influence of the OxR1 antagonist SB334867 on c-Fos expression in the brain stress system (NAc shell, BNST and CeA; Figure 31) as well as on the PVN and NTS noradrenergic cell group (Figure 32).

Post hoc analysis (Figure 31J) revealed that morphine withdrawal increased ($P<0.001$) c-Fos expression in the extended amygdala: NAc shell; (Figure 31B), BNST (Figure 31E) and CeA (Figure 31H). We observed similar significant increase in c-Fos expression in the PVN ($P<0.001$) and NTS ($P<0.01$). We also observed that the main effect of SB334867 administration was to decrease c-Fos expression. This effect was significant across extended amygdala areas (Figure 31C, F, I, J; NAc, BNST,) and the PVN (Figure 32B, C).

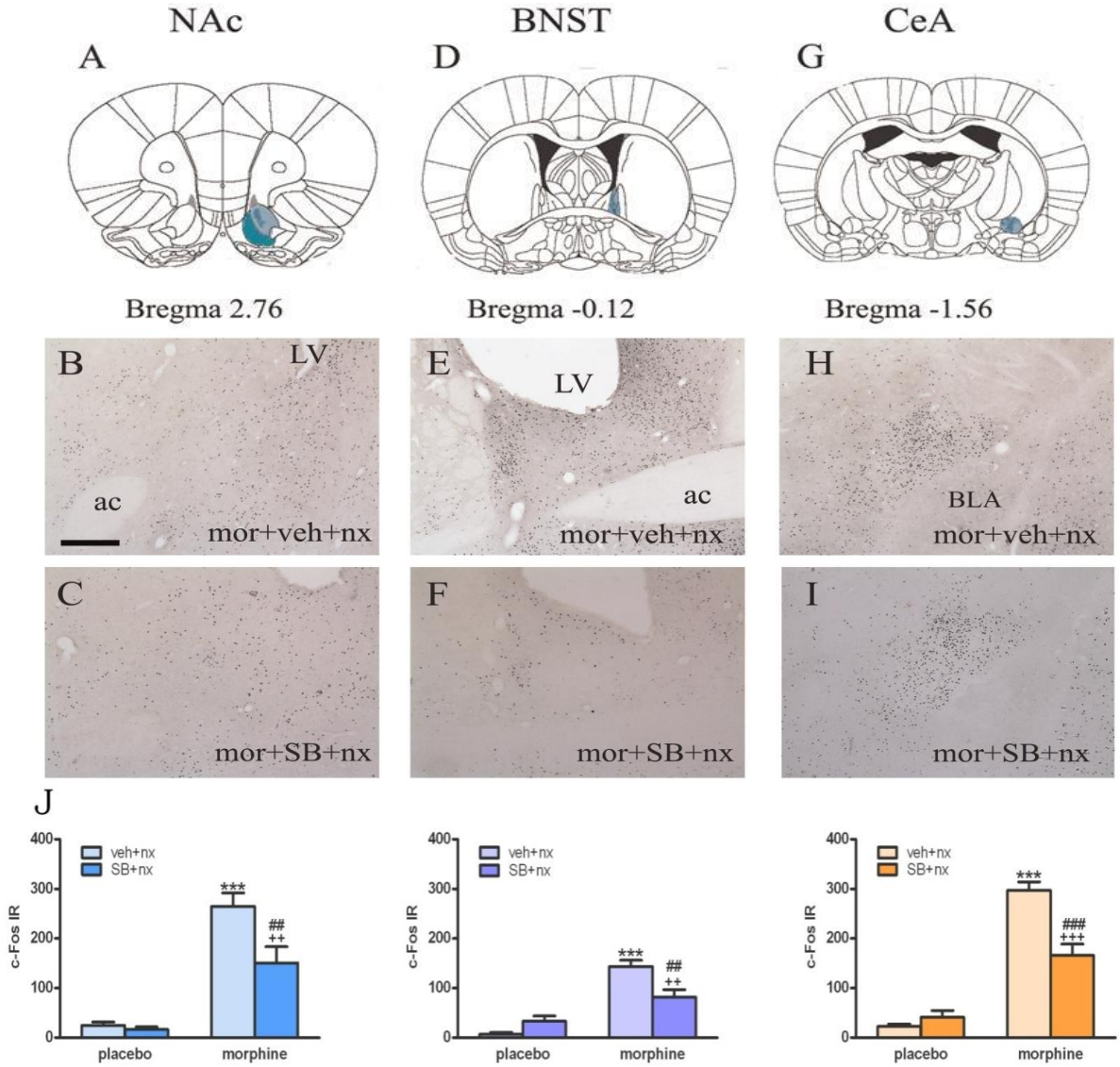

Figure 31. Naloxone-induced morphine withdrawal results in activation of extended amygdala. SB-334867 attenuated c-Fos expression in the NAc, BNST and CeA in morphine-withdrawn rats. Rats were pretreated with the selective OxR1 antagonist SB334867 20 min prior naloxone injection and were sacrificed 120 min after naloxone administration. (A, D, G) Schematic anatomic representation of NAc shell, BNST and CeA adapted from (193). Labeled areas delineate regions where c-Fos expression was examined. (B-I) Representative photographs of c-Fos expression in the NAc, BNST (oval) and CeA in animals pretreated with morphine-vehicle-naloxone (B, E, H) or with morphine-SB-334867-naloxone (C, F, I). LV, lateral ventricle; BLA, basolateral amygdala. Scale bar, 200 mm. (J) Quantification of neurons expressing c-Fos in the NAc shell, BNST (oval) and CeA. Bars represent mean 6 SEM. ***p,0.001 versus placebo + vehicle (veh) + naloxone (nx); ++p<0.01; +++p<0.001 versus placebo + SB-334867+ naloxone; ##p<0.01, ###p<0.001 versus morphine + vehicle + naloxone.

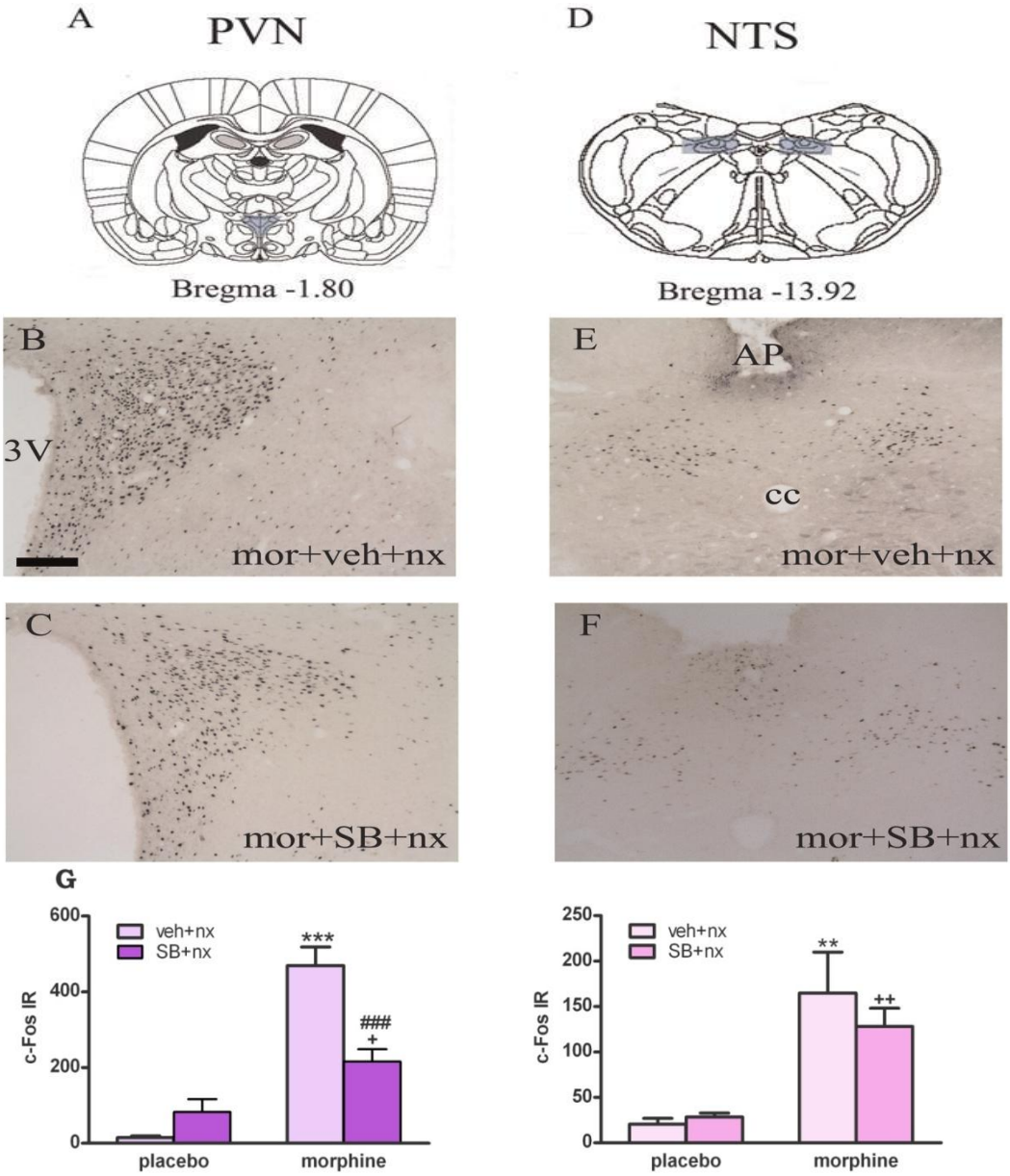

Figure 32. SB334867 attenuated naloxone-induced morphine withdrawal induction of c-Fos expression in the PVN but not in the NTS. (A, D) Schematic anatomic representation of the PVN and NTS adapted from (193). Labeled areas delineate regions where c-Fos expression was examined. (B–F) Representative photographs of c-Fos expression in the PVN and NTS in animals pretreated with morphine-vehicle-naloxone (B, E) or with morphine-SB334867-naloxone (C, F). Animals were sacrificed 120 min after naloxone administration. 3V, third ventricle; AP, area postrema. Scale bar: 100 mm. (G) Quantification of neurons expressing c-Fos in the PVN (parvocellular subdivision) and in the NTS catecholaminergic cell group. Bars represent mean 6 SEM. **p<0.01 ***p<0.001 versus placebo + vehicle + naloxone; +p<0.05; ++p<0.01 versus placebo + SB334867+ naloxone; ###p<0.001 versus morphine + vehicle + naloxone.

3.7. Ox-A fibers project to the extended amygdala, PVN and NTS

Whereas OxA cell bodies are restricted to the LH, Ox-A nerve fibers project widely into the extended amygdala (NAc shell, BNST and CeA), PVN, and NTS, areas that are critically involved in addiction and brain stress system (Figure 33). A double-label immunohistochemical staining was carried out to investigate the overlap between CRF, TH- and pro-dyn-immunopositive cells and Ox-A containing fibers. Figure 33 shows close opposition of Ox-A-immunoreactive fibers and CRF-, TH and pro-dyn immunoreactive pericarya. Distributed throughout the NTS catecholaminergic cell group, and intermingled with TH-containing cells numerous Ox-A-immunoreactive axons were found (Figure 33A). In shell region of the NAc Ox-A containing varicosities were observed in close apposition with pro-dyn containing neurons (Figure 33C). At more caudal level, in the BNST, thin Ox-A-containing fibers have been revealed in overlap with CRF-containing neurons (Figure 33B). Ox-A-immunoreactive axons juxtaposed to CRF-containing cells were present in the CeA (Figure 33E). Ox-A-immunoreactive axons densely innervated the hypothalamic PVN and were found in close apposition to CRF neurons in the medial parvocellular subdivision. (Figure 32D).

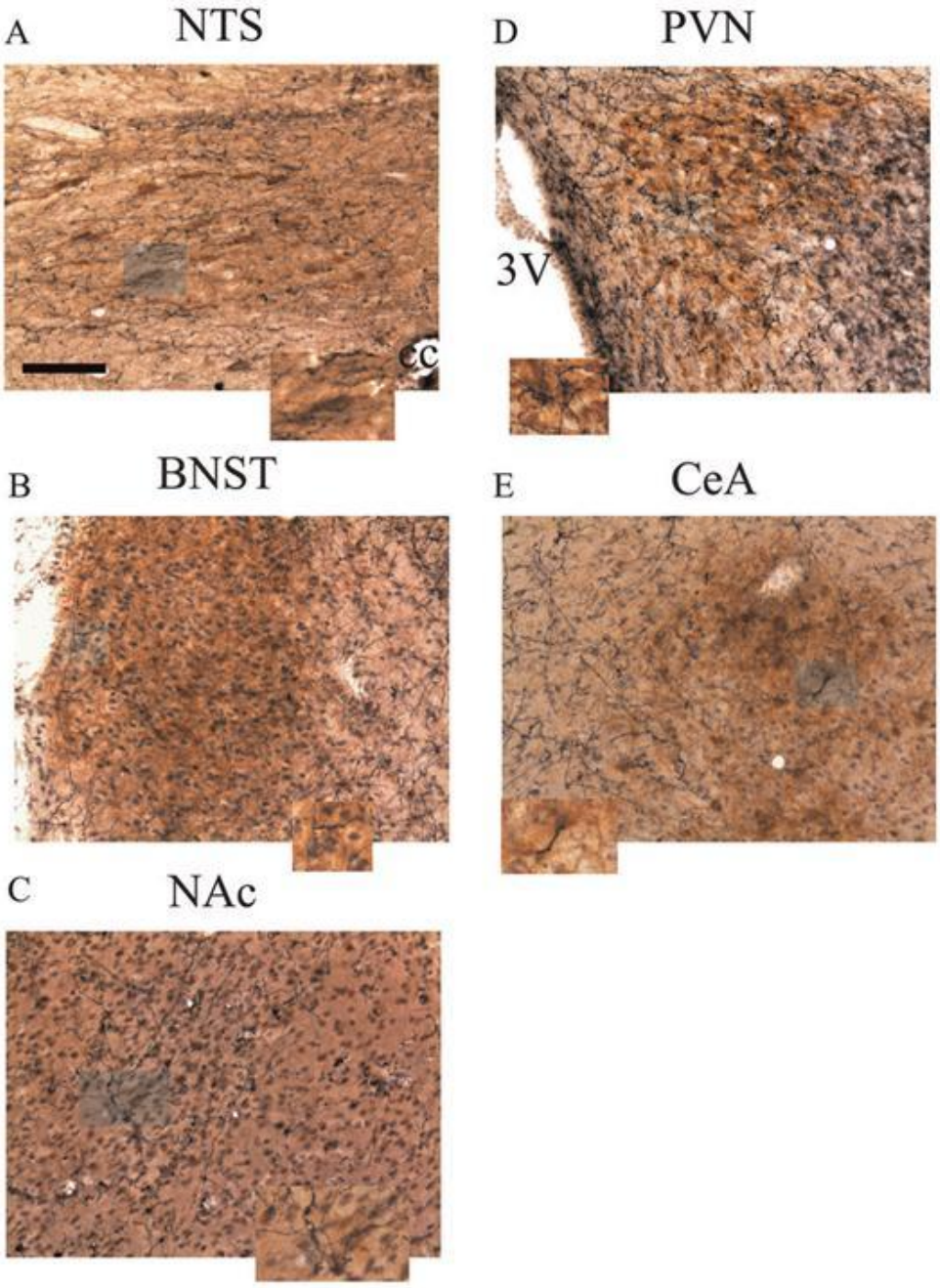

Figure 33. Immunohistochemical localization of orexin A nerve fibers in the NTS, BNST, NAc, PVN and CeA in placebo implanted control rats. Representative photographs showing double-label immunohistochemistry for TH and Ox-A (NTS, A), CRF and Ox-A (BNST, B; PVN, D; and CeA, E) and pro-dyn and Ox-A (NAc, C). Scale bar, 50 mm. Images depict the orexin A innervations (blue-black) of the CRF, pro-DYN and TH neurons (brown) in the extended amygdala, NTS and PVN. Higher magnification images of the boxes areas depicted in each photomicrograph show examples of the areas where Ox-A axons are opposed to the soma of CRF, TH and pro-dyn cells.

3.8. OxR1 antagonism decreases CeA CRF activity during naloxone-precipitated morphine withdrawal

We also tested the influence of the OxR1 antagonist SB334867 on activation of CRF neurons (as assessed by double immunostaining with anti-c-Fos and anti-CRF antibodies) in the PVN, BNST, and CeA (Figure 34).

Post hoc analysis revealed that morphine withdrawal increased ($P<0.001$) the number of CRF-containing neurons expressing c-Fos in the PVN (n= 8), which indicates an activation of CRF neurons, compared with placebo controls receiving naloxone. In these neurons, there was no effect of SB334867 administration on c-Fos expression (Figure 34 A–D, I). In the CeA (Figure 34 G, H, I), there was a significant increase in c-Fos expression in CRF-positive neurons ($P<0.01$). The SB334867 pretreatment significantly attenuated ($P<0.05$) the increase in c-Fos expression in CRF CeA neurons, which indicates that there is a greater Ox-A input to CeA CRF neurons during morphine withdrawal.

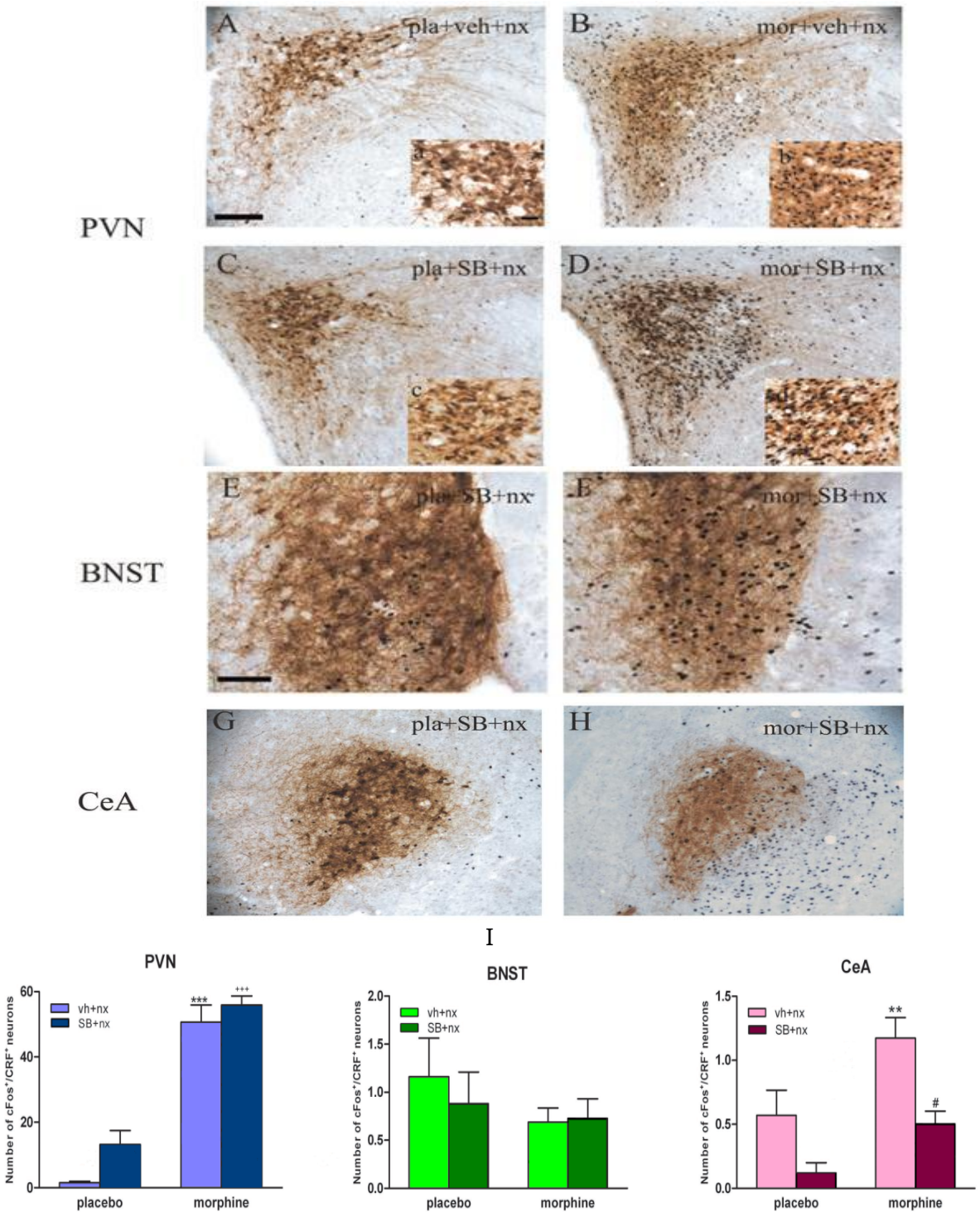

Figure 34. OxR1 antagonist SB334867 attenuates activation of CRF-containing neurons in the CeA but not in either the PVN or the BNST. Microscopy showing double-label immunohistochemistry for c-Fos and CRF in the PVN (A–D), BNST (E, F) and CeA (G, H). (a–d): higher magnification from (A–D). Scale bars, 100 mm (A–H); 50 mm (a–d). (I): mean 6 SEM. **p<0.01, ***p<0.001 versus placebo + vehicle + naloxone; +++p<0.001 versus placebo + SB334867 + naloxone; #p<0.05 versus morphine + vehicle + naloxone.

3.9. OxR1 blockade does not antagonize naloxone-precipitated morphine withdrawal-induced HPA axis activation

We measured plasma corticosterone concentrations (as HPA axis activation marker) in blood samples obtained from morphine dependent or control rats 2 h after injection of naloxone.

As shown in Figure 35, plasma corticosterone levels increased significantly ($P<0.001$) in vehicle pretreated, morphine withdrawn rats. To evaluate if there is a link between OxR1 activation and HPA axis hyperactivity during morphine withdrawal, plasma corticosterone concentrations were measured in animals made dependent on morphine and pretreated with SB334867 before naloxone administration. Corticosterone levels in SB334867 plus naloxone-treated morphine-pelleted animals were significantly ($P<0.01$) higher than those observed in the placebo group also administered SB334867 plus saline. No significant differences were seen between morphine-dependent rats receiving vehicle before naloxone and those receiving SB334867. In addition, placebo-pelleted rats receiving SB334867 showed significant ($P<0.05$) higher plasma corticosterone concentrations compared with its control receiving vehicle instead of SB334867 (n =6).

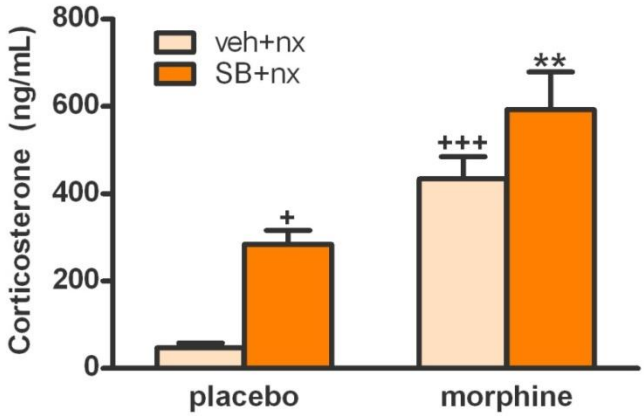

Figure 35. Effects of OxR1 antagonism on the morphine withdrawal-induced increase in plasma corticosterone levels. SB334867 did not attenuate the plasma corticosterone response to naloxone-induced morphine withdrawal. Data represent the mean 6 SEM of corticosterone concentration 120 min after naloxone (nx) injection to placebo- or morphine-treated rats receiving vehicle (veh) or SB334867 (SB) 20 min before naloxone administration. Mean 6 SEM. +$p<0.05$, +++$p<0.001$ versus placebo + vehicle + naloxone **$p<0.01$ versus morphine + vehicle + naloxone.

3.10. Ox2R antagonism increases CRF activity in the PVN during naloxone-precipitated morphine withdrawal

We also tested the influence of the OxR2 antagonist TCSOX229 on the activation of CRF neurons (as assessed by double immunostaining with anti-c-Fos and anti-CRF antibodies) in the PVN (Figure 36). *Post hoc* analysis revealed that morphine withdrawal increased ($p < 0.001$) the number of CRF-containing neurons expressing c-Fos in the PVN (n= 6), which indicates an activation of CRF neurons, compared with placebo controls receiving naloxone (n= 6). In these neurons, TCSOX229 administration to control animals (n = 7) and to morphine-dependent rats (n = 7) significantly ($p < 0,001$) increased c-Fos expression in the PVN (Figure 36B–G). Additionally, the number of c-Fos-positive/CRF-positive neurons in morphine-withdrawn rats receiving the OxR2 antagonist was significantly ($p < 0,001$) higher than in morphine-withdrawn rats receiving vehicle.

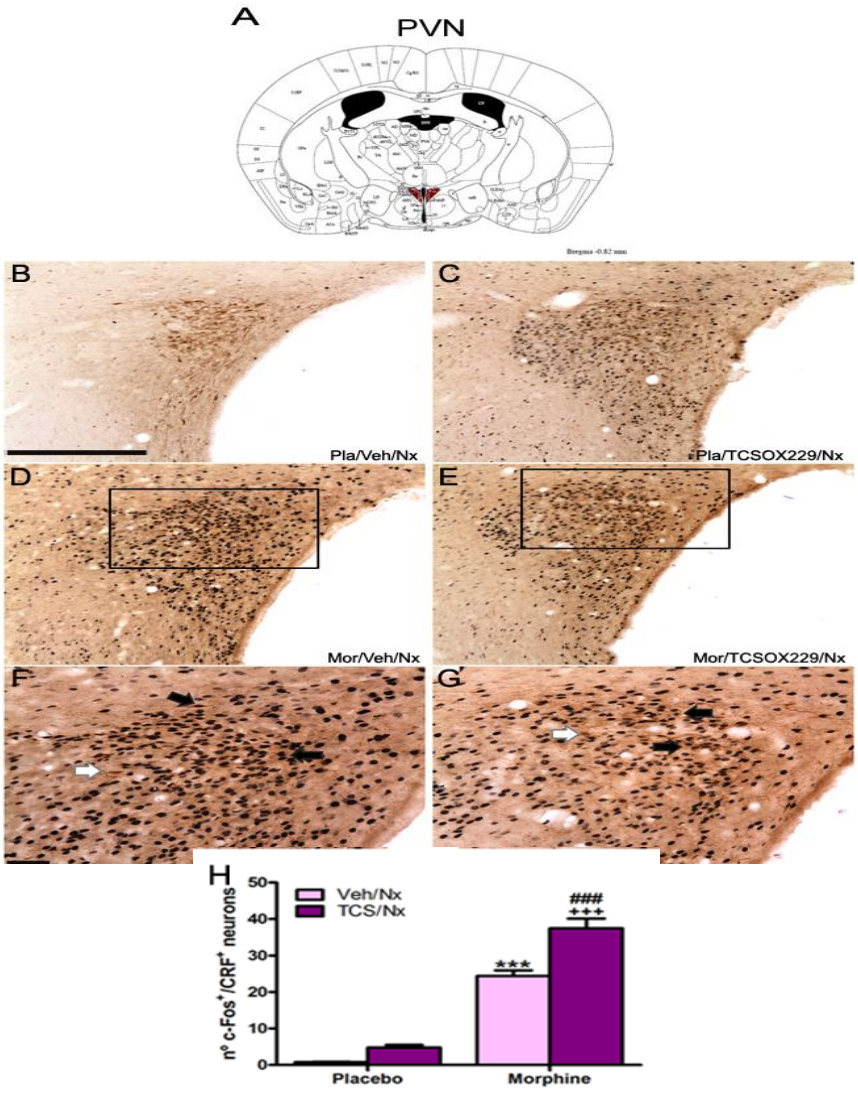

Figure 36. TCSOX229 administration increased the morphine withdrawal-induced activation of the CRF neurons of the PVN. (A) Schematic anatomic representation of the PVN adapted from Paxinos and Watson Rat Brain Atlas(187). (B-E) Representative photographs showing double-label immunohistochemistry for c-Fos and CRF in the PVN of animals treated with placebo + vehicle + naloxone (B), placebo + TCSOX229 + naloxone (C), morphine + vehicle + naloxone (D) and morphine + TCSOX229 + naloxone (E). (F-G) High magnification images showing c-Fos-positive (black)/CRF-positive (brown) neurons of rats treated with morphine + vehicle + naloxone (F) and morphine + TCSOX229 + naloxone (G). Black arrows indicate double-labeled neurons. White arrows indicate CRF-positive neurons. (H) Quantitative analysis of c-Fos-positive/CRF-positive cells in the PVN. Bars represent the mean ± SEM. $^{+++}p<0.001$ versus morphine + vehicle + naloxone; ***$p<0.001$ versus placebo + vehicle + naloxone; $^{###}p<0.001$ versus placebo + TCSOX229 + naloxone.

3.11. OxR2 blockade does not antagonize naloxone-precipitated morphine withdrawal-induced HPA axis activation

We measured plasma corticosterone concentrations (as HPA axis activation marker) in blood samples obtained from morphine-dependent or control rats 2 h after injection of naloxone. As shown in Figure 37, plasma corticosterone levels increased significantly ($p < 0.01$) in vehicle pretreated, morphine withdrawn rats ($n = 6$). To evaluate if there is a link between OxR2 activation and HPA axis hyperactivity during morphine withdrawal, plasma corticosterone concentrations were measured in animals made dependent on morphine and pretreated with TCSOX229 before naloxone administration. Corticosterone plasma levels in TCSOX229 plus naloxone-treated morphine-pelleted animals ($n = 5$) were significantly ($p < 0.05$) higher than those observed in the placebo group also administered TCSOX229 plus saline ($n= 5$). No significant differences were seen between morphine-dependent rats receiving vehicle before naloxone and those receiving TCSOX229, and between placebo-pelleted rats receiving vehicle before naloxone and those receiving TCSOX229 ($n = 6$).

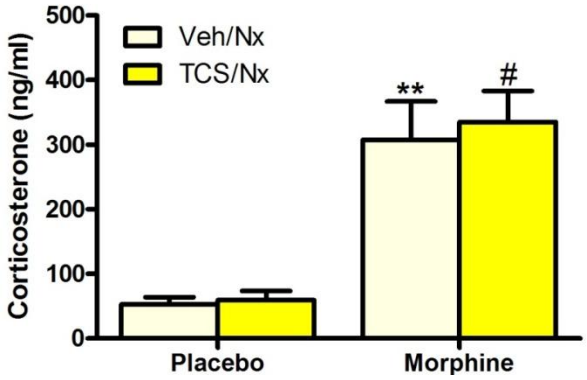

Figure 37. TCSOX229 did not attenuate the plasma corticosterone response to naloxone-induced morphine withdrawal. Data represent the mean ± SEM of plasma corticosterone concentration 120 min after naloxone (Nx) injection to placebo- or morphine-treated rats receiving vehicle (veh) or TCSOX229 (TCS) 20 min before naloxone administration. #$p<0.05$ versus placebo + TCS +nx; **$p<0.01$ versus placebo + vehicle + naloxone.

DISCUSSION

1. ENHANCED TYROSINE HYDROXYLASE PHOSPHORYLATION IN THE NUCLEUS ACCUMBENS AND NUCLEUS TRACTUS SOLITARIUS-A2 CELL GROUP AFTER MORPHINE-CONDITIONED PLACE PREFERENCE

CPP is an important behavioral assay that is widely used to assess the reward value of drugs and natural reinforces. This behavior has been used to guide an extensive body of research into the anatomic, pharmacologic, genetic, and molecular substrates of reward (196;197). The present data indicate that in the dose range of 0.5–8 mg/kg, morphine produces a significant CPP for the drug-associated place. These findings supported previous studies (196;198-200) and demonstrated that morphine induced rewarding effects which, through a mechanism of associative learning, become connected to the environment in which these effects occurred (201).

It is well-known that an opioid-induced place preference depends on activation of the mesolimbic DA system (202). The association of environmental cues with drug reinforcement appears to involve plasticity within the mesocorticolimbic system, and glutamatergic transmission has been implicated in mediating plasticity. VTA and NAc receive noradrenergic innervations from the NTS and are modulated by NA. However, there are a few studies investigating the possible role of NA in morphine CPP. Our results show an increase in the DOPAC and DOPAC/DA ratio indicating an increased turnover of DA after morphine-induced CPP; the increased turnover of DA in the NAc would be associated to expression of conditioned bias toward morphine-paired stimuli. In agreement with these data, elevation of DA and its metabolites was observed in the NAc in parallel with morphine doses–effects on expression of CPP (199).

Although DA signaling has been primarily implicated in the rewarding effects of opioids, a role for NA in opioid reward has been suggested (93;203-205). Recent research indicates the necessity of noradrenergic function in the establishment of morphine CPP (203;206-209). Our results demonstrated an increase in the MHPG levels and NA turnover in the NAc in morphine-induced CPP, suggesting a role of NA in the rewarding properties of opioids. How noradrenergic neurotransmission from the NTS regulates opioid reward remains to be explored.

Changes in the state of phosphorylation of TH, the rate-limiting enzyme in the synthesis of catecholamines, are critically involved in the regulation of catecholamines synthesis and function. In particular, increases in the phosphorylation of Ser40 and Ser31 accelerate TH activity, thereby simulating production of neurotransmitter in catecholamine terminals (210). The results of this work provides evidence for TH phosphorylation after morphine-induced CPP in both: catecholaminergic cell bodies in the NTS, and terminals innervating the NAc. Using phosphorylation state-specific antibodies directed toward Ser40 or Ser31, we have shown that morphine-induced CPP greatly increased the level of TH phosphorylation at Ser40 in NAc, without changes at Ser31. We also found that morphine-induced CPP increased levels of TH phosphorylated at Ser31 and Ser40 in the NTS. Together, these data suggest that Ser40 phosphorylation of TH may be an important modulator of TH activity and might be directly involved with regulating NA and DA turnover in the NAc of mice conditioned to morphine.

It is well-known that phosphorylation of Ser40 develops a considerable increase in TH activity (210). Association of Ser40 phosphorylation with TH activity and catecholamine synthesis in vivo has been shown. Striatal Ser40 phosphorylation was increased concomitantly with enhanced TH activity and DOPAC biosynthesis (211). In the present work, an increased number of TH Ser31-positive cells were seen in the NTS, suggesting that phosphorylation at Ser31 could be the mechanism for maintenance of catecholamine synthesis to replace those released in response to morphine-induced CPP. In the present study, it should be noted that TH protein expression was similar in morphine- or saline-paired groups in the NTS or NAc. In agreement with a previous study (212), present results suggest that phosphorylation of TH and its ability to cause TH activation and catecholamine synthesis is independent of TH protein expression.

Because the mesolimbic dopaminergic system that projects from the VTA to the NAc is critical for initiation of opioid reinforcement and reward-related effects of drugs of abuse, it may be surprising that similar number of TH Ser40- or TH Ser31-positive cells were seen in the VTA from saline- and morphine-paired groups. However, it is possible that morphine-induced CPP lead to adaptive changes in key elements controlling DA levels in the synapse, and these changes could produce alterations in the amount of DA released without altering the

activity of VTA DA neurons. One possible adaptive change of this kind is the DA transporter (DAT) (213). DAT plays a central role in determining the duration and amplitude of DA action by rapidly recapturing extracellular DA into presynaptic terminals after release (214). Moreover, voltammetry studies have demonstrated that mice with a genetic disruption of the DAT, morphine produced a dramatic supernormal increase in the level of extracellular DA (213). Thus, adaptive changes at the level of the DA transporter could produce the long-lasting changes in morphine-induced mesolimbic DA release (215) without altering the activity of VTA DA neurons (216). This hypothesis is indirectly supported by studies showing that a history of opioid dependence can enhance the reinforcing effects of cocaine in a CPP (217;218). In addition, it has been suggested the involvement of VTA in the acquisition but lesser in the expression of morphine-induced CPP (200).

In conclusion, the present study suggests that morphine-induced CPP might stimulate TH activity and accelerate DA and NA turnover in the NAc via a mechanism involving phosphorylation of TH. The novel signaling events reported here provide additional mechanisms by which morphine-induced CPP may modulate TH phosphorylation in the NAc and NTS, one of the primary sources of NA in the NAc.

2. CORTICOTROPIN-RELEASING FACTOR 1 RECEPTOR MEDIATES THE ACTIVITY OF THE REWARD SYSTEM EVOKED BY MORPHINE-INDUCED CONDITIONED PLACE PREFERENCE

Our work supports a critical role of the CRF system in the behavioral and molecular changes in the brain reward neurocircuitry induced by morphine associated stimuli. To our knowledge, present data suggest, for the first time, a critical role CRF_1-R, in the positive reinforcement properties of morphine.

Nowadays, addiction is considered as a cycle where the dysregulation of the brain reward and anti-reward mechanisms results in a negative emotional state, which contributes to the compulsive use of drugs. Thus, it has been proposed that the Antireward system, which has opposing actions, is activated by the reward circuit, limiting the reward function. The brain anti-reward system is composed by the brain stress system. The role of CRF in the later phases of the addiction cycle has been widely studied (for review, see (219)). However, less is known about the role of the stress systems in the earlier phases of drug dependence. Acute administration of drugs of abuse activates the HPA axis, which constitute the hypothalamic stress system (9). The activation of the hypothalamic stress system has been associated with first facilitation of the brain motivational circuits and drug reward, thus facilitating acquisition of drug-seeking behaviour (9). On the other hand, CRF in the extrahypothalamic stress system seems to have more important role in the motivational effects of both protracted abstinence and stress-induced reinstatement (96).

It is well known that opiates induce strong CPP over a wide range of experimental conditions (220). Accordingly, present results show that morphine evoked significant CPP for the drug-associated environment. Different neurobiological substrates have been involved in the rewarding properties of drugs of abuse, although the mesolimbic DA pathway has been pointed out to be the critical system for drug reward. In agreement, our work shows that morphine-paired mice exhibit activation, as shown by c-Fos expression, of the VTA and its projecting area, the NAc. Past and recent hypothesis confer DA an important function in behavior, given the ability of conditioned stimuli to release DA in the

NAc. When measured DA turnover in the NAc we found an increase in DOPAC/DA ratio, which indicates that morphine-induced CPP augmented dopaminergic neurotransmission in the brain reward system. The increased DA release in the NAc has been related with the strong incentive-arousing and incentive-learning properties of drug-conditioned stimuli (18).

VTA receives CRF afferents from the BNST, CeA and PVN (180;221), and both VTA and NAc express high levels of CRF_1-R but not CRF_2-R mRNA in rodents (106). Since VTA and NAc play a main role in reward processing and effects of drugs of abuse, these inputs could indicate that CRF might be involved in the reinforcement effects of addictive substances, and concretely, opiates. Supporting this hypothesis, present data show that administration of CP-154,526 30 min before morphine conditioning sessions blocked the acquisition of the preference for the drug-associated compartment. It has been previously published that CP-154,526, but not antisauvagine-30 (a selective CRF_2-R antagonist), prevented the cocaine-induced CPP (222). Thus, these data point out how CRF_1-R is essential for the acquisition of CPP induced by drugs of abuse, such as morphine and cocaine. We observed as well that CRF_1-R antagonism blocked the activation of the VTA and NAc in morphine-paired mice, so a decreased dopaminergic neurotransmission during morphine-induced CPP would be expected. Nonetheless, the administration of CP-154,526 prior drug-conditioning sessions had no effect on the increased DA turnover in the NAc evoked by morphine-induced CPP. It has been described that morphine induces DA release in the NAc shell, but not in the NAc core (18). Moreover, dopaminergic and non-dopaminergic (GABAergic and glutamatergic) neurons coexist in the VTA (223). The NAc dissection technique used for this study did not discriminate between NAc shell and core. In addition, from our experiments in the present work we cannot state that activated cells in the VTA are exclusively dopaminergic neurons. These facts could explain the conflicting data obtained for c-Fos expression in the VTA and NAc and dopaminergic neurotransmission in the reward system during morphine-induced CPP. On the other hand, high DA turnover was found in the NAc from saline-paired mice receiving CP-154,526. A number of cellular and molecular mechanisms may be involved in these effects. One might include a

compensatory activation mediated by other neurotransmitters because of the blockade of the CRF_1-R.

There are evidences supporting that NA influences the response to drug reward by positive or negative reinforcement mechanisms. Recent research indicates the necessity of noradrenergic function in the establishment of morphine CPP (93). Supporting this hypothesis, present study demonstrates that morphine-induced CPP evokes an increase in c-Fos expression in both NTS and LC noradrenergic systems, as well as an enhancement in noradrenergic neurotransmission in the NAc. It is known that the activity of both LC and NTS is increased by CRF (94), and CRF_1-R has been localized in the LC (94) and in the NTS (224). Our work showed that the blockade of CRF_1-R antagonized the increase of c-Fos expression evoked by morphine-induced CPP in the LC but not in the NTS. Additionally, CP-154,526 administration returned to basal levels the noradrenergic neurotransmission in the NAc of morphine-paired mice. These results would indicate that the noradrenergic inputs to the NAc involved in morphine reward come from the LC. However, it has been previously demonstrated that, although both LC and NTS project to the NAc (225), only the projections from the NTS neurons release NA as a neurotransmitter in the NAc shell (226). Therefore, and given that in present experiments we have not used a marker of noradrenergic neurons to identify the neural populations that are activated in the LC and in the NTS, we would suggest that the blockade of CRF_1-R antagonized the activation of noradrenergic neurons evoked by morphine-induced CPP in the NTS, which would result in diminished noradrenergic neurotransmission in the NAc.

Recent evidences suggest that orexinergic projections from the LH to the VTA are essential for the acquisition and expression of morphine-induced CPP (141;163;227;228). Different functions of orexins neurons have been described in previous studies. Thus, orexinergic neurons located in the DMH and PFA have been suggested to control arousal and modulate stress responses, whereas neurons in the LLH has been related to positive reinforcement effects of drugs (129). In agreement, present work shows activation, revealed by c-Fos expression, of the orexinergic neurons in the LLH, but not in the DMH and PFA, from morphine-paired animals. The existence of CRF boutons on orexin-expressing

neurons has been found in the LH and it is known that CRF activates the release of orexins (176). After CRF_1-R antagonist administration, we observed lower c-Fos expression in the three subdivisions of the LH from saline- and morphine-paired animals compared with vehicle-injected mice, which indicates that CRF function is not exclusively restricted to the LLH, but this neuropeptide also would act on DMH and PFA in goal-directed behaviors. Our data might suggest that the orexinergic system involvement in drug reward might be conducted by CRF.

It is known that acute administration of drugs of abuse activates the HPA axis, which has been related to facilitated drug reward (9). Supporting this, present and other works (229) revealed that morphine-induced CPP evokes an increase in corticosterone release. CRF_1-R is the main CRF receptor subtype found in the pituitary corticotrope cells (230;231), which synthesize and release adenocorticotropic hormone, which in turn stimulates the adrenal gland for GC secretion. In our work, CRF_1-R blockade, not only did not decrease corticosterone release in morphine-conditioned animals, but also increased GC plasma levels during morphine-induced CPP. Recent studies from this and other laboratories have shown that CRF_1-R and CRF_2-R antagonists have no effect on the increased corticosterone plasma concentration induced by morphine withdrawal (224;232). Different cellular and molecular processes might be involved in the increased corticosterone levels after CRF_1-R antagonism in morphine-conditioned animals. These mechanisms can include AVP actions in the pituitary in response to CRF_1-R blockade, resulting in HPA axis over-activation.

In summary, present work demonstrates the activation of different catecolaminergic and non-catecolaminergic brain areas related to drug reward in the expression of morphine-induced CPP. Additionally, this study provides data pointing out the critical role of CRF, through CRF_1-R, in the positive reinforcement effects of morphine and in morphine-conducted behaviors.

Finally, our work supports a potential therapeutic treatment of CRF_1-R antagonists in addictive disorders, as has been previously proposed (219).

3. HYPOTHALAMIC OREXIN A NEURONS ARE INVOLVED IN THE RESPONSE OF THE BRAIN STRESS SYSTEM TO MORPHINE WITHDRAWAL

This report provides evidence in rats of the critical involvement of orexinergic system via OxR1 and OxR2 in activation of brain stress system during morphine withdrawal.

In agreement with previous data Georgescu et al. (2003) (128), Sharf et al. (2008) (154) o Plaza-Zabala et al. (2012) (192), present results show an important role for Ox, through OxR1 and OxR2 pathways, in the somatic expression of drug withdrawal as well as in molecular changes within the brain stress systems induced by morphine withdrawal. Naloxone administration to morphine dependent rats induced robust withdrawal symptoms. Our results indicate that the blockade of OxR1 or OxR2 significantly decreased several of the somatic signs of naloxone-precipitated morphine withdrawal as well as the global score, suggesting that activation of OxR1 and OxR2 pathways might positively modulate the somatic expression of opiate withdrawal. These data are in agreement with previous results showing that administration of SB334867 directly into LC to rats attenuates signs of morphine withdrawal (233). In addition, mice lacking the pre-propeptide encoding for orexins or pretreated with SB334867 display markedly reduced symptoms of opiate withdrawal (128), (154). Moreover, Plaza-Zabala et al. (192) demonstrated that OxR2 antagonism decreased nicotine withdrawal symptoms. In the present study, we demonstrate the involvement of Ox, at least in part, in morphine withdrawal somatic symptoms through OxR1 and OxR2.

Previous study on orexin-T-LacZ reporter mice indicated that morphine withdrawal stimulates the transgenic expression. Using quantitative real time PCR measurement, we provide a direct evidence for activation of Ox-A gene expression in the rat hypothalamus during morphine withdrawal. Furthermore, Ox-A mRNA levels peak between 30–60 min after naloxone administration. This timing of transcriptional activation is compatible with CRE-mediated transcription, as it has been shown in the orexinergic neurons of morphine-

withdrawn CRE-LacZ reporter mice (128). Additionally, naloxone-precipitated morphine withdrawal turned into an activation of lateral hypothalamus orexinergic neurons, as revealed by immunocytochemical detection of c-Fos protein. In contrast to the situation seen in morphine withdrawn mice, cells in all the three functionally distinct subpopulations of hypothalamic orexinergic neurons became c-Fos positive upon morphine withdrawal in rats.

Present study demonstrated that morphine withdrawal induces c-Fos expression in orexinergic neurons in the LLH subdivision. This result suggests that LLH may also be a critical region contributing to morphine withdrawal response in rats. Interestingly, present work also shows hyperactivation of the brain stress system during morphine withdrawal that was attenuated by SB334867. Given that the PVN receives orexinergic inputs arising from both the PFA and the LLH subdivisions of the LH (234), our data might suggest the involvement of Ox-A neurons arising not only from the DMH and PFA, but also from the LLH, in the activation of the brain stress system during opiate withdrawal.

Both the HPA axis and the extended amygdala are dysregulated by chronic administration of drugs of abuse (9;82;103). Furthermore, acute drug withdrawal may also increase the activity of noradrenergic pathways innervating the CeA and the PVN (102;103;235). All these processes are involved, at least partially, in the negative motivational states during drug withdrawal. Present results show an enhancement of c-Fos expression during morphine withdrawal in the main nuclei of the extended amygdala, and in the PVN, the apex of the HPA axis. The NTS, the main noradrenergic cell group innervating the brain stress system (99), was also activated after naloxone-precipitated opiate withdrawal. Present data show that SB334867 administration attenuates this activation of the extended amygdala and the PVN, suggesting that the orexinergic system, via OxR1, might play a main role in regulating the activation of the brain stress system during acute opiate withdrawal. The presence of orexinergic fibers as well as OxR1 and/or OxR2 within all these nuclei showed in the present work and others (110) supports this hypothesis (119). The present study shows that the Ox-A system provides anatomical input to the brain stress system, including the extended amygdala, the PVN and the NTS. Light microscopic double immunocytochemical analysis

revealed close apposition between Ox-A fibers and CRF-, pro-dyn- and TH-expressing in the PVN, CeA, NAc and NTS respectively. These findings provide an anatomical basis for potential modulation of CRF, noradrenergic and dyn neurons by Ox-A.

NAc shell has been related to the expression of morphine withdrawal somatic symptoms (154), together with the CeA and BNST (236;237). Supporting this hypothesis, present results showed that attenuation of somatic signs during opiate withdrawal were accompanied by a decrease of c-Fos expression in these sites.

The results of the present study show that morphine withdrawal induced an increase in corticosterone release, which was not blocked by SB334867 or TCSOX229 administration. We first examined the action of SB334867 on the activity of the CRF neurons in the PVN during morphine withdrawal, and we found that both the morphine-dependent rats injected with SB334867 or with vehicle showed similar responses to naloxone injection. It has been reported that Ox-A excites CRF and AVP neurons in the PVN (Samson et al, Peptides, 2002) and induces both CRF and AVP synthesis (Al_Barazanjani et al, J Neuroendocrinol, 2001). Since there was an attenuation of c-Fos expression in the PVN after SB334867 administration in morphine-withdrawn rats, and our results show that this decrease does not occur in the CRF neurons, it could be suggested that the activity of other PVN neurons, such as AVP-containing cells (which also participates in ACTH and then in glucocorticoids release), is inhibited by the OxR1 antagonist. Given that OxR2 mRNA is predominantly expressed in the PVN (119), and that the administration of OxR2 antagonist to rats inhibits ACTH release induced by Ox-A or stress (234), it might be postulated that the action of Ox-A at this level would be mediated mainly by the OxR2. Thus, it seems logical that the responsiveness of the PVN CRF neurons and the HPA axis to morphine withdrawal did not change after blocking OxR1. Additionally, SB334867 administration induced a slight activation of CRF neurons in the PVN and glucocorticoids release in control rats. Since Ox-A binds to OxR1 and OxR2 with similar affinity (109), and we have blocked OxR1, Ox-A might bind exclusively to OxR2. Thus, it would be tempting to suggest that OxR2 stimulation in the PVN would result in CRF-containing cells activation and in glucocorticoids

release. Nevertheless, when we blocked the OxR2 to study its role in the HPA axis activity during opiate withdrawal we found that, after TCSOX229 administration, there was an over-activation of CRF neurons of the PVN and similar corticosterone plasma levels compared to vehicle injected morphine-withdrawn rats. These responses might be due to OxR1 activation. However, Steiner et al. (238) have just demonstrated that dual OxR antagonists systemically administered do not interfere with endocrine HPA axis function in the rat. All together, these data suggest that endogenous orexin signaling plays a minor role in GC release.

Numerous studies have supported the importance of the brainstem noradrenergic afferents in regulating the brain stress system during opiate withdrawal (102;237;239;240). Since SB334867 administration did not block the activation of the NTS, our data do not support a role of the OxR1 pathways in regulating the activity of the noradrenergic A_2 cell group (NTS).

Extended amygdala nuclei and their neurotransmitter systems have been related with the positive reinforcing effects of drugs, as well as with the negative reinforcing effects of drugs withdrawal (9). It has been previously reported that Ox-A has an excitatory action on the CeA (147), and that this activation occurs in CRF-containing neurons (241). Accordingly, our results show an increase in c-Fos expression in CRF-positive neurons in the CeA during naloxone-induced morphine withdrawal, which was blocked by SB334867. These data indicate the involvement of the orexinergic system in modulating the response to opiate withdrawal syndrome in the CeA via OxR1.

Present data show that BNST CRF neurons were not activated after naloxone administration to morphine dependent rats, which is in agreement with previous data showing no increase in c-Fos expression in BNST CRF neurons in morphine-withdrawn rats (242). Just like in the PVN, SB334867 administration did not alter the response of BNST CRF neurons to morphine withdrawal. It should be taken into account that these two nuclei receive important noradrenergic inputs from the NTS (240;243), whose activation in morphine-withdrawn rats is not attenuated by the OxR1 antagonist.

In summary, this study provides evidences of activation of orexinergic in the three subdivisions of the LH during naloxone-induced morphine withdrawal that is accompanied with increased Ox-A mRNA transcription and increased Ox-A input to brain stress system. Furthermore, present data reveal a critical involvement of the orexinergic system in the physical symptoms of opiate abstinence syndrome, supporting a potentially therapeutic application of OxR1 antagonists in addictive disorders, as has been proposed (154;244). Present findings highlight the pivotal role of orexins in the activation of the hypothalamic and extrahypothalamic brain stress systems during opiate withdrawal in rats.

CONCLUSIONS

1. Present work demonstrates the activation of different catecolaminergic and non-catecolaminergic brain areas related to drug reward during morphine-induced CPP.

2. Morphine-induced CPP might stimulate TH activity and accelerate DA and NA turnover in the NAc via a mechanism involving phosphorylation of TH. The novel signalling events reported here provide additional mechanisms by which morphine-induced CPP may modulate TH phosphorylation in the NAc and NTS, one of the primary sources of NA in the NAc.

3. This study provides data pointing out the critical role of CRF, through CRF_1-R, in the positive reinforcement effects of morphine and in morphine-conducted behaviours, supporting a therapeutic potential of CRF_1-R antagonists in addictive disorders, as has been previously proposed.

4. Naloxone-induced morphine withdrawal activates orexinergic neurons in the three subdivisions of the LH, which is accompanied with increased Ox-A mRNA transcription and increased Ox-A input to the brain stress system.

5. Present data reveal a critical involvement of the orexinergic system in the physical symptoms of opiate abstinence syndrome, supporting a therapeutic potential of OxR1 and OxR2 antagonists in addictive disorders, as has been proposed.

Present findings highlight the pivotal role of orexins in the activation of the hypothalamic and extrahypothalamic brain stress systems during opiate withdrawal in rats.

RESUMEN EN ESPAÑOL

La exposición crónica a sustancias de abuso da lugar a cambios adaptativos moleculares y/o neuroquímicos en el cerebro, y como consecuencia a alteraciones en el comportamiento del individuo que caracterizan el estado de adicción.

Para identificar tanto los cambios adaptativos cerebrales, como las alteraciones comportamentales inducidas por las sustancias de abuso (drogas), es importante comprender los mecanismos implicados en el desarrollo y establecimiento de la adicción, ya que desde una perspectiva clínica la terapia farmacológica puede ser utilizada con mayor efectividad.

1. NEUROBIOLOGÍA DE LA ADICCIÓN

La adicción a una droga se puede definir como un desorden crónico del comportamiento, caracterizado por (a) una búsqueda y un consumo compulsivo de la droga, (b) una pérdida de control en el consumo de la misma, y (c) la aparición de un estado emocional negativo (por ejemplo: disforia, ansiedad, irritabilidad,…) que refleja un síndrome de abstinencia cuando el acceso al consumo de dicha droga queda restringido (3).

La adicción se describe cómo una serie de eventos iniciados por un efecto de recompensa o refuerzo positivo tras el consumo inicial de la droga, que provoca que el individuo vuelva a consumir, generando así el uso crónico de dicha sustancia. Adicionalmente, cuando este consumo es interrumpido a menudo se producen estados aversivos de índole tanto fisiológica como psicológica. Así pues, el consumo de la droga se convierte en un medio para evitar dicho estado de aversión (refuerzo negativo), que caracteriza el estado de abstinencia (5;6). Se pueden establecer tres estados diferentes que caracterizan al ciclo de la adicción: consumo agudo (refuerzo positivo), abstinencia (refuerzo negativo) y preocupación (lo que lleva a la recaída de nuevo).

1.1. Circuitos Neuronales de la Adicción

Durante el proceso adictivo existe una evolución del individuo, que va desde el comportamiento de búsqueda de bienestar tras el consumo de la droga, a una búsqueda y consumo compulsivo de la misma.

La adicción implica plasticidad en áreas cerebrales específicas. En función de la fase del ciclo de la adicción en la que se encuentre el individuo, los distintos circuitos neuronales implicados sufren diferentes alteraciones. Diversos estudios demuestran que las sustancias adictivas generan adaptaciones sinápticas, siendo éstas el primer paso de una cascada de eventos celulares que dan lugar a una serie de modificaciones en los circuitos neuronales y a alteraciones del comportamiento (12;13).

Las sustancias de abuso tienen diversos perfiles farmacológicos, pudiendo alterar el comportamiento del individuo de varias formas, aunque todas tienen una característica común, generar un aumento en la actividad dopaminérgica mesocorticolímbica tras su consumo (16;17). Las sustancias adictivas, en general, provocan la activación de los circuitos de recompensa con un incremento en la liberación de dopamina (DA) en el núcleo accumbens (NAc). Varios estudios relacionan esta liberación de DA con los refuerzos positivos que generan las sustancias de abuso, iniciando así el ciclo de la adicción (18;19). Aunque la DA ha sido ampliamente considerada el principal neurotransmisor de la adicción, se ha propuesto la implicación de otros neurotransmisores, como noradrenalina (NA), glutamato, endocannabinoides, ácido γ-aminobutírico (GABA), péptidos opioides endógenos y orexinas, en las diferentes fases del ciclo de la adicción (9;20).

Los efectos de recompensa inducidos por opioides, generan principalmente la activación de receptores tipo μ. Estos receptores están localizados en interneuronas GABAérgicas, que sinaptan con los cuerpos dopaminérgicos del área tegmental ventral (VTA). Apoyando esta hipótesis estudios previos muestran cómo la activación de receptores opioides tipo μ (MOR) en el VTA provocan un aumento de los niveles de DA en el NAc y en el córtex (33).

Por otra parte, se ha propuesto que el estrés y la adicción están muy relacionados. La exposición a agentes estresantes puede exacerbar el consumo de

drogas, de forma que dicho consumo se vuelva crónico y se desarrolle un síndrome de abstinencia en el momento en el que el consumo de dicha sustancia quede restringido. Se ha descrito que el síndrome de abstinencia desencadena los mismos signos fisiológicos que la exposición aguda a situaciones estresantes. Paralelamente, existen evidencias de que tanto los agentes estresantes como las sustancias adictivas activan las mismas áreas cerebrales, incluyendo los sistemas dopaminérgicos mesolímbico y mesocortical (35-37).

La exposición crónica a drogas supone un descenso en la funcionalidad de los neurotransmisores que comunican los circuitos neuronales implicados en los refuerzos positivos. Hipótesis recientes sugieren que los sistemas dopaminérgicos quedan comprometidos en ciertas fases del ciclo de la adicción, como en el síndrome de abstinencia, lo que lleva a disminuir la motivación hacia estímulos no relacionados con la droga (38). En estudios hechos con animales, se ha observado un descenso de la actividad dopaminérgica mesolímbica en el NAc durante el síndrome de abstinencia generado por cualquier sustancia adictiva (39;40;40).

En un intento de superar la presencia crónica de la droga y de recuperar la actividad normal de la transmisión neuronal, otro componente del síndrome de abstinencia es la adaptación neuronal de sistemas intermedios con diferentes neurotransmisores implicados en la modulación de la respuesta al estrés, y en la generación de los síntomas aversivos. En esta modulación de la respuesta al estrés intervienen, el sistema hipotalámico del estrés, compuesto por el eje hipotálamo-hipófisis-adrenal (HHA), y el sistema extrahipotalámico del estrés, también llamado amígdala extendida y compuesto por el núcleo de la estría terminal (BNST), amígdala central (CeA) y NAc (shell). Los núcleos que componen el eje HHA se activan durante síndrome de abstinencia generado por las sustancias de abuso dando lugar a un aumento del factor liberador de corticotropina (CRF), hormona adrenocorticotropa (ACTH) y de corticosterona (14;41). Por otro lado, durante este mismo síndrome de abstinencia también se produce un estado aversivo y ansiogénico en el que el CRF extrahipotalámico y otros sistemas relacionados con el estrés (incluido el noradrenérgico) cumplen importantes funciones (3).

El CRF, neuropéptido central en la modulación de la respuesta al estrés, podría ser clave en las recaídas. Este neuropéptido modula la ansiedad tanto aguda como prolongada, generada por la ausencia de las sustancias de abuso, convirtiéndolo en una diana terapéutica en el tratamiento de la adicción (48). El CRF es el principal activador de la respuesta endocrina al estrés. De esta forma, la exposición ante agentes estresantes inicia la síntesis de CRF a dos niveles, el núcleo paraventricular (PVN), el cual es el ápice del eje HHA, y en la amígdala extendida. Se ha demostrado que el CRF extrahipotalámico juega un papel clave en la respuesta comportamental ante agentes estresantes (49).

El eje HHA es un complejo sistema neuroendocrino implicado en varias funciones fisiológicas, tanto centrales como periféricas, la mayoría relacionadas con situaciones estresantes (55). Este eje, está compuesto por tres importantes estructuras: PVN hipotalámico, el lóbulo anterior de la glándula pituitaria y la glándula adrenal (56). La síntesis y liberación de ACTH es una respuesta fisiológica como consecuencia de la activación de los receptores de CRF: CRF_1-R y CRF_2-R (71;72) situados en la hipófisis; ambos tipos de receptores están acoplados a proteínas G transmembrana que principalmente funcionan interactuando con G_s, y en consecuencia aumentando los niveles de AMPc, aunque también es sabido que estos receptores son capaces de interaccionar con otros tipos de proteínas G (48). El ACTH estimula la síntesis de glucocorticoides (GC) en la glándula adrenal, y la secreción de éstos al torrente sanguíneo. Por otra parte, los GC circulantes ejercen una regulación negativa sobre todo el eje HHA (73). Esta regulación es provocada por la activación de receptores de GC y de mineralocorticoides (MC), los cuales difieren tanto en la distribución anatómica, como en propiedades farmacológicas (74).

Desde la perspectiva de la adicción, se ha comprobado que tienen lugar cambios progresivos en el eje HHA durante la transición de consumo agudo de sustancias de abuso, a crónico. La administración aguda de la mayoría de las drogas en animales activa el eje HHA y por tanto la secreción de GC al torrente sanguíneo, los cuales facilitan la motivación hacia un nuevo consumo de la sustancia, dando lugar en consecuencia a la progresión hacía un comportamiento de búsqueda crónica y compulsiva de la droga (76). Estas consecuencias de la

progresión del consumo agudo a crónico, son desarrolladas tras la administración repetida de cocaína, opioides, nicotina y alcohol (41;77;78).

La exposición crónica a opioides desarrolla tolerancia y una dependencia física a los efectos endocrinos generados por el eje HHA durante un consumo agudo, de forma que tras la administración de un antagonista opioide a animales expuestos crónicamente a estas sustancias es característico el incremento de la secreción de ACTH, y por lo tanto de GC (79-81). De esta forma, estudios previos de nuestro grupo de investigación han mostrado un incremento en la expresión de RNA heteronuclear (hn-RNA) para CRF en el PVN durante el síndrome de abstinencia a morfina (82).

Además de estos eventos neuroendocrinos, la dependencia de opiáceos da lugar a cambios a largo plazo en la expresión génica (83;84). Estudios previos de nuestro laboratorio han descrito que el síndrome de abstinencia a morfina induce la expresión neuronal de c-Fos en la división parvocelular del PVN (85). Además, investigaciones recientes han demostrado que la sobreexpresión crónica de CRF en el CeA produce una hiperactividad en el eje HHA y un comportamiento ansiogénico asociado con cambios en la expresión génica en el PVN (86). También ha sido descrito el desarrollo de un estado aversivo durante el síndrome de abstinencia, que fue revertido tras la administración de antagonistas de CRF (87). Sin embargo, las rutas de señalización intracelular encargadas de la activación del eje HHA siguen siendo desconocidas. Estudios previos muestran un aumento de proteinquinasa-A (PKA) y proteinquinasa-C (PKC) en el PVN durante la abstinencia a morfina (88;89), lo que podría significar el primer paso para la estimulación de diferentes factores de transcripción, como el c-Fos.

El sistema noradrenérgico, que inerva la amígdala extendida está implicado en la sintomatología negativa, y por lo tanto, en el incremento del consumo de la droga unido a la dependencia de la misma. Mediante estudios de autoadministración, de condicionamiento de preferencia de plaza (CPP), de recaída y de actividad locomotora también se ha evidenciado la implicación de la NA en los refuerzos positivos que produce el consumo de drogas. El sistema noradrenérgico regula la actividad de las vías dopaminérgicas ascendentes, mediante proyecciones desde el locus coeruleus (LC) (90) hasta el VTA, dónde

neuronas noradrenérgicas modulan la liberación de DA a través de la activación de receptores adrenérgicos α_1 (91). Desde el LC también se regula el sistema dopaminérgico mesencefálico de forma indirecta, a través de la vía de la corteza prefrontal (PFC). Cuando la liberación de NA se bloquea, la liberación de DA se atenúa de forma paralela. Si el bloqueo de NA se vuelve crónico, el sistema de DA lo compensa gradualmente aumentando la afinidad de los receptores postsinápticos de DA, o mediante un aumento de la densidad de los mismos en la membrana postsináptica (93). Este proceso genera una hipersensibilidad a psicoestimulantes y a cualquier otra sustancia, cuya función sea el aumento de los niveles de DA (30).

Por otra parte, se ha postulado la existencia de interacciones entre el CRF y el sistema noradrenérgico en el sistema nervioso central (SNC). Existen evidencias a nivel farmacológico, fisiológico y anatómico de la interacción CRF-NA en el LC tras la exposición a un agente estresante. Incluso se ha demostrado que las neuronas de CRF podrían activar la transmisión noradrenérgica, y a su vez, la transmisión noradrenérgica podría estimular la liberación de CRF (95;96). La NA también aumenta la liberación de CRF en otros núcleos como el PVN, el BNST y el CeA (97), y el CRF liberado por estos núcleos induciría la liberación de NA por las áreas noradrenérgicas del bulbo.

Paralelamente, la liberación de CRF por las neuronas del PVN está asociada a la creciente actividad de las neuronas noradrenérgicas del núcleo del tracto solitario (NTS), el cual también está conectado con el BNST, el CeA y el NAc shell (98). Las aferencias noradrenérgicas desde el NTS hacia la amígdala extendida y el PVN median tanto en el desarrollo de los signos propios del síndrome de abstinencia a opioides, como en las recaídas inducidas por la exposición a agentes estresantes, que desencadenan un comportamiento de búsqueda compulsiva de la droga (87;99). De esta forma, las vías noradrenérgicas juegan un papel importante en las recaídas inducidas por agentes estresantes a los que se expone el individuo durante la adicción (100). Además, ha sido estudiado cómo la administración de agonistas adrenérgicos α_2 atenúa estas recaídas en los casos de adicción a alcohol, cocaína y heroína (30).

2. SISTEMA OREXINERGICO

Las orexinas, también identificadas con las abreviaturas Ox-A y Ox-B, son neuropéptidos derivados de una molécula prepro-orexinérgica sintetizada en neuronas hipotalámicas (108;109). Las neuronas orexinérgicas se localizan exclusivamente en el área perifornical (PFA), el hipotálamo dorsomedial (DMH) y en el lateral (LLH) del hipotálamo lateral (LH) (110-112). Sakurai et al., en 1998, describió dos tipos de receptores de las orexinas denominados OxR1 y OxR2. OxR1 se une a la Ox-A con una afinidad muy superior a la que muestra por las Ox-B, sin embargo, el OxR2 se une a ambos péptidos orexinérgicos con la misma de afinidad.

Numerosos estudios han demostrado que las orexinas juegan un importante papel en el comportamiento de búsqueda de la droga en modelos animales de adicción. Sin embargo, la naturaleza exacta de su función está por concretar. Los receptores de orexinas (118;119;132) y las proyecciones orexinérgicas desde el LH (112;133) se encuentran distribuidas por todo el encéfalo, incluidas regiones conocidas por su implicación en la recompensa a drogas y en la adicción. Estudios previos han demostrado que muchas de las proyecciones orexinérgicas incluyen a las neuronas dopaminérgicas del VTA (110;134;135). Por otra parte, investigaciones sobre los circuitos de recompensa y las adaptaciones neurológicas que éstos experimentan durante el consumo de drogas se han centrado en los efectos de las orexinas en el VTA y en las regiones mesocorticolímbicas inervadas por éste, como son el NAc, la amígdala (AMG) y la PFC (136-138).

Las orexinas envían proyecciones excitatorias al VTA, activando tanto células dopaminérgicas (127;140) como no dopaminérgicas (127) a través de un efecto postsináptico directo. La administración directa de orexinas sobre el VTA provoca un aumento en la expresión de c-Fos en las neuronas dopaminérgicas de la región caudomedial del VTA (142). Dicha administración también incrementa los niveles de DA en el shell del NAc, pero no en el core ni en la PFC (141;143).

El uso crónico de las sustancias de abuso también influye en los sistemas de orexinas a través de cambios en la expresión del RNA mensajero (mRNA) para orexinas y en el de sus receptores. La administración crónica de nicotina está

asociada con la creciente expresión del mRNA para prepro-orexinas en el LH (152). La exposición crónica a anfetaminas resulta en un incremento de la expresión de c-Fos en las neuronas orexinérgicas (153). En contraposición, el tratamiento crónico con morfina (128;154;155) o con cocaína (156) no afecta los niveles de mRNA para orexinas, ni a la expresión de c-Fos en las neuronas productoras de orexinas del LH.

La evaluación de la implicación de las orexinas en la dependencia a drogas, mediante el bloqueo del receptor OxR1 con la administración de SB-334867, un antagonista selectivo del OxR1, ha sido probada con gran eficacia (158). Además, los ratones KO para orexinas, incapaces de expresar el gen prepro-orexinas, han sido útiles en la investigación sobre el papel de las orexinas en la respuesta locomotora y en la sensibilización a drogas. El paradigma de CPP se ha convertido en una técnica ampliamente utilizada para el estudio del comportamiento relacionado con la recompensa. Se ha demostrado que los animales que prefieren un medio, previamente asociado con comida, morfina o cocaína muestran un aumento de la expresión de c-Fos en las neuronas orexinérgicas (163). Incluso, la cantidad de c-Fos expresado en estas neuronas del LLH, está directamente relacionada con la amplitud del periodo de tiempo pasado en el medio asociado a la droga (163). La fase de condicionamiento en el paradigma de CPP asociado a morfina, también está relacionada con el incremento específico de la estimulación de las neuronas orexinérgicas del LLH (163).

Investigaciones previas sugieren la implicación de las orexinas en la formación de preferencia de plaza asociada al consumo de morfina. Lesiones bilaterales citotóxicas en el LH bloquearon la adquisición de la preferencia de plaza a morfina; además, lesiones unilaterales en el LH junto con la administración de SB-334867 directamente sobre el VTA del lado contrario a la lesión, también bloqueó el desarrollo de preferencia de plaza asociada a morfina (164). Todos estos datos sugieren que la actividad orexinérgica en el VTA es esencial para el desarrollo del CPP asociado a morfina (164).

El síndrome de abstinencia a sustancias de abuso también puede alterar el sistema orexinérgico, y el primer estudio que conectó las orexinas a este tipo de sustancias demostró la implicación de las orexinas en el síndrome de abstinencia

a morfina (128). La administración de naloxona o naltrexona precipita el síndrome de abstinencia a morfina, lo que induce la actividad del CRF (128) y la expresión de c-Fos en las neuronas orexinérgicas (128;154). Curiosamente, dicho incremento en la expresión de c-Fos se limitó al DMH y al PFA, mientras que no se observó en el LLH (154). El síndrome de abstinencia a morfina espontáneo aumenta el mRNA para orexinas en el LH (155). La activación de las neuronas de orexinas en respuesta a dicho síndrome parece ser específica de opioides, ya que no se observa en la abstinencia espontánea a cocaína (156). Como ha sido mencionado anteriormente, la acción de las orexinas en el VTA está relacionada con la recompensa generada durante el proceso del paradigma de CPP (164), pero en la respuesta al síndrome de abstinencia, el VTA no se ve alterado por el sistema orexinérgico (154). De esta forma, se queda expuesto cómo el papel que ejercen las orexinas durante la recompensa o durante la exposición a agentes estresantes está modulado por diferentes poblaciones orexinérgicas del LH, dando lugar a la activación de diferentes circuitos neuronales.

El sistema orexinérgico, con sus respectivas funciones tanto en el estrés como en el aprendizaje asociado a la recompensa y a la memoria, podría representar una importante diana para futuras terapias farmacológicas diseñadas para prevenir la recaída a las drogas.

3. OBJETIVOS Y MÉTODOS

Los objetivos del presente trabajo han sido:

1. Investigar los posibles cambios en la expresión de tirosina hidroxilasa (TH) total y en dos de sus formas fosforiladas: pSer40 y pSer31, durante el CPP inducido por morfina en el NAc, VTA y NTS. Para ello, los niveles de dichas formas de la enzima se cuantificaron mediante Western blot (WB) e inmunohistoquímica (IHQ), usando anticuerpos específicos; además, se cuantificaron la DA y NA, con sus respectivos metabolitos ácido dihiroxifenilacético (DOPAC) y 3-metoxi-4-hidroxifenil-etilenglicol (MHPG), en el NAc mediante cromatografía líquida de alta resolución (HPLC).

2. Evaluar el papel del CRF_1-R en: (i) las alteraciones comportamentales producidas por la administración de morfina, mediante el paradigma del CPP; (ii) la activación (evidenciada por la expresión de c-Fos; IHQ) de diferentes núcleos cerebrales implicados en la recompensa asociada a la administración de morfina; (iii) la respuesta del sistema dopaminérgico y noradrenérgico, durante el CPP inducido por morfina mediante la cuantificación del turnover de ambas catecolaminas por HPLC; y (iv) la actividad del eje HHA durante el CPP inducido por morfina, mediante la cuantificación de corticosterona en plasma (radioinmunoanálisis; RIA).

3. Evaluar la activación y la implicación del sistema orexinérgico en el síndrome de abstinencia a morfina. Para ello, en primer lugar se midieron los midieron los niveles de mRNA para orexina y la actividad de las neuronas orexinérgicas en el LH. Posteriormente, se administraron antagonistas de OxR1 (SB-334867) y de OxR2 (TCSOX229) y se cuantificaron los signos somáticos generados por dicho síndrome. Para obtener un índice representativo de todos los signos examinados durante el síndrome de abstinencia, se calculó un parámetro global ponderando cada signo por separado, tal y como describe Maldonado et al. (190). Por otra parte, se cuantificó la expresión de c-Fos en el sistema cerebral del estrés, tanto hipotalámico como extrahipotalámico, tras la administración de SB-334867 (IHQ) así como la expresión de mRNA para orexinas en el hipotálamo (PCR a tiempo real; qPCR).

4. Evaluar el papel de los receptores OxR1 y OxR2 en la activación del eje HHA producido durante el síndrome de abstinencia a morfina. Para ello, se valoró la actividad (expresión de c-Fos; IHQ) de las neuronas de CRF en la región parvocelular del PVN, así como los niveles de corticosterona en plasma en animales a los que se les indujo el síndrome de abstinencia a morfina, tratados previamente con los antagonistas respectivos (SB-334867 y TCSOX229), mediante un RIA.

4. RESULTADOS Y DISCUSIÓN

4.1. INCREMENTO DE LA FOSFORILACIÓN DE LA TIROSINA HIDROXILASA EN EL NAc Y EN EL NTS DURANTE EL CPP INDUCIDO POR MORFINA

El CPP es un importante paradigma que permite analizar el comportamiento de un individuo ante un estímulo de recompensa, generado bien por el consumo de sustancias de abuso, bien de forma natural. Los datos obtenidos en este estudio indican que en un rango de dosis de 0.5-8 mg/kg, la morfina produce un CPP para el lugar asociado a la droga alcanzando la máxima expresión al administrar una dosis de 6 mg/kg. Esta información, apoyada por investigaciones previas (196;198;199;200), demuestra que los efectos reforzadores positivos inducidos por morfina, a través de mecanismos de aprendizaje asociativo, están relacionados con el medio en el que dichos efectos se generan (201).

Es bien conocido cómo la preferencia de plaza inducida por morfina depende de la activación del sistema dopaminérgico mesolímbico (202). Nuestros resultados muestran un incremento en los niveles de DOPAC, de MHPG, del turnover de DA y del de NA en el NAc, después del CPP inducido por morfina. De acuerdo con estos datos se ha observado una elevación de DA y de NA en el NAc, paralela a los efectos de las dosis de morfina en la expresión de CPP (199).

Aunque la DA es el principal neurotransmisor implicado en los efectos reforzadores de opioides, se ha sugerido la implicación de la NA en la recompensa a estas sustancias (93;203-205). Nuestros resultados sugirieren un papel de la NA en la recompensa a opioides.

Los cambios en el estado de fosforilación de la TH, enzima limitante de la síntesis de catecolaminas, están profundamente implicados en la regulación de la síntesis de catecolaminas. En particular, la fosforilación de la Ser40 y de la Ser31 acelera la actividad de la TH (210). Los resultados de este estudio proporcionan evidencias de la fosforilación de la TH después del CPP inducido por morfina tanto en las neuronas catecolaminérgicas del NTS, como en las terminales que inervan el NAc. Se observaron niveles aumentados de la TH fosforilada en la

Ser40 en el NAc, mientras que en el NTS se obtuvieron niveles aumentados de la TH fosforilada tanto en Ser40 como en Ser31 tras el CPP inducido por morfina. Estos datos sugieren que la fosforilación de la TH en la Ser40 puede ser un importante modulador de la actividad de dicha enzima durante el CPP y que podría estar implicada en la regulación del turnover tanto de DA como de NA en el NAc de ratones condicionados a morfina.

En este trabajo se cuantificó, un elevado número de neuronas positivas para TH pSer31 en el NTS, sugiriendo que la fosforilación de la Ser31 podría ser el mecanismo de mantenimiento de la síntesis de catecolaminas para reemplazar aquellas liberadas en respuesta al CPP inducido por morfina. Nuestros resultados reflejan una expresión de TH total similar al comparar los animales que recibieron morfina con los que recibieron salino, tanto en el NTS como en el NAc. De acuerdo con un estudio previo (212), estos resultados sugieren que la fosforilación de la TH y en consecuencia su activación y la de la síntesis de catecolaminas, es independiente de la expresión de la proteína.

Como el sistema dopaminérgico mesolímbico es crítico para la inducción de los refuerzos positivos producidos por el consumo de sustancias de abuso, llama la atención encontrar un número similar de neuronas expresando tanto TH pSer40 como TH pSer31 en el VTA en animales tratados bien con morfina o con salino. Sin embargo, es posible que el CPP inducido por morfina genere cambios adaptativos en factores clave que controlan los niveles de DA en la sinapsis, y que estos cambios puedan producir alteraciones en los niveles de DA liberada en la sinapsis sin alterar la actividad de las neuronas dopaminérgicas del VTA. Un posible cambio adaptativo de este tipo sería la modificación en la activación del transportador de DA (DAT) (213).

En conclusión, este trabajo sugiere que el CPP inducido por morfina podría estimular la actividad de la TH y acelerar el turnover de DA y NA en el NAc a través de mecanismos que implican la fosforilación de este enzima. Estos resultados proporcionan un mecanismo adicional a través del cual el CPP inducido por morfina podría modular la fosforilación de la TH tanto en el NAc como en el NTS, una de las principales fuentes de NA para el NAc.

4.2. CRF MEDIA LA ACTIVACIÓN DEL SISTEMA DE RECOMPENSA DURANTE EL CPP INDUCIDO POR MORFINA

Es conocido que los opioides inducen un fuerte CPP sobre una amplia variedad de condiciones experimentales (220). En concordancia, nuestros resultados muestran cómo la administración de morfina induce a preferir el medio asociado a la administración de la droga. Diversos sustratos neurobiológicos están implicados en la recompensa que se experimenta tras la ingesta de drogas, aunque hay que resaltar la decisiva implicación del sistema dopaminérgico mesolímbico en los circuitos de recompensa. Este trabajo muestra cómo los ratones que recibieron morfina reaccionan con una activación del VTA y del NAc cuantificada mediante la expresión de c-Fos. Diversas hipótesis adjudican a la DA una importante función en la modulación del comportamiento a través de su liberación en el NAc asociada a un estímulo. Cuando se cuantificó el turnover de DA en el NAc, encontramos un aumento del ratio DOPAC/DA, lo que indica que el CPP inducido por morfina aumentó la transmisión dopaminérgica en el sistema cerebral de recompensa. El incremento de la liberación de DA en el NAc ha sido relacionado con la capacidad de un estímulo condicionado de generar excitación y aprendizaje (18).

El VTA recibe inervaciones de CRF desde BNST, CeA y PVN (180;221), y ambos, VTA y NAc expresan elevados niveles de mRNA para CRF_1-R, pero no de CRF_2-R en roedores (106). Tanto el VTA como el NAc juegan un papel principal en los procesos de recompensa y en los efectos que generan las sustancias de abuso, por lo que dichas inervaciones de CRF podrían indicar que este neurotransmisor interviene en los efectos reforzadores de las sustancias adictivas, en concreto de opioides. Avalan esta hipótesis los datos aportados, los cuales muestran que la administración de CP-154,526 30 min antes de la administración de morfina bloquea la adquisición de preferencia por el entorno asociado a la droga. Anteriormente, ha sido demostrado que la administración de CP-154,526 bloquea el CPP a cocaína, mientras que la antisauvagina-30 (un antagonista selectivo del CRF_2-R) no lo bloquea (222). De esta forma, los datos muestran cómo el CRF_1-R es esencial para la adquisición del CPP inducido por sustancias de abuso, tales como cocaína o morfina. Paralelamente, también se observa que el antagonismo del CRF_1-R bloqueó la activación del VTA y del NAc en los ratones que recibieron

morfina, por lo que se esperaba un descenso en la neurotransmisión dopaminérgica durante el CPP; sin embargo, la administración de CP-154,526 previa a la sesión de condicionamiento no tuvo efecto sobre el aumento del turnover de DA en el NAc generado durante el CPP a morfina. Por otro lado, ya ha sido descrito cómo la DA se libera concretamente en el shell del NAc, pero no en el core (18). Además, en el VTA coexisten neuronas dopaminérgicas y no dopaminérgicas (GABAérgicas y glutamatérgicas) (223). La técnica empleada para la disección del NAc no discriminó entre el shell y el core del NAc, por lo que con nuestros resultados no se puede afirmar que las neuronas activadas en el VTA fueron las dopaminérgicas exclusivamente. Estos hechos podrían explicar los datos obtenidos sobre la expresión de c-Fos en VTA y en NAc y de la transmisión dopaminérgica en el sistema de recompensa durante el CPP inducido por morfina.

Hay evidencias apoyando que la NA influye en la respuesta de los circuitos de recompensa al consumo de una droga a través de mecanismos positivos o negativos. Investigaciones recientes, indican la necesidad de la función noradrenérgica para la adquisición de CPP inducido por morfina (93). Apoyando esta hipótesis, nuestros estudios demuestran que el CPP inducido por morfina genera un incremento de la expresión de c-Fos en los principales núcleos noradrenérgicos (NTS y LC), así como un incremento en la transmisión noradrenérgica en el NAc. Es sabido que la actividad de ambos, LC y NTS, es incrementada por el CRF (94), y que el CRF_1-R ha sido localizado en ambos núcleos (94;224). Nuestro trabajo muestra que el bloqueo del CRF_1-R antagoniza el incremento en la expresión de c-Fos provocado por el CPP inducido por morfina en el LC, pero no en el NTS. Además, la administración de CP-154,526 mantuvo la neurotransmisión de NA en el NAc a niveles basales en los animales que recibieron morfina. Estos resultados podrían indicar que las proyecciones noradrenérgicas al NAc implicadas en la recompensa a morfina provienen del LC. Sin embargo, previamente se ha demostrado que aunque tanto el LC como el NTS proyectan al NAc (225), solo las proyecciones desde las neuronas del NTS liberan NA en el shell del NAc (226). Ciertamente, hay que tener en cuenta, que en los presentes experimentos no se ha usado un marcador de neuronas noradrenérgicas para identificar las poblaciones neuronales que activan estas áreas, y dado que la cuantificación del turnover de NA disminuye al tratar los

animales con morfina y CP-154,526, quizás podríamos sugerir que el bloqueo del CRF_1-R antagonizó la activación de neuronas noradrenérgicas durante el CPP inducido por morfina en el NTS, lo que tendría como resultado una disminución de la neurotransmisión noradrenérgica en el NAc.

Evidencias recientes sugieren que las proyecciones orexinérgicas desde el LH hasta el VTA son esenciales para la expresión de CPP inducido por morfina (141;163;227;228). Diversos estudios previos proponen que las neuronas orexinérgicas tienen diferentes funciones. Las localizadas en el DMH y en el PFA controlan la excitación y modulan la respuesta al estrés, mientras que las localizadas en el LLH se han relacionado con los refuerzos positivos de los efectos de las drogas (129). De acuerdo con ello, el presente trabajo muestra una activación de las neuronas orexinérgicas en el LLH, pero no en el DMH ni en el PFA de los animales tratados con morfina. Otros estudios han demostrado la presencia de terminaciones nerviosas de neuronas de CRF en las células orexinérgicas del LH, así como que el CRF activa la secreción de orexinas (176). Nuestros resultados muestran cómo tras antagonizar el CRF_1-R, se observa una menor expresión de c-Fos en las tres subdivisiones del LH. Esto indica que la función del CRF_1-R no es exclusiva del LLH, y que el CRF podría actuar también en el DMH y en el PFA durante el CPP.

Es conocido que la administración aguda de sustancias de abuso activa el eje HHA, lo que se ha relacionado también con los efectos de recompensa de las drogas. Partiendo de esta afirmación, el presente trabajo muestra cómo el CPP inducido por morfina genera un incremento de la liberación de corticosterona. CRF_1-R es el tipo de receptor mayoritario presente en las células pituitarias corticotropas (230;231), las cuales sintetizan y liberan ACTH, la cual a su vez estimula a la glándula adrenal y la secreción de GC al torrente sanguíneo. En nuestro trabajo, el bloqueo de CRF_1-R, no solo no disminuyó la liberación de corticosterona en los animales que recibieron morfina, sino que aumentó los niveles de GC plasmáticos de los ratones que expresaron CPP inducido por dicha sustancia. Estudios recientes de éste y otros laboratorios han demostrado que la administración de antagonistas de ambos tipos de receptores de CRF no interfieren en el incremento de GC plasmáticos inducido por opioides (224;232). Diferentes procesos celulares y moleculares pueden estar implicados en el

incremento de los niveles de corticosterona tras la administración del antagonista del CRF_1-R en animales condicionados a morfina. Estos mecanismos podrían incluir la acción del neuropéptido arginina-vasopresina (AVP) en la glándula hipofisaria como respuesta al bloqueo del CRF_1-R, resultando en una hiperactivación del eje HHA.

4.3. IMPLICACIÓN DE LA Ox-A EN LA RESPUESTA DEL SISTEMA CEREBRAL DEL ESTRÉS AL SÍNDROME DE ABSTINENCIA A MORFINA

De acuerdo con los datos anteriores como los de Georgescu et al. (2003) (128), Sharf et al. (2008) (154)o Plaza-Zabala et al. (2012) (192), nuestros resultados muestran el importante papel de las orexinas, a través del OxR1 y el OxR2, en la expresión somática de los signos característicos del síndrome de abstinencia a drogas, así como cambios moleculares del sistema cerebral del estrés inducidos por la abstinencia a morfina. La administración de naloxona a ratas dependientes de morfina da lugar a la expresión de dichos síntomas somáticos de una forma evidente. Nuestros resultados muestran cómo el bloqueo tanto de los OxR1 como de los OxR2, inhibió de forma significativa la expresión de la mayoría de estos signos sugiriendo que la activación de las vías orexinérgicas podría modular la expresión somática de la abstinencia a opiáceos. Estos datos están de acuerdos con experimentos previos en los que tanto la administración local de SB-334867 en el LC durante la abstinencia a morfina (233), como el bloqueo del OxR2 durante la abstinencia a nicotina (192)(Plaza-Zabala et al (2012?) atenúan la manifestación del síndrome de abstinencia a las respectivas drogas.

Estudios previos sobre ratones portadores del T-LacZ orexinérgico indicaron que durante el síndrome de abstinencia, se estimula su expresión génica. Mediante la aplicación de q-PCR, nuestros resultados demuestran la activación de la expresión génica de Ox-A en el LH durante el síndrome de abstinencia a morfina. Por otra parte, dicho síndrome, inducido por la administración de naloxona activó las neuronas orexinérgicas de las tres

subpoblaciones del LH tal y cómo se aprecia en el análisis inmunocitoquímico realizado para c-Fos.

Nuestro estudio también muestra una hiperactivación del sistema cerebral del estrés durante el síndrome de abstinencia a morfina, que se atenúa con la administración de SB-334867. Debido a que el PVN recibe proyecciones orexinérgicas desde el PFA y el LLH (234), siendo ambas subpoblaciones del LH, nuestros datos sugieren la implicación de las neuronas de Ox-A procedentes de las tres áreas del LH en la activación del sistema cerebral del estrés durante el síndrome de abstinencia a opioides.

La actividad tanto del eje HHA, como de la amígdala extendida se ve alterada durante la administración de sustancias de abuso (9;82;103). Además, el síndrome de abstinencia agudo incrementa la actividad de las vías noradrenérgicas que inervan el CeA y el PVN (102;103;235). Todos estos procesos están implicados parcialmente en los refuerzos negativos característicos del síndrome de abstinencia. Nuestros resultados muestran el aumento de la expresión de c-Fos durante la abstinencia a morfina en los núcleos de la amígdala extendida, PVN y NTS. Con la administración de SB-334867, la activación de la amígdala extendida y del PVN se ve atenuada durante la abstinencia a morfina, sugiriendo que el sistema orexinérgico a través de la vía de OxR1, podría representar un papel importante en la modulación de la actividad del sistema cerebral del estrés durante el síndrome de abstinencia a opioides. La presencia de fibras orexinérgicas, así como de OxR1 y de OxR2 en todos los núcleos estudiados en este trabajo y en otros (110), refuerzan esta hipótesis (119), pudiendo relacionar así el sistema orexinérgico con otros sistemas de neurotransmisores encargados de modular el sistema cerebral del estrés, como CRF, NA y dyn.

Los resultados de este trabajo muestran que el síndrome de abstinencia a morfina induce un incremento de la secreción de corticosterona, la cual no se ve atenuada con la administración de SB334867, ni con la administración de TCSOX229. Primero se examinó la respuesta de las neuronas de CRF localizadas en el PVN durante la abstinencia a morfina, y se observó una respuesta similar entre los ratones que recibieron vehículo y los que recibieron el antagonista para

OxR1 previamente a la administración de naloxona. Es sabido que las Ox-A estimulan las neuronas de CRF y de AVP del PVN (182)(Samson et al, Peptides, 2002), induciendo la síntesis de ambos péptidos (245)(Al_Barazanjani et al, J Neuroendocrinol, 2001). Como se observó una disminución de la expresión de c-Fos en el PVN tras la administración de SB-334867 durante el síndrome de abstinencia a morfina, y nuestros resultados muestran que dicha disminución no se produce en las neuronas de CRF, se podría sugerir que la actividad de otras neuronas del PVN como las secretoras de AVP, son inhibidas con la presencia del antagonista de OxR1. Dado que el mRNA para OxR2 se expresa predominantemente en el PVN (119), y que la administración del antagonista de dicho receptor inhibe la secreción de ACTH inducida por Ox-A o por estrés (234), se podría postular que la actividad de la Ox-A en este nivel estaría mediada por el OxR2 basándonos en la afinidad de la molécula por sus receptores. De esta forma, sería fácil sugerir que la activación del OxR2 en el PVN sería la responsable de la activación de las neuronas de CRF y en consecuencia de la liberación de GC. Sin embargo, cuando se administró el antagonista de OxR2 observamos una hiperactividad de las neuronas de CRF en el PVN, así como similares niveles de corticosterona que en los animales control. Steiner et al. (238), recientemente, ha demostrado que la administración sistémica dual de antagonistas de OxR, no interfiere con la actividad endocrina del eje HHA, lo que podría indicar que la señalización endógena de orexinas no interfiere en la liberación de GC.

La amígdala extendida y sus sistemas de neurotransmisores han sido relacionados con los refuerzos tanto positivos como negativos generados por el consumo de sustancias de abuso (9). Previamente, ha sido descrito que la Ox-A tiene una actividad excitatoria en la CeA (147), y que esta actividad ocurre en neuronas de CRF (241). De acuerdo con ello, nuestros resultados muestran una mayor expresión de c-Fos en neuronas de CRF localizadas en el CeA durante el síndrome de abstinencia a morfina inducido por naloxona, y este aumento se atenúa con la administración de SB-334867.

Por otra parte, nuestros datos muestran que las neuronas de CRF localizadas en el BNST no se activaron tras la administración de naloxona a ratas dependientes de morfina, lo que concuerda con estudios previos paralelos (242), y

que las neuronas de CRF del PVN sí que se activaron significativamente con el mismo tratamiento, aunque la administración de SB-334867 no alteró la respuesta de las neuronas de CRF de ninguno de los núcleos. Hay que tener en cuenta que tanto el BNST como el PVN reciben una importante inervación noradrenérgica del NTS (240; 243), y que dicha activación en ratas abstinentes a morfina no es atenuada con la administración del antagonista de OxR1.

5. CONCLUSIONES

1. Nuestro estudio demuestra que durante el CPP inducido por morfina se produce una activación de diferentes áreas cerebrales catecolaminérgicas y no catecolaminérgicas, relacionadas con la recompensa.

2. El CPP inducido por morfina podría estimular la actividad de TH y acelerar el turnover de DA y NA en el NAc, a través de un mecanismo que implicaría la fosforilación de TH. Nuestros resultados aportan mecanismos adicionales por los que el CPP inducido por morfina podría modular la fosforilación de TH en el NAc y NTS, una de las principales fuentes de NA para el NAc.

3. Este estudio aporta datos que destacan la importante implicación del CRF, a través del CRF1-R, en los refuerzos positivos generados por la administración de morfina, lo que podría implicar un futuro potencial terapéutico, como ha sido propuesto en otros estudios.

4. El síndrome de abstinencia a morfina inducido por la administración de naloxona, activa las neuronas orexinérgicas de las tres subdivisiones del LH, concomitantemente con un aumento en la transcripción del mRNA para Ox-A y en la transmisión orexinérgica al sistema cerebral del estrés.

5. Nuestros datos revelan el papel crítico que juega el sistema de orexinas en los síntomas físicos que se ponen de manifiesto durante el síndrome de abstinencia a opiáceos.

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