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Ainhoa Plaza-Zabala

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Thesis directors:

Dr. Fernando Berrendero

Dr. Rafael Maldonado

Departament de Ciències Experimentals i de la Salut

UNIVERSITAT POMPEU FABRA

Abstract

Hypocretin-1 and hypocretin-2, also known as orexin-A and orexin-B, are 2 neuropeptides that are exclusively expressed by a small subset of neurons of the lateral hypothalamic area. Despite their restricted expression pattern, hypocretin-containing axons project widely throughout the brain and exert their physiological functions acting on 2 G protein coupled receptors, hypocretin/orexin receptor-1 and hypocretin/orexin receptor-2. Initially, the hypocretin system was related to the regulation of sleep/wake cycles and feeding behavior. Nevertheless, a growing body of evidence has accumulated over the last decade indicating a role for these neuropeptides in drug addiction. In the present thesis, we have evaluated the involvement of hypocretin transmission in the addictive properties of nicotine, the main psychoactive component that sustains tobacco addiction, by using behavioral and biochemical approaches. Our results indicate that hypocretin peptides, mainly through their actions upon hypocretin receptor-1, influence the severity of nicotine withdrawal and modulate the relapse to nicotine-seeking after prolonged periods of abstinence. Given the importance of withdrawal and relapse on t he pathophysiology of nicotine addiction, we propose hypocretin receptor-1 as a promising therapeutic target for the development of novel smoking cessation therapies.

Resumen

Las hipocretinas 1 y 2, también conocidas como orexinas A y B, son 2 neuropéptidos que se expresan exclusivamente en una pequeña población neuronal del área hipotalámica lateral. A pesar de su patrón de expresión restringido, los axones de las neuronas de hipocretina proyectan a lo largo del sistema nervioso central y ejercen sus acciones fisiológicas mediante la unión a 2 receptores acoplados a proteínas G, el receptor de hipocretina/orexina-1 y

el receptor de hipocretina/orexina-2. Inicialmente, las hipocretinas se relacionaron con la regulación de la alimentación y de los ciclos de sueño y vigilia. Sin embargo, durante la última década se ha descrito que estos neuropéptidos participan en el desarrollo de la adicción a drogas de abuso. En esta tesis, hemos evaluado el papel de las hipocretinas y sus receptores en las propiedades adictivas de la nicotina, el principal componente psicoactivo que mantiene la adicción al tabaco, mediante el uso de técnicas comportamentales y bioquímicas. Nuestros resultados indican que las hipocretinas, principalmente mediante su unión al receptor de hipocretina-1, regulan el síndrome de abstinencia a la nicotina y modulan la recaída a la búsqueda de nicotina después de largos períodos de abstinencia. Dado el papel que la abstinencia y la recaída juegan en el mantenimiento de la adicción a la nicotina, los hallazgos de la presente tesis proponen el receptor de hipocretina-1 como una posible diana terapéutica para el desarrollo de nuevos tratamientos para la adicción al tabaco.

INDEX

Abbreviations

Abbreviations

VTA: ventral tegmental area

NAc: nucleus accumbens

DA: dopamine

GABA: γ-aminobutyric acid

CeA: central nucleus of the amygdala

BNST: bed nucleus of the stria terminalis

CRF: corticotrophin-releasing factor

PFC: prefrontal cortex

ICSS: intracranial electric self-stimulation

CPP: conditioned place preference

CPA: conditioned place aversion

nAchR: nicotinic acetylcholine receptor

CNS: central nervous system

NMDA: N-methyl-D-aspartate

mGlu receptor: metabotropic glutamate receptor

AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate

MOR: mu opioid receptor

DOR: delta opioid receptor

KOR: kappa opioid receptor

NRT: nicotine replacement therapy

Hcrtr-1: hypocretin receptor-1

Hcrtr-2: hypocretin receptor-2

PKC: protein kinase C

PFA: perifornical area

DMH: dorsomedial hypothalamus

LH: lateral hypothalamus

PVT: paraventricular nucleus of the thalamus

HPA: hypothalamic-pituitary-adrenal axis

PVN: paraventricular nucleus of the hypothalamus

AVP: arginine-vasopressin

MAPK: mitogen-activated protein kinase

INTRODUCTION

1. Addiction

1.1. Drug addiction: a chronic brain disease

Drug addiction is a chronic brain disease characterized by compulsion to seek and take the drug, loss of control in limiting intake, the emergence of a negative emotional state (e.g. dysphoria, irritability, anxiety) reflecting a motivational withdrawal syndrome when access to the drug is prevented, and the relapse to drug-seeking even after long periods of abstinence (Koob and Le Moal, 1997; Koob and Le Moal, 2008a). Addicted individuals show an intense desire for the drug, with an impaired ability to control the urges to take the drug even at the expense of serious adverse consequences.

Addiction to drugs of abuse has been traditionally underappreciated as a disease rooted in neurophathology (O'Brien, 2003). Although the aberrant behavioral manifestations of addiction have long been viewed as bad "choices", preclinical studies in animal models and clinical studies using imaging technology have demonstrated that addiction is a chronic brain disease. Indeed, current views acknowledge that drug addiction is based on pathological changes in brain function produced by repeated pharmacological insult to specific brain circuits by drugs of abuse (Kalivas and O'Brien, 2008). Hence, addictive drugs strongly affect the function of brain structures that are involved in learning processes and behavioral adaptations to important environmental stimuli, such as how to best approach rewards like food and sex, or to avoid dangerous situations (Kelley, 2004; Everitt and Robins, 2005). The repeated stimulation of motivational circuitries by addictive drugs impairs the development of behavioral strategies towards biological stimuli in favor of progressively greater orientation of behavior towards drug-seeking and drug-taking (Kalivas and Volkow, 2011). These adaptive changes are long-lasting and give rise to the repeated relapse to drug-taking that characterizes addiction.

Although many people experience with both licit and illicit drugs over their lifetime, not all individuals become addicted. Indeed, it is estimated that the percentage of consumers that develop addiction as a function of ever having tried a drug varies from approximately 9% for marijuana to 31% for tobacco (Anthony et al, 1994). Accordingly, 3 stages of drug use have been differentiated: (1) the occasional, controlled or social use, (2) drug abuse or harmful use, and (3) drug addiction. A major goal of current neurobiological research is to understand the progressive neuroadaptive changes that contribute to the transition from occasional drug use to the chronic drug-dependent state known as addiction (Koob and Volkow, 2010).

An individual's propensity to progress through drug use to misuse, and the development of addiction depends on the interplay of a broad range of vulnerability factors (Swendsen and Le Moal, 2011) (Figure 1). Similarly to other complex diseases, addiction has a multi-factorial and polygenic component that involves the interplay between multiple genes and distinct environmental variables (Enoch, 2012; Swendsen and Le Moal, 2011). Indeed, a large number of genes (Ducci and Goldman, 2012; Bierut, 2011) and environmental settings (Swendsen and Le Moal, 2011) have strongly been associated with addiction susceptibility. Among the most important ones are found the comorbidity with other psychopathological conditions, temperamental and personality traits, genetic factors involving drug sensitivity and metabolism, developmental factors such as age of onset, socioeconomic status, stress and adverse life events (Koob and Le Moal, 2008b). Although addiction presumably arises from complex interactions between all these factors at distinct levels during the life, it is considered that environmental factors have a st ronger effect on initiation, whereas biologic/genetic factors play a larger role in the transition from regular use to the development of addiction (Vink et al, 2005).

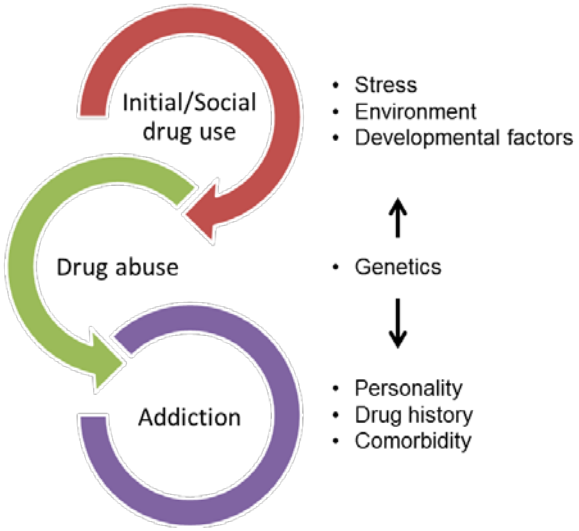

Figure 1. Stages of drug addiction. Vulnerability factors that affect the transition through different stages of drug addiction. Stress, developmental factors and environment are thought to have an early influence in the process. Comorbidity, personality traits and drug history are hypothesized to have a later influence. Genetics interact at all levels with the rest of the factors (adapted from Koob and Le Moal, 2008b).

The 4th edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV; APA, 1994) and revisions of the forthcoming DSM-5 (APA), which is expected to be released on May 2013, describe the clues for the diagnosis of addictive disorders. A remarkable innovation proposed for DSM-5 is that the diagnostic criteria will be organized according to substance or drug of abuse (http://www.dsm5.org), whereas on the currently available 4th edition of the psychiatric manual "core" diagnosis criteria for addiction are described regardless of the drug of abuse. The "core" features described for drug addiction in DSM-IV are summarized in Box 1.

Box 1.

The DSM-IV defines drug addiction as a a maladaptive pattern of substance use, leading to a clinically significant impairment or distress, as manifested by 3 (or more) of the following criteria, occurring at any time in the same 12-month period:

1. Tolerance symptoms, as defined by either of the following:
a. A need for increased amounts of the substance to achieve desired effects
b. Diminished effects with the same amount of the substance
2.Withdrawal, as manifested by either of the following:
a. Appearance of the characteristic withdrawal syndrome
b. The substance is taken to relieve or avoid withdrawal symptoms
3. The substance is taken in larger amounts or over longer periods than intended
4. Persistent desire or unsuccessful efforts to cut down or control substance use
5. A great deal of time spent in activities necessary to obtain or use the substance and recover from its effects
6. Important social, occupational, or recreational activities are given up or reduced because of substance use
7. Substance use is continued despite knowledge of having a persistent or recurrent physical or psychological problem that is likely to have been caused or exacerbated by the substance

Although addiction is often viewed as a unique disorder, the repetitive use of each drug of abuse will lead to different patterns of addiction. For instance, alcohol addicted individuals often show daily episodes or prolonged days of heavy drinking, whereas tobacco addiction displays a highly titrated intake of the drug only during waking hours. Similarly, abstinence in alcoholics induces a s evere somatic and emotional withdrawal syndrome, whereas tobacco abstinence is predominantly characterized by negative emotional states such as dysphoria, irritability and intense craving. The differences observed in the behavioral effects of drugs are mainly due to their pharmacological properties (Camí and Farre, 2003). Although all drugs of abuse are known to target common neurobiological substrates (Nestler, 2005), the mechanisms by which drugs act on these substrates differ, and lead to specific neurobiological effects that are translated to pathological behaviors depending on the type of drug used. Moreover, the potential for abuse of the various substances largely depends on dr ug kinetics and the route of administration. Hence, routes of administration and substances that quickly reach high levels in the brain usually show high abuse potential. The plasmatic half-life of the drug is also directly related to its addictive properties. Thus, high self-administration rates and early emergence of withdrawal symptoms have been observed in drugs with a rapid elimination rate (Camí and Farre, 2003).

1.2. Stages of addiction: from controlled drug use to compulsive drug-taking and relapse

The complex behaviors that define an addicted state arise from time-dependent, drug-induced neuroadaptations in specific brain circuits that contribute to the enduring nature of the addictive disorder. The development of addiction is often achieved through repeated social or controlled use of the drug, and typically involves many relatively short-term changes in brain chemistry and physiology based on the pharmacological effects of the drug (Nestler, 2005). Nevertheless, repeated drug insults eventually induce long-term changes in synaptic physiology of brain circuits regulating cognitive and emotional responding to important environmental stimuli (Kalivas, 2009), leading to the enduring nature of drug addiction through the emergence of chronic relapsing episodes.

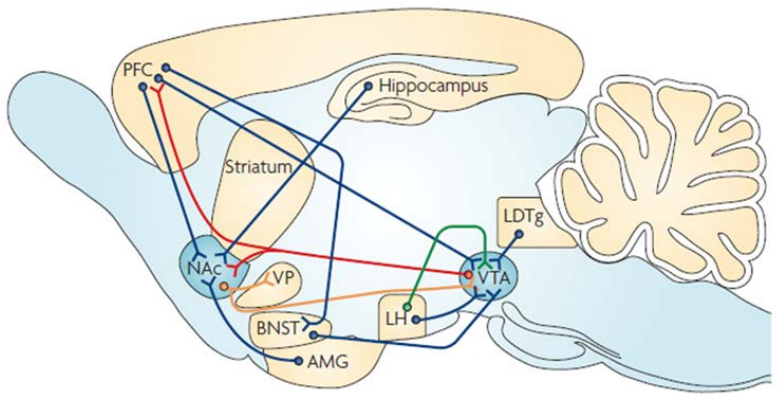

Figure 2. Mesocorticolimbic dopamine system. Simplified schema of the mesocorticolimbic system circuitry in rodent brain highlighting the major inputs to the nucleus accumbens (NAc) and ventral tegmental area (VTA) (glutamatergic projections blue, dopaminergic projections red, GABAergic projections orange, and hypocretin/orexin projections green). AMG, amygdala; BNST, bed nucleus of the stria terminalis; LTDg, laterodorsal tegmental nucleus; LH, lateral hypothalamus; PFC, prefrontal cortex; VP, ventral pallidum (Kauer and Malenka, 2007).

1.2.1. Acute drug-taking: reward and positive reinforcement

The initial acute rewarding effects of drugs of abuse are mainly achieved by the enhancement of the activity of the mesocorticolimbic circuit, which is composed of the ventral tegmental area (VTA), containing the dopaminergic cell bodies, and the terminal areas in the basal forebrain (nucleus accumbens (NAc), olfactory tubercle, amygdala and frontal and limbic cortices), where dopamine (DA) is released (Figure 2). All major drugs of abuse induce DA release in the terminal structures of the mesocorticolimbic system, where DA outflow reinforces reward-learning (Kelley et al, 2004). Thus, the release of DA by addictive drugs facilitates learning of drug-taking behaviors that are important for progressively shaping drug use into drug-seeking behaviors that are difficult to control (Kelley et al, 2004; Cardinal and Everitt, 2004; Di Chiara, 1999; Wise, 2004). However, DA transmission does not fully account for the acute reinforcing effects of all drugs of abuse. Other neurotransmitter/neuromodulator systems such as opioid peptides, γ-aminobutyric acid (GABA), glutamate, endocannabinoids, and serotonin play also key roles in the rewarding effects of addictive drugs (Koob and Le Moal, 2008b). Additionally, brain areas that are interconnected with the mesocorticolimbic DA system play essential roles in acute drug reinforcement. These regions include the amygdala and related structures of the so-called "extended-amygdala" (which comprise the central nucleus of the amygdala (CeA), the bed nucleus of the stria terminalis (BNST) and a transition zone in the medial (shell) subregion of the NAc), the hippocampus and the hypothalamus, among others (Nestler, 2005).

1.2.2. Chronic drug intake: tolerance, sensitization and the emergence of withdrawal

After chronic drug use, neuroadaptations occur in the mesocorticolimbic system in an attempt to restore brain reward homeostasis. Thus, baseline DA function is progressively reduced and a drug may be less effective in producing typical increases in dopaminergic transmission, a

phenomenon known as tolerance (Nestler, 2005). Individuals that have developed tolerance will need to increase the amount of drug taken in order to re-experience the intensity of the drug's initial effect, and this phenomenon may in some cases contribute to the negative emotional symptoms observed between drug exposures or upon drug withdrawal (Acquas et al, 1991). Tolerance might also be connected to the development of physical dependence (Aghajanian, 1978). Nevertheless, the physical symptoms that accompany withdrawal from some drugs of abuse are not etiologically related to the neuropathology of addiction (Volkow and Li, 2004). At the same time, some drugs also produce an opposing response of inverse tolerance, termed sensitization (Nestler, 2005). Thus, greater increases are observed in dopaminergic transmission in response to the drug or drug-conditioned cues (Robinson and Berridge, 2000; Everitt and Wolf, 2002; Vezina and Leyton, 2009). Sensitization is associated with the emergence of glutamatergic synaptic plasticity mechanisms in mesocorticolimbic brain circuitry. Thus, sensitization can last long after drug taking ceases and may relate to drug craving and relapse.

Although tolerance and sensitization can concurrently exist and likely involve different properties of the same dopaminergic neurocircuits, long-term drug use is characterized by the appearance of an aversive state when drug intake is withheld, that results in continued drug use as a means of avoiding the negative consequences of drug abstinence (Koob and Le Moal, 1997; Koob and Le Moal, 2008a). Acute withdrawal from all major drugs of abuse is characterized by a si gnificant decrease in mesocorticolimbic dopaminergic activity (George et al, 2012), and the consequent decrease in accumbal DA transmission contributes to the anhedonia experienced by drug-abstinent individuals. Activation of brain stress systems is also critical for the negative affective state that arises upon cessation of drug intake. Thus, elevations of extracellular corticotrophin-releasing factor (CRF) levels in the CeA are typically observed during acute withdrawal from all major drugs of

abuse (Koob, 2008). Therefore, although the positive reinforcing effects of abused drugs are critical for the initiation of drug consumption, negative reinforcing effects are probably more important for maintaining drug use once addiction has been established.

1.2.3. Long-term abstinence: relapse to drug-seeking and taking

The act of engaging drug-seeking following a period of abstinence is termed relapse, and vulnerability to relapse endures for many years even in the absence of repeated drug intake. Three stimuli have been identified to trigger relapse in humans: (1) the re-exposure to drugs of abuse (De Wit, 1996), (2) drug-associated environmental cues (Carter and Tiffany, 1999), and (3) stressful situations (Shiffman et al, 1996). In contrast to the acute stimulation of DA transmission produced by drugs of abuse, the enduring vulnerability to relapse arises from pervasive, long-term neurodaptations in the corticostriatal glutamatergic circuitry in which the DA axon terminals are embedded (Kalivas, 2009). Glutamatergic inputs from cortex and allocortex (e.g. amygdala and hippocampus) into the striatal motor circuit (including the NAc) are critical for the execution of learned behaviors, and drug-induced changes in synaptic plasticity mechanisms of this neuronal circuitry underlie the maladaptive relapse to drug-taking that characterizes addiction (Kalivas and Volkow, 2011). Furthermore, as a behavior is repeatedly executed and the individual continues relapsing recurrently, the role of glutamate projecting from the prefrontal cortex (PFC) and amygdala into the NAc becomes less important in favor of glutamate projecting from sensory motor cortical areas to the dorsal striatum (Everitt and Robbins, 2005). This transition from prefrontal circuitry to habit motor circuitry is thought to further promote loss of control and compulsive relapse.

1.3. Behavioral models to evaluate drug addiction

Whereas clinical research in human addicts has been helpful to elucidate the extent, demographics and the stages of addiction, much of the recent progress in understanding the neurobiology of addiction has derived from a variety of increasingly sophisticated animal models. Although no animal model of addiction totally emulates the human condition, critical features of the process of drug addiction can be reliably measured in animal studies. Examples of models able to measure these specific features include the intracranial electric self-stimulation paradigms, the place conditioning methods, and the self-administration techniques, among others (Sanchís-Segura and Spanagel, 2006). These experimental models have long been used to characterize the neurobiological substrates underlying the rewarding effects of drugs of abuse and, in some cases, the aversive aspects of drug withdrawal. In addition, over the last decades, some of them have been adapted to study some features related to drug relapse in human addicts. The majority of animal studies on drug relapse are based on reinstatement models (Davis and Smith, 1976; Shaham et al, 2003; Epstein et al, 2006). Reinstatement refers to the recovery of a learned response that occurs when a subject is exposed to some particular stimuli after the extinction of such a response (Bouton and Swartzentruber, 1991).

1.3.1. Intracranial electric self-stimulation

Early work using the intracranial electric self-stimulation (ICSS) paradigm was fundamental for the identification of the brain reward circuitry (Olds and Milner, 1954). Although reward self-stimulation involves widespread brain circuits, the most sensitive sites involve the trajectory of the medial forebrain bundle that connects the VTA to the basal forebrain (Olds and Milner, 1954). In the ICSS model, animals previously implanted with intracranial electrodes into reward-related brain areas are trained to maintain an operant behavior to obtain an electric pulse through these electrodes (Figure 3). During these sessions, the threshold of the minimal current needed

to promote intracranial self-stimulation is estimated. Typically, rewarding stimuli such as drugs of abuse decrease the self-stimulation threshold, whereas aversive drugs or stimuli, such as drug withdrawal, elevate the threshold for self-stimulation (Markou et al, 1993). Thus, ICSS methods are useful to investigate drug reward and withdrawal, but have not been used as animal models of reinstatement of drug-seeking behavior.

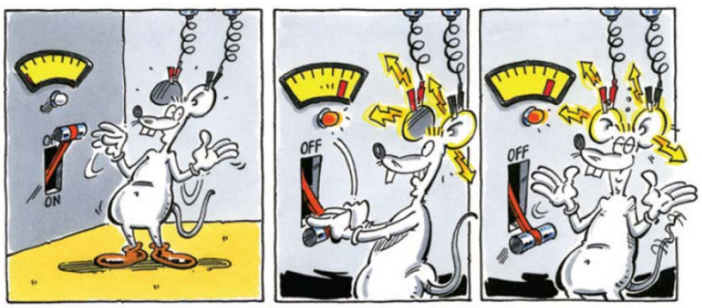

Figure 3. Intracranial self-stimulation paradigm (adapted from Sanchís-Segura and Spanagel, 2006).

1.3.2. Conditioned place preference and conditioned place aversion

In the place conditioning models, animals are exposed to an apparatus generally consisting on 2 initially neutral environments that can differ in characteristics, such as colour, texture, odour and lighting (Bardo and Bevins, 2000) during 3 consecutive experimental phases (Figure 4). Initially, animals are allowed to freely explore the 2 distinct environments and the time spent in each compartment is recorded. In the conditioning phase, animals are exposed to one environment after drug pretreatment, whereas the other compartment is paired with vehicle pretreatment. After several conditioning sessions, animals associate the drug to a specific environment that now acts as a conditioned stimulus. During the test day, the animals (usually in a drug-free state) are allowed free access to the apparatus and the preference/avoidance for 1 of the 2 environments is evaluated. Results are usually expressed with a score calculated as the difference of the time spent in the drug-paired compartment

during the post-conditioning and the pre-conditioning phase. A drug with rewarding properties will typically induce place preference (positive score), whereas a d rug with aversive effects or withdrawal from chronic drug administration will mainly produce place aversion (negative score). Although drug consumption in humans can induce conditioned approach/avoidance to specific drug-related stimuli, conditioned place preference (CPP) and conditioned place aversion (CPA) are not intended to model any particular feature of human behavior (Sanchís-Segura and Spanagel, 2006). These paradigms mainly represent an indirect measure of the rewarding or aversive effects of a drug by measuring the response of the animal towards the conditioned stimulus. Two variables with different implications are usually analyzed using this paradigm: the acquisition and the expression of place preference. The acquisition phase has been proposed to be mainly related to learning and memory mechanisms, whereas the expression phase would be more linked to incentive motivation, memory recall or sign tracking.

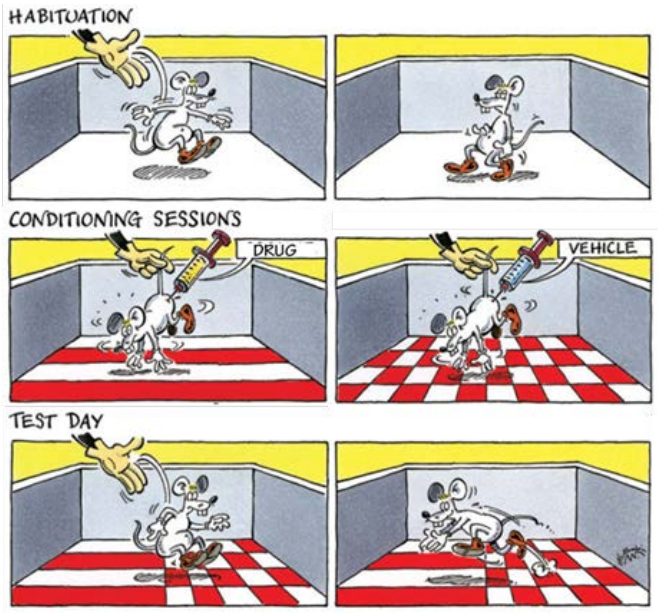

Figure 4. Conditioned place preference paradigm (adapted from Sanchís-Segura and Spanagel, 2006).

During the past decade, drug reinstatement procedures have also been developed using the CPP model (Aguilar et al, 2009), suggesting that this paradigm might also serve in some particular circumstances for modeling relapse in human addicts. Reinstatement studies usually involve an extinction phase after the acquisition period. In this particular model, extinction is performed by exposing the animals to the previously drug-paired context while in a drug-free state (Aguilar et al, 2009). Using this model, an extinguished CPP has been shown to be robustly reinstated by a non-contingent administration of a drug or by exposure to stressful stimuli (Aguilar et al, 2009). However, some of the effects obtained in the place conditioning paradigms may reflect state-dependent learning due to discriminative properties of the test drug rather than rewarding effects (Tzschentke, 2007), which represents a limitation for the interpretation of the reinstatement models based on these paradigms.

1.3.3. Operant drug self-administration

Self-administration methods are considered to be the most reliable models in addiction research. The main advantage of this technique is that the animal self-administers the drug and experimenter intervention is minimal. Consequently, this technique is widely used in preclinical research to directly evaluate the primary reinforcing effects of drugs of abuse as well as relapse after prolonged periods of abstinence. Additionally, the neural chemistry and the anatomical substrates underlying drug self-administration are assumed to be similar in these experimental animal models and in human addicts. Therefore, these procedures seem to be adequate models to identify common neural mechanisms and search for useful strategies for the treatment of drug addiction (Sanchís-Segura and Spanagel, 2006).

In drug self-administration procedures, the reinforcer can be delivered by different routes of administration. Accordingly, intravenous, intraventricular, intracranial, intragastric and oral delivery of drugs sustain operant behavior. For intravenous drug self-administration studies, animals

are implanted with an intravenous catheter and are trained to self-administer the drug for various days in an operant box (Figure 5). Typically, the operant chamber consists of 2 manipulandi that transmit the operant response and devices that deliver the drug reinforcer. The manipulandi are defined as "active" or "inactive" and typically consist of levers or nose-pokes. The response in the active manipulandum is associated with the delivery of the drug whereas activation of the "inactive" manipulandum lacks any programmed consequences. Additionally, the active manipulandum can be paired with other stimuli (e.g. light or tone), which improves learning of the operant behavior. The most common schedules of reinforcement used in these studies are the fixed-ratio and the progressive-ratio programs. Under a fixed-ratio schedule, the drug is delivered each time a preselected number of responses are completed. In contrast, under a progressive-ratio schedule, the required ratio to deliver a drug increases following an arithmetic progression. When using the latter schedule, the "breaking-point" value is commonly measured, which refers to the highest response rate accomplished to obtain a single infusion of the drug. Thus, the "breaking-point" is considered to be a measure of the motivation of the animal to obtain the drug. The analysis of the patterns of self-administration using both the fixed-ratio and the progressive-ratio schedules of reinforcement provide valuable information about the different factors that determine drug consumption. Furthermore, the abuse potential of drugs in humans can be predicted by the presence or absence of intravenous drug self-administration in animals (Collins et al, 1984).

The study of relapse in animal models has been mainly performed using the drug self-administration reinstatement model. The main strength of this method is that reinstatement in laboratory animals is induced by the same 3 conditions reported to provoke relapse in humans. Accordingly, craving and relapse to drug intake in human addicts are mainly triggered by the re-exposure to the drug of abuse (De Wit, 1996), drug-associated cues (Carter

and Tiffany, 1999), and stressful situations (Shiffman et al, 1996), which are exactly the same stimuli used to induce reinstatement in animal models.

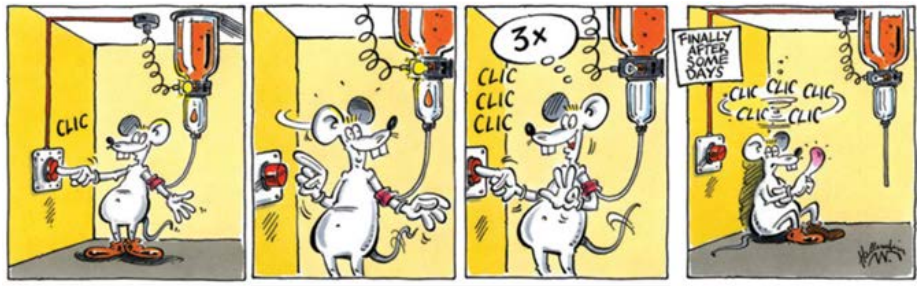

Figure 5. Intravenous drug self-administration (adapted from Sanchís-Segura and Spanagel, 2006).

The reinstatement model of relapse is divided in 3 experimental phases (Figure 6). Initially, animals are trained to acquire and maintain a drug self-administration behavior in operant conditioning chambers. Subsequently, drug-reinforced behavior is extinguished by substituting the drug solution with saline or by disconnecting the infusion pumps. Hence, during the extinction period animals perform the operant response without programmed consequences. After the extinction of drug-reinforced behavior, the ability of drug priming, drug-associated stimuli, and stress to trigger reinstatement of drug-seeking behavior is determined. During drug priming-induced reinstatement, a non-contingent injection of the drug is frequently administered in order to induce drug-seeking behavior. Discrete cues such as lights or tones associated to the reinforcer delivery during the training period have also been widely used to elicit drug-seeking behavior (See, 2002). In addition, contextual-stimuli have been applied to induce reinstatement. Under these situations, rodents are first trained to self-administer the drug in a context with specific cues that reveals the availability of the reinforcer, and the operant behavior is extinguished in a different context that contains other specific cues. The re-exposure of the animal to the drug-paired context

reinstates drug-seeking behavior (Crombag et al, 2008). On the other hand, stress-induced reinstatement of drug-seeking has been induced by intermittent footshock stimuli, restraint stress, pharmacological agents that induce stress (e.g. yohimbine), and food deprivation (Shalev et al, 2010).

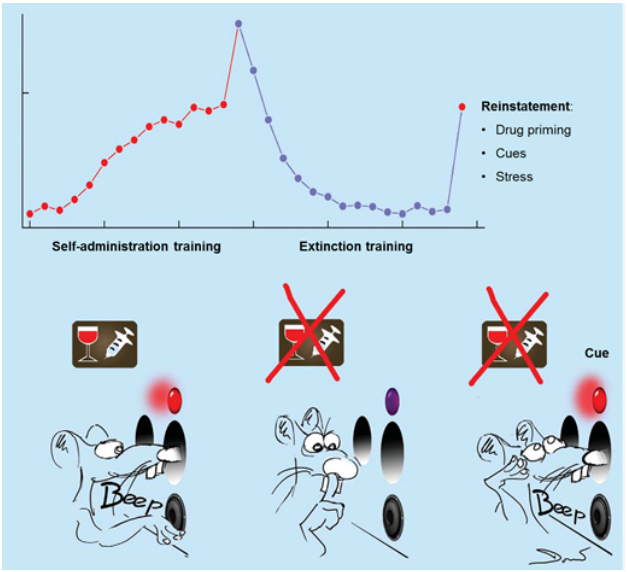

Figure 6. Drug self-administration reinstatement model. The reinstatement model of relapse is divided in 3 phases: acquisition of self-administration behavior, extinction and reinstatement. The cartoon above depicts a typical cue-induced reinstatement procedure. During drug priming- and stress-induced reinstatement of drug-seeking no cues are available (adapted from De Vries and Shoffelmeer, 2005).

2. Nicotine

2.1. Tobacco smoking: a public health concern

Tobacco use is the leading global cause of preventable and premature mortality in the world and poses a substantial and costly health burden (WHO, 2011). Annually, 6 million people die around the world from tobacco-associated illnesses and, if the present trend continues, by 2030 tobacco will kill 8 million people worldwide each year (WHO, 2011). Indeed, the chance for a lifelong smoker to die prematurely from a complication related to smoking is of 50% (Doll et al, 2004).

Tobacco smoke contains over 4000 chemicals, many of which have marked irritant, carcinogenic and toxic properties (Brennan et al, 2010). As a consequence, chronic tobacco use harms almost every organ of the body (USHHS, 2004), contributing to the development of lung and other cancers as well as cardiovascular and chronic lung disease, among others. Although most of the smoking-associated diseases appear after prolonged exposure to the toxic constituents of cigarette smoke, it is generally accepted that nicotine is the main component of tobacco responsible for its addictive properties (Grenhoff and Svenson, 1989; West, 1992; Stolerman and Jarvis, 1995). Indeed, commercial cigarettes are engineered to be optimal delivery devices for nicotine, and also contain other ingredients aimed to enhance or maintain nicotine availability and increase the addictive properties of nicotine (Rabinoff et al, 2007). Accordingly, several studies indicate that sugar combustion products, such as acetaldehyde (Talhout et al, 2007) and others with monoamine oxidase inhibitor properties that are present in tobacco smoke (Lewis et al, 2007), might act synergistically with nicotine, enhancing its addictive properties.

Smoking cessation produces immediate and significant health benefits and reduces the risk of serious disease within a few years of quitting (USHHS,

1990; Doll et al, 2004). However, the powerful addictive qualities of nicotine hinder smoking cessation, even for the people with a strong desire to quit. Indeed, 80% of smokers who attempt to quit on their own, relapse within the first month of abstinence and only 3% of them remain abstinent after 6 months (Benowitz, 2010). Although the currently available pharmacological and behavioral treatments do improve cessation rates, they exhibit limited efficacy and the rate of relapse to smoking remains high, revealing a need for more efficacious, alternative pharmacotherapies (Dwoskin et al, 2009).

2.2. Nicotine: pharmacological properties

Nicotine is a naturally occurring alkaloid present in tobacco (*Nicotiana tabacum*) leaves as well as other members of the solanaceous plant family, where it acts as a natural insecticide (Soloway, 1976; Dome et al, 2010). Chemically, nicotine is a tertiary amine composed by a pyridine and a pyrrolidine ring (Figure 7), and was first isolated from the tobacco plant in 1828 by German chemists Posselt and Reimann (Henningfield and Zeller, 2006). Tobacco leaves predominantly contain the pharmacologically active (S)-isomer of nicotine, (R)-nicotine only accounting for 0.1 to 0.3% of the total nicotine content (Armstrong et al, 1998).

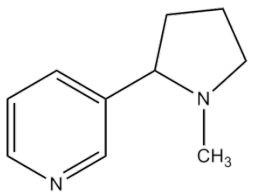

Figure 7. Chemical structure of nicotine [3-(1-methyl-2-pyrrolidinyl)pyridine].

2.2.1. Pharmacokinetics and metabolism

Nicotine is a weak base (pKa = 8.0) (Fowler, 1954) and its absorption across biological membranes depends on the pH of the medium (Hukkanen et al, 2005). In acidic environments, nicotine is in its ionized state and does not readily cross membranes. On the contrary, in more alkaline environments, deionized nicotine is rapidly absorbed. In humans, cigarette smoke (pH: 5.5-6) is inhaled into the lungs (pH: 7.4), where the alkaline fluid of the surface of the alveoli buffers the pH of smoke, allowing nicotine to be absorbed into the pulmonary circulation. After absorption, nicotine enters the bloodstream (pH: 7.4) and is distributed throughout body tissues, such as brain, liver, kidney, spleen and lungs (Hukkanen et al, 2005).

Tobacco smoking is the most reinforcing and addictive route of nicotine administration in humans. A puff of smoked tobacco delivers high levels of nicotine to the brain in 10 to 20 seconds, faster than with intravenous administration, producing rapid rewarding effects through the activation of the mesocorticolimbic DA system (Benowitz, 1990). Other forms of nicotine administration, including smokeless tobacco and nicotine replacement therapy, gradually increase blood levels of nicotine, resulting in lower nicotine levels on the brain and poor abuse liability. Therefore, when setting up an animal model of nicotine addiction, it is important to select a route of administration that approximates human exposure (Matta et al, 2007). The rapid rate of nicotine delivery by smoking can be mimicked by intravenous injections, which presents similar distribution kinetics (Hukkanen et al, 2005). In agreement, it has been shown that rats will readily self-administer nicotine if delivered as a rapidly injected intravenous bolus, rather than a slower infusion (Corrigall and Coen, 1989).

Nicotine is predominantly metabolized in the liver by the action of several enzymes (Hukkanen et al, 2005). In humans, approximately 70% of nicotine is converted to cotinine (Benowitz and Jacob, 1994). Similarly, primary nicotine metabolism yields high levels of cotinine in other animal

species, including mice, dogs, rabbits and monkeys (Matta et al, 2007). This metabolic step is mainly controlled by CYP2A6, a member of the cytochrome P450 family of liver enzymes (Hukkanen et al, 2005; Matta et al, 2007). Interestingly, polymorphisms in *CYP2a6* gene in humans play an important role in cigarette consumption (Schoedel et al, 2004; Minematsu et al, 2006), smoking cue-reactivity (Tang et al, 2012) and the vulnerability to develop nicotine addiction (Audrain-McGovern et al, 2007), suggesting that nicotine metabolism contributes to the addictive properties of nicotine.

The plasma half-life ($t_{1/2}$) of nicotine in humans averages about 2 hours (Hukkanen et al, 2005). Nevertheless, rodents display faster nicotine metabolism and are less sensitive to the effects of nicotine ($t_{1/2}$ rat: 45 min; $t_{1/2}$ mouse: 6-7 min). Thus, when using a murine model of nicotine addiction, it is important to adjust doses in order to get a similar response to humans (Matta et al, 2007).

2.2.2. Mechanism of action: nicotinic acetylcholine receptors

The addictive properties of nicotine arise from its capability to bind nicotinic acetylcholine receptors (nAchRs) in the brain. These receptors are highly conserved across species (Le Novere and Changeux, 1999) and are widely distributed throughout the central nervous system (CNS) (Figure 8), although they are also abundantly found in the peripheral nervous system (Gotti et al, 2009) and non-neuronal tissues (Albuquerque et al, 2009).

The nAchRs belong to the superfamily of ligand-gated ion channels and are endogenously activated by the neurotransmitter acetylcholine (Millar and Gotti, 2009). These ionotropic receptors form homo- and hetero-oligomeric receptors by the combination of 5 membrane-spanning subunits arranged around a central cationic pore that is permeable to Na^+, K^+, and sometimes, Ca^{2+} ions (Albuquerque et al, 2009) (Figure 9). Twelve different nAchR subunits have been identified in the mammalian CNS, each encoded by a single gene (Gotti et al, 2006). Thus, 9 genes encode for the subunits of α

subtype (α2-α10) and 3 genes encode for the subunits of β subtype (β2-β4). These receptor subunits arrange in diverse combinations to form a variety of pentameric nAchRs that exhibit different pharmacological and kinetic profiles (Taly et al, 2009) as well as different cellular and subcellular localization in the brain (Gotti et al, 2009).

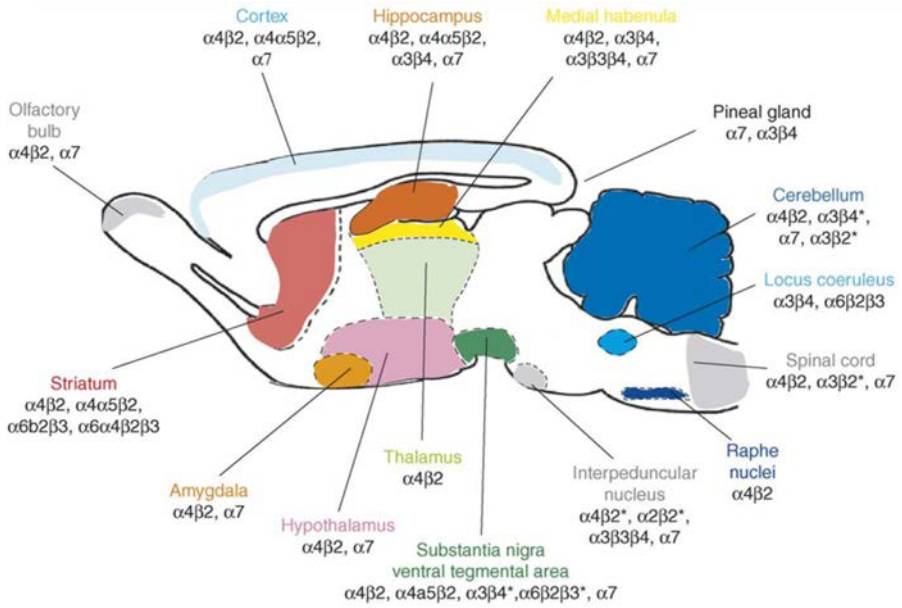

Figure 8. Distribution of nicotinic acetylcholine receptors in the rodent central nervous system (Gotti et al, 2006)**.**

At the present moment, 2 main subfamilies of neuronal nAchRs have been identified: α-bungarotoxin-sensitive and -insensitive receptors. The α-bungarotoxin-sensitive receptor subtype may form homo-oligomers (α7, α8, α9) or hetero-oligomers (α7α8 and α9α10) of α subunits (Gotti and Clementi, 2004). In turn, α-bungarotoxin-insensitive receptors contain both α (α2-α6) and β (β2-β4) subunits organized exclusively as hetero-oligomers, and exhibit high affinity for nicotinic agonists (Gotti and Clementi, 2004). Homo-oligomeric receptors contain 5 identical ligand-binding sites (one on each subunit interface), whereas hetero-oligomeric receptors exhibit 2 ligand-

binding sites at the interface between α and β subunits (Le Novere et al, 2002) (Figure 9). Thus, functional hetero-oligomeric receptors usually comprise 2 α subunits carrying the principal component of the ligand-binding site (α2, α3, α4 or α6), 2 β subunits carrying the complementary component of the ligand-binding site (β2 or β4) and a fifth subunit that does not participate in ligand binding (usually α5 or β3) (Gotti et al, 2009).

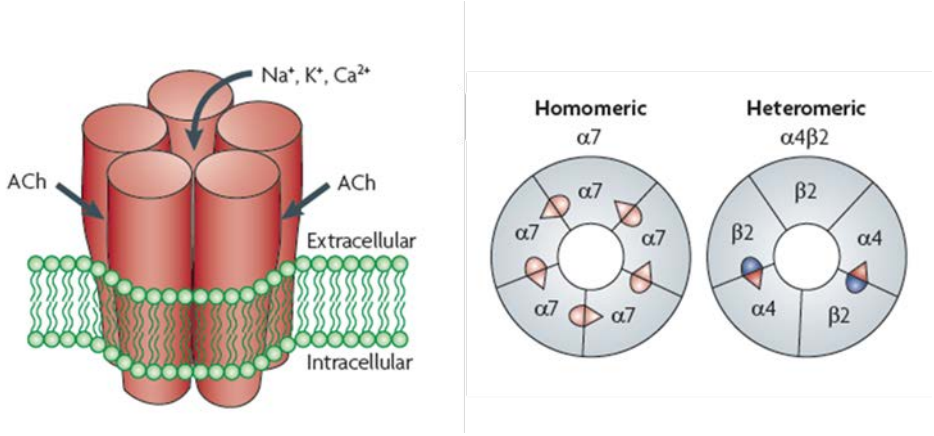

Figure 9. Structure of nicotinic acetylcholine receptors (nAchRs). Nicotinic receptors are transmembrane oligomers that consist of 5 equal (homomeric) or different (heteromeric) subunits. The most abundantly expressed nAchRs in the brain are the α7 homo-oligomeric and the α4β2 hetero-oligomeric. Homomeric receptors contain 5 ligand-binding sites whereas heteromeric receptors exhibit 2 nicotine-binding sites (adapted from Changeux, 2010).

Three main functional states have been described for nAchRs upon agonist binding: closed at rest (before agonist arrives), open pore (when conducting cations in response to agonist) and closed desensitized (unresponsive to agonist) (Dani and Heinemann, 1996). The likelihood of being in a particular state depends on many factors including the nAchR subtype, the agonist concentration and the rate of the agonist application (Dani and Heinemann, 1996). Under physiological conditions, when an agonist is released (e.g. acetylcholine), nAchRs are activated allowing ion flux, then desensitize (in the range of milliseconds) and subsequently recover

upon agonist removal (Giniatullin et al, 2005). However, chronic exposure to a low agonist concentration (e.g. brain nicotine levels in smokers) causes a gradual decrease in the rate of this ionic response (taking seconds to minutes) and produces significant desensitization, which stabilizes the receptor in a closed high-affinity state unresponsive to agonists (Picciotto et al, 2008; De Biasi and Dani, 2011). Long-term forms of nAchR inactivation could explain several aspects of nicotine addiction. Thus, tolerance and withdrawal might be a consequence of the slow recovery of nAchRs into functional states from different levels of desensitization and inactivation (Dani and Heinemann, 1996). Together with desensitization, long exposure (hours to days) to an agonist (e.g. nicotine in smokers) can produce compensatory changes in receptor sensitivity that eventually promote up-regulation of brain nAchRs (Giniatullin et al, 2005; Picciotto et al, 2008; De Biasi and Dani, 2011) (Figure 10).

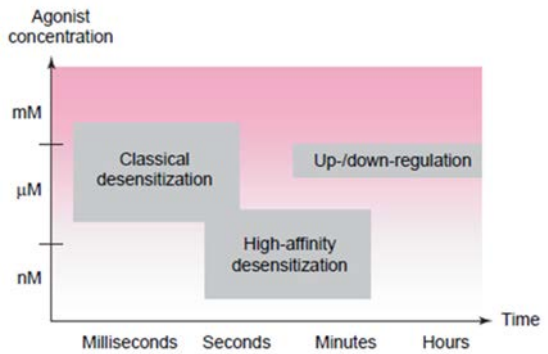

Figure 10. Nicotinic acetylcholine receptor desensitization depends on agonist concentration and exposition time (Giniatullin et al, 2005).

In parallel to the brain cholinergic pathways, nAchRs present a widespread distribution throughout the CNS, including brain areas associated with nicotine addiction (Tuesta et al, 2011) (Figure 11). The most abundant nAchR subtypes in the brain are the α7 homo-oligomer, characterized by a fast activation, a low affinity and a high Ca^{2+} permeability (Taly et al, 2009;

Changeux, 2010); and the α4β2* hetero-oligomer (the asterisk denotes a nAchR that contains the indicated subunits but the complete composition is unknown), which is typified by a high affinity and slow desensitization (Taly et al, 2009; Changeux, 2010), and accounts for more than 90% of the high affinity nAchRs in mammalian brain (Gotti et al, 2009, Alburquerque et al, 2009).

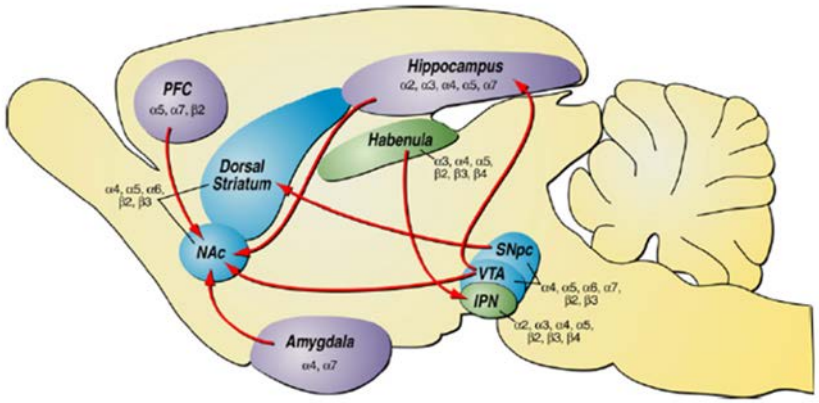

Figure 11. Expression of nicotinic acetylcholine receptor (nAchR) subunit mRNA and protein in brain regions relevant for nicotine reward and reinforcement. The red arrows indicate neuronal projections. There is dense expression of nAchRs in the ventral tegmental area (VTA) and substantia nigra pars compacta (SNpc), which are 2 major sites of ascending dopamine projections. Sites of dopaminergic input, including the nucleus accumbens (NAc), dorsal striatum, prefrontal cortex (PFC) and hippocampus are also enriched in nAchRs. Other brain sites with nAchR expression that have been involved in nicotine addictive properties include the medial habenula, interpeduncular nucleus (IPN) and amygdala (Tuesta et al, 2011).

At the neuronal level, the activation of nAchRs elevates intracellular Ca^{2+} concentrations (Dajas-Bailador and Wonnacot, 2004), which increases neuronal excitability and induces depolarization. Most nicotinic receptors exhibit a pre-synaptic localization, where they facilitate the release of multiple neurotransmitters (Dani and Bertrand, 2007; Dajas-Bailador and Wonnacot, 2004). However, they have also been found at dendritic, somal, axonal and post-synaptic sites (Albuquerque et al, 2009), where they mediate neuronal

excitation as well as activity-dependent modulation of circuits and intracellular enzymatic processes (McKay et al, 2007). By modulating activity-dependent events, nAchRs participate in fundamental aspects of synaptic plasticity that are involved in attention, learning and memory (Dani et al, 2001; Ge and Dani, 2005). Multiple subtypes of nAchRs can be expressed by the same neuronal population and overlapping distribution of different nAchR subtypes can be found in different brain areas.

2.3. Nicotine: addictive properties

Similarly to other addictive processes, nicotine addiction is a chronic brain disease characterized by compulsive tobacco use, loss of control over tobacco consumption despite its harmful effects, the appearance of withdrawal symptoms upon cessation of tobacco smoking, and relapse after periods of abstinence (adapted from Koob and Le Moal, 2008a).

2.3.1. Acute effects: reward and positive reinforcement

In humans, nicotine intake through tobacco smoking produces a mild pleasurable rush, mild euphoria, increased arousal, decreased fatigue and relaxation (Henningfield et al, 1985). Such positive effects play an important role in the initiation and maintenance of tobacco smoking (Markou, 2008). Similar to humans, several other species such as non-human primates (Goldberg et al, 1981), dogs (Risner and Goldberg, 1983), rats (Corrigall and Coen, 1989) and mice (Galeote et al, 2009; Martín-García et al, 2009) also exhibit behavioral evidence of nicotine reinforcement by reliably self-administering intravenous nicotine.

Similar to other drugs of abuse, nicotine induces its rewarding effects through the modulation of the mesocorticolimbic reward system. Intravenous nicotine self-administration decreases ICSS reward thresholds (Kenny and Markou, 2006), indicating a facilitatory role for nicotine in brain reward function. Consistently, acute nicotine administration increases the firing-rate

of mesolimbic DA neurons (Grenhoff et al, 1986; Schilstrom et al, 2003; Mameli-Engvall et al, 2006), and elevates DA extracellular levels in the NAc (Imperato et al, 1986; Di Chiara and Imperato, 1988; Benwell and Balfour, 1992), particularly in the shell (Pontieri et al, 1996). Evidence indicates that the effects of nicotine on DA transmission are due to direct stimulation of nAchRs within the VTA. Thus, intra-VTA infusion of a nAchR antagonist reduced nicotine-elicited DA outflow in the NAc, whereas infusion of the same antagonist into the NAc did not have any effect (Nissell et al, 1994). In agreement, nAchRs from the VTA, but not the NAc, modulate nicotine self-administration behavior in rats (Corrigall et al, 1994). Consistent with a central role of the VTA in nicotine reinforcement, lesions of the mesolimbic DA projection from the VTA to the NAc (Corrigall et al, 1992), systemic administration of DA receptor antagonists (Corrigall and Coen, 1991) and lesions of the cholinergic pedunculopontine nucleus (which projects to the VTA) (Lança et al, 2000) reduce intravenous nicotine self-administration in rats.

The nAchRs located in DA-containing neurons of the VTA desensitize rapidly (Pidoplichko et al, 1997). Hence, post-synaptic activation of nAchRs in DA neurons cannot explain alone the persistent DA release observed in the NAc after nicotine administration (Di Chiara and Imperato, 1988). Amassing evidence indicates that indirect mechanisms involving glutamate and GABA transmission in the VTA may control the mesocorticolimbic dopaminergic tone after nicotine administration (Markou, 2008) (Figure 12). Indeed, nicotine activates nAchRs located on VTA glutamatergic terminals and facilitates glutamate release (Mansvelder and McGehee, 2000; Jones and Wonnacot, 2004), which in turn increases burst-firing of post-synaptic VTA DA neurons (Mansvelder and McGehee, 2000; Mao et al, 2011). Nicotine also excites nAchRs located in GABAergic pre-synaptic terminals within the same brain nucleus (Mansvelder et al, 2002), resulting in a transient increase in inhibitory GABAergic transmission. However, different nAchR subtypes with

distinct desensitization profiles modulate GABAergic and glutamatergic synaptic inputs to the VTA (Mansvelder et al, 2002). Nicotine can enhance glutamatergic transmission while nAchRs on GABA neurons are desensitized, thus shifting the balance of synaptic inputs to excitation and inducing a net activation of the dopaminergic neurons of the VTA (Mansvelder et al, 2002). Cholinergic afferents from brainstem nuclei also regulate the activity of nAchRs located in glutamate and GABA terminals in the VTA (Maskos, 2008). Nevertheless, nicotine rapidly desensitizes nAchRs on GABA neurons, which removes the modulation of endogenous cholinergic drive over GABA neurons, further disinhibiting DA neurons (Mansvelder et al, 2002).

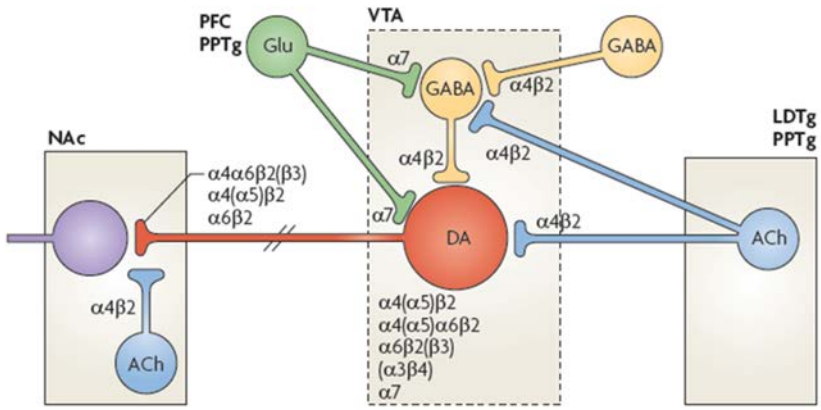

Figure 12. Nicotine-glutamate-GABA-dopamine-acetylcholine interactions in the ventral tegmental area. Schematic depicting neurotransmitter interactions in the VTA, which are hypothesized to be critically involved in nicotine addictive properties. Dopaminergic neurons (DA; shown in red) in the ventral tegmental area (VTA) receive 2 main types of excitatory inputs. First, from cholinergic neurons (shown in blue) of the laterodorsal tegmental nucleus (LDTg) and pedunculopontine nucleus (PPTg); and second, from glutamatertic neurons (shown in green) of various sources, including the prefrontal cortex (PFC) and PPTg. Dopaminergic neurons also receive inhibitory input from GABA neurons (shown in yellow) and send projections to the nucleus accumbens (NAc), where they receive inputs from intrinsic cholinergic interneurons (shown in blue) (Changeux, 2010).

Although the role of the VTA in the acute rewarding effects of nicotine has been extensively studied, other structures may also participate in nicotine

reinforcing effects (Markou, 2008). Thus, structures of the extended amygdala have been widely related to the neurobiological substrates of drug addiction (Koob and Le Moal, 2008b). In agreement, it has been shown that acute nicotine administration induces DA outflow in the BNST (Carboni et al, 2000a). Additionally, nicotine increases N-methyl-D-aspartate (NMDA) receptor mediated excitatory post-synaptic currents in rat CeA slices, and intra-CeA administration of a NMDA receptor antagonist decreases nicotine self-administration in rats (Kenny et al, 2009). A recent study has also shown that α5* nAchRs located in the habenulo-interpeduncular pathway limit intravenous nicotine self-administration in rodents (Fowler et al, 2011), suggesting that deficits in α5* nAchR signaling in the habenulo-interpeduncular tract increases vulnerability to the motivational properties of nicotine. Finally, damage to the insula, a cortical brain region involved in processing interoceptive information related to emotional and motivational states, disrupts abruptly tobacco addiction in humans (Naqvi et al, 2007). Consistently, insular hypocretin/orexin transmission is essential for the reinforcing properties of nicotine (Hollander et al, 2008).

2.3.2. Chronic effects: tolerance and sensitization

Repeated nicotine administration is associated with neuronal adaptations that include prolonged desensitization of nAchRs and their subsequent up-regulation, the need of progressively higher doses of nicotine to obtain the same effects (tolerance), and the increased ability of the drug to activate dopaminergic neurotransmission and trigger appetitive behaviors (sensitization) (De Biasi and Salas, 2008).

The effects of nicotine on DA transmission can similarly undergo tolerance or sensitization. Thus, repeated nicotine exposure results in reciprocal changes in dialysate DA release; sensitization occurs in the core while tolerance is developed in the shell (Cadoni and Di Chiara, 2000). However, tolerance is more likely to be induced by continuous and prolonged

exposure to nicotine, whereas sensitization is normally induced by intermittent exposure to the drug (Di Chiara, 2000).

2.3.2.1. nAchR desensitization and tolerance

Nicotine concentrations reached after smoking excite DA-containing neurons in the VTA to fire action potentials (Pidoplichko et al, 1997). However, smokers maintain steady low levels of plasmatic nicotine concentrations throughout the day (Dani and Heinneman, 1996; Benowitz et al, 1989), and longer exposure to nicotine can cause severe desensitization of nAchRs in the VTA (Pidoplichko et al, 1997). The rate of desensitization and recovery varies depending on which subtypes of nAchR are expressed in a neuron or in different areas of the brain. Thus, different desensitization and recovery could underlie aspects of tolerance leading to the fact that a second dose of nicotine after an initial dose does not elicit the same effects. Additionally, the continuous plasmatic levels of nicotine on smokers and the slow recovery of nAchRs from profound levels of desensitization may explain why smokers report that the first cigarette is the most pleasurable of the day (Dani and Heinneman, 1996).

2.3.2.2. nAchR up-regulation and sensitization

Chronic exposure to an agonist of a particular receptor usually results in receptor down-regulation, whilst chronic antagonist exposure produces up-regulation of receptors (Creese and Sibley, 1981; Overstreet and Yamamura, 1979). Paradoxically, chronic nicotine exposure increases nicotine-binding sites in the brain, a phenomenon that has been termed up-regulation of nAchRs (Wonnacott, 1990). Prolonged desensitization of nAchRs may contribute to the up-regulation of nAchRs (Picciotto et al, 2008), however the relationships between desensitization and up-regulation have not still been clarified. Initially, up-regulation of nAchRs was attributed to an increased number of extracellular receptors in the plasma membrane (Dani and Heinemann, 1996). Nevertheless, more recent reports indicate that up-regulation might not be the result of increased receptor numbers. Instead, it

has been proposed that up-regulation of nAchRs entails conformational changes to receptors that alter the affinity for nicotine (Vallejo et al, 2005). Indeed, up-regulated receptors were typically activated by low agonist concentrations, exhibited slow rates of desensitization, and showed increased responsiveness and sensitivity to agonists (Vallejo et al, 2005). Since nAchRs modulate DA transmission, it would be expected that enhanced sensitivity might facilitate dopaminergic neurotransmission. Consistent with this idea, chronic nicotine produced sensitization of dialysate DA release in the ventral and dorsal striatum and enhanced locomotor responses to nicotine challenge (Cadoni and Di Chiara, 2000, R eid et al, 1998; Shim et al, 2001). Furthermore, chronic infusions of nicotine directly into the VTA induce locomotor sensitization (Panagis et al, 1996), suggesting a role for VTA nAchRs in the chronic effects of nicotine.

2.3.3. Cessation of nicotine intake: acute withdrawal

Chronic nicotine use, similar to other drugs of abuse, results in the development of nicotine addiction. Thus, nicotine-dependent smokers must continue nicotine intake to avoid distressing somatic and affective withdrawal symptoms. Upon smoking cessation, abstinent individuals experience symptoms such as depressed mood, irritability, anxiety, severe craving for nicotine, loss of concentration, bradycardia, gastrointestinal discomfort, impatience, insomnia, increased appetite and weight gain (Hughes and Hatsukami, 1986; Hughes, 2007). Experimental animals, such as mice and rats, exhibit a withdrawal syndrome similar to humans that also includes both somatic signs and a negative affective state. The somatic signs of nicotine withdrawal in rodents include teeth chattering, palpebral ptosis, tremor, wet dog shakes, changes in locomotor activity, and other behavioral effects (Malin et al, 1992; Castañé et al, 2002), whereas the affective changes consist of increased anxiety-like behavior, aversive effects, and reward deficits (Jackson et al, 2008; Johnson et al, 2008). Withdrawal symptoms in animals can be assessed by the sudden discontinuation of chronic nicotine administration or,

alternatively, withdrawal can be precipitated on chronic nicotine treated rodents by administering nAchR antagonists. In rodents, the systemic injection of nAchR antagonists with preferential effects on either α3β4*, α4β2*, or α7* nicotinic receptors precipitates nicotine withdrawal (Damaj et al, 2003).

Similar to other drugs of abuse, the negative affective state of nicotine withdrawal is accompanied by several neuroadaptations in brain reward and stress systems. Cessation of nicotine intake is associated with a decrease in extracellular DA levels in the NAc (Hildebrand et al, 1998; Rada et al, 2001; Rahman et al, 2004) and the CeA (Panagis et al, 2000). Conversely, an increase in PFC DA levels has been observed during nicotine withdrawal (Carboni et al, 2000b). Such increases in PFC DA release may be important in mediating aversive aspects of nicotine abstinence since enhanced DA transmission in this brain region has been observed after exposure to stressful and aversive stimuli (Thierry et al, 1976) and appears to mediate anxiety-like behaviors (Bradberry et al, 1991). Nicotine withdrawal is also associated with deficits in brain reward function (Epping-Jordan et al, 1998), as measured by elevation of ICSS reward thresholds. Interestingly, experimental evidence suggests a relationship between hyperactivity of brain stress systems and a decrease in brain reward function (Bruijnzeel and Gold, 2005; Bruijnzeel, 2012). Indeed, amassing evidence indicates that CRF in the CeA is a pivotal mediator of the negative emotional states associated with nicotine withdrawal. Thus, augmented CRF levels were observed in the CeA of nicotine-abstinent rats (George et al, 2007). Moreover, intra-CeA pretreatment with the CRF_1 receptor selective antagonist R278995/CRA0450 prevented the elevations of ICSS reward thresholds observed during precipitated nicotine withdrawal (Bruijnzeel et al, 2012). CRF signaling in the shell of the NAc has also been related to the anhedonic state observed during nicotine abstinence. Hence, systemic and intra-accumbal infusion of the non-selective $CRF_{1/2}$ receptor antagonist D-Phe CRF_{12-41} prevented mecamylamine-induced deficits in brain

reward circuits in nicotine-dependent rats (Brujnzeel et al, 2007; Marcinkiewcz et al, 2009).

As opposed to the affective symptoms of nicotine withdrawal, the mechanisms and brain regions underlying physical symptoms of nicotine abstinence are not yet clear. However, a recent report has demonstrated a role for the habenula and the interpeduncular nucleus in the somatic symptoms of nicotine withdrawal (Salas et al, 2009). Thus, the nAchR antagonist mecamylamine precipitated nicotine withdrawal when microinjected into the habenula or the interpeduncular nucleus whereas microinfusion of this antagonist into the cortex, VTA and hippocampus did not induce any effect (Salas et al, 2009). Interestingly, α2, α5 and β4 nAchR subunits show an overlapping expression pattern along the habenulo-interpeduncular pathway (Baldwin et al, 2011) and knockout mice for these nicotinic subunits exhibit attenuated physical signs of nicotine abstinence (Salas et al, 2009; Jackson et al, 2008; Salas et al, 2004). Therefore, it has been proposed that signaling through α2, α5 and β4 containing nAchRs in the habenulo-interpeduncular pathway participate in the manifestation of somatic symptoms of nicotine withdrawal.

2.3.4. Long-term abstinence: relapse to nicotine-seeking

Abstinent smokers remain vulnerable to relapse even years after cessation of tobacco smoking. Reinstatement models of relapse in animals have shown that nicotine-seeking can be triggered by nicotine-associated conditioned cues (Martín-García et al, 2009), stressors (Bilkei-Gorzo et al, 2008), and re-exposure to nicotine (Dravolina et al, 2007), which are the same events that trigger resumption of smoking behavior in humans. The neurobiological mechanisms involved in reinstatement of nicotine-seeking are poorly understood. Based largely on w ork with cocaine, it has been hypothesized that drug-induced long-term neuroadaptations impair synaptic plasticity in the prefronto-accumbal glutamatergic projection, and thereby dysregulate the ability of addicts to control their drug taking habits (Kalivas

and Volkow, 2011). In agreement, attenuation of glutamatergic synaptic transmission has been proven effective to prevent reinstatement of nicotine-seeking in animal models. Accordingly, pharmacological blockade of post-synaptic metabotropic glutamate 1 (mGlu1) (Dravolina et al, 2007) and mGlu5 receptors (Tessari et al, 2004; Bespalov et al, 2005) prevent cue- and nicotine-induced reinstatement. Conversely, stimulation of pre-synaptic mGlu2/3 receptors, which diminishes glutamatergic transmission, blocks cue-induced reinstatement of nicotine-seeking (Liechti et al, 2007).

2.4. nAchRs involved in the addictive properties of nicotine

Several studies on mRNA and protein expression levels have shown that different areas of the brain express specific subsets of nAchR subunits. Nevertheless, due to the lack of receptor agonists and antagonists with selectivity for all putative nAchR subtypes, the precise identification of functional nAchR subtypes that regulate the behavioral and physiological actions of nicotine *in vivo* remain unclear. The recent development of genetic animal models with knockout, knockin or selective expression of nAchR subunits has enabled the investigation of the role of specific nAchRs in complex behaviors, including those related to nicotine addiction (Mineur and Picciotto, 2008) (Table 1).

2.4.1. α4β2-subunit-containing nAchRs

The α4β2* nAchR is the most abundant nicotinic receptor subtype expressed in the CNS (Gotti et al, 2009), and considerable evidence supports a role for these receptors in the regulation of the primary reinforcing effects of nicotine (Tuesta et al, 2011). In the VTA, α4β2* nAchRs are located in GABAergic pre-synaptic terminals (Mansvelder et al, 2002), where they regulate the release of the inhibitory neurotransmitter. However, nicotine binding rapidly desensitizes nicotinic receptors containing α4 and β2 subunits (Mansvelder et al, 2002), and disruption of inhibitory GABAergic

transmission in the VTA may contribute, at least in part, to the reinforcing properties of nicotine.

2.4.1.1. β2-subunit-containing nAchRs

Mice with a null mutation of the β2 subunit gene exhibit decreased responsiveness to nicotine-elicited dopaminergic firing in the VTA (Picciotto et al, 1998; Besson et al, 2007). Additionally, nicotine-evoked DA release in the NAc is attenuated as compared to their wild-type counterparts (Picciotto et al, 1998). The inability of nicotine to stimulate the mesocorticolimbic dopaminergic system in these mice is consistent with the absence of nicotine self-administration and place conditioning observed in the same animals (Picciotto et al, 1998; Pons et al, 2008; Walters et al, 2006). Viral vector-mediated re-expression of the β2 subunit in the VTA, but not in the adjacent substantia nigra pars compacta, rescues nicotine addictive properties in β2 knockout mice. Accordingly, nicotine-induced neuronal firing in the VTA, the subsequent DA release in the NAc, and intra-VTA as well as intravenous nicotine self-administration are normalized after β2 subunit re-expression in the VTA (Maskos et al, 2005; Pons et al, 2008; Orejarena et al, 2012). Taken together, these observations suggest that β2* nAchRs play a pivotal role in nicotine reward and reinforcement, consistent with the high density of these subunits in the VTA and other reward related brain regions.

Affective signs of nicotine withdrawal also depend on β2* nAchR signaling. Indeed, nicotine withdrawal induced anxiety-like behavior and place aversion are absent in mice lacking the β2 subunit gene (Jackson et al, 2008). Moreover, the deficits observed in fear conditioning in nicotine abstinent mice are diminished in β2 knockout mice (Portugal et al, 2008). In contrast, β2* nAchRs do not participate in the expression of the somatic signs of nicotine withdrawal (Salas et al, 2004).

2.4.1.2. α4-subunit-containing nAchRs

Comparable to β2 knockout mice, α4 subunit knockout mice fail to show nicotine-induced enhancement of DA levels in the NAc (Marubio et al, 2003) and do not readily self-administer nicotine (Pons et al, 2008). Additionally, following virus mediated re-expression of α4 subunits in the VTA, nicotine self-administration behavior is established again (Pons et al, 2008). In this study, nicotine self-administration was evaluated in mice placed in an apparatus to restrain their movement and prepared with a temporary catheter in their lateral tail vein that typically lasts for 1 day. Although this procedure has a number of notable confounds, these results suggest that α4* nAchRs in the VTA are crucial for acute nicotine reinforcement. In opposition, a recent study has shown that mice lacking α4 subunits exhibit similar self-administration and place conditioning to nicotine than their wild-type counterparts (Cahir et al, 2011). This study used a traditional self-administration paradigm, in which mice were trained during several days to obtain nicotine infusions through chronic indwelling intravenous catheters in their jugular vein. Consequently, methodological differences such as the acute or chronic nature of nicotine self-administration might have contributed to the discrepant results observed in these 2 studies. Knockin mice expressing hypersensitive α4* nAchRs have also been developed. These mice self-administer nicotine more vigorously than their wild-type counterparts (Cahir et al, 2011), and develop a nicotine CPP at lower doses than those necessary to support a place preference in wild-type mice (Tapper et al, 2004). Overall, these data suggest that α4* nAchRs play a role in nicotine rewarding and reinforcing properties.

2.4.2. α7-subunit-containing nAchRs

Similar to α4 and β2 subunits, the α7 subunit exhibits widespread expression throughout the brain. Unlike other nAchR subunits in the brain, the α7 subunit forms functional homopentameric receptors (Coutourier et al, 1990). In the VTA, α7 nAchRs regulate pre-synaptic glutamate release onto

DA neurons (Masvelder and McGehee, 2000; Jones and Wonnacott, 2004). Most of the work concerning the role of α7 receptors in nicotine addiction has been performed using the relatively selective α7 nAchR antagonist methyllycaconitine and contradictory results have been obtained. Intra-VTA infusion of this antagonist attenuated nicotine-induced lowering of ICSS thresholds as well as nicotine elicited CPP (Panagis et al, 2000; Laviolette and van der Kooy, 2003). In agreement, systemically administered methyllycaconitine decreased nicotine self-administration in rats (Markou and Paterson, 2001). In contrast, another study found that methyllycaconitine had no effect on nicotine self-administration (Grottick et al, 2000) and, consistently, α7 knockout mice developed a nicotine place preference and self-administered nicotine similarly to wild-type mice (Walters et al, 2006; Pons et al, 2008). Therefore, the precise role of α7* nAchRs in nicotine reinforcement remains unclear.

The α7* nAchRs do not appear to importantly contribute to nicotine withdrawal. Thus, α7 knockout mice show an intermediate phenotype in the somatic expression of nicotine withdrawal (Salas et al, 2007; Jackson et al, 2008) and do not participate in the affective component of nicotine abstinence (Jackson et al, 2008).

2.4.3. α5-subunit-containing nAchRs

Genome wide association studies have revealed that allelic variation in several nAchR subunits influences the vulnerability to develop tobacco addiction in humans. The most robust genetic finding that alters the risk of developing heavy smoking is located in the chromosome 15q25 region, which contains the α5/α3/β4 gene cluster (CHRNA5, CHRNA3 and CHRNB4) (Bierut, 2011). This genetic association is marked by multiple single nucleotide polymorphisms (Bierut, 2010), including a non-synonimous polymorphism in the CHRNA5 gene, which encodes the α5 nAchR subunit, that decreases the function of α5* receptors (Bierut et al, 2008). This polymorphism has been shown to increase the risk of tobacco dependence by

approximately 30% in individuals carrying a single copy of the gene variant, and more than doubles the risk in those carrying 2 risk alleles (Saccone et al, 2009). In addition, the α5 subunit gene variant is a major risk factor for lung cancer and chronic obstructive pulmonary disease in smokers (Bierut, 2010). However, it is not clear whether the connection between the genetic variant and pulmonary disease is direct or indirect through increasing levels of tobacco addiction in individuals carrying the risk allele (Thorgeirsson et al, 2008). As such, much interest has centered on the role of α5 subunits in the reinforcing properties of nicotine. The function of the α5 nAchR subunit is restricted to a modulatory role when incorporated into functional nAchRs, and regulates the ligand-binding and desensitization kinetics, among other characteristics of nAchRs (Gotti et al, 2009). The α5 subunit knockout mice develop a CPP for high doses of nicotine that are otherwise aversive in wild-type mice (Jackson et al, 2010). Additionally, α5* nAchRs play a critical role in nicotine self-administration behavior. Thus, the α5 subunit knockout mice consume more nicotine than their wild-type counterparts when high doses of nicotine are available (Fowler et al, 2011). These mutant mice also respond more vigorously for high nicotine doses than wild-type mice when tested under a progressive-ratio schedule of reinforcement (Fowler et al, 2011), indicating a higher motivation of these mutant mice to self-administer nicotine. Therefore, it appears that α5 subunit knockout mice are less sensitive to the aversive effects of nicotine that normally serve to limit responding for the drug (Fowler et al, 2011). The expression profile of α5 nAchRs is discrete in the brain, with the highest densities found in the medial habenula and interpeduncular nucleus, among other structures (Wada et al, 1990; Sheffield et al, 2000). Re-expression of the α5 subunit by lentiviral expression in the habenulo-interpeduncular pathway of these knockout mice, rescues the phenotype of wild-type mice (Fowler et al, 2011). Conversely, knockdown of these subunits in the habenulo-interpeduncular pathway of rats increases nicotine intake, especially at high doses of the drug (Fowler et al, 2011). Nevertheless, ICSS studies indicate that knockdown of the α5 subunits in the

habenulo-interpeduncular pathway does not alter the stimulatory effects of nicotine on brain reward systems (Fowler et al, 2011). Instead, the absence of α5 subunits diminishes the inhibitory effect of high nicotine doses in brain reward function (Fowler et al, 2011). Moreover, it has been shown that nicotine aversive effects depend on the balance between β4 and α5 subunits in the medial habenula (Frahm et al, 2011). In summary, these data indicate that nicotine, through stimulation of α5* nAchRs of the habenulo-interpeduncular brain circuit, decreases the motivation to further consume nicotine, limiting its intake.

As mentioned before (chapter 2.3.3.), α5* nAchRs in the habenulo-interpeduncular pathway play a key role in the manifestation of physical dependence. In contrast, α5 subunit containing nAchRs do not appear to mediate affective symptoms of nicotine abstinence (Jackson et al, 2008).

Table 1. Brief summary of the role of nAchR subunits in nicotine addictive properties.

Subunit	Reward/Reinforcement	Withdrawal
β2	Dopamine firing in the VTA Dopamine release in the NAc Intravenous and intra-VTA self-administration	Anxiety-like behavior Conditioned place aversion Deficits in fear conditioning
α4	Dopamine release in the NAc Conditioned place preference Intravenous self-administration	Not evaluated
α7	Lowering of ICSS thresholds Conditioned place preference Intravenous self-administration	Do not participate
α5	Conditioned place preference (aversive doses of nicotine) Intravenous self-administration (high nicotine doses) Motivation for self-administration	Somatic signs
α6	Dopamine release in the NAc Conditioned place preference Intravenous self-administration Motivation for self-administration	Anxiety-like effects Conditioned place aversion
β4 and α2		Somatic signs

2.4.4. α6-subunit-containing nAchRs

Much interest has centered in the potential role of α6 nAchR subunits in regulating nicotine reinforcement processes. Recent evidence from genome wide association studies indicates that genetic variation in the CHRNA6-CHRNAB3 gene cluster, encoding the α6 and β3 subunits respectively, increases vulnerability to tobacco smoking (Thorgeirsson et al, 2010; Hoft et al, 2009). In the VTA, α6* nAchRs appear to regulate GABA release onto DA neurons (Yang et al, 2011), and up-regulation of α6 mRNA occurs following nicotine self-administration (Parker et al, 2004). In the striatum, dopaminergic terminals express α6β2β3* and α4α6β2β3* nAchR subtypes (Zoli et al, 2002; Champtiaux et al, 2003), with evidence suggesting that these α6* nAchRs regulate the stimulatory effects of nicotine on DA release in this region (Champtiaux et al, 2003). Consistent with α6* nAchR expression in the mesoaccumbal dopaminergic pathway, several reports indicate a role for α6 subunit in the reinforcing effects of nicotine. Thus, α6 knockout mice do not acquire nicotine self-administration behavior in the previously described acute model of reinforcement in restraint mice (Pons et al, 2008). Moreover, lentiviral mediated re-expression of α6 subunits in the VTA of these mutant mice re-establishes the sensitivity to nicotine, and knockout mice respond for nicotine at the same rate as their wild-type counterparts (Pons et al, 2008). In line with these findings, infusion of a selective α6β2* nAchR antagonist α-conotoxin MII into the shell compartment of the NAc decreases the motivation to self-administer nicotine in rats (Brunzell et al, 2010). Furthermore, intra-VTA infusion of this antagonist decreases nicotine self-administration behavior and attenuates nicotine-induced DA release in the NAc (Gotti et al, 2010). In agreement, nicotine-induced CPP is blocked in mice pretreated with α-conotoxin MII (Jackson et al, 2009). Systemic administration of another selective antagonist for α6β2* nAchRs, N,N-decane-1,10-diyl-bis-3-picolinium diiodide (bPiDI), also decreases nicotine-evoked striatal DA release and nicotine self-administration in rats (Wooters et

al, 2011). Hence, these findings support a key role for α6* nAchRs in the mesoaccumbens pathway in regulating nicotine reward and reinforcement.

The α6 subunit is also involved in the affective component of nicotine abstinence. Thus, the previous administration of α-conotoxin MII prevents nicotine withdrawal induced anxiety-like effects and CPA (Jackson et al, 2009). On the contrary, the same antagonist does not influence the somatic signs associated with abrupt cessation of nicotine intake (Jackson et al, 2009).

2.5. Other neurotransmitter systems involved in the addictive properties of nicotine

Nicotine addiction is a complex behavioral disease that involves the participation of several neurotransmitter systems (Berrendero et al, 2010). Thus, different transmitters appear to mediate the rewarding properties of nicotine and the adaptations that occur in response to chronic nicotine exposure that ultimately give rise to the addictive process (Markou, 2008).

2.5.1. Glutamatergic system

Glutamate is the major excitatory transmitter in the mammalian brain and plays a critical role in the acute and long-term effects of nicotine (Liechti and Markou, 2008). Nicotine-elicited DA outflow in the NAc is blocked by systemic administration of NMDA and α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate (AMPA) receptor antagonists (Kosowski et al, 2004). Consistent with these observations, compounds that reduce glutamate transmission usually attenuate nicotine rewarding properties. Thus, the systemic injection of the NMDA receptor antagonist LY235959 prevents nicotine self-administration-induced lowering of ICSS reward thresholds (Kenny et al, 2009). In agreement, systemic, intra-VTA, and intra-CeA administration of the same NMDA receptor antagonist decreases nicotine self-administration in rats (Kenny et al, 2009). Moreover, the blockade of post-

synaptic mGlu5 receptors with MPEP decreases intravenous nicotine self-administration at fixed- and progressive-ratio schedules of reinforcement (Paterson et al, 2003; Paterson and Markou, 2005; Liechti and Markou, 2007), suggesting that mGlu5 receptors not only affect nicotine primary reinforcing properties but also the motivation to obtain the drug. In line with these findings, MPEP inhibited nicotine-induced CPP (Yararbas et al, 2010). In contrast, a r ecent report indicates that this antagonist potentiates nicotine-induced CPP, lowering the effective dose of nicotine needed to induce place preference (Rutten et al, 2011). Differences in the experimental procedures used to induce CPP might explain the contradictory results obtained in these 2 studies. Finally, systemic injection or directed microinfusion of the pre-synaptic mGlu2/3 receptor agonist into the VTA and the shell of the NAc dose-dependently reduces the rate of responding in a nicotine self-administration paradigm (Liechti et al, 2007). Interestingly, the same nicotine self-administration regime down-regulates mGlu2/3 receptor coupling to G proteins in a [35S]GTPγS binding assay, further supporting the participation of these pre-synaptic receptors in the reinforcing properties of nicotine (Liechti et al, 2007).

Glutamatergic excitatory transmission also contributes to the negative affective symptoms and somatic signs observed during nicotine abstinence. However, opposing glutamatergic mechanisms underlie nicotine reinforcement and withdrawal. While acute nicotine has stimulatory effects on glutamate cell firing and release (Mansvelder and McGehee, 2002), the acute withdrawal phase seems to be characterized by decreased glutamate transmission (Kenny and Markou, 2001). Indeed, the administration of mGlu2/3 receptor antagonists, which increase pre-synaptic glutamate release, attenuate reward deficits associated with nicotine withdrawal in rats (Kenny et al, 2003). In contrast, treatment with the mGlu5 receptor antagonist MPEP exacerbates brain ICSS reward deficits and the somatic signs associated with nicotine abstinence in rats (Liechti and Markou, 2007). Contrasting with these

results, mGlu5 receptor knockout mice do not manifest somatic sings of nicotine withdrawal nor the associated elevations in brain reward thresholds (Stoker et al, 2012). However, the lack of mGlu5 receptor in the knockout mice might have prevented the development of nicotine dependence, reflected by an attenuation of the anhedonic and somatic aspects of nicotine withdrawal (Stoker et al, 2012).

The hypoglutamatergic state resulting from the cessation of tobacco smoking is thought to promote nicotine-seeking behavior (Markou, 2007). Therefore, restoring glutamatergic transmission to normal levels through manipulation of glutamate transporters may help smokers remain abstinent. N-acetylcysteine binds to the cystine-glutamate exchanger on glial cells and stimulates extracellular glutamate release (Kalivas, 2009). Interestingly, nicotine self-administering rats show decreased expression of this transporter in the NAc, and N-acetylcysteine treatment decreases the number of cigarettes smoked in nicotine-dependent individuals (Knackstedt et al, 2009). Additionally, the first cigarette after an abstinence period is significantly less rewarding in subjects receiving N-acetylcysteine as compared to placebo (Schmaal et al, 2011).

The participation of the glutamatergic system in the re-initiation of nicotine-seeking behaviors during relapse is summarized in chapter 2.3.4.

2.5.2. GABAergic system

GABA is the major inhibitory neurotransmitter in the mammalian CNS and is critically involved in the reinforcing effects of nicotine (Markou, 2008). An increase in GABA levels in the VTA reduces the activity of mesocorticolimbic DA neurons, thus limiting nicotine reward and reinforcement (D'Souza and Markou, 2011). Several studies using γ-Vinyl GABA (vigabatrin), which increases GABAergic transmission by inhibiting GABA metabolism, support a role for GABA in nicotine reward and reinforcement. Increased GABAergic transmission by vigabatrin significantly

attenuates nicotine-elicited elevations of DA in the NAc and abolishes the acquisition and expression of nicotine CPP (Dewey et al, 1999). Nicotine self-administration is also reduced after treatment with this GABAergic compound (Paterson and Markou, 2002). The use of receptor-selective agonists suggests the participation of $GABA_B$ receptors in these effects (Markou, 2008). Thus, systemic, intra-VTA and intra-pedunculopontine tegmental nucleus injections of $GABA_B$ agonists substantially decrease nicotine self-administration behavior (Corrigall et al, 2000; Corrigall et al, 2001; Paterson et al, 2004; Paterson et al, 2005). Moreover, $GABA_B$ stimulation reduces the motivation to work for nicotine infusions, measured by the progressive-ratio schedule of reinforcement (Paterson et al, 2005). GABAergic inhibitory transmission also plays a key role in relapse-like behaviors since pretreatment with $GABA_B$ agonists prevents cue-induced reinstatement of nicotine-seeking in rats (Paterson et al, 2005). Moreover, reinstatement of nicotine-seeking elicited by a priming injection of nicotine is blocked by preadministration of the $GABA_B$ agonist baclofen (Fattore et al, 2009). In line with these preclinical findings, baclofen has been shown to reduce the number of cigarettes smoked as well as craving associated with abstinence in humans (Franklin et al, 2009).

The main disadvantage of using GABAergic compounds is the appearance of undesirable side effects such as severe motor impairment and other unspecific effects (D'Souza and Markou, 2011). Therefore, $GABA_B$ receptor positive allosteric modulators have been developed, which present fewer side effects than full agonists (D'Souza and Markou, 2011). Similarly to $GABA_B$ agonists, these compounds reduce nicotine self-administration levels at both fixed- and progressive-ratio schedules of reinforcement and block the reward-enhancing effects of nicotine in the ICSS paradigm (Paterson et al, 2008). Additionally, these allosteric modulators prevent reinstatement of nicotine-seeking after presentation of nicotine-conditioned cues (Vlachou et al, 2011).

GABAergic transmission might be involved in the affective and somatic manifestations of nicotine withdrawal. Thus, baclofen has been shown to attenuate somatic signs of nicotine abstinence in mice (Varani et al, 2011). In addition, pregabalin, a GABA analogue, reduces several affective symptoms of the nicotine withdrawal syndrome in humans (Herman et al, 2012). In contrast, a recent study has shown that both stimulation and blockade of GABA transmission enhances the anhedonic state associated to nicotine abstinence in both control and nicotine-dependent rats (Vlachou et al, 2011).

2.5.3. Endogenous opioid system

The endogenous opioid system is widely distributed throughout the CNS and multiple animal models and human studies support a role for this system in nicotine addiction. Three different types of opioid receptors have been identified: mu (MOR), delta (DOR), and kappa (KOR) (Kieffer and Evans, 2009). Three families of endogenous opiod peptide precursors have also been characterized: proopiomelanocortin, proenkephalin and prodynorphin (Xue and Domino, 2008). These precursors give rise to several active peptides that bind opioid receptors with different affinities, including β-endorphin, met- and leu- enkephalins, and dynorphins, respectively (Kieffer and Gavériaux-Ruff, 2002). β-endorphin is the main endogenous ligand for MOR, and binds with higher affinity MOR than DOR or KOR. The affinity of met- and leu- enkephalin for DOR is 20-fold higher than for MOR and dynorphins are the main endogenous ligands for KOR (Roth-Deri et al, 2008). Different subtypes of opioid receptors selectively influence rewarding or aversive properties of drugs of abuse. Thus, while MOR and DOR mainly modulate the rewarding properties of drugs of abuse (Shippenberg et al, 2008; Hutcheson et al, 2001), KOR regulates the aversive effects induced by these drugs (Hasebe et al, 2004).

Opioid receptors and peptide precursors are highly expressed in brain reward areas, such as the mesocorticolimbic system and the extended amygdala (Delfs et al, 1994; Mansour et al, 1995) and they contribute to the

rewarding properties of nicotine. Systemic and intra-VTA infusion of a MOR antagonist abolishes nicotine induced elevations in DA extracellular levels in the NAc (Tanda and Di Chiara, 1998). Similarly, nicotine-elicited enhancement of DA transmission in the NAc is absent in mice lacking proenkephalin (Berrendero et al, 2005). Pharmacological inhibition of MOR signaling blocks acquisition and expression of nicotine-induced CPP (Göktalay et al, 2006). In agreement, nicotine-elicited CPP is abolished in genetically modified mice lacking MOR and proenkephalin (Berrendero et al, 2002; Berrendero et al, 2005), and attenuated in mice null for β-endorphin (Trigo et al, 2009), indicating that the activation of MOR by endogenous enkephalins and β-endorphin is required to obtain the rewarding effects of nicotine. More recent studies using the nicotine self-administration model further suggest a role for MOR in nicotine reinforcement. Thus, naloxone, a non-selective opioid antagonist, and the MOR antagonist naloxonazine decreased the number of nicotine infusions self-administered by rats, whereas the DOR antagonist naltrindole and the KOR antagonist guanidinonaltrindole did not affect nicotine self-administration levels (Liu and Jernigan, 2011; Ismayiloba and Shoaib, 2010). In contrast to these findings, a recent study has revealed a role for DOR in nicotine reinforcement. Hence, nicotine-induced elevations of DA extracellular levels in the NAc as well as nicotine self-administration were prevented in mice lacking DOR (Berrendero et al, 2012). Consistently, the selective DOR antagonist naltrindole dose-dependently attenuated nicotine self-administration behavior in the same study (Berrendero et al, 2012), suggesting that DOR contributes to the reinforcing effects of nicotine. Consistent with animal data, some polymorphisms in the MOR gene have been linked to smoking initiation, reward and dependence (Zhang et al, 2006; Perkins et al, 2008). Additionally, the non-selective MOR antagonist naltrexone has been found to reduce the acute reinforcing value of nicotine in humans (King and Meyer, 2000; Rukstalis et al, 2005; Epstein and King, 2004). In contrast, kappa/dynorphin system seems to play an opposite role in nicotine reward. Accordingly, treatment with the KOR agonist U50,488

reduced the number of infusions obtained by nicotine self-administering rats (Ismayiloba and Shoaib, 2010), and mice lacking prodynorphin showed enhanced sensitivity to nicotine self-administration (Galeote et al, 2009), which suggests that kappa/dynorphin system would mediate nicotine aversive effects.

The endogenous opiod system is also involved in the nicotine withdrawal syndrome. Hence, the opioid antagonist naloxone has been shown to induce withdrawal symptoms in heavy smokers (Krishnan-Sarin et al, 1999) and to precipitate abstinence in nicotine-dependent mice (Balerio et al, 2004; Biala et al, 2005) and rats (Malin et al, 1993). On the contrary, morphine attenuates the severity of the spontaneous (Malin et al, 1993) and mecamylamine-precipitated nicotine withdrawal syndrome in the rat (Ise et al, 2000). Additionally, naloxone has been shown to be more effective than the nAchR antagonist mecamylamine in precipitating the negative emotional manifestations of nicotine withdrawal in mice, measured in the place aversion conditioning paradigm (Balerio et al, 2004). However, naloxone also binds to nAchRs (Tomé et al, 2001), which could have some influence in the behavioral effects of this antagonist in nicotine-dependent animals and humans. On the other hand, MOR, DOR and KOR agonists have been shown to attenuate mecamylamine-induced CPA during nicotine withdrawal (Ise et al, 2000; Ise et al, 2002). In contrast, a recent study has shown that KOR antagonists attenuate the anxiety-like behavior and CPA induced by the nicotine withdrawal syndrome as well as the associated somatic symptoms (Jackson et al, 2010). Other components of the endogenous opioid system have also been related to the physical manifestations of nicotine abstinence. Thus, mecamylamine-precipitated nicotine withdrawal was significantly attenuated in mice lacking the MOR (Berrendero et al, 2002) and the proenkephalin gene (Berrendero et al, 2005). In contrast, knockout mice for prodynorphin (Galeote et al, 2009) and β-endorphin (Trigo et al, 2009) express comparable somatic signs to wild-type mice during nicotine

abstinence. These results indicate that activation of MOR by endogenous enkephalins is necessary for the somatic manifestations of nicotine withdrawal. In agreement, an increase in proenkephalin mRNA expression has been observed in the hippocampus (Houdi et al, 1998) and the striatum (Houdi et al, 1998; Isola et al, 2002) of nicotine-abstinent rodents.

A few recent studies have addressed the involvement of the endogenous opioid system in nicotine relapse. The opioid receptor antagonist naltrexone blocks reinstatement of nicotine-seeking elicited by nicotine-conditioned cues (Liu et al, 2009). Another report using the CPP reinstatement model has demonstrated a role for KOR in relapse to nicotine-seeking (Jackson et al, 2012). Hence, pretreatment with the KOR antagonist norbinaltorphimine attenuated stress-induced reinstatement of place preference elicited by nicotine whereas it had no effect on nicotine-induced reinstatement of place preference (Jackson et al, 2012).

2.5.4. Endocannabinoid system

Extensive evidence indicates that the endocannabinoid system is a crucial neurobiological substrate for the development of nicotine addiction. Nicotine-enhanced DA extracellular levels in the NAc (Cohen et al, 2002; Cheer et al, 2007) and the BNST (Cheer et al, 2007) are blocked by pretreatment with the selective CB1 receptor antagonist rimonabant (SR141716A). Consistent with these observations, nicotine-induced CPP is absent in rats and mice pretreated with rimonabant (Le Foll and Goldberg, 2004; Merritt et al, 2008), and mice lacking CB1 receptors (Castañé et al, 2002; Merritt et al, 2008). Furthermore, CB1 receptor antagonists reduce nicotine self-administration in rats (Cohen et al, 2002; Shoaib, 2008), indicating that signaling through CB1 cannabinoid receptors is necessary for the rewarding and reinforcing properties of nicotine. The endocannabinoid system also plays a role in reinstatement of nicotine-seeking. Rimonabant attenuates cue- (Cohen et al, 2005; De Vries et al, 2005) and context-induced (Diergaarde et al, 2008) nicotine-seeking in rats. In addition, nicotine

priming- induced reinstatement of CPP is dependent on CB1 transmission (Biala et al, 2009; Budzynska et al, 2009). On the contrary, CB2 receptors do not participate neither in nicotine-taking nor -seeking behaviors (Gamaleddin et al, 2012).

The participation of the endocannabinoid system in nicotine withdrawal has not still been clarified. Thus, somatic signs of abstinence are blocked by rimonabant (Merritt et al, 2008), whereas physical withdrawal symptoms remain unaffected in mice lacking CB1 receptors (Castañé et al, 2002; Merritt et al, 2008).

2.5.5. Other neurotransmitter systems

Monoamine systems other than the dopaminergic system also appear to be involved in nicotine addiction. Thus, several studies suggest the participation of serotonin in nicotine reinforcement, withdrawal and relapse. Accordingly, the 5-HT2c receptor agonist Ro-60-0175 attenuates nicotine self-administration under both fixed- and progressive-ratio schedules of reinforcement (Fletcher et al, 2012), suggesting a role for these receptors on the reinforcing and the motivational properties of nicotine. Interestingly, 5-HT2c receptor agonists and 5-HT2a receptor antagonists also reduce nicotine-primed and cue-induced reinstatement of nicotine-seeking in rats (Fletcher et al, 2012). Similarly, both 5-HT2c agonists and 5-HT2a antagonists attenuate the depressive-like symptoms of nicotine withdrawal (Zaniewska et al, 2010).

Monoamine oxidase inhibitors such as tranylcypromine have also been shown to enhance nicotine self-administration at low nicotine doses (Villégier et al, 2006), suggesting that inhibition of monoamine oxidase induces a potentiation of nicotine addictive properties. Consistent with this idea, commercially available cigarette smoke contains monoamine oxidase inhibitory activity, which is thought to contribute to tobacco addiction by preventing the degradation of monoamines and increasing the reward signal generated by nicotine (Lewis et al, 2007).

Other central neuromodulatory systems are also arising as new targets for the study of nicotine addiction. Particularly, the hypocretin/orexin system seems a promising substrate to mediate the behavioral effects of nicotine during addictive processes. Thus, the anatomical distribution of the hypocretin/orexin system not only permits the regulation of the mesocorticolimbic dopaminergic function, but also allows mediation of arousal and attentional processes that influence nicotine use, abstinence and relapse (Corrigall, 2009).

2.6. Therapeutic strategies for nicotine addiction

Nicotine addiction is a chronic disease that usually requires repeated intervention and multiple attempts to quit. The most promising approaches for achieving long-term abstinence after smoking cessation include a combination of behavioral approaches along with pharmacotherapies (Fiore et al, 2008). Counseling and medication are each effective in treating tobacco addiction, but the combination of both is more effective than either alone, probably because counseling improves medication adherence (Polosa and Benowitz, 2011). The pharmacologic effect of nicotine in the brain is pivotal in the development of tobacco addiction. Therefore, pharmacotherapy can be useful to decrease the short-term reinforcing effects of tobacco after initial cessation and to reduce withdrawal symptoms and craving during abstinence, which eventually might improve smoking cessation rates (Figure 13).

2.6.1. Currently available pharmacotherapies

Present therapeutical options for tobacco addiction include nicotine replacement therapy (NRT), bupropion and varenicline. Compared to placebo, these medications are modestly effective, but they can significantly enhance quit success rates when combined with counseling (Fiore et al, 2008). NRT and bupropion seem to have similar efficacy, however varenicline has been shown to offer notable improvement in abstinence rates over bupropion (Fiore

et al, 2008). Combinations of these medications have also been proven to increase smoking cessation rates (Polosa and Benowitz, 2011).

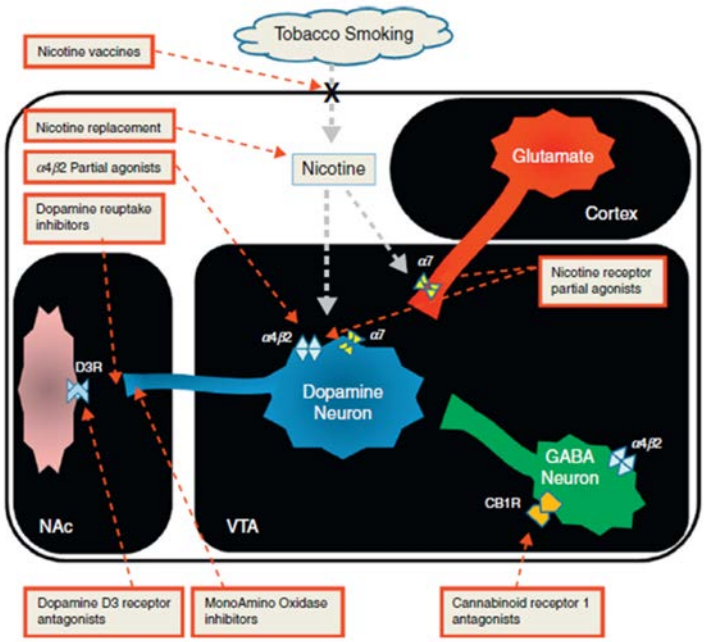

Figure 13. Classic neuronal pathways involved in nicotine addictive properties and related mechanism-based pharmacological rationale for the treatment of nicotine addiction. Nicotine binds principally to α7 and α4β2 nicotinic acetylcholine receptors located on dopaminergic, glutamatergic and GABAergic neurons in the ventral tegmental area, which in turn regulate dopamine release in the nucleus accumbens. Based on this model, several pharmacological strategies that target acetylcholine, dopamine, glutamate, GABA and endocannabioid systems have been proposed and studied for their potential use in the treatment of nicotine addiction. Approaches to reduce the rate and the quantity of nicotine entry into the brain (e.g. nicotine vaccines) could also be of significant benefit (Caponnetto et al, 2012).

2.6.1.1. Nicotine replacement therapy

NRT is a common therapeutic option to support smoking cessation (George, 2007). The objective of NRT is to replace the nicotine formerly obtained from smoking, thus helping achieve abstinence by attenuating the reinforcing effects of smoked nicotine and reducing the severity of withdrawal and cravings (Gross and Stitzer, 1989). However, the use of NRT does not completely prevent withdrawal symptoms since NRT methods deliver

nicotine to the systemic circulation and the brain at slower rates than smoked tobacco (Benowitz, 1993). Five types of NRT exist with 3 different modes of application: oral (gum, lozenge and inhaler), intranasal (nasal spray) and transdermal (patch). A *Cochrane Review* article recently found that all forms of NRT approximately double the likelihood of long-term abstinence from smoking (Stead et al, 2008). Although not a regulated therapeutic option, electronic cigarettes (e-Cig) can also deliver nicotine. Electronic cigarettes are devices that resemble a cigarette and no tobacco or combustion is necessary for its operation. By delivering nicotine, these devices can help smokers remain abstinent or reduce their cigarette consumption. Several large prospective studies are underway in different countries to evaluate efficacy and safety of e-Cigs.

In general, NRT is considered to be safe and adverse events are generally related to the nicotine delivery system (Henningfield, 2005). Due to the slower distribution rate of nicotine, NRT has been shown to have a low liability for abuse and low dependence potential with little to no discomfort when patients discontinue treatment (West et al, 2000). Contraindications for NRT include cardiovascular disease and diabetes mellitus.

2.6.1.2. Bupropion

Bupropion hydrochloride was initially developed and marketed as an antidepressant. It was found later that bupropion was effective as a smoking cessation therapy. Its mode of action is not completely understood, but the inhibition of DA re-uptake by neurons together with a weak nAchR antagonist effect are thought to contribute to the reported reduction in the severity of nicotine cravings and withdrawal symptoms (Miller et al, 2002; Jorenby, 2002). According to a *Cochrane Review* article bupropion doubles the possibilities of quitting smoking compared with placebo (Hughes et al, 2007) and pooled analyses of studies with bupropion generally show quit rates similar to those obtained by NRT (Stead et al, 2008; Hughes et al, 2007).

Nevertheless, one study reported higher cessation rates with bupropion when compared to NRT (Jorenby et al, 1999).

The most common adverse events with bupropion are insomnia, dry mouth and nausea (Hughes et al, 2007) and prescription is contraindicated in people with a history of seizures. Additionally, bupropion carries a "black-box" warning based on observations that antidepressants might increase the risk for suicidal ideation in children and adolescents with certain psychiatric disorders.

2.6.1.3. Varenicline

Varenicline, launched in 2006, became the first new prescription drug for the treatment of tobacco addiction in approximately 10 years. It is a partial agonist for α4β2* nAchRs present in the VTA, among other brain structures. Varenicline has dual effects: partial stimulation of nAchRs, without inducing the full effect of nicotine, and blocking nAchRs, which prevents nicotine to bind them (Coe et al, 2005; Rollema et al, 2007). These effects may provide relief of withdrawal and craving symptoms during abstinence and can also reduce smoking satisfaction, thereby potentially reducing the risk of relapse. After 1 year of abstinence, it has been shown that smokers treated with varenicline have approximately 2.5 greater odds of quitting than placebo, and 1.7 times higher odds compared with bupropion (Gonzales et al, 2006; Jorenby et al, 2006; Cahill et al, 2012).

Varenicline is generally well tolerated and it is also safe and effective in patients with pulmonary and cardiovascular disease (Tashkin et al, 2011; Rigotti et al, 2010). The most common adverse effects are nausea, insomnia, gastrointestinal discomfort and headache (Gonzales et al, 2006; Jorenby et al, 2006). Similarly to bupropion, varenicline carries a black-box warning highlighting an increased risk of psychiatric symptoms and suicidal ideation in patients reporting any history of psychiatric illness.

2.6.2. New therapeutic approaches

As described above, currently available tobacco cessation products increase the chance of quitting smoking. Nevertheless, the success rates after using these compounds remain quite low (Hatsukami et al, 2008), indicating a modest efficacy. Additionally, some pharmacotherapies are associated with significant adverse side effects. Consequently, there is a compelling need for more effective smoking cessation therapies. Several new pharmacotherapies that interfere with nicotine signaling are currently in clinical development. The most active areas of research probably include the use of natural nicotinic ligands with partial agonist activity and the development of therapeutic vaccines (Caponnetto et al, 2012). However, other approaches including new delivery systems for NRT, monoamine oxidase inhibitors and $GABA_B$ agonists, among others, are also under development (Raupach and van Schayck, 2011; Caponnetto et al, 2012).

2.6.2.1. nAchR partial agonists

nAchR partial agonists have the potential to provide relief from withdrawal symptoms and to reduce nicotine reinforcement, thus optimizing benefit and minimizing adverse side effects. Several partial agonists have been evaluated as possible smoking cessation therapies (Hogg and Bertrand, 2007). Three examples of α4β2* nAchR partial agonists that are being currently tested in preclinical animal and human clinical studies include dianacline, sazetidine-A and cytisine (Rollema et al, 2010; Levin et al, 2010).

Cytisine, a structural analogue of nicotine, is a natural alkaloid found in plants such as *Cytisus laburnum* (Etter et al, 2008). It has been used as a smoking cessation therapy in central and eastern European countries for many years, however conclusive clinical data supporting the effectiveness and safety of cytisine are still lacking (Etter et al, 2008). The main advantage of cytisine is that it is inexpensive to manufacture, and its lower price as compared with that of other pharmacotherapies could lead to greater accessibility by smokers. However, it shows a relatively poor brain

penetration, requiring high doses to achieve a pharmacological effect. Additionally, it has a short half-life, thus requiring frequent dosing during the day. A recent randomized phase I clinical study has shown that cytisine is more effective than placebo for smoking cessation and induces similar abstinence rates to NRT (West et al, 2011).

2.6.2.2. Therapeutic vaccines

An innovative approach to facilitate smoking cessation is the development of nicotine therapeutic vaccines. These vaccines produce antibodies against nicotine obtained from tobacco smoking, reducing the rate and the quantity of nicotine entry into the brain (Maurer and Bachmann, 2007). This effect reduces the rewarding effects of nicotine and, consequently, it is expected to assist patients in preventing relapse. Due to the small size of the nicotine molecule, its immunogenicity is scarce, therefore the vaccines consist of nicotine conjugated to a larger carrier protein to form a complete immunogen and elicit antibody formation against it. The main advantage of the nicotine vaccine is its prolonged effect on the immune system (6-12 months), which can be recovered by occasional booster shots. Drawbacks include the necessity of multiple injections and the time delay before an immune response is achieved. There have also been inconsistencies in the degree of antibody response, with some people not achieving adequate antibody titers. In the following lines, the main therapeutic vaccines that are being developed as smoking cessation therapies will be summarized.

TA-NIC, a d evelopmental vaccine by Celtic Pharmaceuticals', has completed Phase I/II trials. This vaccine shows a significant increase in self-reported quit rates at 12 months among patients receiving the vaccine (20-40% depending on t he dose) compared with those receiving placebo (8%) (http://hugin.info/133161/R/982993/146255.pdf). Another large phase II trial at 6 m onths has also been completed, but the results have not yet been announced (http://clinicaltrials.gov).

NIC002 (also known as Nicotine QB or CYT002-NicQB) from Cytos Biotechnology has also been tested in phase II trials. This vaccine only promotes tobacco abstinence in smokers who achieve high levels of antibodies (Cornuz et al, 2008) and side effects such as flu-like symptoms are commonly reported. In cooperation with Novartis, a new phase II trial began in 2008 using a reformulated vaccine with fewer side-effects. An interim analysis released in 2009 showed that continuous abstinence was not achieved, probably because NIC002 was not able to induce sufficiently high antibody titers (http://www.cytos.com/userfiles/file/Cytos_Press_E_091015.pdf).

Positive results had been reported from a phase II clinical trial with NicVAX (Nabi Biopharmaceuticals) (Hatsukami et al, 2005). NicVAX was safe and well-tolerated, and generated high anti-nicotine antibody levels. Moreover, a correlation was observed between the antibody levels and the ability of patients to stop smoking and remain abstinent over the long-term. However, results released on 2011 from phase III clinical trials have been disappointing, since no differences have been observed between NicVAX- and placebo-receiving groups in nicotine abstinence rates (more info in http://phx.corporate-ir.net/phoenix.zhtml?c=100445&p=irol-news&nyo=1).

Another nicotine vaccine in development is Niccine (Independent Pharmaceutica AB). In 2008, the enrollment of 355 smokers into a phase II clinical trial was completed (http://www.independentpharma.com). The objective of this study is to test the ability of the vaccine in preventing relapse in smokers that have recently stopped smoking.

3. The hypocretin/orexin system

3.1. Introduction

It has been more than a decade since the hypocretin/orexin system was discovered simultaneously by 2 independent groups (de Lecea et al, 1998; Sakurai et al, 1998) and extensive knowledge has accumulated over these years regarding the anatomical distribution and the function of this endogenous system in physiological and pathophysiological conditions. Although initially described as exclusive CNS peptides, recent evidence suggests that hypocretins and their receptors are also present in peripheral tissues, including the intestine, pancreas, adrenals, kidney, adipose tissue and reproductive tract (Heinonen et al, 2008). Nonetheless, the hypocretin sources and functions in the periphery still remain unclear and are out of the scope of this thesis. Therefore, in the next sections, the components, distribution and function of the central hypocretin system will be summarized.

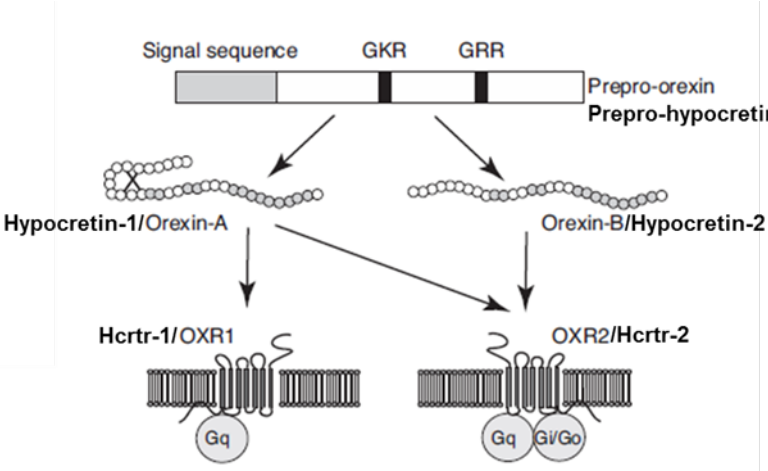

Figure 14. Hypocretins and their receptors (modified from Sakurai and Mieda, 2011).

3.1.1. Hypocretins and their receptors

Hypocretin-1/Orexin-A (33 amino acids) and hypocretin-2/orexin-B (28 amino acids) are 2 endogenous neuropeptides that arise from the proteolytic cleavage of the same precursor peptide, preprohypocretin/preproorexin (131 amino acids) (de Lecea et al, 1998; Sakurai et al, 1998) (Figure 14). A single gene encodes preprohypocretin and is localized to chromosome 17 in humans (Sakurai et al, 1998). Hypocretin peptides are present from fish to mammals and are well conserved across mammalian species (Álvarez and Sutcliffe, 2002; Wong et al, 2011), suggesting a strong evolutionary pressure to preserve structure, and ultimately function. Thus, human hypocretin-1 and -2 share 46% sequence homology. Moreover, hypocretin-1 is identical in a series of mammalian species and hypocretin-2 only differs in 1 or 2 amino acids from human to other mammalians. After cleavage of 1 preprohypocretin molecule, additional post-translational modifications occur in order to obtain the mature functional hypocretin peptides. Hence, hypocretin-1 and -2 are carboxi-terminally amidated and the amino-terminal glutamine residue of hypocretin-1 is cyclized into a pyroglutamil residue (Sakurai et al, 1998). Although hypocretin-2 remains a linear peptide, hypocretin-1 is further stabilized with 2 intrachain disulfide bridges (Sakurai et al, 1998). In the CNS, both hypocretin peptides exert a neuromodulatory function. Accordingly, they are stored in secretory vesicles, accumulated at axon terminals and released in a Ca^{2+}-dependent manner (de Lecea et al, 1998). So far, 2 G protein coupled receptors that respond to hypocretin stimulation have been cloned, hypocretin/orexin receptor-1 (Hcrtr-1/OxR1) and hypocretin/orexin receptor-2 (Hcrtr-2/OxR2) (Sakurai et al, 1998) (Figure 14). Each hypocretin receptor is encoded by a single gene. Thus, Hcrtr-1 (425 amino acids) and Hcrtr-2 (444 amino acids) are respectively encoded in chromosome 1 and 6 in humans (Kukkonen et al, 2002). Hypocretin receptors, similar to hypocretin peptides, are highly conserved across mammalian species and there is an overall 64% identity between them in humans (Sakurai et al, 1998). Nevertheless, in non-mammalian species only Hcrtr-2 has been

identified, suggesting that Hcrtr-1 probably evolved by gene duplication after the divergence that gave rise to mammals (Wong et al, 2011). The ligand binding affinities of hypocretin receptors are different. Thus, while Hcrtr-2 binds with equal affinity both hypocretin peptides, Hcrtr-1 binds with 100-1000 higher affinity to hypocretin-1 than hypocretin-2 (Sakurai et al, 1998; Smart et al, 1999; Ammoun et al, 2003). As the carboxi-terminal region of the hypocretin peptides is highly conserved, it has been suggested that differences in the amino-terminal region account, at least in part, for the higher selectivity of Hcrtr-1 to hypocretin-1 (Takai et al, 2006). Similarly, specific regions of hypocretin receptors also determine ligand selectivity (Putula et al, 2011).

3.1.2. Signaling through hypocretin receptors

The signaling pathways activated by hypocretin receptor stimulation have been extensively investigated in transfected heterologous cell systems, however the signals triggered in native receptor-expressing neurons still remain unclear. In neurons, the most frequent response after agonist binding to hypocretin receptors is an enhancement of intracellular Ca^{2+} concentrations. A common signal transduction mechanism has been identified for both hypocretin receptors based on the activation of Gq proteins. In neurons of many brain regions, Gq protein activation is followed by phospholipase C and subsequent protein kinase C (PKC) stimulation (van den Pol et al, 1998; Uramura et al, 2001; Kohlmeier et al, 2004; Narita et al, 2007; Xia et al, 2009). In turn, the activation of this kinase phosphorylates and modulates effector ion channels that promote Ca^{2+} entrance (Uramura et al, 2001; Kohlmeier et al, 2004; Xia et al, 2009). Although Hcrtr-1 signaling appears to be mainly mediated by Gq proteins at the neuronal level, a recent report has shown that Hcrtr-1 stimulation leads to cyclic AMP production in primary cultures of rat astrocytes (Woldan-Tambor et al, 2011), indicating the existence of Gs-coupled signal transduction through Hcrtr-1 in glial cells. The intracellular Ca^{2+} elevation characteristic of hypocretin receptor activation explains the frequently reported neuroexcitatory nature of hypocretin peptides

in the brain (de Lecea et al, 1998; van den Pol et al, 1998). Thus, the most common response to stimulation by hypocretins is an increase in action potential frequency that is achieved by both pre-synaptic and post-synaptic mechanisms. PKC activation in the post-synaptic neuron regulates effector ion channels that facilitate Na^+ and Ca^{2+} entry and avoid K^+ influx, leading to depolarization (Yang et al, 2003a; Yang et al, 2003b). At the same time, activated PKC phosphorylates and regulates pre-synaptic Ca^{2+} channels, facilitating neurotransmitter release (van den Pol et al, 1998; Li et al, 2002; Schlicker and Kathmann, 2008). In contrast, a few studies have also related hypocretin transmission to synaptic inhibition (Martin et al, 2002; Ma et al, 2007; Mori et al, 2011). Although the mechanisms underlying synaptic inhibition are not well understood, Hcrtr-2 has been shown to couple to inhibitory Gi proteins that prevent cyclic AMP formation (Zhu et al, 2003; Urbanska et al, 2012).

3.2. Neuroanatomical distribution of the hypocretin system

In the CNS, hypocretin-expressing neurons are restricted to some subregions of the hypothalamus, including the perifornical area (PFA), the dorsomedial hypothalamus (DMH), and the dorsal and lateral hypothalamus (LH) (de Lecea et al, 1998; Sakurai et al, 1998; Peyron et al, 1998) (Figure 15). These neurons, which have been assumed to number around 3,000 in rat brain and 70,000 in human brain (Sakurai and Mieda, 2011), project widely throughout the entire CNS (Peyron et al, 1998) (Figure 15). Hypocretin-containing neurons densely innervate the hypothalamus, which reflects the crucial action of these peptides in energy homeostasis and other autonomic functions. The most prominent extrahypothalamic projections are found in brain stem structures involved in the regulation of sleep-wake cycles, such as the raphe nuclei, the reticular formation, and especially the locus coeruleus (Peyron et al, 1998). The high density of fibers in the locus coeruleus and the tuberomammilary nucleus of the hypothalamus, nuclei related with the

maintenance of the waking-state, suits well with the key role that hypocretin peptides play in behavioral arousal. Hypocretin neurons also send significant efferent projections to structures related to drug reward and addiction, including the paraventricular nucleus of the thalamus (PVT), the septal nuclei, the BNST, the anterior and the central amygdaloid nuclei, and the VTA. The cortex and the most medial part of the NAc shell also receive disperse innervation by hypocretin-containing axons (Peyron et al, 1998; Baldo et al, 2003).

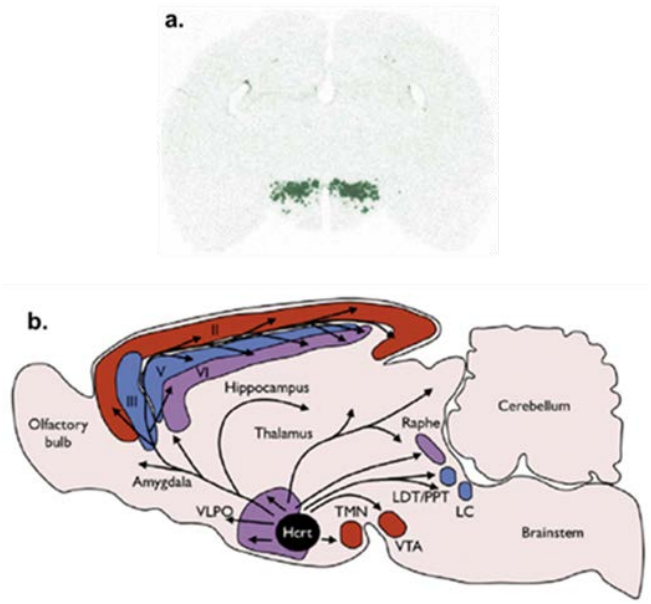

Figure 15. Localization of hypocretin-expressing neurons and their projections in the central nervous system. a. *In situ* hybridization in a coronal section of the rat brain with complementary DNA for prepro-hypocretin. The signal is exclusively localized in the lateral hypothalamic area. b. Lateral hypothalamic hypocretin neurons widely project throughout the brain, including brain areas related to drug addiction (modified from Sutcliffe and de Lecea, 2002; Carter et al, 2009).

Both hypocretin receptors are distributed throughout the projection sites of hypocretin fibers and the expression patterns of Hcrtr-1 and Hcrtr-2 are partially overlapping, although some particular areas express only 1 receptor subtype (Marcus et al, 2001; Trivedi et al, 1998). For instance, the PFC and

the locus coeruleus predominantly express Hcrtr-1 whereas the NAc and the tuberomammilary nucleus mainly express Hcrtr-2 (Marcus et al, 2001). This pattern of expression suggests that Hcrtr-1 and Hcrtr-2 may have different physiological roles.

3.3. The hypocretin system in physiology and disease

The widespread extension of hypocretin fibers and their receptors in the CNS is in agreement with the variety of physiological functions of the hypocretin system, that includes regulation of arousal and sleep/wake cycles, feeding behavior and energy homeostasis, stress, and reward-seeking and reinforcement, among others (Sakurai and Mieda, 2011). Recent evidence also points to a role for the hypocretin system in other CNS disorders, such as Alzheimer's disease (Kang et al, 2009; Fronczek et al, 2011) and panic anxiety disorders (Johnson et al, 2010; Annerbrink et al, 2011; Johnson et al, 2012).

3.3.1. Arousal and sleep/wake cycles

Research during the past decade has established the idea that hypocretins are both sufficient and necessary for the promotion of wakefulness, and they are now generally considered to be "arousal-promoting" peptides (Sakurai, 2007; Saper et al, 2005). Therefore, it is not surprising that the best understood role of hypocretins is the coordination of sleep/wake states. The intraventricular infusion of hypocretin-1 and -2 in rodents during the light (rest) cycle increases the awake time and decreases both REM and non-REM sleep time (Hagan et al, 1999). Similarly, optogenetic activation of hypocretin neurons increases the probability of an awakening event during both REM and non-REM sleep phases (Carter et al, 2009; Adamantidis et al, 2007), whereas optogenetic silencing of these neurons induces slow-wave sleep in mice (Tsunematsu et al, 2011).

Narcolepsy, a chronic sleep disorder, characterized by excessive

daytime sleepiness and episodes of sleep-attacks, is the clearest evidence of aberrant hypocretin signaling in pathophysiological conditions. The first indications that hypocretins were involved in the regulation of sleep/wake cycles and narcolepsy came from the study of animal models. Mice lacking the preprohypocretin gene or dogs with an inactivating mutation of the Hcrtr-2 gene show a phenotype remarkably similar to human narcoleptic patients (Chemelli et al, 1999; Lin et al, 1999). Similarly, hypocretin neuron-ablated (orexin/ataxin-3 transgenic) mice and Hcrtr-2 knockout mice show similar phenotypes with strong similarities to the human condition, characterized by behavioral arrests similar to cataplexy, occasional direct transitions from wakefulness to REM sleep and highly fragmented sleep-wake cycles (Hara et al, 2001; Willie et al, 2003). The link between hypocretin dysfunction and narcolepsy is further supported by studies in human patients. Thus, narcoleptic patients have decreased hypocretin-1 levels in the cerebrospinal fluid (Nishino et al, 2000). Moreover, an 80-100% reduction in the number of neurons containing detectable preprohypocretin mRNA and hypocretin-like immunoreactivity in the hypothalamus has been reported in human narcoleptics (Peyron et al, 2000; Thannickal et al, 2000). Therefore, it has been proposed that hypocretin agonists might be useful for narcoleptic patients, whereas hypocretin receptor antagonists might serve for the treatment of insomnia (Cao and Guilleminault, 2011).

3.3.2. Feeding behavior and energy homeostasis

Hypocretins were initially described as regulators of feeding behavior based on their localization in the lateral hypothalamic area and their effects on food intake. Indeed, the alternative name “orexin” was devised upon t he observation that intraventricular infusion of hypocretins increased food intake in rats (Sakurai et al, 1998). Subsequently, hypocretin effects on food intake have been widely replicated in rodents and, recently, also in zebrafish (Haynes et al, 2000; Thorpe and Kotz, 2005; Yokobori et al, 2011). Moreover, hypocretin mRNA levels have been shown to be increased during fasting

(Sakurai et al, 1998) and the intraventricular administration of an anti-hypocretin antibody or an Hcrtr-1 antagonist attenuates food intake (Haynes et al, 2000; Yamada et al, 2000). Nevertheless, it has been proposed that the wakefulness promoting effects of hypocretins might indirectly contribute to the increase in food intake observed after acute hypocretin administration (Yamanaka et al, 2003). Indeed, hypocretin neuron-ablated mice display an obese phenotype despite exhibiting hypophagia (Hara et al, 2001). Furthermore, narcoleptic humans deficient in hypocretin signaling have increased body mass index although their caloric intake is lower (Schuld et al, 2000; Lammers et al, 1996). Therefore, hypocretin signaling might positively regulate feeding as well as arousal, motor activity and basal energy expenditure, and this might explain why narcoleptic mice and humans show increased body weight despite their hypophagia (Sakurai and Mieda, 2011).

3.3.3. Stress and anxiety

Stimuli that increase arousal/wakefulness often enhance stress and anxiety. Indeed, several lines of evidence support the idea that the hypocretin system is part of the circuitries that mediate responses to acute stress (Winsky-Sommerer et al, 2005). Intraventricular injection of hypocretin stimulates the hypothalamic-pituitary-adrenal (HPA) axis (Kuru et al, 2000) and activates CRF-expressing neurons in the paraventricular nucleus of the hypothalamus (PVN) and the CeA (Sakamoto et al, 2004). In addition, direct reciprocal connections exist between hypocretin and the hypothalamic CRF system (Winsky-Sommerer et al, 2004). Thus, CRF induces depolarization and increases the firing rate of hypocretin neurons, presumably through the direct activation of CRF_1 receptors (Winsky-Sommerer et al, 2004). Similarly, bath application of hypocretin-1 depolarizes and increases spike frequency in magnocellular and parvocellular neurons of the PVN (Samson et al, 2002). These observations suggest that hypocretins may interact with central CRF systems to activate the HPA axis and regulate stress-related processes.

Interestingly, a recent report has revealed a role for hypocretins in panic anxiety disorders. Accordingly, genetic or pharmacological inhibition of hypocretin signaling effectively blocks panic anxiety attacks in rodents (Johnson et al, 2008). Moreover, human patients suffering from panic disorder exhibit elevated levels of hypocretin in the cerebrospinal fluid (Johnson et al, 2008).

3.4. Involvement of the hypocretin system in drug-reward and addiction

The anatomical distribution of the hypocretin system in the CNS supports a role for hypocretin peptides in the modulation of the addictive properties of drugs of abuse. Indeed, reciprocal innervations have been reported between the lateral hypothalamic hypocretin neurons and several structures of the mesocorticolimbic system and extended amygdala, including the VTA, NAc, PFC, CeA and BNST (Peyron et al, 1998; Nambu et al, 1999; Baldo et al, 2003; Yoshida et al, 2006). Consistent with these anatomical data, hypocretins appear to regulate the activity of the mesocorticolimbic dopaminergic system. Thus, bath application of hypocretin-1 and -2 increases the firing rate of VTA DA and GABA neurons (Korotkova et al, 2003). Additionally, intra-VTA infusion of hypocretin-1 enhances DA extracellular levels in the NAc (Narita et al, 2006; Narita et al, 2007; España et al, 2011), PFC (Vittoz and Berridge, 2006), CeA and BNST (Hata et al, 2011) of rodents. Similarly, intra-VTA injection of hypocretin-2 enhances DA outflow in the NAc (Narita et al, 2006; Narita et al, 2007), suggesting that hypocretin peptides might contribute to the regulation of the rewarding properties of drugs of abuse. In agreement, direct infusion of hypocretin-1 and -2 into the VTA leads to the development of a CPP (Narita et al, 2007). In contrast, intraventricular and intra-VTA infusions of hypocretin-1 decrease brain reward activity, as measured by elevated ICSS reward thresholds (Boutrel et

al, 2005; Hata et al, 2011). This effect is similar to that observed after CRF administration (Macey et al, 2000) or during drug withdrawal (Markou and Koob, 1991; Epping-Jordan et al, 1998). Hence, hypocretin transmission might not only influence the positive rewarding effects of drugs of abuse, but also the negative affective state typical of drug withdrawal. Furthermore, hypocretin-1 produces long-lasting plasticity at excitatory synapses of DA neurons in the VTA (Borgland et al, 2006). Thus, hypocretin signaling could also contribute to relapse after long periods of abstinence.

The first reports relating hypocretin transmission to drug addiction were published in 2005 (Harris et al, 2005; Boutrel et al, 2005), and since then, a growing body of evidence has accumulated supporting a role for this system in the rewarding effects of drugs of abuse and addiction.

3.4.1. Role of the hypocretin system in cocaine addiction

The participation of the hypocretin system in cocaine addiction seems to be complex. Although hypocretins appear to participate in the motivational aspects of cocaine addiction, the involvement of these neuropeptides in psychostimulant primary rewarding effects remains controversial. Mice receiving systemic and intra-VTA infusions of the Hcrtr-1 antagonist SB334867 as well as mice null for the preprohypocretin gene show attenuated cocaine-induced enhancement of DA extracellular levels in the NAc (España et al, 2010). Similarly, intra-VTA infusion of hypocretin-1 increases the effects of cocaine on tonic and phasic DA release in the NAc (España et al, 2011). These results suggest that hypocretin signaling modulates cocaine effects in mesocorticolimbic DA transmission. However, amassing evidence indicates that these endogenous neuropeptides do not influence the primary rewarding properties of cocaine. Thus, SB334867 does not prevent the expression of a cocaine-induced place preference (Sharf et al, 2010) nor cocaine-induced enhancement of brain reward function evaluated in the ICSS paradigm (Riday et al, 2012). Furthermore, hypocretins do not contribute to the reinforcing effects of cocaine when using a fixed-ratio 1 schedule of

reinforcement in cocaine self-administration models (Boutrel et al, 2005; España et al, 2010; España et al, 2011; Smith et al, 2009; Zhou et al, 2012). Nevertheless, when access to cocaine self-administration is limited or under conditions that require higher effort to obtain a cocaine infusion, an involvement of the hypocretin system in cocaine reinforcement has been revealed. Hence, SB334867 reduced responding for cocaine when using a 24 hour access paradigm in which the number of infusions that an animal can receive each hour is limited (discrete-trial procedure) (España et al, 2010). Consistently, hypocretin-1 infusion to the lateral ventricles increased lever-pressing under the same experimental procedure (España et al, 2011). Systemic or intra-VTA administration of SB334867 significantly reduced the motivation to work for cocaine, assessed by the progressive-ratio schedule of reinforcement (Borgland et al. 2009; España et al. 2010). Similarly, the same Hcrtr-1 antagonist decreased the number of cocaine infusions obtained by the use of threshold cocaine self-administration paradigm, in which the demand of responding progressively increases to maintain blood levels of cocaine (España et al. 2010). In agreement, intra-VTA infusions of hypocretin-1 increased the breaking-point achieved under a progressive-ratio schedule (España et al. 2011), suggesting that the enhancement of hypocretin transmission in the VTA increases the reinforcing efficacy and the motivational properties of cocaine.

The involvement of the hypocretin system in the reinstatement of cocaine-seeking behavior after presentation of different stimuli has been largely explored. The intraventricular or intra-VTA infusion of hypocretin-1 induces reinstatement of a previously extinguished cocaine-seeking behavior (Boutrel et al, 2005; Wang et al, 2009). Moreover, the systemic injection of the Hcrtr-1 antagonist SB334867 blocks footshock- (Boutrel et al, 2005) and yohimbine- (Zhou et al, 2012) elicited stress-induced reinstatement of cocaine-seeking. Nevertheless, intra-VTA infusion of the Hcrtr-1 antagonist SB408124 does not prevent footshock stress-induced reinstatement of

cocaine-seeking (Wang et al, 2009), suggesting that hypocretins in the VTA do not influence this behavioral response. Signaling through Hcrtr-1 is also necessary for cue- and context-induced reinstatement of cocaine-seeking (Smith et al, 2009; Smith et al, 2010; Aston-Jones et al, 2010; Zhou et al, 2012), but not for cocaine-primed reinstatement (Aston-Jones et al, 2010; Zhou et al, 2012; Mahler et al, 2012). On the contrary, Hcrtr-2 activation is not involved in cocaine-seeking elicited by cocaine-conditioned cues (Smith et al, 2009), indicating functional selectivity for Hcrtr-1 in the regulation of this effect. Hypocretin signaling in the VTA, unlike during stress-induced reinstatement, seems to be essential for reinstatement after presentation of cocaine-associated cues. Thus, the intra-VTA infusion of SB334867 dose-dependently attenuates cue-induced cocaine-seeking (James et al, 2011; Mahler et al, 2012). Conversely, direct infusion of the same Hcrtr-1 antagonist into the VTA does not influence cocaine-primed reinstatement (Mahler et al, 2012).

Notably, cocaine-evoked synaptic plasticity in the VTA also depends on signaling through Hcrtr-1. Accordingly, SB334867 blocked cocaine-induced long-term potentiation in DA neurons of the VTA (Borgland et al, 2006). Consistent with a lack of cocaine-induced synaptic plasticity in the VTA, the systemic or intra-VTA administration of the Hcrtr-1 antagonist prevented the acquisition of cocaine-induced locomotor sensitization (Borgland et al, 2006).

3.4.2. Role of the hypocretin system in opioid addiction

A number of reports have related hypocretin transmission to opioid rewarding effects. Rats that exhibit a p reference for an environment previously paired with morphine in a place conditioning paradigm show an activation of VTA projecting LH hypocretin neurons, as revealed by c-Fos immunohistochemistry (Harris et al, 2005; Richardson and Aston-Jones, 2012). Moreover, excitotoxic lesions of the hypocretin-enriched area in the LH prevent the acquisition of a place preference conditioned by morphine (Harris et al, 2007). These results indicate that LH hypocretin neurons are

critically involved in learning to associate an environment with morphine reward. In line with these findings, systemic (Harris et al, 2005; Sharf et al, 2010) or intra-VTA (Narita et al, 2006) administration of an Hcrtr-1 antagonist attenuates the expression of morphine-induced place preference. Moreover, Hcrtr-1 blockade reduces heroin intake in an operant self-administration paradigm in rats (Smith and Aston-Jones, 2012), suggesting a role for this receptor in opioid reward and reinforcement. These behavioral effects might be explained by the lack of morphine-elicited extracellular DA release in the NAc of preprohypocretin knockout mice (Narita et al, 2006). Nevertheless, conflicting results have been obtained in the CPP paradigm when using these mutant mice. Hence, a suppression (Narita et al, 2006) or a similar (Sharf et al, 2010) morphine-induced place preference was observed in mice with a deletion of the preprohypocretin gene.

Hypocretins also contribute to the affective and somatic symptoms of morphine withdrawal. Thus, the physical signs induced by naloxone-precipitated morphine withdrawal are attenuated in mice and rats receiving the Hcrtr-1 antagonist SB334867 (Sharf et al, 2008; Laorden et al, 2012) or mice lacking the preprohypocretin gene (Georgescu et al, 2003). Moreover, Hcrtr-1 in the locus coeruleus is critical for the somatic expression of morphine withdrawal (Azizi et al, 2010). In contrast, Hcrtr-2 signaling in the PVT is essential for the expression of a CPA after re-exposure to contextual cues associated with morphine withdrawal (Li et al, 2011). Consistent with these behavioral data, naltrexone- or naloxone-precipitated morphine withdrawal leads to the induction of c-Fos expression in hypocretin cells (Georgescu et al, 2003; Sharf et al, 2008; Laorden et al, 2012) and both naloxone-precipitated and spontaneous withdrawal increase hypocretin mRNA levels in the LH (Zhou et al, 2006; Laorden et al, 2012). Furthermore, naloxone-precipitated morphine withdrawal induced c-Fos expression in the NAc shell, BNST, CeA and PVN is reduced by the previous administration of an Hcrtr-1 antagonist (Laorden et al, 2012).

Few studies have examined the implication of the hypocretin system in animal models of opioid relapse. An initial study showed that chemical activation of LH hypocretin neurons reinstated an extinguished morphine place preference, which was completely blocked by prior systemic administration of SB334867 (Harris et al, 2005). In addition, the injection of hypocretin-1 into the VTA, but not in areas surrounding this brain structure, caused a significant reinstatement of the previously extinguished morphine place preference (Harris et al, 2005). In line with these findings, a recent study has confirmed the participation of hypocretin transmission in relapse to opioid-seeking elicited by drug-paired cues using an operant self-administration paradigm. Thus, Hcrtr-1 blockade attenuated cue-induced reinstatement of heroin-seeking, whereas it did not affect responding after a priming injection of heroin (Smith and Aston-Jones, 2012).

3.4.3. Role of the hypocretin system in alcohol addiction

Although the hypocretin system appears to be involved in the addictive properties of alcohol (Lawrence, 2010), conflicting results have been obtained in animal models of alcohol reward. Several studies using the operant self-administration procedure have demonstrated a role for Hcrtr-1 in alcohol reinforcing effects. Thus, the Hcrtr-1 antagonist SB334867 attenuated operant alcohol intake in alcohol preferring and Long-Evans rats (Lawrence et al, 2006; Richards et al, 2008). Similarly, the same antagonist reduced ethanol preference on a two-bottle free-choice paradigm in rats (Moorman and Aston-Jones, 2009). Moreover, a recent study has revealed a role for Hcrtr-1 in the motivation to self-administer alcohol since SB334867 pretreatment reduced the breaking point in a progressive-ratio schedule of reinforcement (Jupp et al, 2011a). Consistently, the infusion of hypocretin-1 into the PVN or the LH, stimulates voluntary ethanol intake (Schneider et al, 2007). Interestingly, Hcrtr-2 also seems to participate in the reinforcing effects of ethanol. Accordingly, the specific Hcrtr-2 antagonist JNJ-10397049 dose-dependently reduced ethanol self-administration in rats (Shoblock et al, 2011). In addition,

the same antagonist attenuated the acquisition and expression of ethanol CPP (Shoblock et al, 2011). Surprisingly, the Hcrtr-1 antagonist SB408124 was ineffective in reducing the reinforcing effects of ethanol in this study (Shoblock et al, 2011). In agreement, Hcrtr-1 blockade by SB334867 did not affect the acquisition and expression of ethanol-induced CPP in mice (Voorhees and Cunningham, 2011), suggesting that Hcrtr-1 does not influence ethanol's primary or conditioned rewarding effects. Differences in animal species and experimental procedures may explain the differences reported in the role of hypocretin receptors in ethanol rewarding responses, and additional research will be necessary to further clarify the role of the hypocretin system in these motivational properties of ethanol.

Several studies have evaluated the involvement of hypocretin neuropeptides in alcohol relapse. Context- (Hamlin et al, 2007) and cue-induced alcohol seeking (Dayas et al, 2008) activate hypocretin-containing neurons in the lateral hypothalamic area. In agreement, Hcrtr-1 signaling is necessary for cue-induced reinstatement of alcohol-seeking after extinction (Lawrence et al, 2006) or protracted abstinence (Jupp et al, 2011b). Moreover, stress-induced reinstatement of alcohol-seeking elicited by the administration of yohimbine is prevented by prior administration of SB334867 (Richards et al, 2008). Consistent with preclinical data, recent studies in humans suggest an involvement of hypocretins in the affective dysregulation that appears in alcohol-dependent patients during alcohol withdrawal and craving (von der Goltz et al, 2011; Bayerlein et al, 2011).

3.4.4. Role of the hypocretin system in nicotine addiction

Emerging evidence suggests that hypocretin transmission may influence the addictive properties of nicotine. An acute nicotine injection increases c-Fos expression in hypocretin neurons (Pasumarthi et al, 2006). In addition, intravenous nicotine self-administration modifies Hcrtr-1 mRNA levels in the arcuate nucleus and the rostral lateral areas of the hypothalamus in rats (LeSage et al, 2010). Similarly, non-contingent chronic nicotine

administration regulates preprohypocretin and hypocretin receptor mRNA levels in the rat hypothalamus (Kane et al, 2000). At the behavioral level, pretreatment with the Hcrtr-1 antagonist SB334867 or the Hcrtr-1/Hcrtr-2 antagonist almorexant decreases intravenous nicotine self-administration in rats under a fixed-ratio 5 schedule of reinforcement (Hollander et al. 2008; LeSage et al. 2010). Moreover, SB334867 decreased the number of nicotine rewards earned under a progressive-ratio schedule of reinforcement (Hollander et al. 2008), suggesting that hypocretins acting on Hcrtr-1 regulate nicotine reinforcement and the motivation to seek the drug. Stroke-associated damage to the insular cortex in human smokers results in spontaneous cessation of the smoking habit and a low urge to smoke (Naqvi et al, 2007). Interestingly, intra-insular infusion of SB334867 decreases nicotine intake in rats in a self-administration paradigm (Hollander et al, 2008), suggesting that insular Hcrtr-1 transmission is crucial for the reinforcing effects of nicotine. Hypocretins may modulate nicotine rewarding and motivational properties by the regulation of the stimulatory effects of nicotine in brain reward systems. Indeed, SB334867 blocks nicotine-induced lowering of ICSS thresholds in rats (Hollander et al, 2008). Consistent with rodent studies, recent evidence in humans also points to a role for hypocretin transmission in tobacco addiction. Thus, a negative correlation between hypocretin plasma concentration and nicotine craving has been shown (von der Goltz et al, 2010).

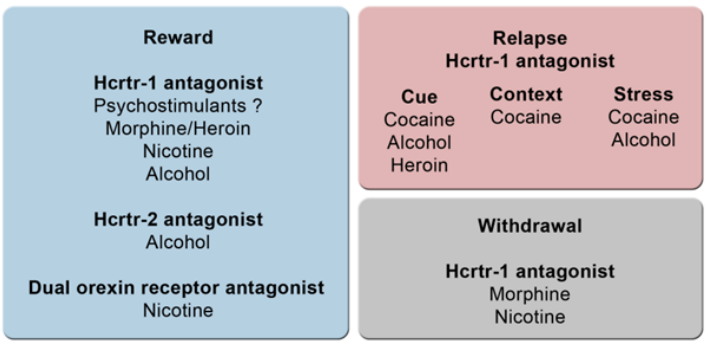

Figure 16. Diagram depicting the potential therapeutic utility of hypocretin receptor antagonists in drug addiction.

In summary, amassing evidence supports a role for the hypocretin system in drug addiction. Indeed, available studies indicate that hypocretin signaling not only contributes to the rewarding and motivational properties of drugs of abuse, but also to the aversive state of drug withdrawal and to relapse following prolonged periods of abstinence. Therefore, hypocretin receptor antagonists might have potential therapeutic utility for the treatment of the various aspects of drug addiction (Figure 16).

OBJECTIVES

General objective

Anatomical, functional and behavioral data point to a role for the hypocretin system in drug addiction. Therefore, the main goal of this thesis is to elucidate whether hypocretin transmission contributes to the addictive properties of nicotine.

Specific objectives

- ✓ To study the participation of the hypocretin system in the processes driving to reinstatement of nicotine-seeking behavior, an animal model of relapse.
    - o Involvement of hypocretin signaling in the anxiety-like effects of nicotine and stress-induced reinstatement of nicotine-seeking (article #1).
    - o Role of hypocretin transmission in cue-induced reinstatement of nicotine-seeking behavior (article #2).
- ✓ To evaluate whether hypocretin signaling participates in the behavioral expression of nicotine withdrawal and to assess the mechanisms and the neuroanatomical basis of the possible role of hypocretins in this nicotine effect (article #3).
- ✓ To update and summarize the role of the hypocretin system in different stages of drug addiction (article #4).

RESULTS

ARTICLE 1

Hypocretins Regulate the Anxiogenic-Like Effects of Nicotine and Induce Reinstatement of Nicotine-Seeking Behavior

Ainhoa Plaza-Zabala, Elena Martín-García, Luis de Lecea,
Rafael Maldonado and Fernando Berrendero

J Neurosci (2010) 30(6):2300-2310

Figure 5

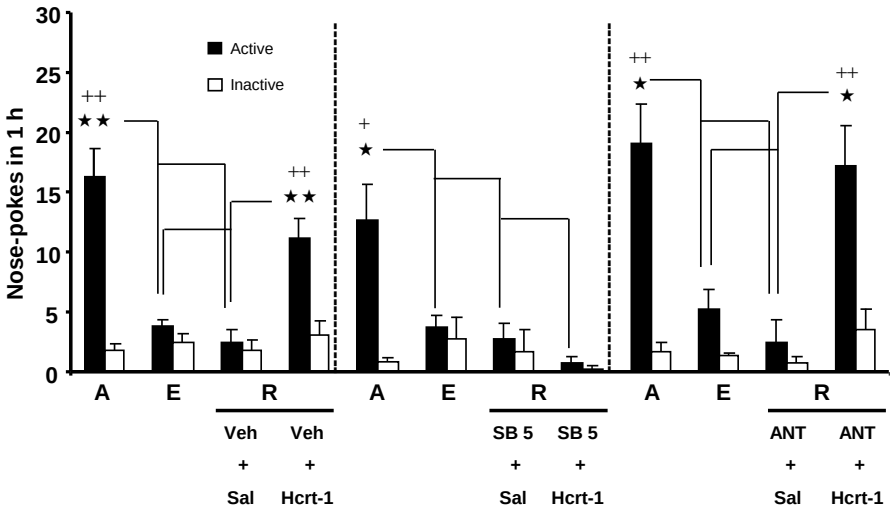

Figure 5. Hcrtr-1, but not CRF1 receptor, regulates the reinstatement of nicotine-seeking behavior induced by Hcrt-1. Mice that achieved the extinction criterion received an intracerebroventricular saline infusion preceded by SB334867 (5 mg/kg, i.p.) (n = 4), antalarmin (30 mg/kg, s.c.) (n = 4), or vehicle (n = 9) 10 min before the reinstatement session. Next day, the same mice were infused with Hcrt-1 (0.75 nmol/1 µl, i.c.v.) preceded by the same SB334867 (5 mg/kg, i.p.) (n = 4), antalarmin (30 mg/kg, s.c.) (n = 4), or vehicle (n = 9) and tested for reinstatement 10 min later. SB334867 and antalarmin were administered 30 min and 1 h, respectively, before intracerebroventricular Hcrt-1/vehicle infusions. Mean number of nose-pokes in the active (■) and inactive (□) holes during the different experimental phases: A, acquisition of nicotine self-administration behavior (mean of 3 d acquisition criteria); E, extinction (mean of 3 d criterion), and R, reinstatement (intracrebroventricular infusion of vehicle/Hcrt-1). ★ $p < 0.05$, ★★ $p < 0.01$, differences between experimental phases when considering the active hole; + $p < 0.05$, ++ $p < 0.01$, differences between holes within the same experimental phase (Newman-Keuls test).

Figure 6

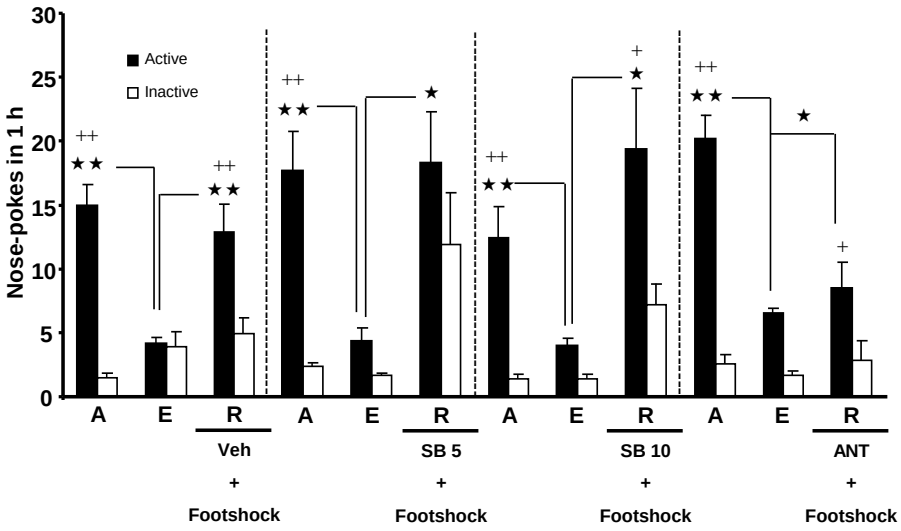

Figure 6. CRF1 receptor, but not Hcrtr-1, regulates footshock stress-induced reinstatement of nicotine-seeking behavior. Mice that achieved the extinction criterion received intermittent footshock stimuli (0.22 mA, 5 min) immediately before the reinstatement session preceded by vehicle (n = 14), SB334867 (5 mg/kg, i.p.) (n = 6), SB334867 (10 mg/kg, i.p.) (n = 7), or antalarmin (30 mg/kg, s.c.) (n = 6). SB334867 and antalarmin were administered 30 min and 1 h, respectively, before electric footshock. Mean number of nose-pokes in the active (■) and inactive (□) holes during the different experimental phases: A, acquisition of nicotine self-administration behavior (mean of 3 d acquisition criteria); E, extinction (mean of 3 d criterion), and R, reinstatement (0.22 mA footshock stress). ★ $p < 0.05$, ★★$p < 0.01$, differences between experimental phases when considering the active hole, + $p < 0.05$, ++ $p < 0.01$, significant differences between holes within the same experimental phase (Newman-Keuls test).

Plaza-Zabala A, Martin-Garcia E, de Lecea L, Maldonado R, Berrendero F. Hypocretins regulate the anxiogenic-like effects of nicotine and induce reinstatement of nicotine-seeking behavior. Supplemental data. J Neurosci. 2010 Feb 10;30(6):2300-2310.

ARTICLE 2

A role for hypocretin/orexin receptor-1 in cue-induced reinstatement of nicotine-seeking behavior

Ainhoa Plaza-Zabala, África Flores, Elena Martín-García, Rocío Saravia, Rafael Maldonado and Fernando Berrendero

(submitted)

A role for hypocretin/orexin receptor-1 in cue-induced reinstatement of nicotine-seeking behavior

Ainhoa Plaza-Zabala, África Flores, Elena Martín-García, Rocío Saravia, Rafael Maldonado and Fernando Berrendero[#]

Laboratory of Neuropharmacology, Department of Experimental and Health Sciences, Universitat Pompeu Fabra, PRBB, C/ Doctor Aiguader 88, 08003 Barcelona, Spain

[#] Corresponding author: Fernando Berrendero, Phone: +34-93-3160890; Fax: + 34-93-3160901; E-mail: fernando.berrendero@upf.edu

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Supplementary material: 1 (Methods, and 1 Figure)

Abstract

Background: Hypocretin/orexin signaling is critically involved in relapse to drug-seeking behaviors. The involvement of the hypocretin system in cue-induced reinstatement of nicotine-seeking behavior has not been evaluated yet.

Methods: Mice were trained to self-administer nicotine for 10 days, and following extinction, they were pretreated with the hypocretin receptor 1 (Hcrtr-1) antagonist SB334867, the Hcrtr-2 antagonist TCSOX229, the PKC inhibitor NPC-15437 or vehicle 30 min before inducing reinstatement with conditioned cues. The activation of hypocretin neurons was investigated by using double label immunofluorescence of c-Fos with hypocretin-1. Changes induced by nicotine-seeking in the phosphorylation levels of NMDA and AMPA receptor subunits as well as some MAPK were analyzed by Western blot assays. The effects of Hcrtr-1 and Hcrtr-2 antagonists on cue-induced reinstatement of food-seeking were also evaluated.

Results: Pretreatment with SB334867, but not with TCSOX229, attenuated cue-induced reinstatement of nicotine-seeking, which was associated with an activation of hypocretin neurons of the hypothalamic perifornical area. In addition, relapse to nicotine-seeking increased the phosphorylation levels of GluR2-Ser880, NR1-Ser890 and p38 MAPK in the nucleus accumbens (NAc). Notably, phosphorylations of NR1-Ser890 and p38 MAPK, but not GluR2-Ser880, were dependent on Hcrtr-1 activation. NPC-15437 reduced nicotine-seeking behavior consistent with the PKC-dependent phosphorylations of GluR2-Ser880 and NR1-Ser890. SB334867 failed to modify cue-induced reinstatement of food-seeking, which did not elicit any biochemical changes in the NAc.

Conclusions: These data are the first to identify Hcrtr-1 and PKC signaling as potential therapeutic targets for the treatment of relapse to nicotine-seeking induced by nicotine-associated cues.

Introduction

Tobacco use is the leading cause of preventable death in developed countries. However, despite the harmful health consequences of tobacco smoking, approximately 80% of smokers attempting to quit on their own relapse within the first month of abstinence, and only around 3% remain abstinent at six months (Benowitz, 2009). The re-exposure to environmental stimuli associated with nicotine consumption is critical to explain the high rates of relapse to nicotine-seeking in humans (Caggiula et al, 2001; Chiamulera, 2005).

Several neurotransmitters are involved in the addictive properties of nicotine, which is considered the main addictive constituent of tobacco (Berrendero et al., 2010). Hypocretin-1 and -2 (also known as orexin A and B) are lateral hypothalamic neuropeptides that project throughout the brain (Peyron et al., 1998), and play an important role in drug addiction (Plaza-Zabala et al., 2012a). Increasing evidence suggests that hypocretin transmission is involved in the addictive effects of nicotine. Thus, the hypocretin receptor-1 (Hcrtr-1) antagonist SB334867 (Hollander et al., 2008) and the mixed Hcrtr-1/Hcrtr-2 antagonist almorexant (LeSage et al., 2010) decreased nicotine self-administration behavior in rats. Hcrtr-1 signaling in the hypothalamic paraventricular nucleus was shown to participate in the somatic signs of nicotine withdrawal (Plaza-Zabala et al., 2012b). In addition, hypocretin-1 induced reinstatement of previously extinguished nicotine-seeking behavior in mice by a mechanism independent of the corticotrophin releasing factor (CRF) (Plaza-Zabala et al., 2010). The potential participation of hypocretin transmission in the reinstatement of nicotine-seeking induced by nicotine-conditioned cues remains largely unexplored.

In this study, we investigated the specific contribution of Hcrtr-1 and Hcrtr-2 in cue-induced reinstatement of nicotine-seeking behavior. Relapse to drug-seeking behavior is associated with changes in synaptic plasticity involving excitatory glutamatergic transmission (Knackstedt and Kalivas,

2009), and MAPK signaling pathway (Wang et al., 2007). Therefore, we investigated the effects of cue-induced reinstatement of nicotine-seeking and the influence of hypocretin transmission in the phosphorylation levels of NMDA and AMPA glutamate receptor subunits as well as MAPK pathway activity in the nucleus accumbens (NAc) and the prefrontal cortex (PFC). To test whether the observed changes were specific for nicotine, we studied the influence of hypocretin signaling on the behavioral and biochemical effects produced by the reinstatement of cue-induced food-seeking.

Methods and Materials

Animals

Experiments were performed using male C57BL/6J mice (8-10 week old) (Charles River), which were single-housed in a temperature (21.1 ± 1 ºC)- and humidity (55 ± 10 %)-controlled room under reversed light/dark cycle conditions (lights off 8 A.M.). Mice were habituated to reversed cycle and handled during 1 week before starting with the operant training sessions. The experiments took place during the dark phase. Food and water were available *ad libitum* except for mice being tested for food-seeking behavior. The observer was blind to treatment in all the experiments. Animal procedures were conducted in accordance with the guidelines of the European Communities Directive 86/609/EEC regulating animal research and approved by the local ethical committee (CEEA-IMAS-UPF).

Drugs

(-)-Nicotine hydrogen tartrate salt [(-)-1-methyl-2(3-pyridyl)pyrrolidine] (Sigma) was dissolved in physiological saline (0.9% NaCl). The pH of the nicotine solution was adjusted to 7.4 and was contingently administered by intravenous (iv) route at the dose of 30 µg/kg per infusion (free base). The Hcrtr-1 antagonist SB334867 (Tocris Bioscience) was dissolved in 1% (2-hydroxypropyl)-β-cyclodextrin (Sigma) and 10% DMSO in distilled water. The Hcrtr-2 antagonist TCSOX229 (Tocris Bioscience) was dissolved in physiological saline. SB334867 and TCSOX229 were administered by intraperitoneal (ip) route in a volume of 5 ml/kg at the doses of 5 and 10 mg/kg. The protein kinase C (PKC) inhibitor NPC-15437 dihydrochloride hydrate (Sigma) was dissolved in physiological saline and administered by ip route in a volume of 10 ml/kg at the dose of 1 mg/kg. Ketamine hydrochloride (100 mg/kg) (Imalgène 1000) and xylazine hydrochloride 20 mg/kg (Sigma) were mixed and dissolved in ethanol (5%) and distilled water (95%). This anesthetic mixture was administered intraperitoneally in a volume of 10 ml/kg

body weight. Thiopental sodium (5 mg/ml) (Braun Medical S.A.) was dissolved in distilled water and it was delivered in a volume of 0.05 ml through the iv catheter.

Cue-induced reinstatement of nicotine-seeking behavior

Jugular vein catheterization. Mice were anesthetized with a ketamine/xylazine mixture and then implanted with indwelling iv silastic catheters in their right jugular vein as previously described (Soria et al. 2005). Briefly, silastic tubing of 6 cm long (0.3 mm inner diameter, 0.6 mm outer diameter (Silastic, Dow Corning) was fitted to a 22-gauge steel cannula (Semat) that was bent at a right angle, and then embedded in a cement disk (Dentalon Plus, Eraeus) with an underlying nylon mesh. The catheter tubing was inserted 1.3 cm into the right jugular vein and anchored with suture. The remaining tubing ran subcutaneously to the cannula, which exited at the midscapular region. All incisions were sutured and coated with antibiotic ointment (Bactroban, GlaxoSmithKline). After surgery, mice were allowed to recover for 4 days before initiation of self-administration sessions.

Apparatus. The experiments were conducted in mouse operant chambers (model ENV-307A-CT; Med Associates Inc.) equipped with two holes, one randomly selected as the active hole and the other as the inactive hole. Pump noise and stimuli lights (environmental cues), one located inside the active hole and the other above it were paired with the delivery of the reinforcer. Nicotine (30 µg/kg/infusion, free base) was delivered in a volume of 23.5 µl over 2 seconds via a syringe that was mounted on a microinfusion pump and connected via Tygon tubing to a single channel liquid swivel and to the mouse iv catheter.

Self-administration training. Slight modifications were applied to the previously described operant model (Martín-García et al, 2009). One hour daily self-administration sessions were conducted consecutively for 10 days. Mice were trained under a fixed ratio 1 schedule of reinforcement with a 10 second time-out. Each daily session started with a priming injection of the

drug. The stimuli light together with the pump noise (environmental cues) signaled delivery of nicotine infusion through the entire self-administration session. During the 10 second time-out period, the cue light was off and no reward was provided after nose-poking on the active hole. Responses on the inactive hole and all the responses elicited during the 10 second time-out period were also recorded. The session was terminated after 50 reinforcers were delivered or after 1 hour, whichever occurred first. The criteria for the acquisition of self-administration behavior were achieved when in 3 consecutive sessions: (1) mice maintained a stable responding with < 20% deviation from the mean of the total number of reinforcers earned (80% stability); (2) at least 75% responding on the active hole, and (3) a minimum of 6 reinforcers per session. The patency of iv catheters was evaluated at the end of nicotine self-administration training by an infusion of 0.05 ml of thiopental sodium through the catheter. If prominent signs of anesthesia were not apparent within 3 seconds of the infusion the mouse was removed from the experiment. Only mice with patent catheter that met all the acquisition criteria were moved to the extinction phase.

Extinction. During the extinction period, nicotine and environmental cues were not available after nose-poking in the active hole. Mice were given 1 hour daily sessions 6 days per week until reaching the extinction criterion during a maximum of 50 days. The criterion was achieved when responses on the active hole were < 30% of the mean responses obtained during the 3 days achieving the acquisition criteria across 3 consecutive extinction sessions. Only mice that reached the extinction criterion were evaluated for cue-induced reinstatement of nicotine-seeking behavior.

Cue-induced reinstatement of nicotine-seeking behavior. One day after reaching the extinction criterion, mice were tested in a single cue-induced reinstatement session that lasted for 1 hour. At the beginning of the session, mice were re-exposed to the pump noise and stimuli lights (environmental cues) for 2 seconds. Subsequently, each active nose-poke led to the presentation of the same environmental cues. Nicotine was not available

through the entire session. In order to evaluate the involvement of Hcrtr-1, Hcrtr-2, and PKC in this behavioral response, different groups of mice were pretreated with the Hcrtr-1 antagonist SB334867 (5 and 10 mg/kg, ip), the Hcrtr-2 antagonist TCSOX229 (5 and 10 mg/kg, ip), the PKC inhibitor NPC-15437 (1 mg/kg, ip) or vehicle 30 minutes before the initiation of the cue-induced reinstatement test. These doses were selected based on previous experiments (Plaza-Zabala et al., 2010; Plaza-Zabala et al., 2012b; Sato et al., 2004), and do not affect locomotor activity (Plaza-Zabala et al, 2010; Figure 4; Supplemental Figure 1).

Cue-induced reinstatement of food-seeking behavior

See Supplement 1

Immunofluorescence

See Supplement 1

Immunoblot analysis

See Supplement 1

Data analysis

Two-way ANOVA with repeated measures was used to analyze the acquisition of nicotine self-administration and food-maintained operant behavior (hole and day as within subject factors). The analysis was followed by one-way ANOVA for each day of training. One-way ANOVA with repeated measures (with experimental phase as within subject factor) was applied to evaluate the reinstatement elicited by presentation of nicotine or food-associated cues in mice extinguished from operant behavior. The evaluation of the effects of the pretreatment with SB334867, TCSOX229 or NPC-15437 on cue-induced reinstatement of nicotine- and food-seeking behavior was performed using one-way ANOVA followed by post-hoc analyses (Newman-Keuls) when required. The results of immunofluorescence

and immunoblot experiments were analyzed by Student's *t* test. The level of significance was $p < 0.05$ in all experiments.

Results

Acquisition and extinction of nicotine self-administration

Mice were trained to self-administer nicotine at the dose of 30 µg/kg per infusion (free base) during 10 consecutive days. Two-way ANOVA with repeated measures revealed an interaction between the day of training and the hole ($F_{(9,450)} = 19.48$, $p < 0.01$), indicating a progressive acquisition of the operant behavior across days (Figure 1A). The acquisition criteria were achieved in 9 ± 0.2 days by 71% of mice trained to self-administer nicotine. We have previously shown using this operant paradigm that mice trained to self-administer nicotine reliably acquired the behavior when compared to mice self-administering saline (Martín-García et al, 2009; Plaza-Zabala et al, 2010). Mice that met the acquisition criteria for nicotine self-administration went through extinction sessions. The extinction criterion was achieved by 89% of mice in 18 ± 1.2 days (Figure 1B). During the first extinction session mice showed a significant increase in the number of responses in the active hole (21 ± 1.6) compared to the last day of nicotine self-administration training (17 ± 0.9) ($p < 0.05$) (Figure 1A and 1B). This "extinction burst" behavior is a confirmation of the reliability of nicotine self-administration training (Cooper et al, 1987). One day after the mice met the extinction criterion, they were tested for cue-induced reinstatement of nicotine-seeking.

Pretreatment with SB334867, but not TCSOX229, attenuated cue-induced reinstatement of nicotine-seeking behavior

Reinstatement studies were performed using a between subjects design. Thus, different groups of mice were tested for cue-induced reinstatement after pretreatment with vehicle, the Hcrtr-1 antagonist SB334867 (5 and 10 mg/kg, ip) or the Hcrtr-2 antagonist TCSOX229 (5 and 10 mg/kg, ip). In order to exclude any bias on the acquisition levels of nicotine self-administration among the different groups of treatment tested for reinstatement, two-way ANOVA with repeated measures was performed. As expected, two-way

ANOVA revealed a significant effect of day of training ($F_{(9,333)} = 13.3$, $p < 0.01$) but no significant effect of the treatment groups tested for reinstatement ($F_{(5,37)} = 0.18$, NS) nor interaction between the two factors ($F_{(45,333)} = 0.9$, NS), indicating that there were no differences on self-administration levels for the different groups tested for reinstatement.

As previously reported (Martín-García et al, 2009), mice presented with nicotine-associated cues reinstated a previously extinguished nicotine-seeking behavior (Figure 1C and 1D). Pretreatment with the Hcrtr-1 antagonist SB334867 dose-dependently attenuated this effect (Figure 1C). Thus, one-way ANOVA revealed a significant effect of SB334867 treatment on reinstatement ($F_{(2,27)} = 7.13$, $p < 0.01$). Subsequent post-hoc analysis confirmed that mice treated with SB334867 showed lower response levels on the active hole at the doses of 5 mg/kg ($p < 0.05$) and 10 mg/kg ($p < 0.01$). Conversely, pretreatment with the Hcrtr-2 antagonist TCSOX229 did not influence responding after presentation of nicotine-conditioned cues ($F_{(2,14)} = 0.12$, NS) (Figure 1D), indicating that Hcrtr-1 plays a specific role in the modulation of this behavior.

Re-exposure to nicotine-paired cues specifically activated hypocretin neurons of the hypothalamic perifornical area

To further explore the involvement of hypocretin transmission in cue-induced reinstatement of nicotine-seeking, we evaluated the possible activation of hypocretin neurons by using double label immunofluorescence of c-Fos with hypocretin-1 in the lateral hypothalamic area. The percentage of c-Fos-positive hypocretin neurons increased after presentation of nicotine-associated cues in the hypothalamic perifornical area (PFA) ($p < 0.05$) (Figure 1E and 1F). In contrast, c-Fos expression in hypocretin cells from the lateral and dorsomedial hypothalamus remained unchanged (Figure 1E), indicating that PFA subpopulation of hypocretin neurons is specifically engaged during cue-elicited reinstatement of nicotine-seeking behavior.

Cue-induced reinstatement of nicotine-seeking was associated with an Hcrtr-1 dependent phosphorylation of NR1-Ser890 and p38 MAPK in the NAc

It is generally accepted that changes on excitatory glutamatergic transmission underlie drug-seeking behaviors (Kalivas, 2009). Phosphorylation of NMDA and AMPA receptor subunits plays a key role in the regulation of trafficking, surface expression and function of these receptors (Gladding and Raymond, 2011; Santos et al, 2009), finally modulating the efficacy and strength of excitatory synapses (Wang et al, 2006). Additionally, the MAPK signaling pathway is vigorously involved in the regulation of synaptic plasticity (Wang et al, 2007). Therefore, we evaluated the possible changes induced by nicotine-seeking in the phosphorylation levels of NMDA and AMPA receptor subunits as well as some MAPK in 2 key regions relevant to drug relapse, the NAc and the PFC. Interestingly, cue-induced reinstatement of nicotine-seeking elicited an increase in the phosphorylation levels of GluR2-Ser880, NR1-Ser890 and p38 MAPK in the NAc ($p < 0.05$) (Figure 2A, 2D and 2F). Other phosphorylations analyzed in the same brain region, such as GluR1-Ser831, GluR1-Ser845, NR1-Ser896 and ERK were not influenced by nicotine-seeking (Figure 2B, 2C, 2E and 2G). Notably, phosphorylation levels of NR1-Ser890 and p38 MAPK ($p < 0.05$), but not GluR2-Ser880, were significantly reduced in the NAc of mice pretreated with SB334867 (10 mg/kg, ip) (Figure 2A, 2D and 2F). SB334867 (10 mg/kg, ip) treatment *per se* did not modify the phosphorylation levels of NR1-Ser890 and p38 MAPK in the NAc (Figure 2H and 2I). On the contrary, analysis of the same phosphorylations in the PFC did not reveal any differences (Figure 3). These results indicate that cue-induced reinstatement of nicotine-seeking increases phosphorylation of GluR2-Ser880, NR1-Ser890 and p38 MAPK in the NAc. However, only NR1-Ser890 and p38 MAPK phosphorylations depend on Hcrtr-1 activation.

The PKC inhibitor NPC-15437 attenuated nicotine-seeking behavior elicited by drug-paired cues

Both GluR2-Ser880 and NR1-Ser890 are PKC-regulated phosphorylation sites (Tingley et al., 1997; Chung et al., 2000). Moreover, cue-induced reinstatement of nicotine-seeking increased phosphorylation levels of PKC target proteins in the NAc ($p < 0.05$) (Figure 4A and 4B) as assessed by an antibody that specifically recognizes PKC phosphorylated substrates. Therefore, we next evaluated whether PKC signaling played a role in the execution of nicotine-seeking behaviors after presentation of nicotine-conditioned cues. A between subjects design was used to perform this experiment. Pretreatment with the PKC inhibitor NPC-15437 (1 mg/kg, ip) reduced cue-elicited reinstatement of nicotine-seeking (Figure 4D). Thus, one-way ANOVA showed a significant effect of NPC-15437 treatment in active nose-poking during reinstatement ($F_{(1,18)} = 11.86$, $p < 0.01$). This effect was not due to changes in locomotion since NPC-15437 (1 mg/kg, ip) did not modify locomotor activity (Figure 4C). Furthermore, the PKC inhibitor was effective in blocking the activity of this enzyme since pretreatment with NPC-15437 attenuated the phosphorylation of PKC substrates in the NAc of mice tested for reinstatement ($p < 0.05$) (Figure 4A and 4B). These results indicate that activation of PKC signaling is involved in cue-induced reinstatement of nicotine-seeking behavior.

Hypocretin receptors do not influence cue-induced reinstatement of food-seeking behavior

To discard any potential non-specific effects of SB334867 and TCSOX229 on the reinstatement of nicotine-seeking behavior as well as the possible modifications that the operant behavior by itself could induce in the biochemical changes observed in the NAc, a new group of C57BL/6J mice were tested for cue-induced reinstatement of food-seeking. Mice were trained to obtain standard food pellets using the same operant paradigm described for nicotine. Two-way ANOVA with repeated measures showed an interaction

between the training session and the hole ($F_{(9,396)}$ = 139.59, p < 0.01), indicating a progress in food-maintained operant responding (Figure 5A). All the mice trained to respond for food achieved the acquisition criteria in 6 ± 0.3 days. Once the training phase was completed, mice underwent extinction sessions. The criterion for extinction was reached by the complete group of mice in 4 ± 0.1 days (Figure 5B). One day after the mice extinguished the behavior, they were tested for cue-induced reinstatement of food-seeking. The reinstatement experiments were performed using a between subjects design. Thus, mice were divided in 4 treatment groups: vehicle, SB334867 (5 and 10 mg/kg, ip), and TCSOX229 (10 mg/kg, ip). Two-way ANOVA with repeated measures revealed that treatment groups tested for reinstatement showed similar levels of responding during the acquisition period. Thus, no interaction was observed between active responding across days and the treatment received on reinstatement ($F_{(27,288)}$ = 0.76, N S). Mice re-exposed to food-associated cues reinstated the previously extinguished food-seeking behavior (Figure 5C and 5D). However, in contrast to mice responding for nicotine cues, pretreatment with SB334867 (5 and 10 mg/kg, ip) did not induce any effect on cue-induced reinstatement of food-seeking ($F_{(2,21)}$ = 0.74, N S) (Figure 5C). Likewise, pretreatment with the Hcrtr-2 antagonist TCSOX229 (10 mg/kg, ip) did not influence reinstatement of nicotine-seeking triggered by food-conditioned cues ($F_{(1,13)}$ = 1.25, NS) (Figure 5D). These results point toward a differential and specific role for Hcrtr-1 in the modulation of cue-induced nicotine-seeking behavior.

Cue-induced reinstatement of food-seeking was not associated with increased phosphorylation of GluR2-Ser880, NR1-Ser890 and p38 MAPK in the NAc

The phosphorylation levels of GluR2-Ser880, NR1-Ser890 and p38 MAPK were analyzed in the NAc of mice that were tested for reinstatement of food-seeking behavior. In opposition to the results obtained for nicotine, cue-induced food-seeking did not induce changes in the phosphorylation

levels of GluR2-Ser880, NR1-Ser890 and p38 MAPK in the NAc (Figure 5E-G). Additionally, pretreatment with SB334867 (10 mg/kg, ip) did not have any effect on the above mentioned phosphorylations in the NAc (Figure 5E-G). These data suggest that different biochemical mechanisms govern food- and nicotine-seeking behavior after re-exposure to reward-associated cues.

Discussion

This report demonstrates that blockade of Hcrtr-1, but not Hcrtr-2, attenuates cue-induced reinstatement of nicotine-seeking behavior. In contrast, hypocretin transmission does not influence reinstatement of food-seeking elicited by food-paired cues, indicating a specific role for Hcrtr-1 in the modulation of cue-induced relapse to nicotine-seeking behavior. Reinstatement of nicotine-seeking was associated with specific changes in glutamate transmission and MAPK signaling in the NAc. Thus, PKC-dependent phosphorylation of GluR2-Ser880 and NR1-Ser890 as well as phosphorylation of p38 MAPK were increased in the NAc of mice re-exposed to nicotine-associated cues. Interestingly, phosphorylation of NR1-Ser890 and p38 MAPK selectively depended on Hcrtr-1 activation. Moreover, inhibition of PKC signaling effectively reduced reinstatement of cue-induced nicotine-seeking.

We have recently shown that the intracerebroventricular infusion of hypocretin-1 reinstates a previously extinguished nicotine-seeking behavior in mice by a mechanism independent of the CRF system (Plaza-Zabala et al., 2010). Thus, the CRF_1 receptor antagonist antalarmin did not block the effects of hypocretin-1 on reinstatement, whereas the Hcrtr-1 antagonist SB334867 did not modify the CRF-dependent footshock stress-induced reinstatement of nicotine-seeking (Plaza-Zabala et al, 2010). In agreement, hypocretins and CRF use independent mechanisms to modulate cocaine-seeking behavior, at least at the level of the ventral tegmental area (VTA) (Wang et al., 2009). In this study, we demonstrate that the blockade of Hcrtr-1 attenuates cue-induced reinstatement of nicotine-seeking behavior in a dose dependent manner, suggesting that presentation of the cues previously associated with nicotine availability is sufficient to evoke the release of endogenous hypocretins to drive this behavior. Consistently, hypocretin neurons from the PFA showed an increase in c-Fos expression following presentation of nicotine-associated cues. In agreement with our results, Hcrtr-

1 signaling has also been reported to modulate cue-induced reinstatement of alcohol (Lawrence et al., 2006), cocaine (Smith et al., 2009) and heroin-seeking (Smith and Aston-Jones, 2012). Moreover, reinstatement of alcohol-seeking elicited by stimuli linked to ethanol availability increased the number of c-Fos-positive hypocretin neurons in the lateral hypothalamic area (Dayas et al, 2008). The present results point to a specific role for Hcrtr-1 in relapse to nicotine-seeking since the Hcrtr-2 antagonist TCSOX229 did not modify this response and both hypocretin antagonists did not prevent cue-induced food-seeking behavior. In agreement, Hcrtr-2 did not participate in cue-induced relapse to cocaine-seeking (Smith et al., 2009) and Hcrtr-1 blockade did not influence the reinstatement of high-fat food-seeking after pellet-priming or stress induction by yohimbine (Nair et al., 2008).

Vulnerability to relapse in addicted individuals can persist after years of abstinence implying that addiction is caused by long-lasting changes in brain function as a result of repeated drug intake (Kalivas and Volkow, 2005). The glutamatergic projection from the PFC to the NAc has been identified as the final common pathway for initiating drug-seeking behaviors, and a variety of molecular adaptations in this brain circuitry have been correlated with the resumption of drug-seeking induced by different classes of addictive drugs (Kalivas and Volkow, 2011). In agreement, attenuation of glutamatergic transmission inhibits the reinstatement of nicotine-seeking in rodents (Knackstedt and Kalivas, 2009). Consistent with the important role of glutamate in drug relapse, we show that re-exposure to nicotine-associated cues modifies glutamatergic transmission in the NAc. Thus, reinstatement of nicotine-seeking increased phosphorylation of GluR2-Ser880 and NR1-Ser890 in this brain region, but not in the PFC. These changes in accumbal glutamatergic transmission are specific since other phosphorylations such as NR1-Ser896, GluR1-Ser831 and GluR1-Ser845 were not modified by the presentation of nicotine-conditioned cues in the NAc or the PFC. In agreement with our results, cocaine-primed reinstatement increased GluR2-Ser880 phosphorylation in the rat NAc shell (Famous et al., 2008). Both

GluR2-Ser880 and NR1-Ser890 phosphorylations depend on PKC activation (Tingley et al., 1997; Chung et al., 2000), a kinase related to glutamate receptor trafficking and plasticity mechanisms (Sanderson and Dell'Aqua, 2011). Upon PKC activation, a rapid dispersal of NMDA receptors from synaptic to extrasynaptic sites has been observed in cultured hippocampal neurons (Fong et al, 2002; Ferreira et al, 2011). Moreover, PKC-induced phosphorylation of Ser890 but not Ser896 disrupts surface-associated NR1 subunit clusters in heterologous cell systems (Tingley et al, 1997), suggesting that PKC stimulation might facilitate lateral diffusion of NMDA receptors out of the synapse, where they could be subsequently targeted for internalization (Carroll and Zukin, 2002). Likewise, PKC-dependent phosphorylation of GluR2-Ser880 induces rapid internalization of GluR2-containing AMPA receptors in neuronal cultures (Chung et al., 2000). Interestingly, the inhibition of PKC-induced GluR2 subunit internalization in the NAc blocks cocaine-primed reinstatement (Famous et al., 2008), suggesting that PKC might be involved in the re-initiation of drug-seeking behaviors. Here, we show that reinstatement of nicotine-seeking after presentation of nicotine-paired cues increased PKC-phosphorylated substrates in the NAc. Moreover, prior administration of the PKC inhibitor NPC-15437 attenuated cue-elicited reinstatement of nicotine-seeking, thus confirming a role for this protein kinase in relapse to nicotine-seeking triggered by re-exposure to nicotine-conditioned cues. The MAPK pathway is also critical for the regulation of synaptic plasticity mechanisms (Wang et al, 2007). In agreement, we observed an increase in the phosphorylation levels of p38 MAPK in the NAc upon presentation of nicotine-associated cues, while phosphorylation of ERK remained unchanged. Interestingly, p38 MAPK pathway plays an active role in the internalization of AMPA receptors during some forms of long-term depression (Zhu et al, 2002; Zhong et al, 2008). Nevertheless, the participation of the p38 MAPK pathway in NMDA receptor trafficking has not been assessed yet. Notably, the changes that we report in accumbal transmission are selectively engaged during the execution of nicotine-seeking

behaviors. Thus, phosphorylation levels of GluR2-Ser880, NR1-Ser890 and p38 MAPK were not modified upon presentation of food-conditioned cues during reinstatement of food-seeking behavior, suggesting that different biochemical mechanisms govern food- and nicotine-seeking behaviors following re-exposure to reward-associated cues. Nevertheless, future investigations will be needed to elucidate the exact contribution of the biochemical changes observed in the NAc to the reinstatement of nicotine-seeking behavior.

The Hcrtr-1 antagonist SB334867 blocks cocaine-induced long-term potentiation in VTA excitatory synapses (Borgland et al, 2006), indicating that Hcrtr-1 activation modulates glutamatergic synaptic transmission. Consistent with this idea, we have shown that SB334867 selectively attenuates the enhanced phosphorylation levels of NR1-Ser890 and p38 MAPK induced by the reinstatement of nicotine-seeking in the NAc. These effects are improbable to be modulated directly by Hcrtr-1 in the NAc since Hcrtr-2 is the main receptor expressed in this brain region (Marcus et al., 2001; Cluderay et al, 2002). Other key areas related to the execution of drug-seeking behaviors such as the VTA or the PFC, where Hcrtr-1 expression is more abundant (Marcus et al., 2001) could be responsible for this effect. Accordingly, cue-induced reinstatement of cocaine-seeking is dependent upon hypocretin and AMPA receptor interactions within the VTA (Mahler et al., 2012).

In conclusion, we demonstrate a selective role for Hcrtr-1 in cue-induced reinstatement of nicotine-seeking and the associated biochemical changes in the NAc. Moreover, we show that PKC signaling modulates relapse to nicotine-seeking behavior triggered by nicotine-conditioned cues. These results could be of relevance for the design of new therapeutic strategies to achieve smoking cessation.

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Financial Disclosures

The authors have no conflicts of interest to declare.

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Figure legends

Figure 1. Hcrtr-1 selectively attenuates cue-induced reinstatement of nicotine-seeking behavior. **(A, B, C, D)** Mean number of nose-poking responses in the active (black) and inactive (white) hole during **(A)** acquisition of nicotine self-administration (n = 51), **(B)** extinction training (n = 51) and cue-induced reinstatement of nicotine-seeking after pretreatment with **(C)** SB334867 (0, 5 and 10 mg/kg, ip) (n = 8-11 mice per group) or **(D)** TCSOX229 (0, 5 and 10 mg/kg, ip) (n = 5 mice per group). E1-E2-E3, 3 days achieving the extinction criterion. **(E)** Percentage of hypocretin cells expressing c-Fos in the lateral hypothalamus (LH), perifornical area (PFA) and dorsomedial hypothalamus (DMH) in mice extinguished from nicotine self-administration or re-exposed to nicotine-associated cues (n = 4 mice per group). **(F)** Representative images of sections of the PFA obtained by confocal microscopy after direct double labeling combining rabbit polyclonal antiserum to c-Fos (red) with mouse monoclonal antibody to hypocretin-1 (green). Arrowheads indicate c-Fos-positive hypocretin-1-expressing neurons. f, fornix. Scale bar, 50 μm. Data are expressed as mean ± S.E.M. **(A, B)** ★★ $p < 0.01$ comparison between holes, **(C, D, E)** ★ $p < 0.05$, ★★ $p < 0.01$ compared to extinction, # $p < 0.05$, ## $p < 0.01$ compared to vehicle pretreatment.

Figure 2. Cue-induced reinstatement of nicotine-seeking increases phosphorylation levels of GluR2-Ser880, NR1-Ser890 and p38 MAPK in the nucleus accumbens (NAc). Hcrtr-1 selectively contributes to NR1-Ser890 and p38 MAPK phosphorylations. **(A, B, C, D, E, F, G)** Phosphorylation of **(A)** GluR2-Ser880, **(B)** GluR1-Ser831, **(C)** GluR1-Ser845, **(D)** NR1-Ser890, **(E)** NR1-Ser896, **(F)** p38 MAPK and **(G)** ERK in the NAc of mice extinguished from nicotine self-administration or re-exposed to nicotine-associated cues after pretreatment with SB334867 (0 and 10 m g/kg, ip) (n = 6 mice per group). **(H, I)** Phosphorylation of **(H)** NR1-Ser890 and **(I)** p38 MAPK in the

NAc of C57BL/6J naive mice after pretreatment with SB334867 (0 and 10 mg/kg, ip) (n = 5 mice per group).). Data are expressed as mean ± S.E.M. ★ $p < 0.05$ compared to the extinction group; # $p < 0.05$ comparison between pretreatments.

Figure 3. Prefrontal cortex (PFC) phosphorylation levels are not affected by cue-induced reinstatement of nicotine-seeking behavior **(A, B, C, D, E, F, G)** Phosphorylation levels of **(A)** GluR2-Ser880, **(B)** GluR1-Ser831, **(C)** GluR1-Ser845, **(D)** NR1-Ser890, **(E)** NR1-Ser896, **(F)** p38 MAPK and **(G)** ERK in the PFC of mice extinguished from nicotine self-administration or re-exposed to nicotine-paired cues after pretreatment with SB334867 (0 and 10 mg/kg, ip) (n = 7 mice per group). Data are expressed as mean ± S.E.M.

Figure 4. Cue-induced reinstatement of nicotine-seeking is modulated by PKC signaling. **(A)** PKC-phosphorylated substrates in the nucleus accumbens (NAc) of mice extinguished from nicotine self-administration or re-exposed to nicotine-associated cues after pretreatment with NPC-15437 (0 and 1 mg/kg, ip) (n = 4 mice per group). **(B)** Representative blot showing PKC-phosphorylated substrates in the NAc of mice after extinction or cue-induced reinstatement. **(C)** Horizontal locomotor activity counts after pretreatment with NPC-15437 (0, 0.5 and 1 mg/kg, ip) (n = 6-7 mice per group). Locomotion was measured during a 1 hour period 30 minutes after NPC-15437 administration. **(D)** Cue-triggered reinstatement of nicotine-seeking after pretreatment with NPC-15437 (0 and 1 mg/kg, ip) (n = 9-11 mice per group). Data are expressed as mean ± S.E.M. ★ $p < 0.05$, ★★ $p < 0.01$ compared to extinction, ## $p < 0.01$ comparison between pretreatments.

Figure 5. Hypocretin transmission does not influence cue-induced reinstatement of food-seeking behavior. Presentation of food-associated cues does not modify phosphorylation levels of GluR2-Ser880, NR1-Ser890 and p38 MAPK in the nucleus accumbens (NAc). **(A, B, C, D)** Mean number of

nose-poking responses in the active (black) and inactive (white) holes during **(A)** acquisition of food-maintained operant behavior (n = 38), **(B)** extinction (n = 38) and **(C, D)** reinstatement of cue-induced food-seeking after pretreatment with **(C)** SB334867 (0, 5 and 10 mg/kg, ip) (n = 7-9 mice per group) or **(D)** TCSOX229 (0 and 10 mg/kg, ip) (n = 6-8 mice per group). **(E, F, G)** Phosphorylation of **(E)** GluR2-Ser880, **(F)** NR1-Ser890 and **(G)** p38 MAPK in mice extinguished from operant behavior or re-exposed to food-conditioned cues after pretreatment with SB334867 (0 and 10 mg/kg, ip). Data are expressed as mean ± S.E.M. **(A, B)** ★★ $p < 0.01$ comparison between holes, **(C, D)** ★★ $p < 0.01$ compared to extinction.

Figure 1

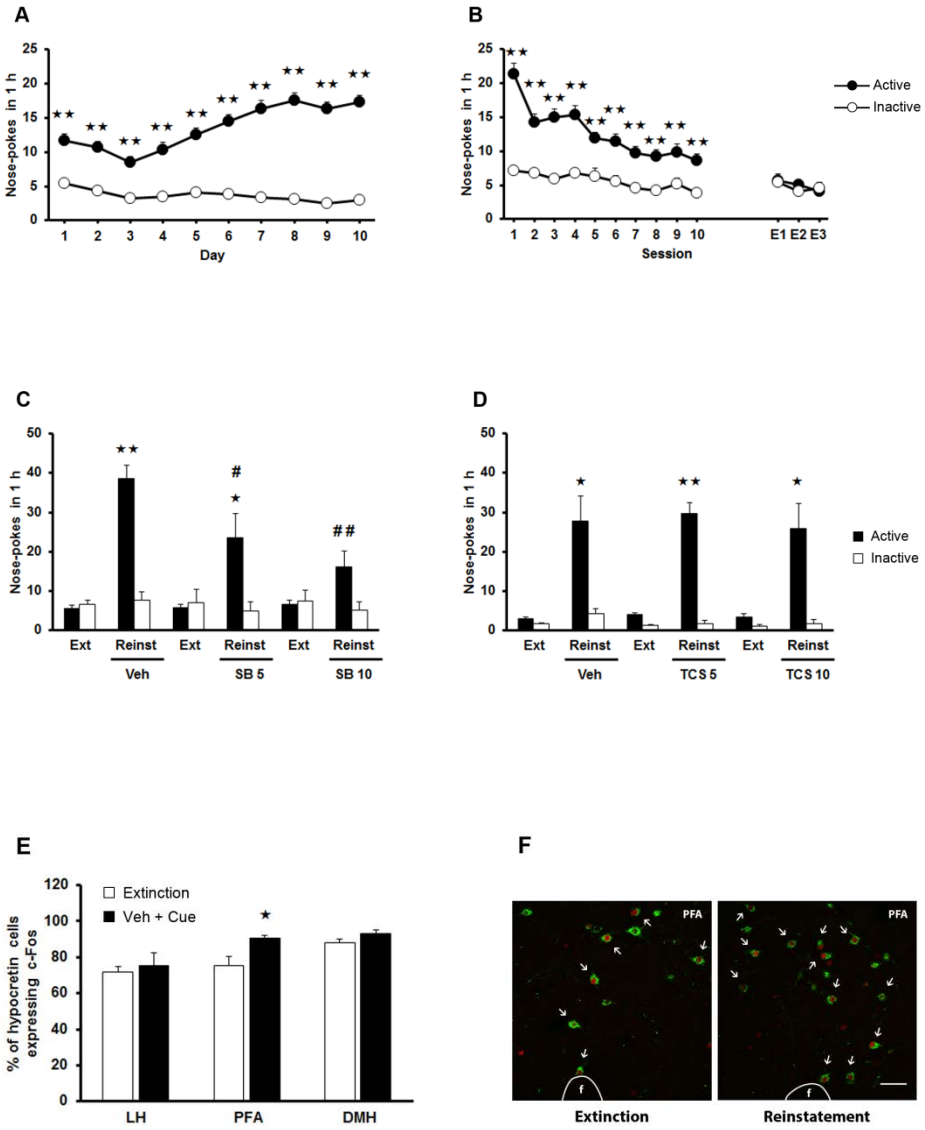
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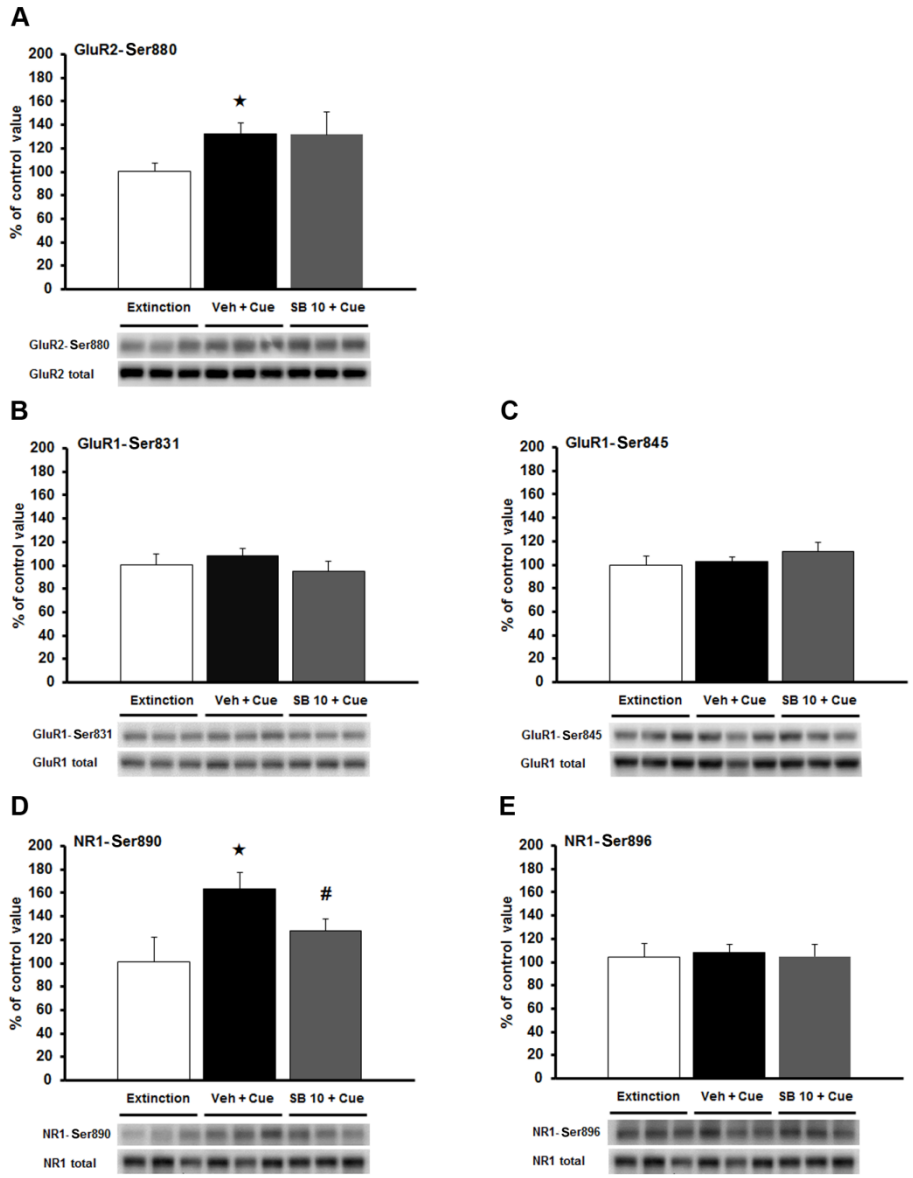
Figure 2



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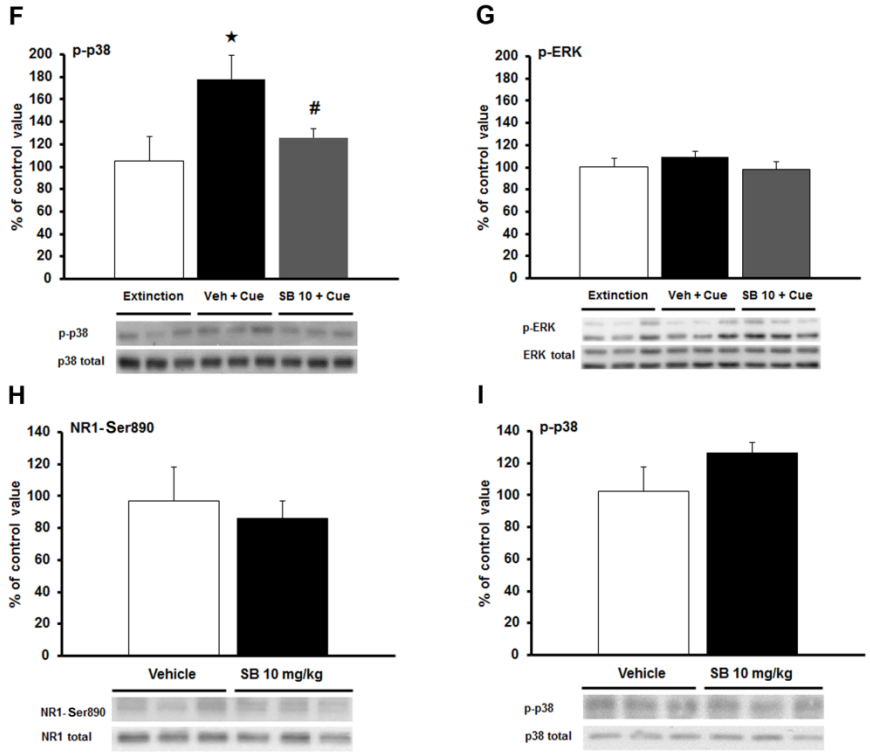
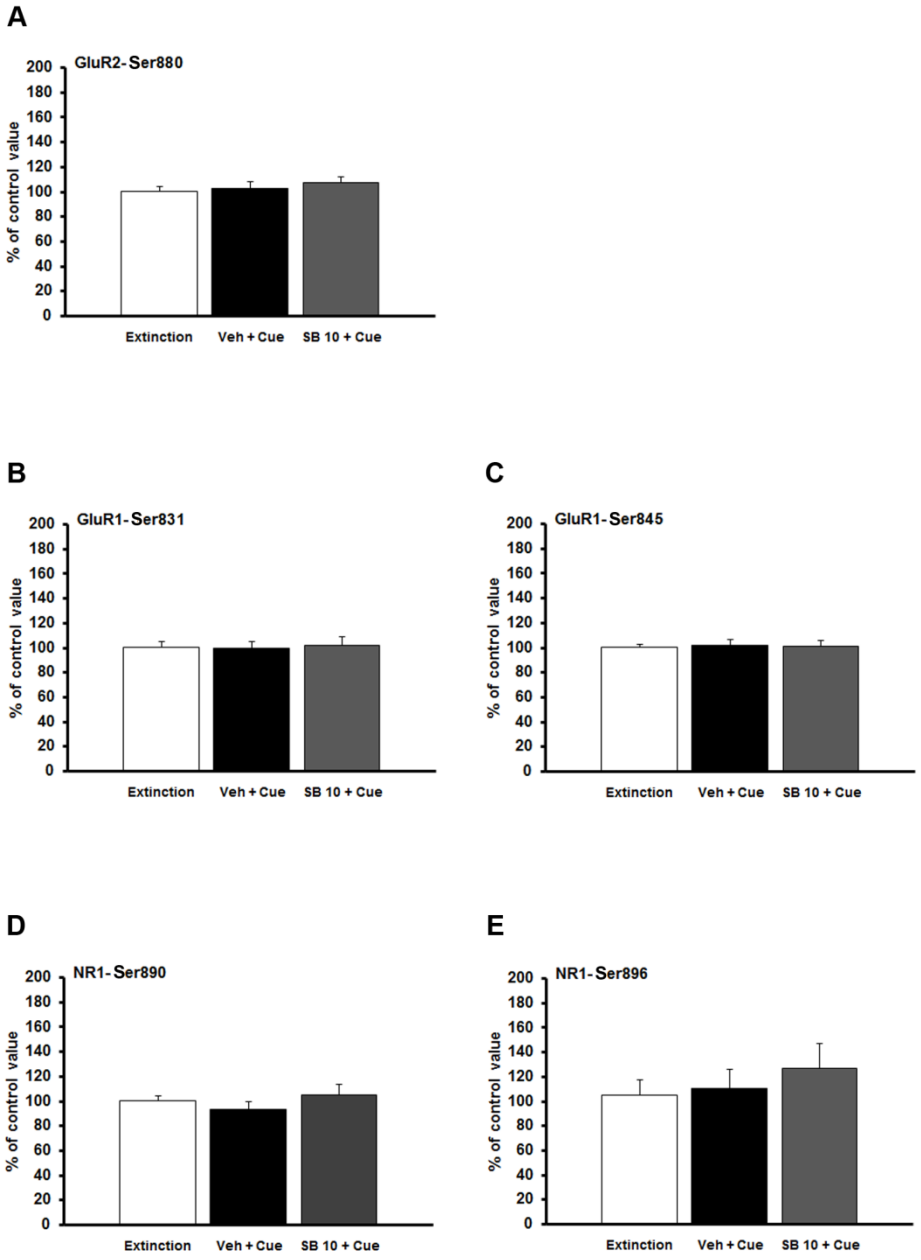


Figure 3



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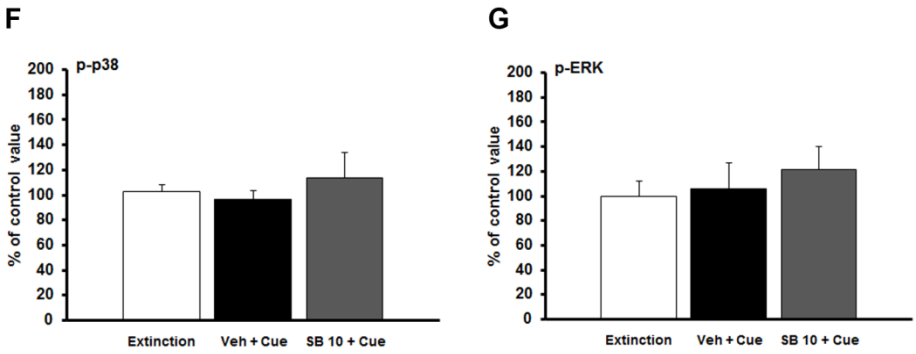

Figure 4

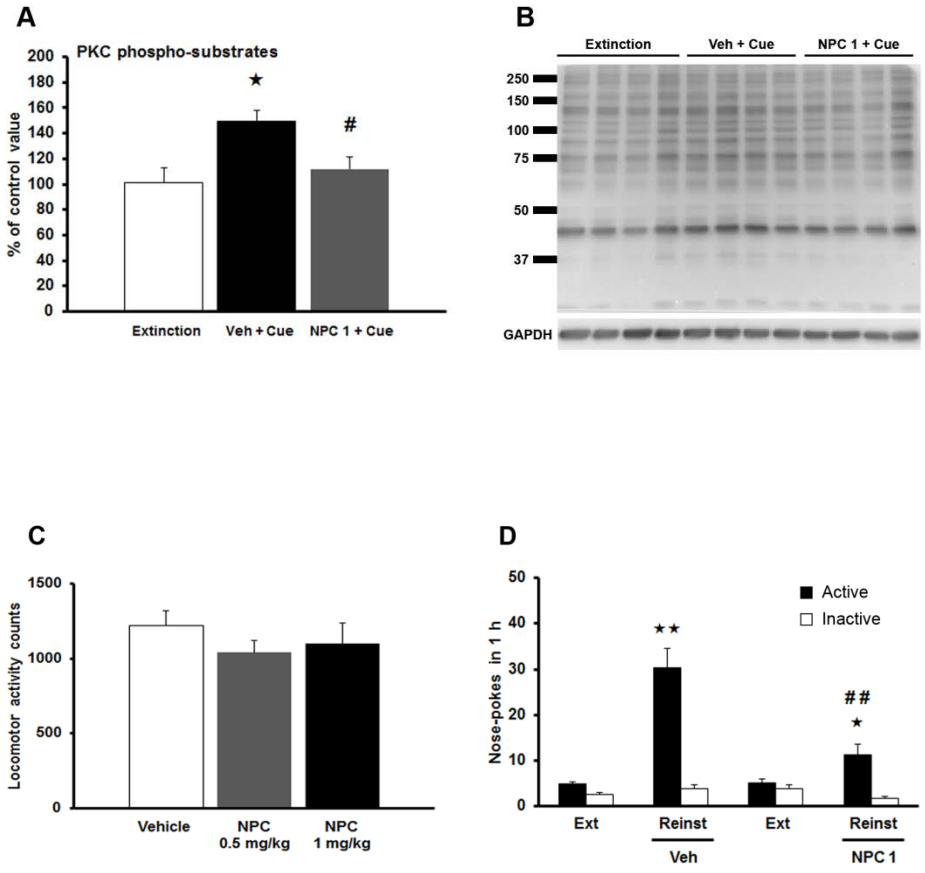
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Figure 5

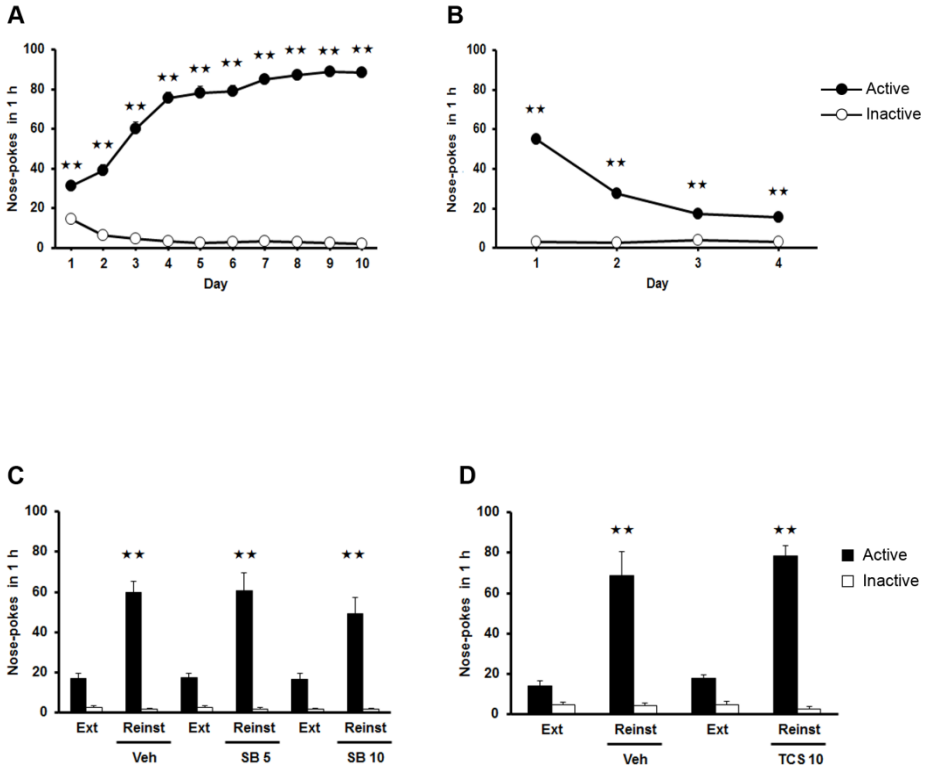

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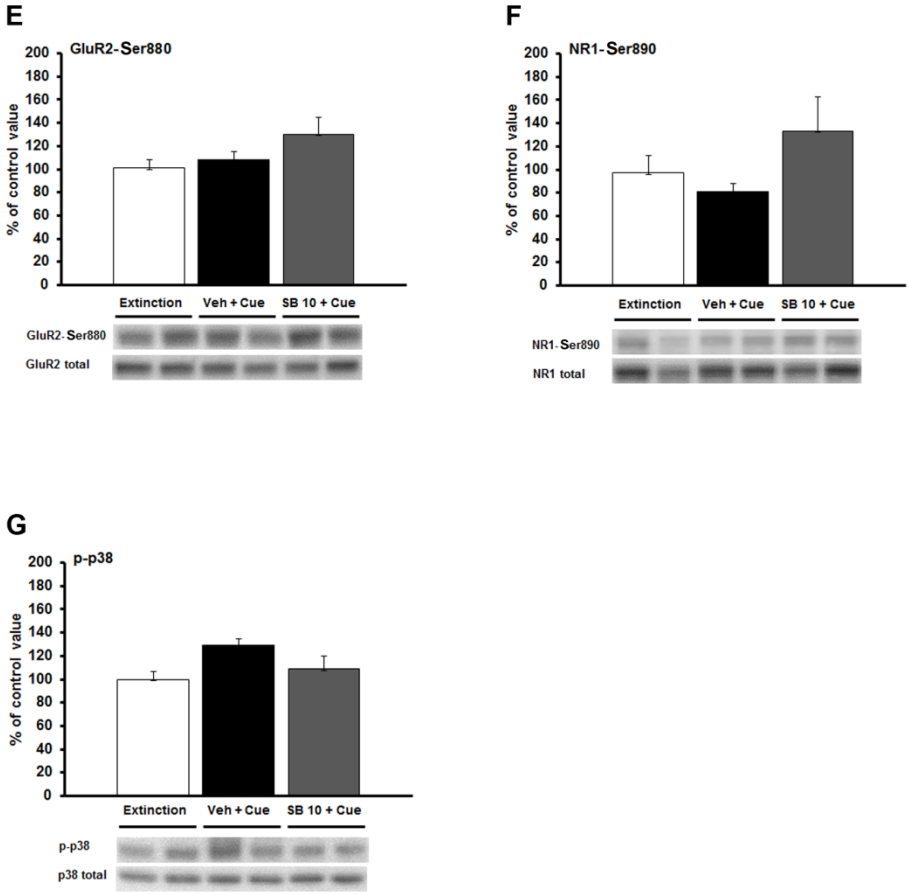
Figure 5 (continuation)



Supplement 1

Cue-induced reinstatement of food-seeking behavior

Slight modifications were performed to a recently described food-seeking model (Martín-García et al, 2011). Mice were food deprived 5 days before starting training sessions and during all the experimental procedure to maintain their weight at 90% of their *ad libitum* initial weight adjusted for growth. Water was provided *ad libitum* during the whole experimental procedure. The same operant chambers described for nicotine were used for the acquisition, extinction and reinstatement of food-seeking behavior except that a food dispenser was located equidistant to the 2 holes and that the behavior was reinforced by a 20 mg standard food pellet. The training sessions were terminated after 100 reinforcers were delivered or after 1 hour, whichever occurred first. The criteria for acquisition of food-maintained behavior were the same as for nicotine except that mice had to obtain at least 10 reinforcers per session. The extinction sessions and the cue-induced reinstatement tests were performed under the same conditions used for nicotine.

Immunofluorescence

The activation of hypocretin neurons was evaluated by double label immunofluorescence of c-Fos with hypocretin-1.

Tissue preparation. One hour after the end of extinction or cue-induced reinstatement sessions, mice were transcardially perfused with cold 4% paraformaldehyde. Subsequently, the brains were extracted and postfixed in the same fixative for 4 hours and cryoprotected overnight in a solution of 30% sucrose at 4ºC. Coronal frozen sections of the lateral hypothalamic area, including the lateral, perifornical and dorsomedial hypothalamus (coordinates relative to bregma, -1.22 mm to -1.82 mm), were made at 30 μm on a freezing microtome and stored in a 5% sucrose solution at 4ºC until use.

Immunofluorescence. Free-floating slices were rinsed in 0.1 M PB, blocked in a solution containing 3% normal goat serum and 0.3% Triton X-100 in 0.1 M PB (NGS-T-PB) at room temperature for 2 hours, and incubated overnight in the same solution with the primary antibody to c-Fos (1:1000, rabbit, Ab-5, Calbiochem) and hypocretin-1 (1:500, mouse, R&D Systems) at 4ºC. The next day, after 3 rinses in 0.1 M PB, sections were incubated at room temperature with the secondary antibody to rabbit Cy3 (1:500, Jackson ImmunoResearch) and mouse Cy2 (1:500, Jackson Immunoresearch) in NGS-T-PB for 2 hours. After incubation, sections were rinsed and mounted immediately after onto glass slides coated with gelatine in Mowiol mounting media.

Image analysis. Confocal images were obtained using a Leica TCS SP2 confocal microscope, adapted to an inverted Leica DM IRBE microscope. Tissue sections were examined at 40x objective with oil-immersion. The images were 8 bit, 1024 x 1024 pixels and they were processed using the Image J analysis software using a fixed threshold interval. Co-expression of c-Fos with hypocretin-1 was quantified using the manual particle counting option in order to delimit the quantification of c-Fos positive nuclei to hypocretin-containing neurons. Four representative brain sections of each mouse were quantified along the rostro-caudal trajectory of the lateral, perifornical and dorsomedial hypothalamus and the average number of c-Fos positive hypocretin neurons was calculated for each mouse. Data are expressed as the percentage of hypocretin neurons that express c-Fos in 4 mice per each experimental condition (extinction and cue-induced reinstatement).

Immunoblot analysis

Prefrontal cortex (PFC) and nucleus accumbens (NAc) tissues were extracted 1 hour after the end of extinction or cue-induced reinstatement sessions. Frozen tissues were processed to obtain the total solubilized fraction, as previously described (Ozaita et al., 2007). Briefly, tissues were dounce-

homogenized in 30 volumes of lysis buffer (50 mmol/l Tris-HCl pH 7.4, 150 mmol/l NaCl, 10% glycerol, 1 m mol/l EDTA, 1µg/ml aprotinin, 1 µg /ml leupeptine, 1µg/ml pepstatin, 1 mmol/l sodium fluoride, 5 mmol/l sodium pyrophosphate and 40 mmol/l betaglycerolphosphate) plus 1% Triton X-100. After 10 minutes of incubation at 4ºC, samples were centrifuged at 16000 *g* for 30 minutes to remove insoluble debris. Protein contents in the supernatants were determined by DC-micro plate assay (Bio-Rad, Madrid, Spain), following manufacturer's instructions. Blots containing equal amounts of protein samples were probed for different primary antibodies: antiphospho-NR1 (Ser890) (Cell Signaling, 1:500) (Ser896) (Abcam, 1:700), NR1 (Novus Biologicals, 1:750), antiphospho-GluR1 (Ser831) (Millipore, 1:1000) (Ser845) (Millipore, 1:1000), GluR1 (Abcam, 1:2000), anti-phosho-GluR2 (Ser880) (Sigma, 1:1500), GluR2 (Millipore, 1:2000), antiphospho-p38 (Thr180/Tyr182) (Cell Signaling, 1: 500), p-38 (Cell Signaling, 1:500), antiphospho-ERK (Thr202/Tyr204) (Cell Signaling, 1:1000), ERK (Cell Signaling, 1:1000), antiphospho-serine (ser) PKC substrate antibody (Cell Signaling, 1:2000) and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Santa Cruz, 1:5000). Bound primary antibodies were detected using horseradish peroxidase-conjugated antibodies to rabbit (Cell Signaling, 1:10.000) or mouse antibodies (Pierce, 1:2.500) and visualized by enhanced chemiluminescence detection (West-Femto-SuperSignal, Pierce). When necessary, Immobilon-P membranes (Millipore) were stripped in buffer containing 100mM glycine (pH 2.5), 200mM NaCl, 0.1% Tween 20 (vol/vol) and 0.1% beta-mercaptoethanol (vol/vol) for 45 minutes at room temperature, followed by extensive washing in 100mM NaCl, 10mM Tris and 0.1% Tween 20 (pH 7.4) before re-blocking and re-probing. The optical density of the relevant immunoreactive bands was quantified after acquisition on a ChemiDoc XRS System (Bio-Rad) controlled by The Quantity One software v4.6.3 (Bio-Rad). For quantitative purposes, the optical density values of phospho-specific antibodies were normalized to the detection of nonphospho-specific antibodies in the same sample. The optical density value of

antiphospho-ser PKC substrate was normalized to the amount of GAPDH. Data are expressed as the percentage of control treatment.

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Ozaita A, Puighermanal E, Maldonado R (2007): Regulation of PI3K/Akt/GSK-3 pathway by cannabinoids in the brain. *J Neurochem* 102:1105-1114.

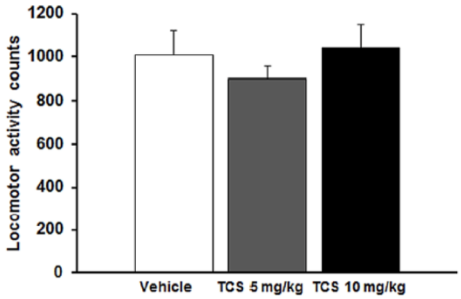

Figure S1. TCSOX229 (0, 5 and 10 mg/kg, ip) effects in horizontal locomotor activity. Locomotion was measured during a 1 hour period 30 minutes after TCSOX229 administration. Data are expressed as mean ± S.E.M. of photocell counts (n = 7 mice per group).

ARTICLE 3

Hypocretin/Orexin Signaling in the Hypothalamic Paraventricular Nucleus is Essential for the Expression of Nicotine Withdrawal

Ainhoa Plaza-Zabala, África Flores, Rafael Maldonado
and Fernando Berrendero

Biol Psychiatry (2012) 71(3):214-223

Plaza-Zabala A, Flores A, Maldonado R, Berrendero F. Hypocretin/orexin signaling in the hypothalamic paraventricular nucleus is essential for the expression of nicotine withdrawal. Supplemental information. Biol Psychiatry. 2012 Feb 1;71(3):214-223.

ARTICLE 4

The Hypocretin/Orexin System: Implications for Drug Reward and Relapse

Ainhoa Plaza-Zabala, Rafael Maldonado and Fernando Berrendero

Mol Neurobiol (2012) 45(3):424-439

DISCUSSION

The anatomical distribution of the hypocretin system supports a role for these neuropeptides in addictive disorders. Indeed, a growing body of evidence indicates that hypocretins contribute to the processes underlying the development of drug addiction (article 4). Nevertheless, the potential participation of hypocretin peptides in nicotine addictive properties has remained largely unexplored. Recent studies have reported that hypocretin transmission (Hollander et al, 2008; Le Sage et al, 2010), mainly through the activation of Hcrtr-1 in the insula (Hollander et al, 2008), is essential for the acute positive reinforcing effects of nicotine. However, the results described in this thesis are the first to demonstrate the specific involvement of the hypocretin system in acute nicotine withdrawal as well as in relapse to nicotine-seeking following prolonged periods of abstinence.

Stress is a key element of the negative emotional states associated with drug withdrawal and represents a crucial factor for the reinstatement of drug-seeking behavior (Koob, 2008). Interestingly, hypocretin neurons may be part of the circuitries that mediate the hypothalamic response to acute stress (Winsky-Sommerer et al, 2004; Pañeda et al, 2005; Winsky-Sommerer et al, 2005), and thereby contribute to the addictive properties of drugs of abuse (de Lecea et al, 2006). Acute stress responses are mainly controlled by the HPA axis, which is composed by 3 structures: the PVN, the anterior lobe of the pituitary gland and the adrenal cortex (Turnbull and Rivier, 1997) (Figure 17). In the present thesis, we confirm that hypocretins and the hypothalamic stress system interact to regulate some aspects of nicotine addiction. Thus, we show that nicotine-induced anxiogenic-like effects and somatic expression of nicotine withdrawal depend on hypocretin-induced activation of CRF neurons within the hypothalamic paraventricular nucleus. In contrast, hypocretin-1 induces reinstatement of nicotine-seeking by a CRF independent mechanism, and CRF-dependent stress-induced reinstatement does not rely on hypocretin transmission, suggesting that hypocretins and CRF do not interact to trigger nicotine relapse. Instead, our results support a

role for the hypocretin system in the reinstatement of nicotine-seeking elicited by nicotine-associated environmental stimuli, presumably through the modulation of glutamatergic synaptic transmission in the NAc.

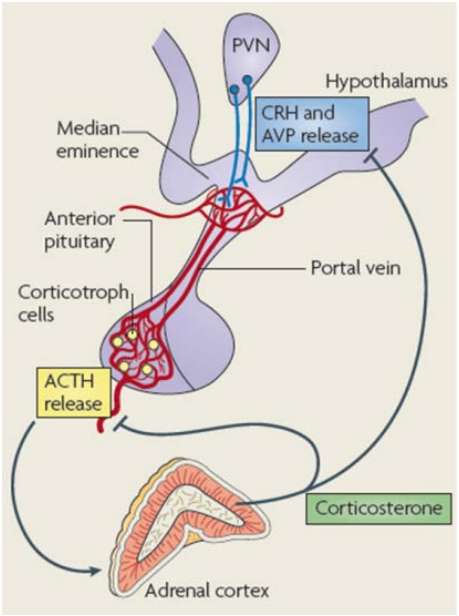

Figure 17. Hypothalamic-pituitary-adrenal (HPA) axis. The HPA axis is defined by 3 major structures: the paraventricular nucleus of the hypothalamus (PVN), the anterior lobe of the pituitary gland, and the adrenal gland. Neurosecretory neurons of the PVN synthesize and release corticotrophin-releasing factor (CRF) into the portal blood that enters the pituitary gland. CRF stimulates pituitary corticotropes and induces the release of adenocorticotropic hormone (ACTH) into the systemic circulation. ACTH, in turn, stimulates corticosterone synthesis and release from the adrenal cortex. Arginine-vasopressin (AVP) released from the PVN produces synergistic effects on ACTH release. The HPA axis is finely tuned via negative feedback from circulating corticosterone, which mainly acts in the PVN and the hippocampus (adapted from Lightman and Conway-Campbell, 2010).

1. Participation of the hypocretin system in nicotine-induced anxiogenic-like effects and the somatic expression of nicotine withdrawal

Acute nicotine injections produce opposing responses in anxiety-like behavior in rodents (Balerio et al, 2005; File et al, 1998). Thus, mice tested on the elevated plus maze after administration of low doses of nicotine exhibit anxiolytic-like responses, whereas higher doses of nicotine induce anxiogenic-like effects (Balerio et al, 2005; Balerio et al, 2006). In a first study, we evaluated the potential participation of hypocretin transmission in acute nicotine-induced anxiety-like responses. Interestingly, mice pretreated with the selective Hcrtr-1 antagonist SB334867 and preprohypocretin knockout mice showed attenuated anxiogenic-like responses in the elevated plus maze, whereas the anxiolytic-like effects of nicotine remained unchanged (article 1). This effect was less robust in the case of knockout mice, probably due to the mild hypolocomotion exhibited by these animals under basal conditions (article 1). In line with our findings, hypocretin-1 infusion into the ventricles, the BNST, and the PVT decreases the percentage of time spent in open arms in the elevated plus maze (Li et al, 2010; Lungwitz et al, 2012; Suzuki et al, 2005), suggesting that hypocretin-1 release in the CNS is associated with anxious emotional states.

Anxiety disorders are frequently characterized by hyperactive and/or dysregulated stress systems with elevated secretion of CRF and arginine-vasopressin (AVP) from the hypothalamic paraventricular nucleus (Bonfiglio et al, 2011; Holsboer and Ising, 2008). Moreover, acute nicotine injections stimulate the PVN and activate the HPA axis in rodents (Valentine et al, 1996; Loughlin et al, 2006; Lutfy et al, 2006; Shram et al, 2007). Consistently, using c-Fos as a marker of neuronal activation (Kovács, 2008), we found that the anxiogenic dose of nicotine, but not the anxiolytic one, increased c-Fos expression in the PVN, particularly in CRF- and AVP-containing neurons (article 1). Notably, the activation of the PVN was specific since c-Fos

expression in other brain regions analyzed such as the CeA, cingulate cortex, medial and lateral septum, dorsal and ventral striatum and BNST was not altered after acute injection of the anxiogenic dose of nicotine (article 1). Furthermore, mice pretreated with SB334867 and preprohypocretin knockout mice exhibited attenuated c-Fos expression in the PVN (article 1). In agreement, the activation of CRF and AVP neurons was blocked by preadministration of the Hcrtr-1 antagonist SB334867 (article 1). These results demonstrate a co rrelation between the anxiogenic-like effects of nicotine and the consequent activation of CRF and AVP neurons in the PVN, which is dependent on hypocretin transmission. Consistent with our data, several reports support the idea that hypocretin peptides influence the activity of CRF and AVP neurons within the hypothalamic paraventricular nucleus. Hence, hypocretin-1 increases c-Fos expression in CRF neurons of the PVN (Sakamoto et al, 2004), and both hypocretin-1 and -2 elevate CRF and AVP mRNA levels in the parvocellular division of the PVN (Al-Barazanji et al, 2001; Kuru et al, 2000). Nonetheless, the possible involvement of other cellular types of the PVN in the modulation that hypocretins exert in the anxiogenic-like effects of nicotine cannot be ruled out since the blockade of the CRF_1 receptor with antalarmin did not prevent this behavioral response (article 1). Similarly, α-helical $CRF_{9-41,}$ a non-selective CRF antagonist, did not block acute nicotine-induced anxiogenic-like responses in rats (Tucci et al, 2003).

Using retrograde tracing, we have shown that direct axonal projections exist from the lateral hypothalamic hypocretin neurons to the PVN (article 3). This is congruent with previous anatomical and electrophysiological data suggesting an interaction between hypocretins and the hypothalamic CRF system. Thus, hypocretin immunoreactive fibers have been identified in the PVN (Peyron et al, 1998; Nambu et al, 1999; Baldo et al, 2003), particularly in CRF-expressing perikarya (Winsky-Sommerer et al, 2004), and cells within the PVN reciprocally project to hypocretin neurons

(Winsky-Sommerer et al, 2004; Yoshida et al, 2006). Moreover, bath application of hypocretin-1 and -2 to hypothalamic slices induces depolarization of PVN neurons (Samson et al, 2002; Follwell and Ferguson, 2002; Shirasaka et al, 2001). In turn, CRF robustly depolarizes and enhances the firing rate of hypocretin neurons (Winsky-Sommerer et al, 2004). These data correlate well with the lack of activation of hypocretin neurons observed in CRF_1 receptor knockout mice after acute stress exposure (Winsky-Sommerer et al, 2004), and confirm that the hypocretin system might influence CRF-mediated behaviors that occur in response to stressful situations (Pañeda et al, 2005; Winsky-Sommerer et al, 2005). In agreement, our data suggest that hypocretin release within the PVN and the subsequent activation of CRF and AVP neurons is associated with the acute anxiogenic-like effects induced by nicotine. Indeed, we have shown that the anxiogenic dose of nicotine, but not the anxiolytic one, increased the percentage of c-Fos expressing hypocretin neurons in the PFA/DMH, but not in the LH (article 1). Consistently, PFA/DMH hypocretin neurons respond to stressful events and might activate CRF systems (Harris et al, 2005; Harris and Aston-Jones, 2006). Moreover, an acute high dose of nicotine activates hypocretin neurons of the lateral hypothalamic area (Pasumarthi et al, 2006; Pasumarthi and Fadel, 2008).

Immunostaining studies have shown that both hypocretin receptors are expressed in the hypothalamic paraventricular nucleus (Hervieu et al, 2001; Cluderay et al, 2002), although only Hcrtr-2 mRNA has been detected (Trivedi et al, 1998; Marcus et al, 2001). In agreement, we and others have shown that Hcrtr-1 (Bäckberg et al, 2002) and Hcrtr-2 protein is present in CRF and AVP neurons of the PVN (article 1), suggesting that direct hypocretin action on these neurons might occur during nicotine-induced anxiety-like responses. Although our behavioral and biochemical data suggest a role for Hcrtr-1 in the acute anxiogenic-like effects of nicotine, the potential participation of Hcrtr-2 cannot be ruled out since the effects of direct

pharmacological blockade of this receptor were not assessed in the present study. Interestingly, the involvement of hypocretin signaling in the acute effects of nicotine appears to be specific for the anxiogenic-like effects, because anxiolytic-like effects, hypolocomotion and antinociception induced by acute nicotine administration were not altered by pretreatment with the Hcrtr-1 antagonist SB334867 nor in preprohypocretin knockout mice.

The transition from acute to chronic drug administration involves progressive changes in the HPA axis (Koob, 2008). Acute administration of most drugs of abuse, including nicotine, activates the HPA axis in humans and animals (Armario, 2010) and this effect has been proposed to facilitate drug reward by the modulation of the brain motivational circuitry (Piazza and Le Moal, 1997). However, chronic drug exposure dysregulates HPA axis activity, which subsequently leads to the impairment of extrahypothalamic brain stress systems and the emergence of withdrawal symptoms after cessation of drug intake (Kreek and Koob, 1998; Koob and Kreek, 2007). In rodents, nicotine withdrawal syndrome is characterized by somatic signs as well as affective changes similar to those observed in humans. Notably, withdrawal symptoms contribute to the maintenance of the smoking habit, and are a potent deterrent for those who are attempting to quit (West et al, 1989; Piasecki et al, 1998; al'Absi et al, 2004). Therefore, the identification of the potential neurobiological mechanisms and neurotransmitter systems underlying nicotine abstinence is essential for the design of new therapeutic interventions. In this thesis, we describe a new mechanism whereby hypocretin signaling through the selective activation of Hcrtr-1 in the PVN mediates the somatic expression of nicotine withdrawal.

Physical manifestations of nicotine abstinence were abolished in mice lacking the preprohypocretin gene (article 3). Furthermore, mice pretreated with the Hcrtr-1 antagonist SB334867, but not with the Hcrtr-2 antagonist TCSOX229, exhibited a dose-dependent decrease in the somatic manifestations of nicotine withdrawal (article 3). The effects of SB334867 on

the behavioral expression of nicotine withdrawal were not biased by unspecific responses since the low doses used in this study did not modify locomotor activity (article 1). In agreement, previous studies have demonstrated that somatic signs of precipitated morphine withdrawal are attenuated in preprohypocretin knockout mice (Georgescu et al, 2003) as well as in mice pretreated with the Hcrtr-1 antagonist SB334867 (Sharf et al, 2008; Laorden et al, 2012). Moreover, SB334867 infusion into the locus coeruleus, but not into the PVT affected the expression of the somatic manifestations of morphine abstinence (Azizi et al, 2010; Li et al, 2011), suggesting anatomical selectivity for the actions of Hcrtr-1 in the expression of this behavioral response. Hypocretin receptors exhibit an overall overlapping, but sometimes complementary distribution in the CNS (Marcus et al, 2001), suggesting that each receptor might serve different physiological functions. Few studies have evaluated the role of Hcrtr-2 in drug withdrawal and addiction, mainly due to the lack of commercially available Hcrtr-2 antagonists. However, a recent study has revealed a selective role for Hcrtr-2 in the affective component of morphine withdrawal. Hence, direct infusion of TCSOX229, but not SB334867, into the PVT attenuated the expression of a CPA associated with morphine abstinence (Li et al, 2011). Interestingly, intra-PVT infusions of the same Hcrtr-2 antagonist did not influence the physical symptoms of naloxone-precipitated morphine withdrawal (Li et al, 2011). This is congruent with our results, which support a selective involvement of Hcrtr-1 in the somatic discomfort associated with cessation of nicotine intake.

In agreement with our behavioral data, hypocretin neurons of the lateral hypothalamic area were activated after precipitation of nicotine withdrawal (article 3). Hence, c-Fos expression was increased in PFA/DMH and LH neurons upon the emergence of the somatic signs of nicotine abstinence (article 3). Likewise, an activation of hypocretin neurons has been observed during naloxone-precipitated morphine withdrawal (Georgescu et al, 2003; Laorden et al, 2012). Interestingly, pretreatment with the Hcrtr-1

antagonist SB334867 attenuated the activation of PFA/DMH neurons, but not that of LH neurons, in mecamylamine receiving nicotine-dependent mice (article 3), which suggests that a local auto-regulatory network of hypocretin transmission might exist within the PFA/DMH. This is consistent with the presence of Hcrtr-1 immunoreactivity in hypocretin neurons (Bäckberg et al, 2002) and the existence of asymmetric synaptic contacts between hypocretin-containing axon terminals and cell bodies (Horvath et al, 1999), supporting the idea that Hcrtr-1 might serve as a somatodendritic autoreceptor that regulates the activity of hypocretin neurons. Alternatively, electrophysiological evidence suggests that pre-synaptic hypocretin receptors in local glutamatergic terminals might also influence the activity of hypocretin-expressing neurons (Li et al, 2002).

Although hypocretin neurons exhibit a restricted localization within the lateral hypothalamic area, they send efferent projections widely throughout the brain (Peyron et al, 1998; Nambu et al, 1999). Therefore, we next evaluated other brain regions potentially related to the manifestations of nicotine withdrawal and the addictive properties of nicotine that could mediate the effects of hypocretins on the behavioral expression of nicotine abstinence. Interestingly, mecamylamine injection in nicotine-dependent mice increased c-Fos expression in 3 out of 10 areas tested (article 3). Thus, an activation of the lateral septum, the BNST and the PVN was observed upon precipitation of nicotine withdrawal (article 3). Similarly, these brain nuclei have been found to be stimulated during naloxone-precipitated morphine withdrawal in rats (Nuñez et al, 2007). The lateral septum plays a critical role in the regulation of motivation, mood (Sheehan et al, 2004), and stress responses (Singewald et al, 2011). Hence, it has been suggested that c-Fos stimulation in the lateral septum during abstinence may reflect not only dysregulation of motivational processes, but also the engagement of stress and anxiety responses associated with drug withdrawal (Sheehan et al, 2004). Remarkably, direct administration of nicotine into the lateral septum induces

anxiogenic-like effects in rats (Ouagazzal et al, 1999; File et al, 2000; Cheeta et al, 2000), suggesting that the lateral septum might play a role in the emotional responses produced by nicotine use and withdrawal. The BNST, together with the CeA and the NAc shell, forms the basal forebrain macrostructure known as the extended amygdala, which contains neurotransmitter systems that modulate the positive reinforcing effects of drugs of abuse as well as major components of the extrahypothalamic CRF system that mediate the aversive aspects of drug withdrawal (Koob, 2010). Acute nicotine injections, similar to other drugs of abuse, elevate DA extracellular levels in the BNST (Carboni et al, 2000a; Cohen et al, 2002). Nonetheless, despite the potential role of this nucleus on nicotine withdrawal, blockade of $CRF_{1/2}$ receptors in the BNST does not prevent mecamylamine-induced elevations of brain reward thresholds in the ICSS paradigm in nicotine-dependent rats (Marcinkiewcz et al, 2009). Furthermore, injections of nAchR and DA D1-like receptor antagonists in the same brain structure do not precipitate abstinence in nicotine-dependent rats (Jonkman and Markou, 2006). Under our experimental conditions, Hcrtr-1 stimulation did not contribute to the activation of the lateral septum and BNST during nicotine withdrawal (article 3). The lack of hypocretin modulation over these brain nuclei could be explained by the initial activation of the lateral septum and the BNST, which subsequently could have influenced hypocretin neuron activity. In support of this hypothesis, direct afferent inputs have been detected from the lateral septum (Yoshida et al, 2006; Sartor and Aston-Jones, 2012) and the BNST (Yoshida et al, 2006) to lateral hypothalamic hypocretin neurons. However, SB334867 prevented the activation of the BNST during naloxone-precipitated morphine abstinence (Laorden et al, 2012). Remarkably, nicotine withdrawal-induced activation of the PVN was significantly attenuated by prior administration of the Hcrtr-1 antagonist SB334867 or the deletion of the preprohypocretin gene, whereas the Hcrtr-2 antagonist TCSOX229 did not modify this biochemical effect (article 3). These data are in agreement with the Hcrtr-1-dependent activation of the PVN reported during morphine

abstinence (Laorden et al, 2012), and suggest that the hypothalamic paraventricular nucleus is a crucial region connecting both nicotine withdrawal and hypocretin function through the activation of Hcrtr-1.

CRF, through its stimulatory action on the HPA axis and extrahypothalamic brain stress system in the extended amygdala, is a key element contributing to the emotional dysregulation occurring during drug addiction (Koob and Kreek, 2007). Withdrawal from all prototypic drugs of abuse increases HPA axis activity, reflected by elevated ACTH and corticosterone plasma levels, and facilitates CRF release from the CeA (Koob and Zorrilla, 2010). In line with these observations, nicotine withdrawal increases HPA axis activity in humans (Pickworth and Fant, 1998; Roche et al, 2010) and animals (Gentile et al, 2011; Skwara et al, 2012). Here, we demonstrate an Hcrtr-1 dependent activation of CRF-expressing neurons of the PVN, which are the main regulators of HPA axis activity, during the somatic manifestation of nicotine abstinence (article 3). In agreement, extensive evidence indicates that the activation of the PVN and the subsequent CRF release are involved in the stressful conditions associated with drug withdrawal, as mainly revealed in the case of opiates. Accordingly, administration of naloxone to morphine-dependent animals induces a prominent and reproducible increase in PVN c-Fos levels (Laorden et al, 2012; Nuñez et al, 2007; Navarro-Zaragoza et al, 2010), presumably through the activation of noradrenergic pathways innervating this hypothalamic nucleus (Laorden et al, 2002). Furthermore, robust increases in the transcriptional activity of CRF have been observed in morphine-dependent rats during opiate abstinence (Nuñez et al, 2007). Consistent with these biochemical observations, CRF_2 receptor blockade decreases the somatic signs associated with precipitation of morphine withdrawal (Navarro-Zaragoza et al, 2011). Nevertheless, contradictory results have been obtained regarding the role of CRF_1 receptor subtype in morphine abstinence (Navarro-Zaragoza et al, 2010; Papaleo et al, 2007).

Using retrograde tracing, we have revealed that the PVN receives direct hypocretinergic afferents from the lateral hypothalamic area (article 3). Notably, hypocretin projections originating from the PFA/DMH were more abundant than those arising from the LH (article 3). This is congruent with previous reports showing that moderate to strong hypocretin inputs from the PFA/DMH innervate the PVN (Qi et al, 2009), and the electrophysiological evidence pointing to the PFA/DMH as the main hypothalamic area providing excitatory drive to PVN neurons (Boudaba et al, 1997). Additionally, we have shown that the Hcrtr-1 antagonist SB334867 selectively blocked the activation of PFA/DMH hypocretin neurons (article 3), suggesting that this specific neuronal subpopulation might play a major role in the activation of the PVN during mecamylamine-precipitated nicotine withdrawal.

Based on the role of Hcrtr-1 in the somatic expression of nicotine abstinence as well as the simultaneous activation of hypocretin neurons and the PVN during this behavioral response, we evaluated whether SB334867 infusion into the PVN might influence the physical manifestations of nicotine withdrawal. Direct bilateral injections of the Hcrtr-1 antagonist into the PVN prevented the somatic manifestations associated with nicotine withdrawal (article 3), indicating that signaling through Hcrtr-1 in this brain area is essential for the behavioral modulation of nicotine abstinence.

In summary, our data reveal a novel role for the hypocretin system in the anxiogenic-like effects induced by acute nicotine administration as well as the somatic manifestations of mecamylamine-precipitated nicotine withdrawal. Supported by previously reported anatomical and functional data, our results suggest that hypocretins are directly released within the PVN to modulate these behaviors. Additionally, the modulatory role of Hcrtr-1 upon this brain nucleus, together with the subsequent stimulation of CRF transmission, provides a new mechanistic insight whereby hypocretin transmission regulates the effects of nicotine on anxiety and withdrawal (Figure 18).

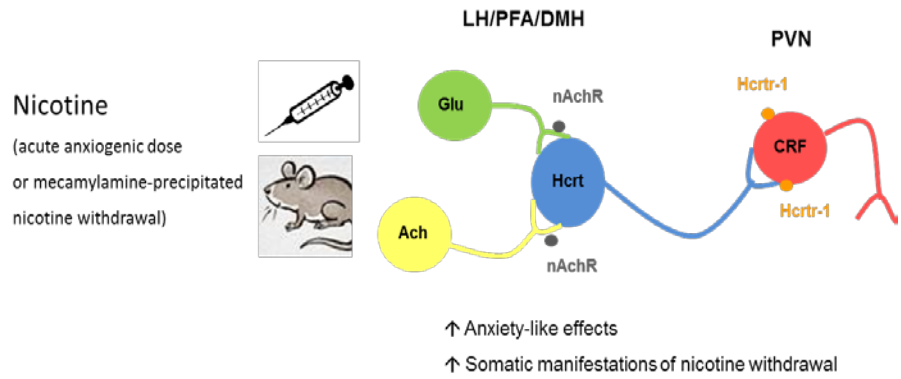

Figure 18. Schematic illustration of the neurobiological mechanisms underlying the expression of acute nicotine-induced anxiety-like effects and mecamylamine-precipitated nicotine withdrawal. Nicotine binds to pre-synaptic nAchRs in glutamatergic and cholinergic afferents in the lateral hypothalamic area to activate hypocretin-expressing neurons (Pasumarthi and Fadel, 2010). Hypocretin axon terminals, mainly arising from the perifornical area (PFA) and the dorsomedial hypothalamus (DMH), directly innervate the hypothalamic paraventricular nucleus (PVN), and corticotrophin-releasing factor (CRF)-containing neurons express hypocretin receptor-1 (Hcrtr-1). Injection of the anxiogenic dose of nicotine and precipitation of nicotine withdrawal increase hypocretin release into the PVN, which in turn activates CRF neurons of this brain nucleus through the selective stimulation of Hcrtr-1. LH, lateral hypothalamus; Glu, glutamate; Ach, acetylcholine; Hcrt, hypocretin; nAchR, nicotinic acetylcholine receptor.

2. Role of the hypocretin system in reinstatement of nicotine-seeking behavior

One of the most insidious aspects of drug addiction is the recurring desire to take drugs even after prolonged periods of abstinence (Kalivas and Volkow, 2005). Accordingly, the enduring vulnerability to relapse has been identified as a p oint where pharmacological intervention may be most effectively employed (O'Brien, 2001). Nevertheless, success rates of achieving long-term abstinence after smoking cessation remains quite low even with the help of the available medications (Hatsukami et al, 2008), and prevention of relapse is still the main need uncovered by present pharmacotherapies (Dwoskin et al, 2009). Therefore, efforts must be made to identify the neurobiological mechanisms and the neurotransmitter systems

that govern re-initiation of nicotine-seeking and -taking behaviors even long after having stopped smoking. Similarly to other drugs of abuse, 3 factors have been identified to promote the resumption of nicotine-seeking behaviors: stress (Bilkei-Gorzo et al, 2008), environmental stimuli previously associated with nicotine use (Martín-García et al, 2009), and the re-exposure to nicotine (Dravolina et al, 2007).

The first reports emphasizing a role for the hypocretin system in drug addiction proposed that these neuropeptides might contribute to the resumption of cocaine- and morphine-seeking behaviors after periods of abstinence (Boutrel et al, 2005; Harris et al, 2005). As a consequence, we first tested the possible intrinsic effects of hypocretin-1 on nicotine relapse using an operant model of reinstatement in mice (Martín-García et al, 2009). Interestingly, the intraventricular infusion of hypocretin-1 reinstated a previously extinguished nicotine-seeking behavior, and this effect was abolished by prior administration of the Hcrtr-1 antagonist SB334867 (article 1). In line with our findings, intraventricular and intra-VTA infusions of hypocretin-1 induced reinstatement in an operant model of cocaine-seeking in rats (Boutrel et al, 2005; Wang et al, 2009). Moreover, hypocretin-1 infusion into the VTA was also effective in reinstating a previously extinguished CPP to morphine (Harris et al, 2005).

Hypocretin-containing axon terminals present a widespread distribution throughout addiction-related brain areas (Kenny, 2011). Therefore, hypocretin-1 could have triggered reinstatement of nicotine-seeking through the activation of differential neurobiological mechanisms. In this sense, hypocretin-1 might have induced a nicotine-like rewarding (priming) effect. Indeed, infusion of this peptide into the VTA elevates DA extracellular levels in the NAc (Narita et al, 2006; Narita et al, 2007; España et al, 2011) and elicits a CPP (Narita et al, 2007). Alternatively, exogenous hypocretin-1 may have evoked a stress-like aversive state, which in turn could have stimulated nicotine-seeking. Supporting this hypothesis, intraventricular

and intra-VTA infusions of hypocretin-1 elevate ICSS reward thresholds (Boutrel et al, 2005) through the activation of the CRF system (Hata et al, 2011). In addition, hypocretin transmission enhances attention and cognitive function through the modulation of the basal forebrain cholinergic system (Fadel and Burk, 2010). Hence, hypocretin-1 might have influenced attentional processing of drug-related external and internal cues (Fadel and Burk, 2010), and elicited reinstatement of nicotine-seeking. As a result, hypocretin-1 appears to have the potential ability to gate any of the 3 modalities of reinstatement that are known to elicit relapse in humans. Therefore, we next sought to examine the involvement of the hypocretin system in stimulus-driven reinstatement of nicotine-seeking behavior.

As previously discussed, we have shown that hypocretin-induced activation of hypothalamic CRF neurons modulates the anxiogenic-like behavior elicited by acute nicotine as well as the stressful conditions associated with nicotine withdrawal. Based on these findings, we initially evaluated the involvement of the hypocretin system in stress-induced nicotine relapse. Prior administration of the Hcrtr-1 antagonist SB334867 did not prevent the reinstatement of nicotine-seeking induced by acute footshock stress under our experimental conditions (article 1). In contrast, a high dose of SB334867 was previously shown to block footshock stress-elicited reinstatement of cocaine-seeking behavior in rats (Boutrel et al, 2005). Nonetheless, the high doses of SB334867 used in this previous study have been shown to produce sedation and behavioral impairments in rats (Rodgers et al, 2001; Nair et al, 2008), which might have interfered with lever-pressing behavior (Nair et al, 2008) and bias the interpretation of the results. Conversely, the low doses of SB334867 used in our study did not induce deleterious effects in water-maintained operant responding in mice (article 1), indicating that SB334867 did not elicit unspecific effects under our experimental conditions.

With repeated cycles of drug withdrawal and relapse, the persistent activation of the HPA axis induces a blunted hypothalamic CRF response and sensitizes the extrahypothalamic CRF system (Koob and Zorrilla, 2010). Indeed, stress-induced relapse, although CRF-dependent, does not rely on the activation of the HPA axis (Shaham et al, 1997; Erb et al, 1998). Thus, adrenalectomy or metyrapone injections, which block corticosterone synthesis, do not prevent stress-elicited relapse to heroin- (Shaham et al, 1997) or cocaine-seeking (Erb et al, 1998) behavior in rats. Instead, the extrahypothalamic CRF system mediates stress-induced reinstatement of drug-seeking (Shalev et al, 2010). Accordingly, mild footshock stress enhances CRF release into the VTA (Wang et al, 2005) and increases CRF expression in the BNST (Funk et al, 2006). Moreover, a CRF-containing projection from the CeA to the BNST plays an essential role in the mediation of the effects of footshock stress on the reinstatement of cocaine-seeking in rats (Erb et al, 2001). Therefore, the switch from hypothalamic to extrahypothalamic CRF systems might have contributed to the lack of hypocretin participation on stress-induced relapse to nicotine-seeking. Consistent with a role for CRF in stress-induced relapse, we found that the CRF_1 receptor antagonist antalarmin effectively blocked footshock-induced reinstatement of nicotine-seeking behavior (article 1). Similarly, previous studies have shown that stress-induced nicotine relapse depends on CRF transmission (Zislis et al, 2007; Bruijnzeel et al, 2009).

Footshock-induced CRF release into the VTA promotes local glutamate and dendritic DA outflow within the same brain nucleus in cocaine-experienced rats (Wang et al, 2005). Notably, hypocretin-1 perfusion into the VTA elicits similar neurochemical changes (Wang et al, 2009), suggesting that hypocretin-1 and CRF might converge in the VTA to induce reinstatement of drug-seeking behavior. Nevertheless, a recent report has shown that hypocretin-1 and CRF do not interact within the VTA to trigger this behavioral response (Wang et al, 2009). Accordingly, perfusion of the

Hcrtr-1 antagonist SB408124 into the VTA blocked the reinstatement of cocaine-seeking as well as the neurotransmitter release induced by intra-VTA hypocretin-1 (Wang et al, 2009). However, perfusion of the same Hcrtr-1 antagonist into the VTA was not effective in attenuating CRF-dependent footshock-induced reinstatement of cocaine-seeking and the associated neurotransmitter release within the same brain area (Wang et al, 2009). Moreover, intra-VTA perfusion of the CRF antagonist α-helical CRF did not modify hypocretin-1 induced behavioral and neurochemical effects within the VTA, while blocked similar responses induced by footshock (Wang et al, 2009). In agreement, we found that hypocretin-1 induced reinstatement was not inhibited by prior administration of the CRF_1 receptor antagonist antalarmin at a dose that blocked footshock stress-induced reinstatement (article 1). Furthermore, the CRF antagonist D-Phe CRF_{12-41} attenuated, but did not abolish, the reinstatement of cocaine-seeking induced by hypocretin-1 (Boutrel et al, 2005). Taken together, these findings suggest that similar to the effects of cocaine, reinstatement of nicotine-seeking elicited by hypocretin-1 and CRF-dependent footshock stress might involve independent mechanisms, both leading to similar biochemical and behavioral changes.

The reinstatement induced by hypocretin-1 was independent of stress-elicited relapse to nicotine-seeking. Therefore, hypocretin-1 could be stimulated under any of the other conditions known to enhance nicotine-seeking behavior (i.e. drug-primed or cue-induced reinstatement). The re-exposure to environmental stimuli associated with nicotine consumption is critical to explain the high rates of relapse to nicotine-seeking in humans (Caggiula et al, 2001; Chiamulera, 2005). Indeed, nicotine is particularly effective in establishing or magnifying the incentive properties and reinforcing effects of associated non-pharmacological stimuli, such as the predictive visual cues typically found in self-administration paradigms or the smell of tobacco smoke in humans (Balfour, 2009; Caggiula et al, 2001; Goldberg and Henningfield, 1988; Rose and Corrigall, 1997). In agreement,

the exposure to environmental cues previously paired with nicotine availability was the most effective condition to elicit relapse in an operant model of reinstatement in mice (Martín-García et al, 2009). Therefore, we next analyzed whether hypocretin peptides play a role in cue-induced reinstatement of nicotine-seeking (article 2). Unlike during stress-induced relapse, pretreatment with the Hcrtr-1 antagonist SB334867 dose-dependently attenuated the reinstatement of nicotine-seeking elicited by nicotine-conditioned cues (article 2), suggesting that re-exposure to nicotine-associated environmental stimuli is sufficient to facilitate endogenous hypocretin release and motivate nicotine-seeking behaviors. In agreement, cue-triggered reinstatement of nicotine-seeking increased the percentage of hypocretin cells expressing c-Fos in the PFA (article 2). In line with our findings, Hcrtr-1 blockade has been shown to prevent cue-induced reinstatement of ethanol- (Lawrence et al, 2006), cocaine- (Smith et al, 2009), and heroin-seeking (Smith and Aston-Jones, 2012) behaviors. Moreover, re-exposure to alcohol-paired environmental stimuli increased c-Fos positive hypocretin neurons in the lateral hypothalamic area (Dayas et al, 2008). Interestingly, direct infusion of SB334867 into the VTA dose-dependently attenuated cue-induced reinstatement of cocaine-seeking in rats (James et al, 2011; Mahler et al, 2012), indicating that Hcrtr-1 in the VTA plays an essential role in the mediation of cue-induced, but not stress-evoked (Wang et al, 2009), reinstatement. In contrast, SB334867 did not prevent reinstatement of food-seeking behavior triggered by food-related cues (article 2), suggesting that Hcrtr-1 might play a specific and differential role in the modulation of drug-seeking behaviors. Consistent with this idea, a previous study showed that Hcrtr-1 blockade was not effective in reducing reinstatement of high-fat food-seeking after pellet priming or the induction of a stress-like state by yohimbine (Nair et al, 2008). On the other hand, Hcrtr-2 blockade with the selective Hcrtr-2 antagonist TCSOX229 did not influence cue-induced reinstatement of nicotine- nor food-seeking behavior (article 2), indicating that Hcrtr-1 plays a selective role in the modulation of nicotine relapse elicited

by drug-paired environmental cues. Similarly, Hcrtr-2 does not participate in cue-induced reinstatement of cocaine-seeking behavior in rats (Smith et al, 2009).

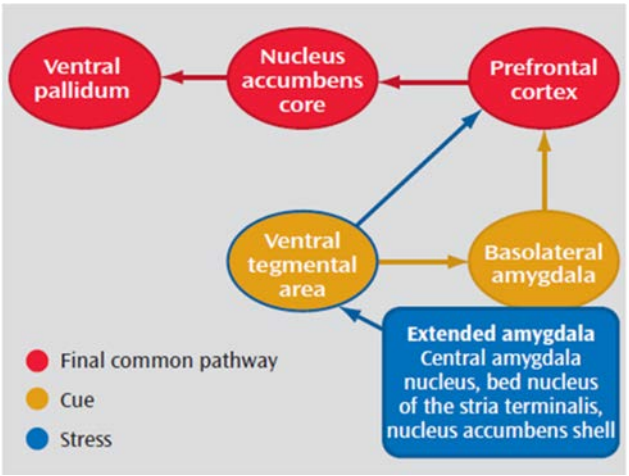

Figure 19. Diagram depicting the neural circuitry that mediates drug-seeking behavior. The series projection from the prefrontal cortex to the nucleus accumbens core to the ventral pallidum is a final common pathway for drug-seeking initiated by stress, drug-associated cues or the drug itself (which increases dopamine release in the prefrontal cortex) (Kalivas and Volkow, 2005).

The fact that vulnerability to relapse in addicted individuals can persist after years of abstinence implies that addiction is caused by long-lasting changes in brain function as a result of repeated drug intake (Kalivas and Volkow, 2005). The activation of the mesocorticolimbic DA system by drugs of abuse is essential to reinforce drug-taking behavior and to facilitate the formation of learned associations. However, the uncontrollable urge to take drugs in addicted individuals seems to arise from plastic changes in excitatory glutamatergic transmission (Kalivas, 2009). During the last few years, the glutamate projection from the PFC to the NAc has been identified as the final common pathway for the initiation of drug-seeking behaviors, and a variety of molecular adaptations in this brain circuitry have been correlated with the resumption of drug-seeking induced by different classes of addictive

drugs (Kalivas and Volkow, 2011) (Figure 19). In agreement, attenuation of excitatory transmission has been shown to reduce nicotine-seeking behavior. Hence, pharmacological blockade of post-synaptic mGlu1 (Dravolina et al, 2007) and mGlu5 receptors (Tessari et al, 2004; Bespalov et al, 2005) prevent nicotine- and cue-induced reinstatement of nicotine-seeking. Conversely, stimulation of pre-synaptic mGlu2/3 receptors blocks cue-induced nicotine relapse (Liechti et al, 2007).

Consistent with this view, we have shown that cue-induced reinstatement of nicotine-seeking is associated with changes in glutamatergic transmission in the NAc. Accordingly, re-exposure to nicotine-associated cues increased phosphorylation levels of NR1-Ser890 and GluR2-Ser880 in the NAc, but not in the PFC (article 2). These changes in accumbal glutamatergic transmission are specific since other phosphorylations, such as NR1-Ser896, GluR1-Ser831 and GluR1-Ser845 were not modified by the presentation of nicotine-conditioned cues in the NAc or the PFC (article 2). In agreement, a recent study has shown that reinstatement triggered by a priming injection of cocaine increases GluR2-Ser880 phosphorylation in the NAc (Famous et al, 2008). The phosphorylation of NDMA and AMPA receptors by protein kinases plays a critical role in the regulation of trafficking, surface expression and function of these receptors (Gladding and Raymond, 2011; Santos et al, 2009), eventually modulating the efficacy and strength of excitatory synapses (Lee, 2006; Wang et al, 2006). Both NR1-Ser890 and GluR2-Ser880 phosphorylations are driven by activation of PKC (Tingley et al, 1997; Chung et al, 2000), an enzyme widely related to plasticity mechanisms (Sanderson and Dell'Aqua, 2011; Sossin, 2007). Upon PKC activation, a rapid dispersal of NMDA receptors from synaptic to extrasynaptic sites has been observed in cultured hippocampal neurons (Fong et al, 2002; Ferreira et al, 2011). Moreover, PKC-induced phosphorylation of Ser890, but not Ser896, disrupts surface-associated NR1 subunit clusters in heterologous cell systems (Tingley et al, 1997; Ehlers et al, 1995), suggesting that PKC stimulation might

facilitate lateral diffusion of NMDA receptors out of the synapse, where they could be subsequently targeted for internalization (Carroll and Zukin, 2002). Similarly, PKC-dependent phosphorylation of GluR2-Ser880 facilitates rapid internalization of GluR2-containing AMPA receptors in neuronal cultures (Chung et al, 2000). Furthermore, inhibition of PKC-stimulated internalization of GluR2 subunits in the NAc blocks cocaine-primed reinstatement (Famous et al, 2008), suggesting that PKC might be involved in re-initiation of drug-seeking behaviors. Here, we show that reinstatement of nicotine-seeking after presentation of nicotine-paired cues increased PKC-phosphorylated substrates in the NAc (article 2). Moreover, prior administration of the PKC inhibitor NPC-15437 attenuated cue-elicited reinstatement of nicotine-seeking (article 2), thus confirming a role for this protein kinase in relapse to nicotine-seeking triggered by re-exposure to nicotine-conditioned cues. The effect of NPC-15437 on r einstatement was specific since the same doses of this antagonist did not impair locomotor activity (article 2). Glutamate receptor-dependent activation of the mitogen-activated protein kinase (MAPK) pathway is critical for the regulation of synaptic plasticity mechanisms (Wang et al, 2007). In agreement, an increase in phosphorylation levels of p-38 MAPK was observed in the NAc upon presentation of nicotine-associated cues, while phosphorylation of extracellular signal regulated kinase (ERK) remained unchanged (article 2). Interestingly, p-38 MAPK pathway plays an active role in the internalization of AMPA receptors during some forms of long-term depression (Zhu et al, 2002; Huang et al, 2004; Brown et al, 2005; Zhong et al, 2008). Nevertheless, the participation of the p-38 MAPK pathway in NMDA receptor trafficking has not been assessed yet. Notably, the changes that we report in accumbal transmission are selectively engaged during the execution of nicotine-seeking behaviors. Thus, phosphorylation levels of NR1-Ser890, GluR2-Ser880 and p-38 MAPK were not modified upon presentation of food-conditioned cues during reinstatement of food-seeking behavior (article 2). Future investigations will be necessary to address the exact functional relevance of

the biochemical changes observed in the NAc as well as their contribution to the resumption of nicotine-seeking behaviors.

Hypocretin-1 produces long-lasting synaptic plasticity in glutamatergic synapses of the VTA (Borgland et al, 2006) and the striatum (Shin et al, 2009). Moreover, the Hcrtr-1 antagonist SB334867 blocks cocaine-induced long-term potentiation in VTA excitatory synapses (Borgland et al, 2006), indicating that Hcrtr-1 activation modulates glutamatergic synaptic transmission. Consistent with this idea, we have shown that SB334867 selectively attenuates the enhanced phosphorylation levels of NR1-Ser890 and p-38 MAPK in the NAc of mice undergoing cue-induced reinstatement of nicotine-seeking (article 2). Nonetheless, it is improbable that these effects are directly mediated by Hcrtr-1 in the NAc since Hcrtr-2 is the main receptor subtype expressed in this brain area (Marcus et al, 2001; Trivedi et al, 1998; Cluderay et al, 2002). Alternatively, Hcrtr-1 modulation of relapse could occur in other key brain areas related to the execution of drug-seeking behaviors, i.e. the VTA or the PFC, where Hcrtr-1 expression is more abundant (Marcus et al, 2001). In agreement, a recent study has shown that reinstatement of cocaine-seeking triggered by cocaine-associated cues depends on Hcrtr-1 and AMPA receptor interactions within the VTA (Mahler et al, 2012).

In summary, our results demonstrate a selective role for Hcrtr-1 in the processes underlying the enduring vulnerability to nicotine relapse (Figure 20). Thus, hypocretin-1 is sufficient to induce nicotine-seeking behavior in an operant model of reinstatement, and this effect is completely abolished by Hcrtr-1 blockade. Nevertheless, Hcrtr-1 does not contribute to CRF-dependent stress-induced relapse, and reinstatement induced by hypocretin-1 does not depend on CRF transmission. Therefore, hypocretin-1 and CRF modulate nicotine reinstatement through independent, but complementary mechanisms. Our results propose a specific involvement of hypocretin-1 in the reinstatement of nicotine-seeking promoted by nicotine-associated cues. Thus,

hypocretin-1, through the specific stimulation of Hcrtr-1, enhances the association between nicotine conditioned-cues and drug availability. We also provide a neurobiological correlate for the effects that Hcrtr-1 exerts on cue-elicited nicotine-seeking behavior. Hence, cue-engaged nicotine-seeking alters glutamatergic synaptic transmission in the NAc and some of these specific changes are influenced by Hcrtr-1 activation. Further research will be needed to evaluate the role of the hypocretin system, if any, in nicotine-primed reinstatement.

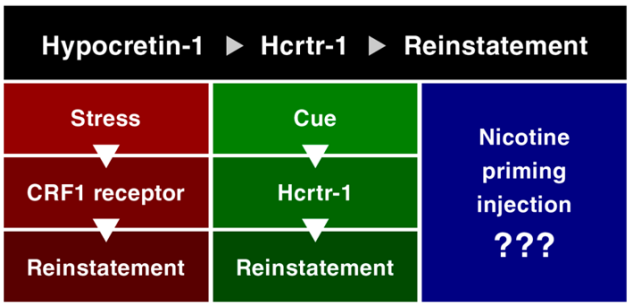

Figure 20. Brief schematic summarizing the role of the hypocretin system in relapse to nicotine-seeking behavior. Hypocretin-1 injections reinstate a previously extinguished nicotine-seeking behavior, and this effect depends on hypocretin receptor-1 (Hcrtr-1) activation. Nonetheless, signaling through Hcrtr-1 does not modulate stress-induced relapse to nicotine-seeking behavior. Instead, corticotrophin-releasing factor 1 (CRF_1) receptor mediates nicotine-seeking after acute exposure to stress. Interestingly, Hcrtr-1 stimulation regulates cue-induced nicotine relapse. Therefore, the presentation of nicotine-conditioned cues might induce endogenous hypocretin-1 release, and the action of this peptide on Hcrtr-1, may influence nicotine reinstatement through the enhancement of the association between nicotine-paired cues and drug availability. Whether hypocretin transmission participates in nicotine-primed reinstatement remains an open question.

Taken together, the current thesis has identified a new neuromodulatory role for Hcrtr-1 in several stages of nicotine addiction, including nicotine withdrawal and relapse (article 4). Taking into account the involvement of Hcrtr-1 in nicotine reinforcement (Hollander et al, 2008), and given the importance of withdrawal and relapse on the pathophysiology of nicotine addiction, Hcrtr-1 antagonists arise as a new therapeutical target that

could improve smoking cessation therapies acting at distinct stages of the addictive process (article 4). Accordingly, these antagonists could facilitate smoking cessation by the attenuation of the reinforcing efficacy of nicotine. Moreover, once quitted smoking, they could ameliorate the discomfort associated with cessation of nicotine intake and reduce the likelihood to relapse following prolonged periods of abstinence.

CONCLUSIONS

The findings revealed in the present thesis allow us to draw the following conclusions:

1. Hypocretin peptides regulate the acute anxiogenic-like effects of nicotine, likely through their actions upon Hcrtr-1. This behavioral effect is associated with a specific activation of the hypothalamic paraventricular nucleus, which is dependent on Hcrtr-1 transmission. Other behavioral effects induced by acute nicotine administration such as the anxiolytic-like effects, hypolocomotion and antinociception are independent of hypocretin signaling.
2. Hcrtr-1, but not Hcrtr-2, contributes to the somatic manifestations of nicotine withdrawal. Mecamylamine injection in nicotine-dependent mice induces an activation of the lateral septum, the BNST and the PVN. Nevertheless, Hcrtr-1 specifically modulates the stimulation of the PVN. In agreement, Hcrtr-1 signaling within the hypothalamic paraventricular nucleus is essential for the somatic expression of nicotine withdrawal.
3. Hypocretin neurons directly innervate the PVN. Hence, the modulatory effects that hypocretins exert on P VN activity during nicotine-induced anxiety-like behavior and withdrawal are presumably achieved through direct hypocretin release within this brain structure. The subsequent activation of Hcrtr-1 in CRF-containing neurons of the PVN, underlies at least in part, the appearance of these behavioral responses.
4. The intraventricular infusion of hypocretin-1 reinstates a previously extinguished nicotine-seeking behavior in an Hcrtr-1 dependent manner. Nevertheless, Hcrtr-1 does not participate in CRF-dependent footshock stress-induced reinstatement of nicotine-seeking. Furthermore, CRF_1 receptor does not influence the reinstatement elicited by hypocretin-1, although it does play a role in footshock stress-induced reinstatement. These results suggest that hypocretin-1

and CRF modulate reinstatement of nicotine-seeking through independent, but complementary mechanisms.

5. Hypocretin peptides, acting selectively at Hcrtr-1, modulate cue-induced reinstatement of nicotine-seeking behavior. In contrast, Hcrtr-1 does not influence reinstatement of food-seeking triggered by food-conditioned cues, suggesting that Hcrtr-1 plays a specific and differential role in the reinstatement of nicotine-seeking behavior.
6. The reinstatement elicited by nicotine-paired cues is associated with increased phosphorylation levels of GluR2-Ser880, NR1-Ser890 and p38 MAPK in the NAc. However, only NR1-Ser890 and p38 MAPK phosphorylations depend on Hcrtr-1 stimulation. In contrast, cue-triggered reinstatement of food-seeking is not accompanied by elevated phosphorylation levels of GluR2-Ser880, NR1-Ser890 and p38 MAPK in the NAc, indicating that different biochemical mechanisms govern nicotine- and food-seeking behaviors.
7. GluR2-Ser880 and NR1-Ser890 are PKC-phosphorylated sites. Additionally, cue-induced reinstatement of nicotine-seeking elicits an increase in PKC phosphorylated target proteins in the NAc. Consistently, inhibition of PKC signaling effectively attenuates the reinstatement of nicotine-seeking induced by nicotine-conditioned cues.
8. Hypocretin peptides, through the selective activation of Hcrtr-1, significantly contribute to behaviors associated with nicotine addiction, including withdrawal and relapse. Therefore, Hcrtr-1 arises as a new promising therapeutical target that might improve smoking cessation therapies both ameliorating withdrawal symptoms and reducing the likelihood to relapse after prolonged periods of abstinence.

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ANNEX

ARTICLE 5

Effects of the Endogenous PPAR-α Agonist, Oleoylethanolamide on MDMA-induced Cognitive Deficits in Mice

Ainhoa Plaza-Zabala, Fernando Berrendero, Juan Suárez, Francisco-Javier Bermúdez-Silva, Emilio Fernández-Espejo, Antonia Serrano, Francisco-Javier Pavón, Loren H. Parsons, Fernando Rodríguez de Fonseca, Rafael Maldonado and Patricia Robledo

Synapse (2010) 64(5):379-389

ARTICLE 6

Effects of repeated MDMA administration on the motivation for palatable food and extinction of operant responding in mice

Ainhoa Plaza-Zabala, Xavier Viñals, Rafael Maldonado
and Patricia Robledo

Psychopharmacology (Berl) (2010) 208(4):563-573

ARTICLE 7

Deletion of the δ Opioid Receptor Gene Impairs Place Conditioning But Preserves Morphine Reinforcement

Julie Le Merrer, Ainhoa Plaza-Zabala, Carolina del Boca, Audrey Matifas, Rafael Maldonado and Brigitte L. Kieffer

Biol Psychiatry (2011) 69(7):700-703

Le Merrer J, Plaza-Zabala A, Del Boca C, Matifas A, Maldonado R, Kieffer BL. Deletion of the delta opioid receptor gene impairs place conditioning but preserves morphine reinforcement. Supplemental information. Biol Psychiatry. 2011 Apr 1;69(7):700-703.

ARTICLE 8

Influence of the δ-Opioid Receptors in the Behavioral Effects of Nicotine

Fernando Berrendero*, Ainhoa Plaza-Zabala*, Lola Galeote*, África Flores*, S Andreea Bura, Brigitte L. Kieffer and Rafael Maldonado

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*Equal contribution

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