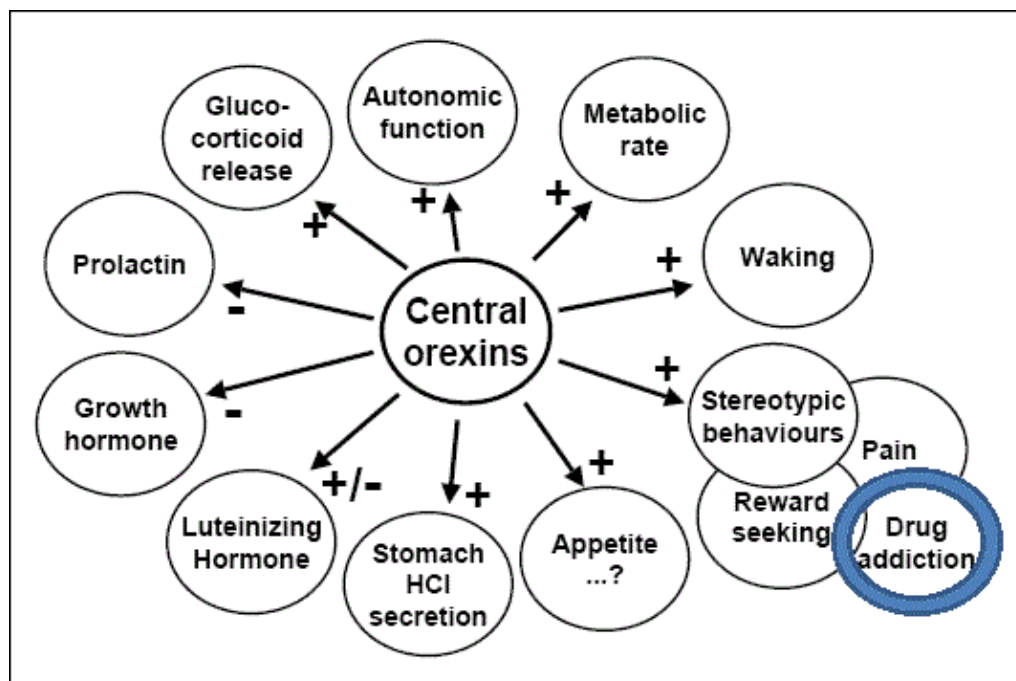UNIVERSITY OF GRONINGEN

Orexin's role in Addiction

Pharmacological agents targeting orexin as a treatment for drug addiction

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28-7-2016

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Abstract

Drug addiction is a problem and its's prevalence and associated burden is highest in relatively wealthy countries. Despite this, there are relatively few effective and approved pharmacotherapies for addiction. Most of the known pharmacotherapies only cover a subset of addictions and the need for more efficient therapies remains. Finding new physiological targets for treatment is therefore desired. One of these targets is the orexin/hypocretin system. This system originates within the hypothalamus and has projections throughout the brain, including the reward system. Targeting this system with pharmacological agents (antagonists for the orexin receptors) has been shown to reduce addiction-like-behavior. The current thesis addresses the current knowledge about the orexin/hypocretin system and its role in substance addiction. The investigated substances are cocaine, nicotine, the opiates and ethanol. Based on the current data the orexin system is indeed a strongly favored target for potential addiction treatment. Not only is it implicated in different substance addictions, the system is well studied and quite well understood. Pre-clinical studies show promising results and perhaps the time has come for the clinical testing of orexin antagonists as a target for addiction treatment.

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CHAPTER 1

Introduction

Addiction is a chronic relapsing disorder characterized by compulsive drug-seeking which persists despite adverse consequences (Khoo et al., 2014). The use of illicit drugs like opiates and cannabis contributes to approximately 20 million disability-adjusted life years (DALYs) and the use of legal drugs including alcohol contributes to another 17.6 million DALYs (Degenhardt et al., 2013; Whiteford et al., 2013; Khoo et al., 2014). The prevalence and associated burden is highest (and increasing) in relatively wealthy countries, like the United States, Australia, United Kingdom and Russia.

Despite all this, there are relatively few effective and approved pharmacotherapies for addiction (box 1.1). Most of the known pharmacotherapies only cover alcohol, nicotine and opiate addiction, whereas treatments for cocaine (and other substances) are still under development (Khoo et al., 2014). Since the current therapies are not or only partly fit for several drug addictions (some even lack proper treatment), it is important that new possible physiological targets are investigated.

One candidate target for the development of novel pharmacotherapies is the orexin/hypocretin system. This system has quite recently been discovered by two different research groups whom each gave a different name to their discovery, one named the peptide ''orexin'' (Sakurai et al., 1997), the other ''hypocretin'' (de Lecea et al., 1997). The system involves two different neuropeptides: orexin-A or hypocretin-1 (OXA or hcrt-1) and orexin-B or hypocretin-2 (OXB or hcrt-2) which both bind to two G-protein coupled receptors (GPCRs): the OX1R and OX2R with different affinities). Orexin containing neurons are located into the hypothalamus and their projections are widespread. Orexin appears to have many functions, centrally as well as peripherally. It's involvement in addiction and the reward system became clear in 2005, when the first publication confirmed that orexin stimulates morphine, food and cocaine reward seeking (Harris et al., 2005). Further studies only strengthened this claim and a new field of research was created. Orexin showed to be involved in cocaine, nicotine, amphetamine, opiate (heroin, morphine) and ethanol addiction and in many more. Targeting this system could therefore potentially be used as a treatment for addiction and the way to go seems to be administering antagonists for the receptors. A hand full of antagonists have already been developed and they can be divided into three groups: dual orexin receptor antagonists (DORA), which block both receptor's signaling, and two single orexin receptor antagonists (SORA), antagonists belonging to group 1-SORA block the OX1R and antagonists belonging to the 2-SORA the OX2R. At the present it is especially the DORAs that are close to or have received clinical approval in usage as treatment for insomnia (one of orexin's implications). However, preclinical animal studies suggest that for addiction, it is primarily the OX1R that is relevant, so developing a 1-SORA should be worth considering (Khoo et al., 2014).

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Addiction is a chronic relapsing disorder characterized by compulsive drug-seeking which persists despite adverse consequences

''Orexin'' and ''hypocretin'' are two names for the same neuropeptides

The orexin/hypocretin system appears to play a role within the reward system

Orexin antagonists have shown to reduce addiction-like behavior

Three types of OX antagonists:
- DORA
- 1-SORA
- 2-SORA

This leads us to the main focus of this thesis: *Could antagonists of the orexin receptors be used as treatment for addiction?* Immediate questions that arise are: (1) what is orexins physiological function within the reward system, (2) what is the reward system, (3) does orexin have other functions, (4) which antagonists are currently available, (4) what exactly is it that the antagonists do, (5) are there differences in effectivity for different drugs, (5) are there any known side-effects, (6) what are the specific differences between both receptors, (8) is there a specific type of antagonist that appears more effective than the others, (9) and most importantly, what is the current status of research on orexin antagonists and their effect on addiction?

> Could pharmacological antagonists of the orexin receptors be used as treatment for addiction?

To answer all of these questions this thesis has been divided into eight different chapters. The first four chapters will form a broad introduction into the orexin/hypocretin system. The first real chapter (chapter two) will address orexins discovery and its main molecular properties. Chapter three will address the location of the orexin producing neurons and their widespread projections. Chapter four will give a short summary of other reported functions of orexin, apart from its function on the reward system. The later four chapters will address the main topic of this thesis, with chapter five giving a short introduction into the reward system. Then, chapter six briefly addresses the discoveries made that let to our current knowledge of orexin and the reward center. Following chapter six, chapter seven will focus on the effects of orexin and its antagonists on addiction. The addressed substances are cocaine, nicotine, the opiates and alcohol, because they are the most researched addictive substances. Finally, chapter eight will inform on the current (pre-)clinical evidence of orexin antagonists in relation to developing a treatment for addiction.

> The first four chapters will address orexins basic properties
>
> The later four the main topic of the thesis

As a service to the reader, every chapter will be summarized at the end. Adding to the summaries, important or just really interesting facts will be stated in colored blocks (at the right side of each page throughout this entire thesis), so that information is easy to retrieve. These blocks will contain the most important information and they will function as a small summary of the text right next to it. Moreover, as already clear form the introduction, the author has added a few ''boxes'' with extra information that is not necessarily related to the main topic of the thesis, but they function to broaden the reader's knowledge on the topic and can therefore be helpful. Since some credit should be given to the ''pilots'' of the orexin research field, publications with a great impact on the field will be addressed more deeply, including their methods, results and discussion. In chapter 7 a publication will be added for all the discussed drugs, again as a service to the reader, for further understanding of the methods used in orexin research.

> Blocks function as a summary
>
> Boxes function to broaden the readers understanding

A quote from Mahler back in 2012
''In general, research on the orexin system since its discovery 14 years ago has indicated that these neurons play important roles in fundamental brain and behavioral processes. In fact, it is hard to identify another neuropeptide system that is as strongly linked to wide-ranging behavioral effects as the orexin system. However, work in this nascent field has generated perhaps as many questions as it has answered. It seems clear that continuing studies will reveal ever more complex and intriguing properties of this key brain system'' (Mahler et al., 2012).

Box 1.1: Current treatments for addiction

At the moment, therapy for addiction is largely focused on psychological and physical rehabilitation. Addicts are usually secluded from daily live and are hospitalized in special rehabilitation centers. The goals of treatment are (1) terminating drug usage, (2) staying drug free and (3) becoming more productive in life. Finding the most effective treatment is challenging, and different for every individual.

Currently the most successful treatments consist of detoxification (the process of which the body gets accustomed to being without the drug), behavioral counseling, medication (only for opioids, tobacco and alcohol addiction), evaluation of treatment for co-occurring mental health issues such as depression of anxiety and long-term follow-up to prevent relapse. These treatments are not as effective as desired, since part of the addicts experiences relapse soon after being returned to the real world (NIDA, 2016).

Currently used medications
The medications used function to manage withdrawal symptoms, prevent relapse and treat co-occurring conditions. There are several pharmacological treatments for some substances (opioids, tobacco and alcohol) and they will shortly be addressed. Treatments for other substances have either not been developed/approved or do not yet exist (NIDA, 2016).

Opioids
For opioids, a few agents have been developed (NIDA, 2016). One of them being Methadone, available in two forms: levomethadone (a opioid μ receptor agonist) and dextromethadone (a NMDA antagonist). Another agent is buprenorphine (Suboxone®), an agonist opioid receptor modulator. Both suppress withdrawal symptoms, relieve cravings, reduce seeking and related criminal behavior and help patients to become more open to behavioral treatments. Naltrexone (Vivitrol®) blocks the opioid receptors and it has similar effects as the other two (apart from reducing withdrawal and craving).

Tobacco
For nicotine addiction, therapies can have several forms but all consist of nicotine replacement therapy. Some of them are available to everyone in normal grocery stores, these are the ones that can come in patches, sprays, gum and lozenges. Furthermore two other medications have been approved by the Food and Drug Administration (FDA): bupropion and varenicline (NIDA, 2016). Bupropion (Zyban®) is a norepinephrine-dopamine reuptake inhibitor. Varenicline (Chantix®) on the other hand, is a nicotinic receptor partial agonist (it stimulates the nicotine receptors more weakly than nicotine itself). Both reduce cravings and help to prevent relapse.

Alcohol
The FDA approved three medications for alcohol addiction and a fourth (topiramate) is looking promising in clinical trials. The first is naltrexone (also used in opioids) and it has been shown to reduce alcohol seeking and relapse. The second is acamprosate (Campral®) and appears to reduce symptoms of long-lasting withdrawal, like insomnia, anxiety, dysphoria and restlessness. The third medication is disulfiram (Antabuse®) and it interferes with the breakdown of alcohol, leading to unpleasant reactions (flushing, nausea, irregular heart beat) if the patient drinks alcohol (NIDA, 2016). …

Goals of treatment:
- Termination of usage
- Staying drug free
- Becoming productive

Opioid:
- Methadone
- Suboxone®
- Vivitrol®

Tobacco:
- Nicotine replacement
- Zyban®
-Chantix®

Alcohol:
- Naltrexone
- Campral®
- Antabuse®

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Interesting new methods
Since addiction is such a problem, and the current treatment methods only work for some people and are not very effective, there is a great interest in targeting other signaling pathways in the brain that may or may not be able to reduce addiction-like-behavior. Many molecular systems are under study and could potentially play a future role in pharmacological therapy for addiction. A few of these molecular targets are phosphodiesterase 7 (PDE7), corticotropin-releasing factor, N/OFQ-NOP, Neuropeptide S, the NK1 receptor and finally, the main subject of this thesis: the orexin/hypocretin system. For more information visit chapter 12 from Neuroscience for Addiction Medicine: From Prevention to Rehabilitation – Methods and Interventions by Ekhtiari and Paulus (Ekthiari et al., 2016).

Molecular targets:
- PDE7
- Corticotropin-
  releasing factor
- N/OFQ-NOP
- Neuropeptide S
- NK1 receptor
- OX/hcrt system

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CHAPTER 2
Orexins discovery

Back in 1996, Gautvik and colleagues were able to clone mRNAs selectively expressed in the hypothalamus (one clone nowadays known to be the precursor peptide of the orexins/hypcretins). They extracted them from the rat lateral hypothalamus by the process of directional tag PCR subtraction cloning (Gautvik et al., 1996; Ebrahim et al., 2002). A few months later, two different research groups ''discovered'' the neuropeptide and published their findings at the same time. They each gave a different name to the newly found neuropeptides, causing some confusion in the field about whether to name them one way or the other. The current chapter will discuss the two publications and further basic understanding of orexins properties.

> Two research groups discovered the neuropeptides independently and at the same time

The hypocretins: Hypothalamus-specific peptides
(de Lecea et al., 1997)

The first publication that will be discussed is the work done by de Lecea and colleagues, published in November 1997 with the title: The hypocretins: Hypothalamus-specific peptides with neuroexcitatory activity. Their group consisted out of researchers from different universities (Stanford University, Yale University and University of Oslo). In their introduction they state the importance of research of the hypothalamus, because ''it has been implicated in the regulation of activities beyond those for which factors have been identified''. They had recently identified 38 rat mRNAs as selectively expressed in the hypothalamus (Gautvik et al., 1996). One of the clones (number 35) was expressed exclusively by a bilaterally symmetry structure within the posterior hypothalamus. They wanted to further investigate this particular clone and that led to the discovery of the neuropeptides nowadays called the hypocretins. They found that clone 35 was a nucleotide sequence which coded for a 130 residue protein, and named it preprohypocretin (hcrt). The authors analyzed the sequence and discovered hcrt yielded two peptides: hypocretin-1 (hcrt-1) and hypocretin-2 (hcrt-2). Hcrt-1 resulted from the 28-66 residues of the preprohypocretin and hcrt-2 from 69-97. Since both hypocretins differ a little in their amino acid sequence, de Lecea argued there might be two different receptors (but they did not investigate this). The authors named the neuropeptides and its precursor hypocretin because of its resemblance (in amino acid identities) with the gut hormone secretin and its family the ''in*cretins*'', and chose ''*hypo*'' because they wanted to show it was a peptide selectively found in the hypothalamus.

> Preprohypocretin (hcrt) is a 130 residue protein and precursor for hypocretin-1 (residues 28-66) and -2 (residues 69-97).

> The name hypocretin comes from their resemblance to the secretin family and hypo from their selectivity for the hypothalamus

The experiment
After investigating the previously mentioned ''basic knowledge'' of the newly discovered peptides, de Lecea and colleagues investigated different properties

of the neuropeptides. The used methods, results and following conclusions will be addressed briefly. See box 2.1 for an overview of their conclusions.

Hypocretin production was restricted to the neuronal cell bodies of the dorsal and lateral hypothalamus. This was shown by immunohistochemical studies with a produced polyclonal antiserum (serum 2050) against the C-terminal 17 amino acids of the preprohcrt. Hcrt was found to be the product of a gene on chromosome 11 by means of an interspecific backcross. After immunocytochemically visualizing the neuropeptides (hcrt-1 and hcrt-2) it became clear the peptides had a peptide neurotransmitter/endogenic-like function: they were stored in secretory vesicles. Moreover, several characteristics of the orexins led the researchers to believe their function was in intercellular communication. Some of these characteristics were the structure of the peptides, their accumulation in vesicles at axon terminals and their excitatory effect on cultured *hypothalamic neurons*. To study this hypothesis they recorded postsynaptic currents in 10 days old cultures of synoptically coupled rat hypothalamic neurons under voltage clamp. Application of a synthetic peptide (corresponding hcrt-2) resulted in an increase in frequency of the postsynaptic currents in 75% of the neurons tested. In the other 25% of the neurons the application had no effect. They did not study hcrt-1's effects. To investigate target selectivity for hcrt they performed the same study (voltage clamp) with synaptically coupled *hippocampal dentate granule neurons* (do not express hcrt in vivo). This time, application of the synthetic peptide elicited no response. Finally, they found the two hypocretins to be conserved between the rat and the mouse (the mouse hcrt molecule differed in 39 positions from the rat hcrt, figure 1). Since their resemblance with the incretin family, de Lecea and colleagues suggested that both peptides might signal through two related G protein coupled receptors, but they were not aware of the specific receptor molecules involved (at the time of publishing). They also suggested an endogenous role for the peptides in the central nervous system as homeostatic regulaters. Moreover they concluded that, after the immunohistochemistry analysis of the revealed hypocretin circuitry, the peptides could play a role in nutritional homeostasis. Furthermore, because hcrt is profoundly found in the dorsal-lateral hypothalamus, they suggested possible functions for the hypocretins in feeding behavior, blood pressure, central regulation of immune function and control of energy balance.

> The hypocretins were thought to have a neurotransmitter-like function in intercellular communication

> Both hypocretins are highly conserved between species

> De Lecea suggested that hypocretin could function in:
> - feeding behavior
> - blood pressure
> - central regulation of Immune function
> - control of energy balance

Figure 2.1. Comparison of the amino acid sequence differences between the hypocretins.
(A) Nucleotide and amino acid sequences of preprohypocretin in rat/mouse. Differences in nucleotide sequences are indicated by asterisk, differences in amino acid sequences by triplets. (B) Alignment of rat hypocretin-1, hypocretin-2 and secretin. The identities between the hypocretins and secretin are indicated by asterisks. The hypocretin-1/2 consensus residues appear above the alignment.

From de Lecea et al (1997)

Orexins and Orexin Receptors: A Family of Hypothalamic Neuropeptides and G Protein-Coupled Receptors that Regulate Feeding Behavior.
(Sakurai et al, 1997)

The other research group that discovered the same set of neuropeptides published their findings (a month later) in December 1997. Their first author was Takeshi Sakurai and the group worked for the University of Texas Southwestern (and together with a company called SmithKline Beecham Pharmaceuticals). They were also investigating the hypothalamus, their interest laying more with the hypothalamic control of feeding and energy homeostasis. Their main interest was with G protein-coupled receptors. According to them ''all of the known small regulatory peptides (small peptide hormones and neuropeptides'' exert their biological actions by acting upon GPCRs'' (Sakurai et al., 1997). However, at that time a large number of cDNA sequences that encoded ''orphan'' GPCRs were without known ligands. They argued that many of these receptors are probably receptors of unidentified peptide hormones and neuropeptides and they therefore wanted to undertake a systematic biochemical search for these endogenous peptide ligands for multiple orphan GPCRs. In order to accomplish this, they used a cell-based reporter system. They found two ligands that bound to two closely related orphan G-protein coupled receptors. Sakurai and colleagues named the peptides ''orexin'' after the Greek word ''orexis'', which means appetite. They distinguished orexin-A (hcrt-2) from orexin-B (hcrt-2). The methods used and results will be discussed below.

''Orexis'' is Greek for appitite

The experiment
High resolution HPLC fractions of various tissue extracts were screened for GPCR –agonist activity. They generated 50 stable transfectant cell lines, each expressing different orphan GPCR cDNA. They challenged the cells with HPLC fractions derived from tissue extracts and monitored transduction read-outs for heterotrimeric G protein activation. Several reverse-phase HPLC fractions of brain extracts elicited a robust increase in cytoplasmatic Ca^{2+} in the cell line with, the by them named, HFGAN72 orphan GPCR. They found two peaks of activation. The ligand that caused the first and major peak was named orexin-A (OXA). It was a ''33-amino acid peptide of 3.562 Da, with an N-terminal pyroglutamyl residue, a C-terminal amidation and two intra-chain disulphide bonds''. A smaller peak was caused by a ''28-amino acid, C-terminally amidated linear peptide of 2,937 Da'' and they named it orexin-B (Sakurai et al., 1997). Because they did not find any meaningful similarities between the orexins and any known peptides, they sought to isolate the cDNA encoding the precursor polypeptide, which they called prepro-orexin. This was accomplished by obtaining a cDNA fragment encoding a part of OXA, and performing 5'-RACE and 3'RACE reactions to obtain the full length cDNA, which they found that encoded both: OXA and OXB. Sakurai and colleagues were also able to isolate the genomic fragments containing the prepro-orexin

Orexin A is a 33 amino acid peptide with an amino-terminal pyroglutaml residue, two intra-chain disulphide bonds and a carboxy-terminal amidation.

Orexin B is a 28 amino acid C-terminally amidated linear peptide.

gene and found the same conversivety: human, rat and mouse prepro-orexin sequence was almost identical.

The two orphan receptors (that OXA and OXB bound to) were named the orexin 1 receptor (OX1R; Hctr$_1$) and orexin 2 receptor (OX2R, Hctr$_2$)(de Lecea et al., 1998; Sakurai et al., 1998). Both receptors are G-protein coupled and have been shown to display a striking distribution by analysis of their mRNA in the rat brain: the OX1R binds OXA with a 100-1000-fold higher affinity than OXB, whereas the OX2R seems to have equal affinities for both OXA and OXB (Trivedi et al., 1998). The receptors have been mapped on human chromosome 1p33 (OXA) and 6cen (OXB).

Later research showed OXA's structure to be completely conserved among several mammalian species (human, rat, mouse, cow, sheep, dog and pig). The amino acid sequence of OXB differs a little between species, although overall OXB is highly conserved (Sakurai et al., 2014).

Orexins role in the regulation of feeding behavior (Sakurai et al., 1997)
Sakurai et al argued orexin to be involved in the regulation feeding behavior and energy homeostasis. Since the prepro-orexin peptide was abundantly and specifically expressed in the lateral hypothalamus and adjacent areas, a region that is strongly associated with the central regulation of feeding behavior and energy homeostasis. To test the hypothesis that orexins played a role in regulation of feeding behavior, Sakurai et al centrally administered orexin to rats that were freely fed. There were five groups: (1) vehicle, (2) OXA 3 nmol, (3) OXA 30 nmol , (4) OXB 3 nmol and (5) OXB 30 nmol. The amounts were administered in a 5µL bolus through a catheter placed in the left lateral ventricle in early light phase. Food consumption was plotted over the period of 4h after injection (see figure 2.2). It is clear that both orexins seemed to increase food consumption and the larger the concentration, the larger the effect. However, this does not necessarily mean that it is orexin that caused the increase, orexin could simply enhance the reward given by the food consumption, and therefore lead to an increase in food consumption, rather than causing a direct effect on food intake. Orexins possible involvement in the reward system will later be discussed. They also found that the orexin production was influenced by the nutritional state of the animal.

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The OX1R binds OXA with 100-1000 fold higher affinity than OXB

The OX2R seems to have equal affinities

Both OXA and OXB are conserved across several mammalian species.
However OXA is completely conserved whereas different species differ in some OXB sequences.

Central administration of OXA and OXB both ''increases food consumption ''
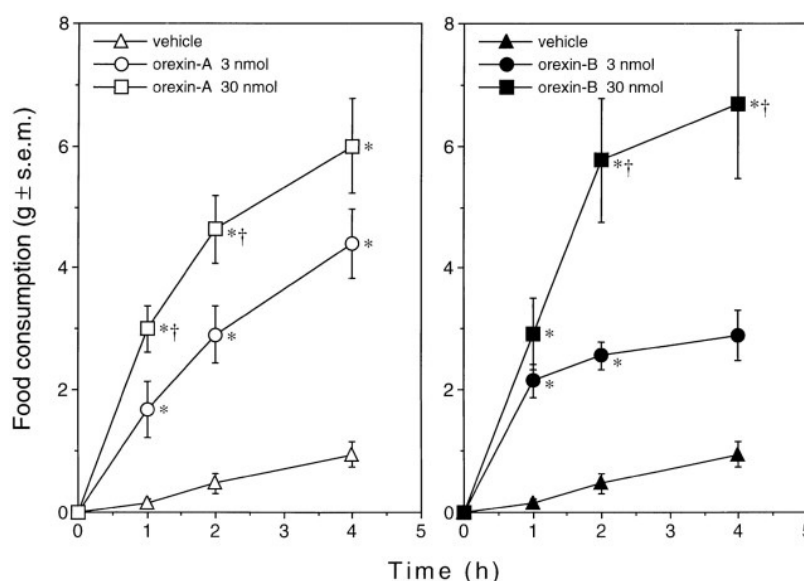
Figure 2.2. Stimulation of Food Consumption by Intracerebroventricular Injection of Orexin-A and –B
There were five groups: vehicle, OXA 3 nmol, OXA 30 nmol , OXB 3 nmol and OXB 30nmol. Asterisks (*) indicate significant difference from vehicle controls Crosses (†) designate significant difference between 3 nmol and 30 nmol injections. There is a clear increase in food consumption when administered OXA or OXB and this increases further when the dosis is higher.
From: Sakurai et al., 1997

Box 2.1: Proposed functions by de Lecea

In short, de Lecea and colleagues concluded that: (1) rat preprohypocretin contains two peptides with related sequences: hcrt-1 and hcrt-2, (2) both peptides were conserved between rat and mouse, (3) the gene for hcrt is located on rat chromosome 11, (4) hcrt production is restricted to neurons in the dorsal and lateral hypothalamus, (5) the hypocretins have neurotransmitter-like/regulatory functions, (6) hcrt-2 is neuroexcitatory and (7) hcrt is target selective. Moreover, they suggested that (1) there might be two different receptors, (2) the hypocretins might be involved in many different processes: from regulating feeding behavior, energy balance, blood pressure, to regulation of central immune function. Sakurai and colleagues confirmed (but they did not know about each other) the existence of two almost identical GPCRs (OX1R and OX2R) and the involvement of the orexins (hypocretins) in the regulation of feeding behavior (de Lecea et al., 1997).

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In summary, orexin/hypocretin is a neuropeptide produced by so-called orexin neurons in the hypothamalus. Its precursor is prepro-orexin which consists of 130 residues. After cleavage two biologically functional peptides are formed: orexin-A (residus 28-66) and orexin-B (residues 69-97). There are two orexin receptors: OX1R and OX2R. The OX1R binds OXA with 100-1000 fold higher affinity than OXB, the OX2R seems to have equal affinities. Both OXA and OXB are conserved across several mammalian species. However OXA is completely conserved whereas different species differ in some OXB sequences. Orexin has been proposed to be involved in many different physiological and psychological processes (chapter 4).

Both, de Lecea and Sakurai have gotten credit for their discoveries and there is no consensus yet on how to name the peptide. It depends on the field of interest and on the personal choice of the author. The author of this thesis has decided to continue with using orexin instead of hypocretin.

CHAPTER 3
Anatomy

Orexin neurons are selectively located in the hypothalamus and have projections throughout the entire brain, resulting in their involvement in many different mechanisms and bodily functions. There are approximately 3.000 orexin neurons in the rat brain and 70.000 orexin neurons in the human brain(Sakurai et al., 2014). The current chapter will address the first article to map orexins projections, enriched with the current knowledge on the location of the neurons and the distribution of both orexin receptors and what this means.

> The rat brain has approximately 3.000 orexin neurons, the human brain 70.000

Neurons containing hypocretin (orexin) project to multiple neuronal systems.
(Peyron et al, 1998)

Back in 1998, Peyron and colleagues were the first to perform an immunonhistochemical study to examine the distribution of the newly discovered orexins and their projections. Their main reason for studying this was the recent discovery by de Lecea's and Sakurai's groups. Peyron, who had been part of the publication by de Lecea wanted to characterize the peptides further and was especially interested in the distribution of the hcrt-immunoreactive neurons and fibers in the brain. For the details on the immonuhistochemisty it is suggested to read Peyron's publication (1998). They exclusively investigated prepro-orexin.

They found the cell bodies producing orexin to be exclusively found in the subregion of the tuberal part of the hypothalamus, the so-called feeding center (Peyron et al., 1998; Ebrahim et al., 2002). The anatomical projections of these neurons are widespread and they project to many important areas in the central nervous system, with major projections towards the noradrenergic (NE) locus coeruleus (LC), lesser projections to the basal ganglia, thalamic regions, medullary reticular formation, and the nucleus of the solitary tract and minor projections to the cortical regions, central and anterior amygdaloid nuclei, and the olfactory bulb (Peyron et al., 1998). For a more detailed overview of the projections see table 3.1. The fiber tracts out of the hypothalamus are divided into four different pathways: the dorsal and ventral ascending pathways and the dorsal and ventral descending pathways. OX neurons sent axons through all of those (table 3.1).

> Orexin cell bodies are located in the feeding center of the hypothalamus.

> Orexin neurons have projections throughout the entire brain, with major projections to the locus coeruleus.

Location of orexin neurons

Orexin neurons are exclusively found in some hypothalamic nuclei
Later research showed that the specific hypothalamic nuclei containing orexin neurons are located in the lateral hypothalamic area (HLA, aka feeding center),

> Hypothalamic nuclei containing orexin neurons are:
> - HLA
> - PFA
> - DMH
> - Posterior hypothalamus

the perifornical area (PFA), the dorsomedial hypothalamus (DMH) and the posterior hypothalamus (Date et al., 1999; Sakurai et al., 2014). The orexin neurons in these areas are critical for receiving and integrating internal and external information, regulating autonomic and neuroendocrine systems, arousal (or vigilance) levels and behavior accordingly. Several findings suggest that the orexin neurons in the different areas exert different functions, however, these findings have yet to be confirmed (Sakurai et al., 2014).

Table 3.1: Projections of orexin neurons through or towards different brain areas.
The figure shows the four way distinction in the four hypothalamic pathways: dorsal ascending, ventral ascending, dorsal descending and ventral descending. Areas through which some projections lead to others are stated under ''through''. ''End'' projections are stated under ''to''. The figure is probably not complete, since it contains all projections found by Peyron and colleagues in 1998.

Pathway	Projection trough	Projection to
Dorsal ascending	zona incerta	paraventricular nucleus (Th)
		central medial nucleus of Th
		lateral habenula
		substantia innominata
		bed nucleus of stria terminalis
		septal nuclei (medial and lateral)
		dorsal anterior nucleus of OB
		cerebral cortex
Ventral acsending		ventral pallidum
		vertical limb of the diagonal band of Broca
		horizontal limb of the diagonal band of Broca
		medial part of the nucleus accumbens
		olfactory bulb (mainly antorior)
Dorsal descending	mesencephalic central gray	colliculi
		pontine central gray
		locus coeruleus
		dorsal raphe nucleus
		laterodorsal tegmental nucleus
	dorsal tegmental area	pedunculopontine nucleus
		parabrachial nucleus
		dorsal subcoerules area
		alpha subcoerulus area
		→ dorsolateral part of gelatinous layer of the caudal spinal trigeminal nucleus
Ventral descending	interpeduncular nucleus	
	ventral tegmental area	
	substantia nigra pars compacta	
	pons	raphe nuclei
	medulla	ventral medullary area

Orexins are thought to act as neurotransmitters
Both orexins are thought to primarily act as excitatory neurotransmitters. (Sutcliffe et al., 2000; de Lecea et al., 1998). They affect serotonin, histamine, dopamine, acetylcholine neurotransmission in an excitatory way, and facilitate gamma-aminobutyric (GABA) and glutamate transmission (Ebrahim et al., 2002). There are projections to the monoaminergic and cholinergic nuclei in

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Orexin neurons affect almost all (neurotransmitter) systems:
- 5-HT
- DA
- ACh
- GABA
- Glutamate
- NE

the brain stem, where both OX receptors are differentially expressed and particular dense (Sakurai et al., 2014).

Projections

Orexin neurons have projections throughout the brain
An overview of the presently known projections is shown in figure 3.1. Orexin neurons receive input (especially salient cues, which have a certain ''conspicuous'' value to the receiver) from the nucleus accumbens (NAc) and several limbic structures, including the bed nucleus of the stria terminalis (BNST) and the central nucleus of the amygdala (CeA). Excitatory projections towards the NAc, nucleus of the solitary tract (NTS), paraventricular nucleus of the hypothalamus (PVN), neuropeptide Y (NPY) neurons in the arcuate nucleus and glucoreceptor (GR) neurons in the ventromedial hypothalamus are thought to be implicated in orexin's regulation of feeding behavior (Sakurai et al., 2014).

Other connections are with autonomic regulatory regions to increase sympathic outflow in respons to salient cues. Another function of orexin lays with its influence on the reward system, and the importance of orexins role in cue-dependency will become clear in the following chapters. (discussed in Orexin is able to excecute its ''reward-promoting'' function by its connections with and between the NAc and the ventral tegmental area (VTA). Moreover, orexin neurons increase arousal to support motivated behavior through the NAc, VTA and monoaminergic centers including the dorsal raphe nuclei (DR), locus coeruleus (LC) and tuberomamillary nucleus (TMN).

> OX neurons receive input from the NAc, BNST, CeA and probably more.

> They hold projections to many different brain areas (figure 3.1).

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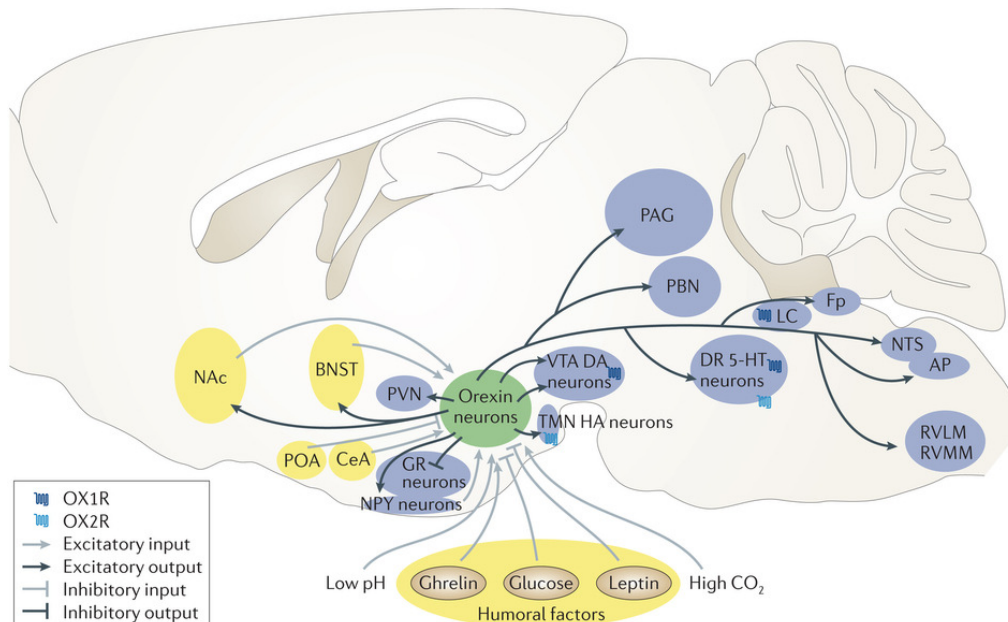
Legend figure 3.1
5-HT, 5-hydroxytryptamine (also known as serotonin); AP, area postrema; Fp, folium-p; HA, histamine; OX1R, orexin receptor type 1; OX2R, orexin receptor type 2; PAG, periaqueductal grey; PBN, parabrachial nucleus; POA, preoptic area; RVLM, rostral ventrolateral medulla; RVMM, rostral ventromedial medulla. (DR), locus coeruleus (LC) and tuberomamillary nucleus (TMN)
From Sakurai et al., 2014

Figure 3.1. Input and output of orexin neurons
The input areas are shown in yellow, under which the nucleus accumbens (NAc), central nucleus of the amgygdala (CeA), bed nucleus of the stria terminalis (BNST). Output areas are shown in blue, including the NAc, nucleus of the solitary tract (NTS), paraventricular nucleus of the hypothalamus (PVN), neuropeptide Y (NPY) neurons in the arcuate nucleus, glucoreceptor (GR) neurons in the ventromedial hypothalamus, ventral tegmental area (VTA), and dorsal raphe nuclei.

Differences between orexin neurons and receptors

Orexin neurons exhibit functional dichotomy
It has now been confirmed that there is a functional dichotomy within the different populations of orexin neurons. Orexin neurons in the lateral hypothalamus have been proposed to be especially involved in reward processing, whereas orexin neurons in the more medial areas (dorsomedial nucleus: DMH and perifornical area: PFA) are more involved in waking and stress responses (Harris et al., 2006; Mahler et al., 2012).

> There is a functional dichotomy between orexin neurons in different populations

The orexin receptors exhibit a different distribution & function
Not only do the orexin neurons innervate many different areas resulting in the broad span of functions, both the OX1R and OX2R receptors exhibit a different and basically complementary distribution. Figure 3.1 shows some areas where either one or the other receptor is present. Where OX_1 mRNA is mainly observed in the prefontal and infralimbic cortex, hippocampus, paraventricular thalamic nucleus, ventromedial hypothalamic nucleus, dorsal raphe nucleus and locus coerolus, the OX_2 mRNA is prominent in a complementary distribution including the cerebral cortex, septal nuclei, hippocampus, medial thalamic groups, raphe nuclei, and many hypothalamic nuclei including the tuberomammillary nucleus, dorsomedial nucleus, paraventricular nucleus and ventral premammillary nucleus (Marcus et al., 2001).

> The OX receptors exhibit a different and complementary distribution in location in some brain areas.
>
> Suggesting they may differ in function

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It has been suggested that each receptor might exert different functions, depending on where in the brain they are located. Recent studies have shown that the OX1R is most closely associated with reward ''function of orexin'', whereas the OX2R is more related to orexins role in arousal. Chapter 4 will give an overview of orexins most prominent roles and functions. Bear in mind however, that since orexin neurons project through almost the entire brain, orexins functions and effects are widespread and not all will be addressed.

In summary, the rat brain has approximately 3.000 orexin neurons, the human brain 70.000. Orexin neurons are exclusively found in hypothalamic nuclei including the HLA, PFA, DMH and Posterior hypothalamus. They receive input from the NAc, BNST, CeA and probably more. The neurons have projections throughout the entire brain, with major projections to the locus coeruleus. There is a functional dichotomy between orexin neurons in different populations. Furthermore, the OX receptors exhibit a different and complementary distribution in location in some brain areas, suggesting they may differ in function.

CHAPTER 4
Functions

As should be clear from the previous chapters, orexin neurons project to almost the entire brain. A logical follow-up would be assuming that, because of its many interactions with other brain areas, orexin could be involved in many different physiological and/or psychological mechanisms. The main goal of this chapter is to give a brief overview of orexin's influences on different systems. Therefore, this chapter will shortly address some known functions of orexin, starting with the suggested functions at the time of orexin's discovery. Since orexin plays a role in many different physiological processes and addressing them all goes beyond the span of this thesis, not all functions will be discussed. For a short introduction of orexin's role in psychiatric disease, visit box 4.2.

Orexins functions

Orexins suggested functions at the time of discovery

At the time of discovery, several characteristics (the structure of the peptides, their accumulation in vesicles at axon terminals and their excitatory effect on cultured hypothalamic neurons) of the orexins led researchers to believe orexins function was in intercellular communication. (de Lecea et al., 1998; Peyron et al., 1998).

Peyron and colleagues (1998) argued that because of the widespread distribution of the OX fibers they found in their study, orexin may play a role in more than just feeding behavior and energy expenditure. They suggested possible roles for orexin in regulation of blood pressure, the neuroendocrine system, body temperature and the sleep-wake cycle. Many of these functions were already suggested by de Lecea, who also proposed a possible function in central regulation of immune function (de Lecea et al., 1998).

It is interesting and important to state that de Lecea and colleagues were well aware of the (possible) energy regulation function of hypocretin, in their discussion they state that hypocretin might have ''orexigenic activity''. This is because they found the location of the hcrt-cell bodies in the posterior hypothalamus to completely overlap with the location of melanin-concentrating hormone (MCH). They argue that hypocretin and MCH could be produced in the same cell bodies, and since MCH was thought to have potential orexigenic activity, so might hypocretin.

> Several characteristics led researchers to believe its function was in intracellular communication

> Peyron suggested functions in regulating:
> - blood pressure
> - neuroendocrine system
> - body temperature
> - sleep-wake cycle
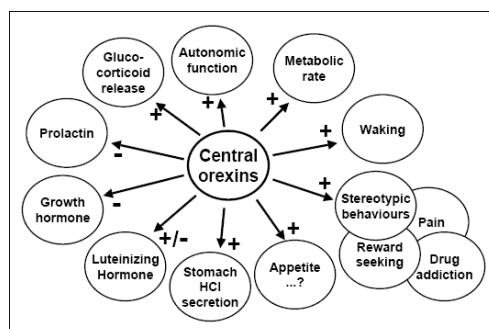
Figure 4. Central orexins functions
Orexin has a stimulating effect on glucocorticoid release, autonomic function (arousal), metabolic rate, waking, appetite, stomach HCL secretion, Luteinizing hormone secretion. Orexin injection has a inhibitory effect on prolactin and growth hormone. Finally orexin also influences ''higher'' brain functions like stereotypic behaviors, pain, reward seeking and addiction. From Korczynski et al., 2006)

Orexin influences not only the brain, but also the periphery
Substantial plasma orexin levels can be found in the periphery (Adam et al., 2002), suggesting peripheral functions of orexin. Orexin's receptors have also been found in several peripheral tissues: including gastrointestinal tract (GIT), endocrine pancreas, adrenal glands and adipose tissue (Digby et al., 2006; Heinonen et al., 2008). The orexin levels in plasma are approximately one-fifth to one-eighth of the orexin CSF values (Snow et al., 2002). The source of the plasma orexin levels is still unclear, it could be released from the brain, or produced directly in the peripheral tissues. It has been shown that some cells in the GIT and pancreas are orexin-immunoreactive, but the origin of the plasma orexin is still under discussion (Messina et al., 2014).

> Orexin has been found in the plasma and it's receptors in the periphery.
>
> Suggesting OX has peripheral functions.

Neuroendocrine effects and the cardiovascular system
Orexin is, as mentioned before, involved in feeding behavior, energy balance and arousal. One of the major influences of orexin is on the plasma lipoprotein profile and insulin glucose homeostasis (Muroya et al., 2001). Orexin A injections increase metabolic rate, insulin secretion, luteinizing hormone levels and cortisol levels. Plasma prolactin and growth hormone levels are decreased (Chemelli et al., 2001; Sutcliffe et al., 2000; Ida et al., 2000; van den Pol et al., 1998). Furthermore, one of the major functions of orexin is promoting arousal in the brain and in the periphery trough stimulation of the (ortho)sympatic nervous system. Orexin injections cause an increase in heart rate and mean arterial blood pressure (MAP) (Kilduff et al., 2000). Interestingly, it seems that especially the OX2 receptor is involved in the arousal-promoting effect of orexin (Sakurai et al., 2011).

> OXA increases (↑):
> - metabolic rate
> - insulin secretion
> - luteinizing hormone Levels
> - cortisol levels
> - heart rate
> - MAP
>
> OXA decreases (↓):
> - plasma prolactin
> - growth hormone

Central regulation of glucose
In addition to the peripheral regulation of glucose via the stimulation of insulin secretion, orexin neurons also directly interact with the glucose-responsive neurons (stimulated by rise in glucose levels) in the VMH and glucose-sensitive neurons (stimulated when the glucose level falls) in the LHA (Messina et al., 2014). Orexin-A stimulates the glucose-sensitive cells of the LHA (Liu et al., 2001), and inhibits the glucose-responsive cells in the VMH (Shiraishi et al., 2000). Moreover orexin neurons also innervate the nucleus of the solitary tract, which receives sensory information such as portal vein glucose levels and gastric distension (Ciriello et al., 2003). Thus, orexin might centrally also give an appetite-stimulating effect, because it activates the neurons that would normally signal the brain (and body) that the glucose level is high enough. It has even been suggested that orexin neurons themselves act as ''conditional glucosensors'' because the electrical activity of orexin neurons is more potently inhibited by glucose when intracellular energy levels are low, whereas higher energy levels attenuate the glucose response in orexin neurons (Venner et al., 2011). Furthermore, it has been shown that insulin-induced hypoglycaemia activates up to one third of all neurons containing orexin (Moriguchi et al., 1999).

> OXA:
> - stimulates glucose-sensitive neurons and
> - inhibits glucose-responsive neurons

> Orexin neurons seem to act as ''conditional glucosensors''

Adrenal gland
Orexins stimulate glucocorticoid secretion from rat and human adrenocortical cells (exclusively through the OX1R). Both receptors are present in the adrenal

> Orexin induces/is:

> glucocorticoid secreation ↑

gland (Ziolkowska et al., 2005). Orexin seems to play an important role within the central nervous system and the peripheral organs through the hypothalamic-pituitary-adrenal/gonadal axis (Korczynski et al., 2006).

Feeding behavior and energy expenditure
Injection of orexin in the PVN, dorsomedial nucleus, LHA and perifornical area result in an increase of food intake (Dube et al., 1999). SB334867, a selective OX1R antagonist significantly reduced OXA-induced food intake, further confirming orexin's possible food-intake-promoting role. Central administration of orexin also leads to an increase in water consumption (Sakurai et al., 2014).

- food intake ↑
- water consumption ↑

Gastrointestinal system
OX1R reactivity has been demonstrated in different parts of the GIT, including in the nerve fibers in the ganglia, smooth muscles, mucosa. Moreover, central administration of orexin has been shown to increase gastric acid secretion and gut mobility in the gastrointestinal system (Sutcliffe et al., 2000; Kirchgessner et al., 1999). Thus, orexin has a stimulating effect on the gastrointestinal tract.

gastric acid secretion↑

Pain
It has also been suggested that orexin plays a role in pain modulation, because there are long descending axonal projections containing orexin at all levels of the spinal cord (van den Pol et al., 1999). A more recent study by Mobarakeh showed that the orexins (when injected) were indeed effective in relieving pain in thermal, mechanical, chemical and nociception-induced behavioral responses. OXA had a more profound effect than OXB (Mobarakeh et al., 2005). Thus, orexin may be a pain relieving substance.

pain relieving

Orexin and BMI
Many studies have found a strong correlation between low levels of circulating orexin and obesity. Patients with disarrangements in their orexin system leading to narcolepsy (a sleeping disorder discussed in box 4.1), have a risk at an increased body mass index (Schuld et al., 2000) and a have higher risk of developing type-II diabetes mellitus (Honda et al., 1996). It has been suggested that orexin (when injected) will activate thermogenesis, without limiting feeding or increasing physical activity and therefore may be a possible '''therapy'' for obese people (Messina et al., 2014). Clinical tests are now being conducted

Narcolepsy (lack of OX) has been associated with increased risk of obesity

OX could potentially be used to ''treat'' obesity

Arousal and orexins role in sleep and waking
Orexin plays an important role in sleep and waking. Short after its discovery it was found that orexin was the molecule behind quite a miraculous disorder: narcolepsy, in which people and animals suffer from what are called ''sleep attacks''. Activation of orexin neurons increases wakefulness, whereas inhibition of these neurons decreases it (Sasaki et al., 2011). The orexins also coordinate goal-directed arousal, such as increased wakefulness following food deprivation or the anticipatory arousal before a rewarding stimulus (Yamanaka et al., 2003; Mieda et al 2004; Muschamp et al., 2007). Since the amount of knowledge about orexin's effect on sleep and waking is a field on its

Orexin is stimulates (goal-directed) arousal

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own and beyond this thesis it will not further be discussed. Narcolepsy and orexin's role in it will be addressed briefly in box 4.1.

Reward seeking
Orexin's role in addiction and its function in the reward system is the main topic of this thesis and therefore will be discussed in detail in the following chapter. Apart from its role in the reward system, orexin appears to play a role in the emotional state of an animal as well. For more information on this topic visit box 4.2.

Orexin plays a role in the reward system

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In summary, orexin is a neuropeptide with a broad range of functions. Centrally it is involved in the regulation of arousal, reward, regulation of glucose and probably in many more. Peripherally orexin stimulates the adrenal glands, feeding behavior and energy expenditure and the GIT. Orexin has also been associated with obesity. Patients with narcolepsy (box 4.1) have an increased risk of being obese and orexin has been thought to activate thermogenesis, making it a possible target for ''obesity therapy''.

Box 4.1: Narcolepsy

In 1999, Lin and colleagues found that a mutation in the OX2R gene caused canine narcolepsy, an until then, unresolved sleeping disorder. This discovery led to an increased interest in the orexins (Ebrahim et al., 2002). Because this discovery was so important for the research on the orexins, the current thesis will address narcolepsy and its relationship with orexin shortly.

In the public eye, narcolepsy is known as a disorder in which people and animals get ''sleep attacks'', causing them to fall asleep in an instant, often triggered by powerful (positive) emotions. It is however, more than that, and the sleep attacks do not necessarily appear (but in the majority of the cases they do). Narcolepsy is a primary disorder of alertness. The presenting symptom is excessive daytime sleepiness with the occurrence of the sleep attacks. They are irresistible and happen during the day. Another symptom is cataplexy: the patient can be struck by brief episodes of muscle weakness or paralysis precipitated by strong emotion (Ebrahim et al., 2002). A third symptom is sleep paralysis, caused by the persistence of rapid eye movement (REM) sleep atonia on waking. Finally, hypnogogic hallucinations or dream like images at sleep onset can also occur. A less prevalent symptom is the occurrence of micro-sleeps (reflections of brief intrusions of sleep), which are short periods of automatic behavior (Krahn et al., 2001; Ebrahim et al., 2002).

As mentioned before, narcolepsy's problem lays with the orexin system. However there are differences among species. Knocking out the canine OX2R is enough to cause canine narcolepsy (Lin et al., 1999), whereas in mice, narcolepsy is only produced by knocking out both receptors, the OX1R and OX2R (Chemelli et al., 1999; Kisanuki et al., 2001; Kisanuki et al., 2000; Takita et al., 2001). Intravenous administration of OXA to narcoleptic Dobermans (canines) reduced cataplexy and normalized their sleep and waking durations (John et al., 2000).

Narcolepsy in Humans
An early study conducted by Nishino and colleagues (2000) found that 7 out of 9 patients with narcolepsy had undetectable levels of orexin-A in their cerebral spinal fluid (CSF). One of the other two had orexin levels in the control range (250-280pg/mL), and the other patient had elevated levels of orexin. The authors suggested that the latter two had problems with the orexin receptor instead of the orexin production itself, because their symptoms they were indistinguishable from the other patients. Another study reported undetectable levels of orexin-A in 32 out of 36 patients with narcolepsy (the other 4 had levels below the control range). This led people to conclude that there are two variants of human narcolepsy: with on one hand patients with a orexin deficiency (majority), and on the other hand patients with a ''resistance'' to orexin, due to abnormal orexin receptor/post-receptor dynamics leading to overproduction of orexin (Chicurel, 2000).

Studies examining orexin cells post mortem found striking reductions in the number of cells: narcoleptic brains had to about 10% of the normal number of orexin cells. One of the studies found cell loss without gliosis or signs of inflammation (Peyron et al., 2002), whereas the other did find evidence for those two (Thannickal et al., 2000). Concluding from their findings, Thannickal and colleagues implied for a degenerative process underlying the

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Narcolepsy is a primary disorder of alertness.

Primary symptoms:
- sleep attacks
- excessive daytime sleepiness

Other symptoms
- cataplexy
- sleep paralysis
- hallucinations
- micro-sleeps

Narcolepsy's problem lays with the orexin system. It is caused by:

- KO OX2R (canine)
- KO OX1R & OX2R (mice)

Human narcolepsy is caused by:
- Lack of OXA
- Problem with OX2R

Some patients have a loss of orexin neurons

orexin cell loss in narcolepsy. More support for this came from the finding that narcoleptic patients had more astrocytes in their hypothalamus than did controls. Other explanations for the loss of the orexin cells can be mechanisms like neurodegeneration, failure of development, reduction in synthesis or release of orexins or a mutation in the coding for orexin (Ebrahim et al., 2002).

Interest of orexins
Because of its involvement in the regulation of sleep and waking, (including narcolepsy) the field of research of orexin has intensified. The orexins are receiving a lot of attention and many research groups are investigating orexins effects on the brain, body and behavior. In sleep and waking studies, the orexins are seen as endogenous, potent and arousal-promoting peptides, their antagonists possibly useful as pharmacological agents for treating insomnia (Sakurai et al., 2014).

> Loss in OX neurons could be caused by a degenerative process

Box 4.2: Psychological state

The orexin system has a wide variety and range of projections to different brain areas. The orexin neurons project densely to the noradrenergic (NE), serotonin (5HT), dopaminergic (DA), cholinergic, and GABA/glutamate areas of the brain. Because of these wide projections and the involved areas, it has been suggested that orexin could be involved in certain psychiatric and neuropsychiatric disorders (Charney et al., 1998; an den Pol et al., 2000). The orexin system may be important in affective disorders like major depression and bipolar affective disorder (Ebrahim et al., 2002). One of the proposed hypothesis for the cause of depression is the monoamine hypothesis (biogenic amine hypothesis), that suggests that dysfunctional or deficient neurotransmission of NE and/or 5HT could underlie depression (Coppen et al., 1967). Especially the involvement of the neurotransmitter systems in the aetiology and treatment of depression is of interest. According to Ebrahim and colleagues (2002), orexin is the only substance known to innervate all the relevant areas of the brain implicated in the neurobiology of depression. It might therefore be interesting to see orexins possible implications to counter depression. Moreover, there is evidence that innervation to the LC and dorsal raphe region, the stimulation of DA and Ach and the prohistaminergic actions all point to an antidepressant effect (Ebrahim et al., 2002).

> Orexin might be involved in affective disorders like major depression and bipolar affective disorder

> Depression is thought to be caused by dysfunctional/deficient NE and/or 5HT neurotransmission.

> Orexin is the only substance known to innervate both systems, implicating orexin as a possible target for innervations

CHAPTER 5
The Reward System

As mentioned in the previous chapter, orexin plays a major role in the reward system. The projections of the orexin cell bodies reach far into this system, with its foremost nuclei the ventral tegmental area (VTA) and nucleus accumbens (NAc). In order to fully understand orexins role on the rewards system and to be able to answer the main question of this thesis: *Could pharmacological agents of the orexin receptors be used as a treatment for addiction?*, it is important to address the reward system first. The current chapter will briefly address the basics of the reward system and examine the process of becoming addicted. The current chapter will be summarized at the end of chapter 6.

The foremost nuclei of the reward system are the VTA and NAc

Dopamine

Dopamine exerts its function on the reward system via the mesolimbic pathway

The foremost neurotransmitter considering the reward system is dopamine (DA). Dopamine producing neurons are necessary for normal functioning of motor behavior, motivation and working memory. Dopamine itself has many functions, and problems with dopaminergic neurons cause a wide diversity of problems, including Parkinson's disease.

The foremost neurotransmitter of the reward system is dopamine

There are two main dopamine signaling pathways in the brain. The mesolimbic pathway and the nigrostriatal pathway (figure 5.1). These originate in two different areas in the brain, one in the ventral mesencephalon and the other in the arcuate nucleus of the hypothalamic median eminence (Volkow et al., 2014). The first pathway playing a major role in the reward system. The DA neurons of the mesolimbic pathway are located in the ventral tegmental area (VTA), located in the ventral mesencephalon and their primary targets are the medium spiny neurons (MSNs) in the nucleus accumbens (NAc) (Volkow et al., 2014). For an overview of the projections by the orexin neurons into the reward system see figure 6.1.

The two main dopamine signaling pathways are: - mesolimbic pathway   - nigrostriatal pathway

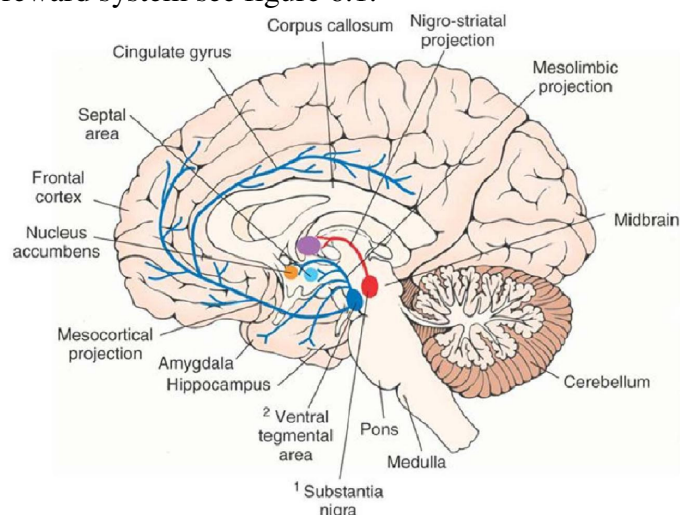| |
|---|
| DA neurons are located in the VTA and project to the NAc and MSNs |

Figure 5.1: Mesolimbic and Nigro-Striatal pathway. *Figure shows the different brain areas involved in the different pathways. Mesolimbic pathway is involved in reward seeking, motivation (blue). Nigro-Striatal pathway is important in motor and other functions (red).*

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Upon activation, DA neurons release DA. Dopamine is re-uptaken by a presynaptic auto-receptor (the dopamine transporter) and excess dopamine is broken down by monoamine oxidase (MAO). This is a crucial modulation mechanism and without the re-uptake additional DA stays behind in the cleft and can cause dramatic effects (Lundbeck Institute).

It has long been thought that dopamine encodes for the reward, but the idea now is that dopamine is more important for the anticipation of a reward, and encodes for a reward prediction signal. The firing frequency of the DA neurons (triggered by environmental cues associated with the reward) is associated with the expected reward value and the possibility of actually obtaining the reward. Firing stops soon after it is clear the reward will not be obtained (Schultz, 2002).

The transition to addiction

''liking becomes wanting''

Addiction emerges gradually, and the rate of the transition of ''liking (controlled) towards wanting (compulsive drug taking: needing the substance)'' is dependent on the type of drug, the pattern of exposure and the developmental state (Volkow et al., 2014). When first exposed to the drug, the body will respond in a certain (often pleasant) way. Following exposures are often experienced as even more pleasant, the same (or a slightly increased) dose will elicit a higher response, this is called sensitization (figure 5.2). However, the body will become accustomed to the drug usage and a tolerance can develop. There are different ways for this to happen, but it most often includes a decreased sensitivity of the receptor (which the drug binds). Which results in a smaller dopamine response, which in its turn results in a decreased sensation of reward. During this process the body eventually begins to crave for the drug and it needs it in order to feel normal. That is usually the breakpoint where someone or something is diagnosed as addicted.

This transition is associated with a shift in the involvement of the striatal subregions of the NAc towards the dorsal striatum, an area important in habit formation (Everitt et al., 2013). Continued exposure to high concentrations of the drug can eventually lead to neuroplastic changes that ultimately change the reactivity of brain DA pathways, resulting in things like tolerance (Volkow et al., 2014). Other changes can be differences in synaptic plasticity, like the strengthening or weakening in various brain reward regions. Most of these are the results of epigenetic changes, changed gene expression and RNA editing modulation (Volkow et al., 2014). The molecular mechanisms in the changing plasticity are the same as in long-term potentiation and depression, that are crucial for memory acquisition, eventually leading to larger synapses and dendrites (De Roo et al., 2008).

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Figure 5.2: The liking-wanting theory
At first the response elicited by the drug increases, the body becomes more sensitive to it and administering the drug gives a pleasant feeling. This is the ''liking'' phase. However, the body often develops a tolerance, requiring a higher dose of the drug in order to get the same results (the dopamine response decreases).

DA is either re-uptaken by the DA transporter or it is broken down by MAO

DA is now thought to resemble the anticipation of a reward, not so much the reward itself

The transition to addiction is dependent on the:
- drug
- pattern of exposure
- developmental state

Drug sensitization (liking) is followed by tolerance (wanting)

The transition is associated with a shift from the NAc towards the dorsal striatum and with molecular changes like epigenetic modifications and synaptic plasticity
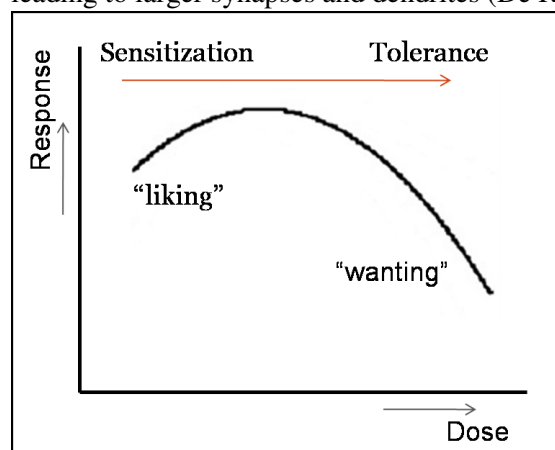
CHAPTER 6
Orexin and the Reward System

Early studies speculated that orexin could play a potential role in the reward system. Orexin neurons send wide projections to the two major areas of the reward system: the ventral tegmental area (VTA) and nucleus accumbens (NAc). Orexins were also thought to play a role in the reward system because of their arousal promoting effect. Since rewards were closely related to arousal (cues that predict rewards increase arousal and reward-seeking behavior is accompanied by arousal), they thought this would be a logical follow-up. In 2005, further investigation of orexin and its potential role in the reward system led to this confirmation.

Rewards are closely related to arousal

A role for lateral hypothalamic orexin neurons in reward seeking
(Harris et al., 2005)

The first publication to confirm orexins role in the reward system was written by Harris and colleagues (2005). Orexins role in sleep and arousal had recently been confirmed, and its food-intake promoting effect had been demonstrated. However, Harris wanted to investigate orexins possible role on the reward system because of the projections to the NAc and VTA. The two major areas involved in processing reward. Harris and colleagues showed that activation of orexin cell bodies in the lateral hypothalamus is strongly linked to preferences for cues associated with food and drug reward and that this activation reinstates extinguished drug-seeking behavior.

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Activation of the orexin neurons in the LHA is strongly linked with cue-dependent reinstatement of extinguished drug-seeking behavior

For their experiment they used a two-chamber nonbiased, conditioned place preference model to measure the rewarding properties of morphine, cocaine or food (Harris et al., 2005). One of the chambers became associated with reward, whereas the other was empty. The rats were given free access to both chambers after conditioning (preference was measured by the amount of time the animals spent in the reward-associated chamber minus the time in the other chamber). They used double-label immunohistochemistry to determine orexin neuron activity (they measured orexin and the immediate early gene protein, Fos) in the lateral hypothalamus, perifornical area (PFA) and dorsomedial hypothalamus (DMH).

Only the conditioned animals that showed a preference (chose the conditioned chamber over the control chamber after the training phase) had higher activation of their orexin neurons (48-52%), for morphine, cocaine and food reward testing (non-conditioned animals had 17% activation, which was not significantly different form naïve untreated animals). And as preference scores increased, so did the percentages of activated lateral hypothalamus orexin neurons (for all three rewards). Another group of animals was given SB 334867, a selective orexin-A antagonist (discovered by Smart and colleagues in 2001) injection after morphine training. The antagonist produced a significant reduction in preference.

SB-22486, an OX1R antagonist, significantly reduced preference

Because of these results the authors wanted to investigate whether stimulation of the orexin cells (that were activated in the previous experiment) could reinstate extinguished preference. They extinguished morphine preference by exposing the animals to empty chambers. To activate the orexin neurons, they injected a molecule (rPP, rat pancreatic polypeptide) into the lateral hypothalamus. The neurons were expected to be activated because they contained the right receptor for rPP and activation of the receptor potently induced Fos activation. rPP injection into the lateral hypothalamus robustly reinstated the extinguished morphine place preference (injection in other areas of the hypothalamus did not reinstate preference).

They finished their publication by stating ''We conclude that the lateral hypothalamus orexin system is an integral part of circuitry that integrates environmental cues with consummatory rewards, and that stimulation of these neurons can drive relapse of drug-seeking behaviour'' (Harris et al., 2005).

Pharmacologic activation of orexin neurons reinstated extinguished preference

Orexin's role in the reward system

Other studies confirm orexins role in the reward system
Later studies further confirmed and strengthened this conclusion. Boutrel and colleagues added information that orexin also contributes to drug seeking behavior and that it exerts its drug seeking function through the activation of stress pathways in the brain. They infused orexin-A into the ventricles of rats and this resulted in dose-related reinstatement of cocaine seeking (without altering cocaine intake). Moreover they found that OXA infusion dramatically elevated intracranial self-stimulation thresholds, which led them to suggest that orexin negatively regulates the activity of brain reward circuitries. Furthermore, blocking the noradrenergic and corticotropin-releasing factor systems prevented the by OXA-induced reinstatement of cocaine seeking. This led them to assume that OXAs effect on drug seeking behavior is due through its induction of a stress-like state. They also used the orexin antagonist SB 334867 and this also blocked the reinstatement of previously extinguished cocaine seeking behavior.

Orexin's effects on drug-seeking could also be through activation of stress pathways in the brain

Another study investigated the effects of OXA and OXB on dopamine levels and its major metabolites in the VTA. They found that dopamine levels in the nucleus accumbens were markedly increased in response to OXA and OXB injection into the VTA and that the antagonist SB334867A significantly suppressed the morphine induced place preference in rats (Narita et al., 2006). Furthermore they showed that deleting the prepro-orexin gene in rats caused a significant reduction in the increase of dopamine levels resulting a morphine injection. These finding provide further evidence of orexins role in reward processing. Furthermore, it has now been confirmed that cues and contexts associated with reward (food, sex, drugs) increase the number of active orexin neurons and prepro-orexin mRNA levels (Harris et al., 2005; Sakurai et al., 1998; Di Sebastiano et al., 2011; Cason et al., 2010).

OXA and OXB injection in the VTA increased DA levels in the NAc

Prepro-orexin KO rats significantly reduced the morphine-induced increase of DA levels

Especialy cues and contexts associated with reward increase the number of active OX neurons and prepro-orexin mRNA levels

Orexin neurons project to the reward system

As mentioned one of the major projection area of orexin neurons is the ventral tegmental area (VTA). The VTA in its turn, contains dopaminergic neurons that innervate the nucleus accumbens (NAc), which sends projections back to the orexin neurons (Yoshida et al., 2006).

There is a differential distribution between the OX1R and the OX2R in the brain (chapter 3). The reward center is no exception. The VTA expresses both receptors (Marcus et al., 2001). However the dopaminergic neurons seem to predominantly express the OX1R (figure 6.1). The dorsal raphne serotonergic neurons have also been reported to express both receptors.
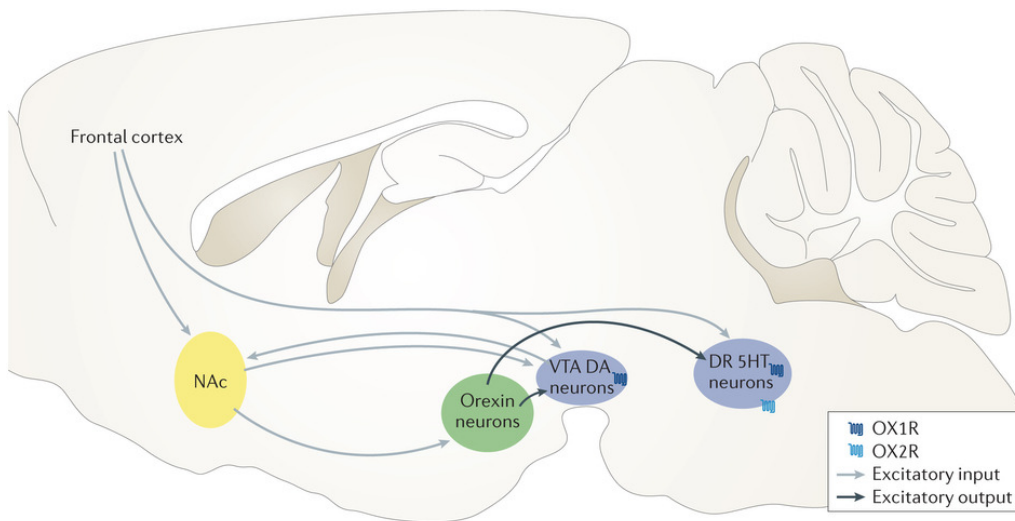
26

Figure 6.1: Projection of orexin neurons on reward system
Input area shown in yellow, output areas in blue. The motor, somatosensory and prefrontal cortices send projections to the dopaminergic VTA, NAc and serotonin neurons in the DR. The NAc sends innervations to both the orexin neurons and the VTA. Output from the OX neurons to the VTA and DR. NAc ;nucleus accumbems, VTA; ventral tegmental area, DA: dopamine, DR: dorsal raphne, 5-HT: serotonin. From Sakurai et al., 2014.

In summary, orexin is clearly involved in the reward system. The reward system is a dopaminergic system, with its most important nuclei the ventral tegmental area (VTA) and nucleus accumbens (NAc). Anticipation on a reward increases dopamine levels, which on its turn, causes a pleasant feeling. At first, this feeling it pleasant and the bodies response towards the drug increases (sensitization). However, after a certain period the body develops a tolerance and a higher dose is required for (the same, or an even lower) response. This transition towards addiction is associated with a shift in the involved brain areas and different kinds of molecular changes.

Orexin is implicated in the reward system: OX antagonists or knocking out the precursor peptide reduce drug-seeking (and reinstatement of extinguished drug-seeking behavior. Furthermore, activation of the OX neurons leads to an increase in dopamine levels in the NAc.

Orexin neurons receive input from the NAc and project towards the VTA, which sends (and receives) information to (and from) the NAc.

CHAPTER 7
Orexin's Role in Addiction

That orexin plays a role in the reward system should be clear. However, this brings a lot of interesting implications for orexin's further functions executed via the reward center. A major field of interest now lays in orexin's role in mediating the processes that accompany addiction. Moreover, it seems that especially the OX1R is important for executing this function, and studies on blocking this receptor with antagonists seem promising in reducing addiction-like behavior. This chapter will address orexins more specific role in addiction process for different substances (cocaine, nicotine, the opiates and alcohol) and the main focus will be on the effects of orexin antagonists on this addiction process. Therefore, the term ''addiction'' will shortly be addressed, followed by a summary of all known orexin antagonists. Finally, to give a clear overview of orexin's and its antagonist's role on the different substance classes, the different substances will be addressed separately. Furthermore, since most studies use certain animal models of addiction, which might not sound familiar for some readers, they have briefly been discussed in box 7.2.

The current chapter will address orexin's involvement in addiction for the following substances:
- Cocaine
- Nicotine
- Opiates
- Alcohol

Addiction according to the DSM-V

In the Diagnostic and Statistical Manual of Mental Disorders, fifth edition (DSM-5), the term ''addiction'' is listed under ''Substance Use Disorders''. It recognizes varies substance related disorders, resulting from the use of ten separate classes of drugs: alcohol (1), caffeine (2) ,cannabis (3), hallucinogens (phencyclidine, arycyclohexylamines) (4), other hallucinogens (LSD, opioids, sedatives, hypnotics, stimulants: amphetamine-type substances, cocaine and others) (6), tobacco (7), and other or unknown substances. As stated in the DSM-5, while the pharmacological mechanisms for each class are different, the resulting activation of the reward system is similar across all substances in producing feelings of pleasure or euphoria. Visit box 7.1 for the list of criteria as stated by the DSM-5. Orexins role on these different substances and their action upon the reward system is the main focus of this chapter.

While the pharmacological mechanisms might differ, the resulting activation of the reward system is similar for each class of substances

Box 7.1: DSM-V Criteria for addiction

1. Taking the substance in larger amounts or for longer than the you meant to
2. Wanting to cut down or stop using the substance but not managing to
3. Spending a lot of time getting, using, or recovering from use of the substance
4. Cravings and urges to use the substance
5. Not managing to do what you should at work, home or school, because of substance use
6. Continuing to use, even when it causes problems in relationships
7. Giving up important social, occupational or recreational activities because of substance use
8. Using substances again and again, even when it puts the you in danger
9. Continuing to use, even when the you know you have a physical or psychological problem that could have been caused or made worse by the substance
10. Needing more of the substance to get the effect you want (tolerance)
11. Development of withdrawal symptoms, which can be relieved by taking more of the substance.

Synthetic orexin ligands: the antagonists

Several orexin receptor antagonists have been developed (table 7.1). The most widely used compounds are small molecules, and are not comparable in size to both orexins (OXA and OXB).

The first antagonist was SB-334867 (SB), a naphthyridine-substituted biarylurea which is selective for the OX1R, but is still able to inhibit the OX2R (Porter et al., 2001). Other more recently developed antagonists are SB-408124 and SB-674042 (Langmead et al., 2004). SB-408124 is urea-based like SB-224867, but it is not as commonly used, although it is slightly better (its dissociation constant (Kb) is 21.7 nM for the OX1R compared to 27.8 nM for the SB-334867). The third, SB-674042 is (in contrast with the ureas) a ketone with three heterocyclic rings and two phenyl groups and it is significantly more potent and more selective than the both ureas, with a Kb of 1.1 nM for the OX1R and 129 nM for the OX2R (Langmead et al., 2004). Another antagonist is ACT-335827, which has been reported to be 10 times more selective for the OX1R than the OX2R (table 7.1). The interesting thing about ACT-225827 is that it is orally bioavailable and it can cross the blood brain barrier (Steiner et al., 2013).

<div>
1-SORAs:
- SB-334867
- SB-408124
- SB-674042
- ACT-335827
</div>

Selective orexin antagonists for the OX2R have also been developed. They have radically different structures. The first OX2R is TCS OX2 29, a tetrahydroisoquinoline, highly selective for the OX2R, while not having any effect on the OX1R (Hirose et al., 2003). Another antagonist is JNJ-10397049, a phenyl-dioxanyl urea with a 600-fold selectivity for the OX2R (McAtee et al., 2004). A quite recently developed antagonist is EMPA, it is an acetamide with a branched structure, with each sulfonamide side chain contains either a pyridine or tobuene group. It inhibits OXA and OXB's responses in the OX2R, but not the OX1R (Malherbe et al., 2009). Finally, another OX2R antagonist, LSN2424100, is strongly selective (200-fold compared with OX1R) for the human OX2R and has been shown to have antidepressant-like activity in mice and rats (Fitch et al., 2014).

<div>
2-SORAs:
- TCSOX229
- JNJ-10397049
- EMPA
- LSN2424100
</div>

Suvorexant is one of the two DORAs which have reached clinical trials for the treatment of insomnia, the other being filorexant. Filorexant is a pyridlpiperdine, with sleep promoting effects in rats, mice and dogs. Clinical studies have shown filorexant to treat insomnia, migraine and painful diabetic neuropathy (Khoo et al., 2014). However, clinical trials to investigate its role in depression have been terminated (Merck et al. 2013). Results with suvorexant (a substituted diazepam) antagonist have been more promising (Khoo et al., 2014). It has been approved by the FDA and PMDA for the treatment of insomnia. Finally almorexant (ACT-078573) was also tested in the clinic, but its use was stopped because of tolerability issues (Boss et al., 2015).

<div>
DORAs:
- Suborexant
- Filorexant
- Almorexant
</div>

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Type	Compound	Affinity K_i (nM)	
		Human OX1R	Human OX2R
DORA	ACT-078573 (almorexant)	13	8
DORA	MK-4305 (suvorexant)	0.6	0.4
DORA	SB-649868	0.3	0.4
1-SORA	SB-410220	8.1 (pKb)	6.3 (pKb)
1-SORA	SB-334867 (SB)	28	1,704
1-SORA	SB-408124	22	1,405
1-SORA	SB-674042	1.1	129
1-SORA	ACT-335827	6 (IC_{50})	417 (IC_{50})
2-SORA	***	5.3-6.1 (pK_i)	6.8-7.1 (pK_i)
2-SORA	JNJ-10397049	1,644	6
2-SORA	C1m	27 (IC_{50})	3 (IC_{50})
2-SORA	EMPA	900	1.1
2-SORA	TCS-OX2-29	ND	7.4 (pK_i)

Table 7.1. Overview of known and developed orexin receptor antagonists *Figure shows three different groups of antagonists: dual orexin receptor antagonists (DORA), single orexin-1 recepor antagonists (1-SORA) and single orexin receptor antagonists (2-SORA). Names and affinities for all antagonists have been shown From Sakurai et al., 2014*

Box 7.2: Animal models of addiction

Since studies on human kind are not feasible, animals are usually chosen as an alternative. However, as the common mind understands, animals are not humans. Therefore it is important to work with different kinds of so called animal models, in which human diseases/disorders have been made somewhat comparable to a specific animal phenotype. In order to understand orexins role in addiction, several animal models of addiction need to be understood. Because most of these models should be familiar with the reader, they will be addressed briefly. The most widely used models (and the ones that are being discussed in this thesis) are: acute withdrawal, sensitization, conditioned place preference, self-administration and relapse to drug seeking.

Acute withdrawal

One of the key symptoms of addiction is acute withdrawal (APA, 2000). The term withdrawal describes the process where one is without the drug, which is something the body does not like. When addicted, several withdrawal symptoms can occur (in humans), from emotional (like anxiety, irritability, depression, social isolation) to physical (like sweating, an increased heart frequency, palpitations, muscle tension, tightness in the chest, difficulty breathing, tremor, nausea, vomiting, diarrhea etc). Acute withdrawal can also out itself in more dangerous symptoms, like heart attacks, strokes, hallucinations or seizures (AaR, 2016).

In animals, acute withdrawal symptoms can be induced in two ways: spontaneous withdrawal and precipitated withdrawal. The first being elicited by cessation of chronic drug exposure, the second being induced by the administration of an antagonist for the receptors the drug interacts with. Widely used animal withdrawal symptoms can again be psychological (anxiety, negative affect, isolation) and physical (piloerection, hunched posture, diarrhea). The acute withdrawal model of animal addiction studies the animals and their behavioral/physiological symptoms (Mahler et al., 2012).

> Spontaneous withdrawal is elicited by cessation of drug exposure
>
> Precipitated withdrawal is induced by administration of antagonists.

Sensitization

Another key process in addiction is sensitization, which is not tolerance (by which the person or animal requires a higher dose of the drug after prolonged/continued usage in order to achieve the same effect). Sensitization occurs when the same dose of the drug causes an increased effect with repeated exposures (Mahler et al., 2012).

In animals, locomotor activation is often used to measure sensitization. Cocaine for instance, has been shown to increase hyper locomotion (for example, upregulation of receptors) in rats with each subsequent injection (Downs et al., 1932). Another measure of sensitization is incentive sensitization and has been linked to the ''wanting'' perspective of addiction (Robinson et al., 1993). It also occurs with repeated dosing. Both kinds of sensitization measures have been correlated with addiction-like behaviors (Mahler et al., 2012).

> Sensitization occurs when patient becomes more sensitive to the drug, resulting in an increased effect of the same dose

> Measures for sensitization are:
> - locomotor activation
> - incentive sensitization

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Conditioned place preference

Conditioned place preference is one of the most physically used models of addiction. It is a paradigm that measures addiction in the form of chamber preference (aka time spent in a certain room). In the majority of cases, the experimental setup consists of two chambers, one with the substance, the other neutral. The animals learn which room contains the substance through Pavlovian conditioning (the chamber contains different kinds of cues). After the association has been made, the ''real'' experiment begins. The animals are placed in between the chambers (now both empty/neutral) and the time measured in the chamber that previously contained the substance is taken as a measure of addiction (Mahler et al., 2012).

Self-administration

Another widely used model of addiction is self-administration, in which the animal has control over the intake of the substance (Mahler et al., 2012). To achieve the substance, the animal is required to perform a certain task, which might be pressing a lever, giving a nose poke, making the wheel turn, pulling a chain, it could be anything. During the training period the animal learns to associate this action with the reward (the substance) on a fixed ratio, meaning for every certain number of actions, the animal receives one reward. The experimental setup can differ, but the major way of performing the experiment is putting the animals on a progressive ratio: the amount of actions they undertake has to be continually increased, to receive the same amount of the substance (for example, during training they learn that every action results in a reward, however on the new ratio, they need to press 3 times, then 6, then 9 etcetera just to receive one reward).

Relapse to drug seeking

A severe problem for rehabilitated people is their risk on drug relapse, which is, not surprisingly, also one of the major ''symptoms'' of addiction. Relapse means that drug seeking is triggered again after a period of non-usage (Mahler et al., 2012). There are three main factors that are known to trigger a relapse in drug seeking: exposure to drug-associated stimuli, the drug itself and stressors. These triggers also trigger relapse in animals. Relapse is often investigated in so-called extinction-reinstatement paradigms after drug self-administration training. During the experimental phase, the animals are restricted from the drug (they had become addicted to during the training phase) for a certain period of time. After the extinction period, the experimenter exposes the animals to cues associated with obtaining the drug, stressors or the drug itself (this is called priming) to trigger relapse behavior (Mahler et al., 2012).

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A CPP procedure consists of 2 chambers, one which will be associated with the drug, the other neutral

Time spent in each chamber is a measure for drug preference

Set-up often contains two levers, one active, one inactive. Animals need to press the active lever to obtain the reward

Three main factors trigger relapse:
- exposure to drug
- exposure to drug associated stimuli
- exposure to stressors

Orexin and cocaine addiction

Orexin fulfills multiple roles in different models of cocaine (and amphetamine) addiction. It is required for locomotor sensitization, the expression of cocaine preference, instrumental cocaine seeking (when driven by motivated states or external cues and stressors) and it seems to increase DA outflow. OX1R antagonists seem to attenuate all these effects. The effects of both orexin and its antagonists on the will be summarized below. Furthermore, since orexins main role in cocaine addiction (and others) seems to be in mediating cocaine seeking, a (fairly recent) study investigating this will be further analyzed (Smith et al., 2010).

Attenuation of sensitization
There have been mixed findings on OX1R antagonists on cocaine sensitization. SB-334867 (which will further be named SB) seems to block sensitization when injected into the VTA, but not the expression of sensitization, which is only blocked when given a period of absence from the drug. The same effects have been found for DORA, an OX1R and OX2R antagonist (Borgland et al, 2006; Winrow et al., 2010).

Conditioned place preference is lowered
As was the case with sensitization, administration of antagonists led to a decrease in preference in this model. SB attenuates both cocaine and amphetamine preference in rats (Gozzi et al., 2011;Hutcheson et al., 2011; Sartor et al., 2012). Interestingly, another study found no effect for a moderate SB dose (20mg/kg) on preference for cocaine, but they did find a decrease in preference for morphine (Sharf et al., 2010).

Self-administration performance is reduced
Orexin has been shown to increase motivation during self-administration, in contrast to its antagonists.
A study with rats showed that administering OXA increased motivation in a drug seeking in which rats were put on a progressive ratio scale. Moreover, adding a selective antagonist (SB-334867) significantly reduced drug seeking (Borgland et al., 2009).
However, other studies found no effect of SB on self-administration of cocaine when the rats were put on a fixed ratio (Smith et al., 2010). However, other studies found no effect of orexin or its receptor on self-administration of cocaine and amphetamine (Hutcheson et al., 2011; Sartor et al., 2012).

Reinstatement of extinguished drug-seeking behavior during relapse is reduced
The orexins have been shown to reinstate extinguished cocaine-seeking behavior. When orexin is infused by means of ICV infusion, it reinstated cocaine seeking (Boutrel et al., 2005).
As expected, SB blocks reinstatement: when SB was injected intraperitoneal into the VTA it blocked reinstatement of cocaine seeking behavior elicited by (conditioned) cues (Smith et al., 2010). This suggests that the orexin system is activated by external cues and that the orexin neurons send that information to the VTA to induce reinstatement (Sakurai et al., 2014).

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Effects of antagonists: (cocaine)

Sensitization:↓
(1-SORA)
(2-SORA)
(DORA)

CPP: Preference:↓(SB)

Drug-seeking:↓↔(SB)

Reinstatement:↓ (SB)

Interestingly, the Boutrel (2005) also found that administration of an antagonist for the corticotropin-releasing hormone (CRH) receptor reduced reinstatement. Likewise, administering the a2 receptor agonist clonidine even blocked orexins effect on reinstating the extinguished behavior. This suggests that orexins role on reward-seeking behavior is mediated by CRH and noradrenergic systems (Boutrel et al., 2005; Sakurai et al., 2014).

DA outflow is attenuated
Orexin administration into the VTA leads to enhanced dopamine responses to cocaine and it promotes self-administration of cocaine (Espana et al., 2011). Moreover OXA in the VTA seems critical for the induction of synaptic plasticity and behavioral sensitization to cocaine (Borgland et al, 2006).

An in vivo microdialysis study in rats by Quarta in 2010, showed that acute administration of SB (30 mg/kg SC) reduced amphetamine-evoked (1 mg/kp IP) dopamine outflow in the shell of the NAc and decreased the expression of amphetamine sensitization, measured as locomotor activity. The authors concluded that administering a selective OX1R antagonist reduces both the acute effects of amphetamine on DA outflow in the NAc and the expression of locomotor sensitization (Quarta et al., 2010).

DA outflow: ↓(SB)

Orexin increases the motivational properties of cues, not the reinforcing properties of the drug itself
In general, orexin seems to increase the incentive motivational properties of conditioned cues and to enhance highly motivated seeking of stimulants, but not to affect the direct reinforcing properties of these drugs themselves. This is shown in figure 7.1. A self-administration program showed that SB (30mg/kg) significantly lowered reinstatement (lever responding) in rats when cues, context or stress were available. SB had no effect on reinstatement when the drug itself was given (prime) during the reinstatement phase. Moreover, SB also reduced cocaine seeking after a period of abstinence. The OX1Rs involvement in cocaine-seeking behaviors, like the expression of preference in the CPP (Harris et al., 2005) and relapse to cocaine seeking behavior (Mahler et al., 2013), is completely dependent on the availability of cocaine-associated cues. When cues are absent, or when the behavior is less cue dependent, OX1R signaling is not involved. Behaviors that are less cue-dependent can be low-effort self-administration or cocaine-primed reinstatement of cocaine seeking (Bentzley et al., 2015).

Orexin increases the motivational properties of cues, not the reinforcing properties of the drug itself

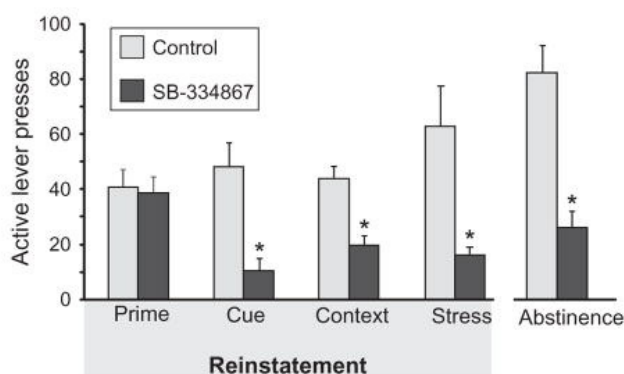Orexin's effects are cue/context/stress-dependent

Figure 7.1 Effect of SB on cocaine reinstatement
SB attenuates drug seeking when reinstatement is cue- context- or stress dependend, not when it is primed. SB also attenuated lever pressing after a period of abstinence. Adapted from Mahler et al., 2012

Orexin/Hypocretin is necessary for context-driven cocaine seeking

(Smith et al., 2010)

Since it became clear that orexins effects on addiction-like behavior were most prominent in context-dependent drug seeking and relapse behavior, the goal of Smiths study was to investigate orexins role in context/cue-driven cocaine seeking. Since most studies only address orexins role in seeking-behavior following extinction, the current study also wanted to investigate the effect of orexin on seeking behavior following a period of abstinence. Abstinence models are thought to more directly reflect relapse in humans because addicts rarely undergo explicit extinction of drug-associated stimuli during drug abstinence. In order to test orexins role in this, they administered SB prior to drug seeking following 1 day or 2 weeks of cocaine abstinence from chronic cocaine self-administration, or prior to context-induced reinstatement of extinguished drug-seeking using ABA design.

Methods

The study used naïve male Sprague-Dawley rats under a reversed 12-h light-dark cycle, with ad libitum food and water. The rats learned to lever press on a fixed ratio of one, and would receive cocaine (0.2mg/50 μL) through an intravenous catheter during 2-h daily sessions, for a total of 10 self-administration sessions in which they received more than 10 infusions. Following training, rats would undergo one of three different experiments. The first to investigate cocaine seeking following periods of abstinence, the third context-induced reinstatement of cocaine seeking following extinction.

Experiment 1: self-administration + cues

During the first experiment, the rats had learned to associate cocaine infusions with a discrete tone and light cues. After training, they experienced 7 daily extinction sessions (lever presses had no consequence, no drug, no cue). Thirty minutes prior to the first extinction session, rats were treated with SB (10, 20, 30 mg/kg) or vehicle.
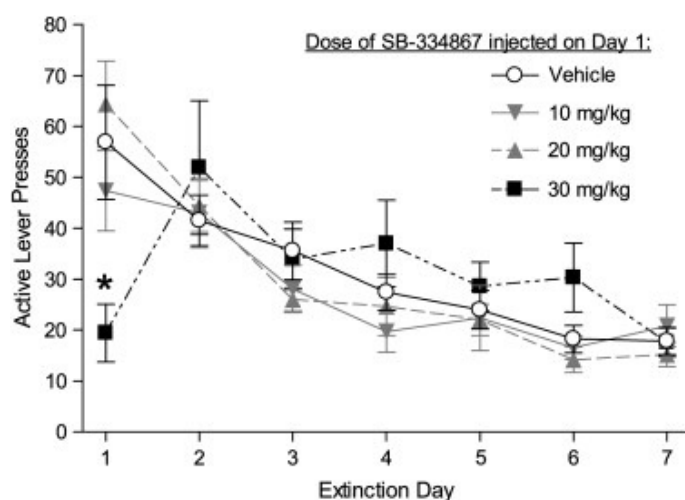
Experiment 1: drug-seeking behavior

Figure 7.2. Effects of SB334867 on cocaine seeking during extinction. *Rats were pretreated with SB in different concentrations (legend) 30 minutes prior to the first extinction session only. Responding during the first extinction session was significantly lower for the group with 30 mg/kg compared to vehicle. *: P< 0.05.*

Only a 30 mg/kg SB dosage at the first extension day significantly attenuated active lever pressing on the first extinction session, but it had no lasting effect on responding in subsequent sessions (figure 7.2).

Only a dose of 30 mg/kg attenuated cocaine seeking

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Experiment 2: Self-administration - cues
During the second experiment, rats were trained to self-administer without
cues. Rats then were given a 2-week abstinence period, followed by a single
extinction session (relapse test), during which lever presses had no effect. As
with the first experiment, SB was injected thirty minutes prior to the session in
the same concentrations (10, 20, 30 mg/kg or vehicle).

Results on the second experiment were significant. Increasing SB dosage
significantly decreased active lever presses (7.3A) during self-administration,
but not during inactive (7.3B) self-administration (where lever presses did not
result in reward). During session times, SB pretreatment only affected lever
pressing during the first hour (Figure 7.3D). Figure #D shows the latency for
the first lever press per animal, again, the 30mg.kg SB group showed a
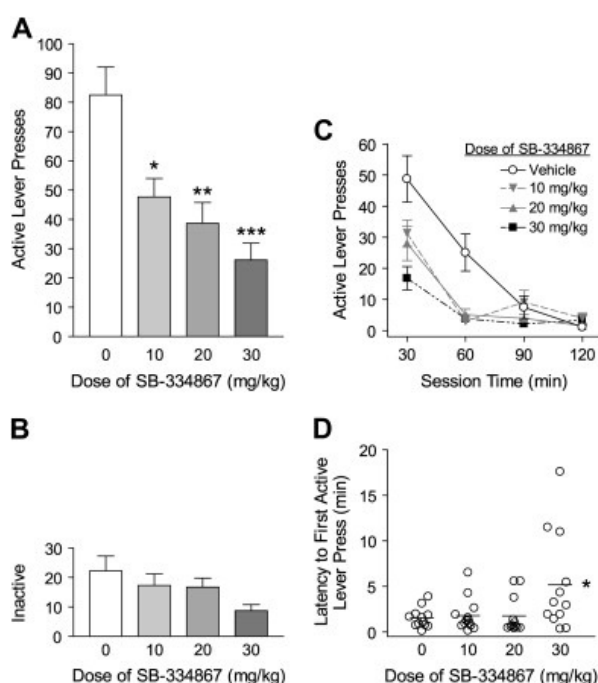significantly longer latency.

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Figure 7.3. Effects of
SB334867 on cocaine seeking
after two weeks of abstinence.
*Rats were pretreated with SB in
different concentrations (legend)
30 minutes prior to the first
extinction session only. Mean
number of presses on active (A)
and inactive (B) lever. SB (dose-
dependently) significantly reduced
responding on the active lever, not
on the inactive lever. Mean
number of active lever presses
during 30 minutes intervals (C).
SB significantly affected active
lever presses during the 30 and 60
minute interval. Latency of first
active lever press (D). Latency was
significantly higher in the 30
mg/kg compared to all other
groups.*

Experiment 3: Self-administration + context
The third experimental setup used an ABA design: two separate environments
(named context A and B) with different visual, auditory, olfactory and tactile
cues. Rats were randomly assigned to either context A or B and could self-
administer cocaine in that context. For extinction, rats were put in the other
context (A-rats to B, B-rats to A), in which lever presses had no effect (no
drug, no cues), until two consecutive sessions with more than 25 active lever
presses. Rats were then returned to their original context for two 2-h context
induced reinstatement tests, were lever presses again, had no effect. Thirty
minutes prior to reinstatement, SB was injected in the known concentrations.

SB pretreatment had a significant effect on active lever responding (A), and as expected, not on inactive responding (B). However, SB dosage did not matter, there were no significant differences between the dosages.
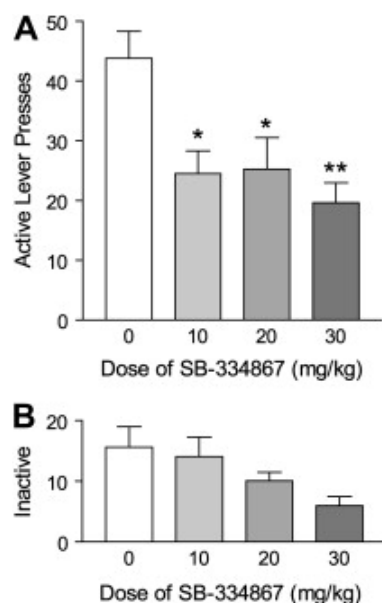
Figure 7.2. Effects of SB334867 on context-induced reinstatement of cocaine-seeking
Animals were trained to self-administer cocaine in a distinct context and then given extinction training in an alternate context. Rats were pretreated with SB (10, 20, 30 mg/kg) or vehicle 30 minutes prior to re-exposure to the original self-administration context. SB decreased active lever presses (with 30mg/kg having the biggest effect), and did not influence the number of inactive lever presses. *: P< 0.05, **: P<0.01

Discussion

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As stated by Smith ''the current studies show that orexin signaling at OX1R is involved in context-elicited cocaine-seeking following abstinence or extinction. The antagonist SB-224867 attenuated seeking for 1 day (only the 30 mg/kg dosage, during the first session) and 2 weeks of abstinence (all doses). Context-induced reinstatement was also reduced for all dosages. Moreover, SB dosage had no effect on inactive lever pressing. This led the authors to suggest that attenuation of cocaine seeking by SB is not due to sedation or decreased locomotion, but really because of a decrease in the motivation to obtain the drug. Importantly, this study has focused not only on orexins role on cocaine seeking following extinction, but also following abstinence and has shown that its role is critical for both. Smith concludes by stating ''these findings indicate that orexin is necessary for conditioned reinforcement or conditioned motivation for cocaine in the absence of drug, and that orexin is acting within the neural pathways that are generally involved in conditioned cocaine-seeking.

In summary, orexin fulfills multiple roles in different models of cocaine (and amphetamine) addiction, and it is the OX1R that plays the dominant role. Orexin is required for locomotor sensitization, the expression of cocaine preference, instrumental cocaine seeking (when driven by motivated states or external cues and stressors) and it seems to increase DA outflow. Orexin does not seem necessary for the primary reinforcing or priming effects of cocaine

Orexin and nicotine addiction

Tobacco use is the leading cause of preventable death in developed countries, and even though approximately 80% of smokers attempts to quit on their own, only about 3% remain abstinent at 6 months (Plaza-Zabala et al., 2013). Recent studies showed that orexin is also involved in nicotine addiction. However, whereas with cocaine its role lay mainly in drug seeking behavior, with nicotine its major involvement is with the reinforcing aspects of nicotine and its aversive effects like withdrawal and anxiety (Mahler et al., 2012).

Withdrawal is reduced
Intra-PVN infusion of SB and prior intraperitoneal administration of SB have both been shown to attenuate nicotine withdrawal (to same levels as orexin knock-out mice). Interestingly, a OX2R antagonist (TCSOX229) failed to reduce the withdrawal effects, suggesting that the OX1R has the upper hand when addressing withdrawal (Plaza-Zabala et al., 2010; Plaza-Zabala et al., 2013).

Antagonists:
(nicotine)

Withdrawal:↓(SB)

It has now been suggested that especially orexin signaling through the PVN is important in signaling nicotine's aversive effects (Mahler et al., 2012), since pretreated SB mice or KO mice do not show a nicotine-induced Fos activation in this region, and as mentioned SB injection in the PVN drastically reduced withdrawal (Plaza-Zabala et al., 2010).

Nicotine-induced activation of orexin neurons is reduced
Orexin neurons respond to the administration of nicotine: acute nicotine administration increased fos expression (a measure for activation) in orexin neurons, without a significant effect on other neurons (Pasumarthi et al., 2006). When using two different kind of nicotine antagonists, these activational effects were attenuated. Pasumarthi suggests that ''the orexin system is likely to play a role in the coordination of physiological and behavioral responses to acute nicotine treatment'' (Pasumarthi et al., 2006).

Activation of OX
neurons:↓

Self-administration performance is reduced
As with cocaine, orexin also promotes the motivation to perform certain behaviors to obtain nicotine (when previously exposed). Several self-administration studies show that where orexin stimulates seeking behavior, its OX1R antagonists attenuate it (Hollander et al., 2008; LeSage et al., 2010).

Drug-seeking:↓
(SB)
(Almoxerant)

Pretreatment with SB (and not with a OX2R antagonist), attenuated cue-induced nicotine-seeking behavior in mice. Especially the orexin neurons in the lateral and perifornical hypothalamic areas were involved (Plaza-Zabala et al., 2013). Furthermore, in a study by LeSage (2010), SB and Almorexant (Antagonist for both OX1R and OX2R) reduced nicotine self-administration in rats dose dependently.

The insular cortex has been implicated in cigarette smoking in humans. For this reason Hollander and colleagues investigated the effects of orexin and SB on the insular cortex. They found that local administration of SB to this area decreased nicotine self-administration. This shows the insular cortex's importance in possible nicotine addiction treatment.

Reinstatement of extinguished nicotine-seeking behavior is reduced
SB334867 attenuated reinstatement of nicotine seeking behavior, whereas
TCSO229 (a OX2R antagonist) had no effect (Plaza-Zabala et al., 2013). An
interesting finding is that SB did not attenuate reinstatement of food-seeking,
suggesting different mechanisms for drug addiction and food addiction (Sharf
et al., 2010). Plaza-Zabla (2013) also found that relapse increased
phosphorylation levels in the NAc, but not in the prefrontal cortex and almost
all phosphorylation levels were OX1R dependent.

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Reinstatment:↓(SB)

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A Role for Hypocretin/Orexin Receptor-1 in Cue-Induced Reinstatement of Nicotine-Seeking Behavior
(Plaza-Zabala et al., 2013)

Plaza-Zabala's motivation to investigate orexin came from the recent results of studies all pointing in the direction of a possible pharmacological treatment for nicotine: usage of orexin antagonists provided promising results in attenuating addiction-like behavior. Rising support came for Orexin's role to be primarily involved in increasing cue-dependent drug seeking behavior. However nicotine-seeking induced by associated cues, and which of the orexin receptors was involved, remained largely unexplored. This led the research group to investigate the specific contribution of the OX1R and OX2R cue-induced reinstatement of nicotine-seeking behavior. Seeking behavior was investigated with the self-administration model of addiction. Phosphorylation levels were also measured but will not be addressed.

Methods

All experiments were performed using male C57BL/6J mice (single-housed, room temperature, reversed dark/light cycle and food and water were available ad libitum). Nicotine was administered intravenous at a dose of 30μg/kg per infusion. SB334867 was used to block the OX1R and TCSOX229 for the OX2R. Both were administered by intraperitoneal (ip) route (5ml/kg or 10ml/kg).

The mice had 1 hour daily self-administration training sessions (fixed ratio of one, with an active (reward) and inactive (no reward) lever) for 10 days. Each session started with a priming injection of the drug. Light cues and a pump noise signaled prior to every nicotine delivery. Each session was terminated when either 50 reinforcers were delivered or after one hour (whichever occurred first). ''The criteria for the acquisition of self-administration behavior were achieved when in three 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''(Plaza-Zabala et al., 2013). After training, an extinction period followed. Mice were again 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 active responses 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 reinstatement'' (Plaza-Zabala et al., 2013). One day after extinction, mice were tested in a single-cue induced reinstatement session that lasted for 1 hour. Mice were re-exposed to the pump noise and the light cues for 2 seconds at the beginning of the session. Each nose poke would again lead to both cues, however, nicotine would not be delivered. To assess the role of the orexin receptors, one group of mice was pretreated with SB334867 (5 or 10 mg/kg, ip), TCSOX229 (5 or 10 mg/kg, ip).

Experiment: self-adminsitration

Results

Pretreatment with SB334867, but not TCSOX299 attenuated cue-induced reinstatement of nicotine seeking behavior. Mice differentiated the active lever from the inactive lever and responded significantly more on the active lever during training (figure 7.5, left). Mice showed a significant increase in the number of responses at the first extinction session compared to the last day of nicotine self-administration (figure 7.5). Responding quickly decreased during extinction, and the extinction criteria were met for 89% of the mice within approximately 18 days.

SB334867 significantly reduced responding on the active lever compared to vehicle and this was dose dependent (figure 7.6, left), the effect was more significant for a dosage of 10 mg/kg (5 mg/kg also gave a significant reduction compared to vehicle). TCSOX229 (both 5 and 10 mg/kg) had no significant effect on responding compared to vehicle (figure 7.6, right). Responding on the inactive lever stayed the same for all groups during all phases. There was no difference between the active and inactive lever at the end of the extinction phase for all groups.

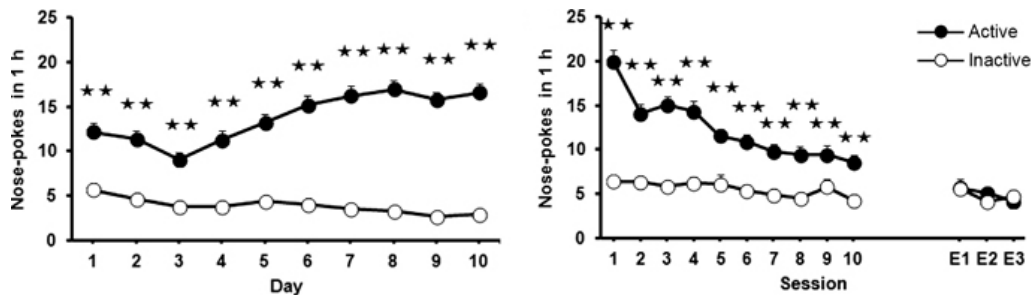SB (not TCS) reduced cue-induced reinstatement of nicotine-seeking behavior

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Figure 7.5. Nicotine seeking during acquisition of nicotine and during extinction training.
Mean number of nose-pokes per training day (left) or extinction session (right). E1, E2, E3 are means of three days on which extinction criteria were met. During training mice increased nose-poking on the active lever with the days. Responses on the inactive lever stayed the same (left). Extinction training causes responding on the active lever to decrease (right) to the level of responding on the inactive lever (E1, E2, E3). **: P<0.01
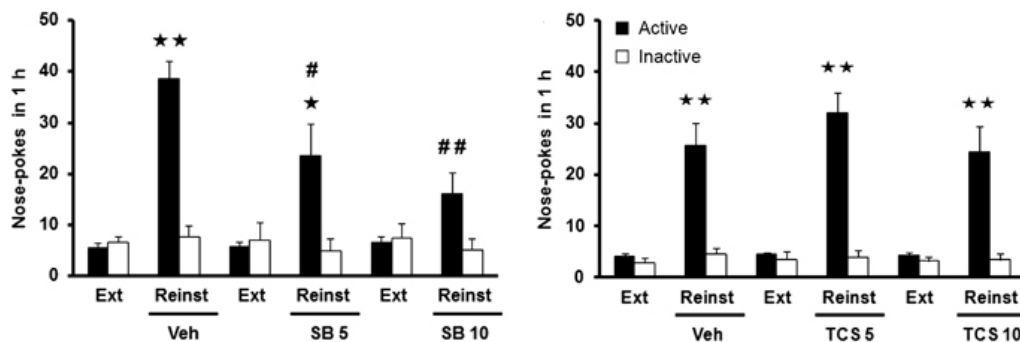

Figure 7.6 . Effects of SB334867 and TCSOX229 on nicotine seeking during reinstatement
Effects of SB334867 on mean number of nose-pokes during the reinstatement session (left), effects of TCSOX229 (right). */** comparison between the inactive and active hole, #/## comparison between agent and vehicle. SB significantly reduced mean number of nose-pokes compared to vehicle injection and this was dose dependent (SB 10 > SB5). TCS did not have a significant effect. There was no significant difference for responding at both leversduring extinction for all groups. *: P<0.05, **: P<0.01, #: P<0.05, ##: P<0.01.

Discussion

The OX1R seems primarily involved in attenuating cue-induced nicotine-seeking behavior. SB334867 attenuated seeking behavior, whereas TCSO229 (a OX2R antagonist), had no effect. Plaza-Zabala concludes that ''the present results point to a specific role for Hcrtr-1 in relapse to nicotine-seeking as the Hcrtr-2 antagonist TCSOX229 did not modify this response. In agreement, Hcrtr-2 did not participate in cue-induced relapse to cocaine-seeking behavior'' (Plaza-Zabala et al., 2013). The authors did not only investigate the antagonist's role on seeking behavior, but also investigated phosphorylation levels in different brain areas. For more information about brain activation and phosphorylation it is suggested to read the publication.

The OX1R is primarily involved in attenuating cue-induced nicotine-seeking behavior

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In summary, orexin seems to play a role in both the aversive as the rewarding properties of nicotine. Orexin is involved in withdrawal, reinstatement of previously extinguished behavior and nicotine-seeking behavior. It is primarily the OX1R that mediates these effects. As with cocaine, orexin's effects are mostly cue-dependent.

Orexin and opiate addiction

Approximately 50 percent of the orexin neurons express the μ opioid receptor (Georgescu et al., 2003). It should not be surprising that orexin is also involved in opiate addiction. As with nicotine, its effects are mainly visible on self-administration and withdrawal protocols (Mahler et al., 2012). However, orexin appears to play different roles in heroin and stimulant reinforcement (Mahler et al., 2012).

Approximately 50% of the orexin producing neurons contain the μ opioid receptor

Conditioned place preference is reduced
Intraperitoneal injection with SB reduced motivation: mice with SB showed a reduction in morphine-induced motivation, measured by a place preference test (Sharf et al., 2010). The same effects were found with heroin: heroin self-administration was reduced after intraperitoneal infusion of SB (Smith et al., 2012), however another study found that it does not completely block heroin self-administration (Smith et al., 2012). Orexin KO mice did not show any morphine induced conditioned place preference and had reduced morphine-induced hyperlocomotion (Narita et al., 2006).

Antagonists:
(opiate)

CPP: Preference:↓(SB)

Withdrawal is reduced
Orexin knock-out mice and wild-type mice that received SB both showed reduced morphine withdrawal responses (Sharf et al., 2010).
        Morphine withdrawal caused an increase in activity of orexin neurons and increase of orexin mRNA (Georgescu et al., 2003). Systematic SB administration reduced withdrawal in mice. Another study found that SB was ineffective, and that TCSOX229 (a OX2R antagonist) was effective in reducing withdrawal symptoms in a conditioned place aversion experiment (Li et al., 2011). Moreover there have been a lot of studies finding different effects of orexin and its antagonists on different brain areas. This indicates that orexins actions differ in varying brain areas, and that it therefore may be involved in different aspects of the withdrawal process. Where SB (OX1R antagonist) seems to reduce physical symptoms, OX2R antagonists seem more involved in reducing the aversiveness of withdrawal.

Withdrawal:↓
(SB)
(TCS)

Reinstatement of extinguished opiate-seeking is reduced
The VTA appears an important site for orexins role in opiate reward: local administration of orexin or pharmacologic activation of LH orexin neurons triggered reinstatement of extinguished morphine CPP (Richardson et al., 2012). Moreover, SB blocked the effect the LH orexin neurons had on reinstatement. For heroin, SB reduced cue-induced reinstatement of extinguished seeking behavior, but it did not attenuate heroin-primed reinstatement, which has also been shown in cocaine research (Smith et al., 2012; Mahler et al., 2012). Other studies again point to orexins variable function on distinct brain areas depending on the animal's state of opiate dependence/withdrawal (Richardson et al., 2012).

Reinstatement:↓(SB)

Orexin increases the motivational properties of cues, not the reinforcing properties of the drug itself

As with cocaine, orexins role in opiate reward appears to mainly be in associations between context/cues and morphine, rather than in mediating the intrinsic rewarding properties of the morphine itself (Georgescu et al., 2003; Harris et al., 2007; Mahler et al., 2012). Accordingly, SB blocked acquisition of morphine CP (Harris et al., 2007; Narita et al., 2006).

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Orexin/hypocretin 1 receptor antagonist reduces heroin self-administration and cue-induced reinstatement

(Smith et al., 2012)

Several studies had previously found a relationship between the orexin OX1R and the acquisition and expression of conditioned place preference, withdrawal and other addiction models. However, most of these studies investigated morphine addiction and not heroin. So the authors soughed to ''investigate the role of OX1R signaling in heroin self-administration behaviors by evaluating the effects of SB-334867 on drug intake under FR and PR schedules, as well as the reinstatement of extinguished heroin seeking elicited by cues or a heroin prime'' (Smith et al., 2012).

Methods

Male Sprague Dawley rats (pair-housed, temperature-controlled, reversed light/dark cycle, and ad libitum food and water) were used. Rats were implanted with chronic indwelling intravenous catheters for heroin administration. For the self-administration experiment, operant chambers were used with two levers (one active, one inactive).

Experiment 1: FR self-administration + cues

Presses on the active lever resulted in a heroin infusion (FR-1, 50µL) paired with a tone and light cues, inactive lever presses had no effect. Daily sessions lasted 2 hours. Prior to the experiment rats were given two sessions of FR-1 self-administration with a higher heroin dose (0.04 mg/infusion), afterwards they received 10-12 sessions with a lower dose (0.02 mg/infusion). One group was given SB-334867 (30 mg/kg) 30 minutes prior to the tenth session followed by two additional sessions of self-administration. Following the FR-1, separate groups of rats were moved to a PR self-administration or extinction/reinstatement procedure.

Experiment 1: FR self-administration

SB reduced responses on the active lever (figure 7.7C) and thus heroin intake (figure 7.7A/B) when administered prior to session 10 of established the FR-1 self-administration.

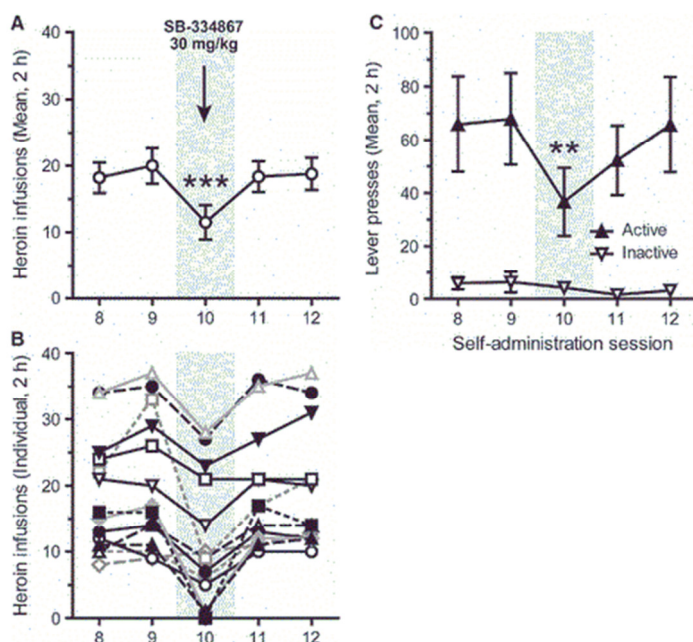| SB reduced heroin-seeking |
|---|

Figure 7.7. Effect of SB-334867 on heroin self-administration (FR-1). *Rats were given 30 mg/kg SB-334867 prior to session 10 (Gray area). Mean number of heroin infusions per session (A). Individual heroin infusions (B). Mean number of lever presses during the different sessions. SB signigicantly reduced mean number of heroin infusions and its effects were present for all individual rats (B). SB also decreased responses on active lever and had no effect on responses on inactive lever. **: P<0.01, ***: P<0.001*

Experiment 2: PR self-administration + cues
After the 1-FR, a subset of rats was placed on a PR schedule of reinforcement. Successive heroin infusions required an increasing ratio of lever presses (on an adapted logarithmic function, ratio: 1, 6, 15, 20, 25 etc). However, before these ratios rats were put on a modified step sequence (1, 4, 7, 8, 9, 10) to reduce sedation or satiation from excessive heroin exposure. ''Once rats showed stable daily PR self-administration, the role of orexin in PR responding was evaluated during three test sessions, for which SB-334867 (10 or 30 mg/kg, intraperitoneal) or vehicle was given 30 min prior'' (Smith et al., 2012).

SB also reduced heroin intake under a PR schedule of self-administration, it reduced the breakpoint (the highest step completed, figure 7.8A). There was no effect of SB on the time of the last infusion, indicating that rats worked for a similar amount of time but earned fewer infusions overall (figure 7.8B). This goes together with the lower rate of intake observed for the FR-1 sessions following SB administration.
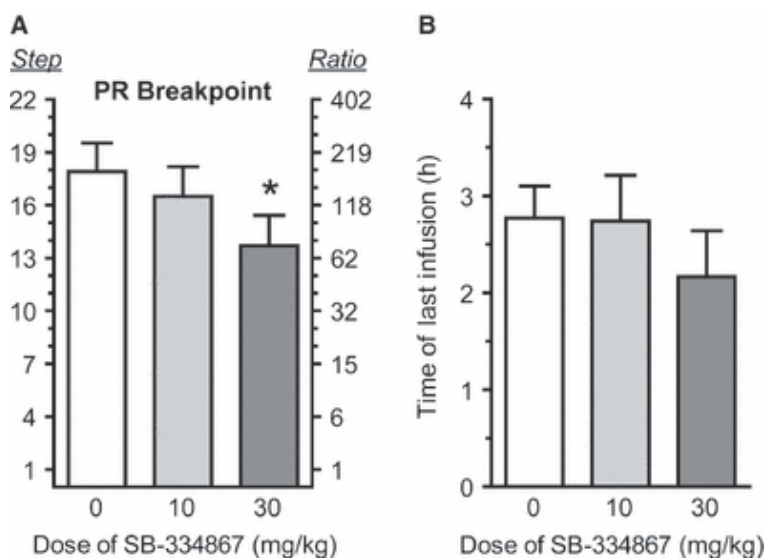
44

Figure 7.8. Effect of SB-334867 on heroin self-administration (PR). *Rats were given 10 or 30 mg/kg SB-334867 prior to the three test sessions. Highest number of steps reached (breakpoint, A) and time of last infusion (B). A dosage of 30 mg/kg SB significantly reduced breakpoint. There was no effect of SB on time of last infusion.* *: $P<0.05$

Experiment 3: Reinstatement + cues
Another subset of rats underwent a reinstatement procedure. They were first given daily extinction sessions (active lever presses did not result in rewards and cues were absent). ''Some of these rats had previously received SB-334867 in the tenth session of FR-1 self-administration; these animals had an additional 2 days of self-administration after SB-334867 treatment before extinction sessions commenced. Prior to reinstatement testing, rats were required to meet an extinction criterion of ≤25 active lever presses for 2 consecutive days, with at least seven extinction sessions prior to the first reinstatement session and two extinction sessions prior to subsequent reinstatement sessions'' (Smith et al., 2012). During the reinstatement phase, for cue-induced reinstatement, active lever presses resulted in presentation of tone and light cues and for heroin-induced reinstatement, the rats were injected with heroin (25 mg/kg subcutaneous). SB was administered 30 minutes prior to reinstatement sessions. ''Each rat was given only two reinstatement sessions of

each type (two cue, followed by two heroin prime), for which they were pretreated with only one dose of SB-334867 for one session and vehicle for the other session for each type of reinstatement in a counterbalanced order'' (Smith et al., 2012).

SB showed to reduce reinstatement when elicited by cues (figure 7.9A), but not when elicited by a heroin prime (figure 7.9B). Only a dosage of 30 mg/kg S reduced reinstatement significantly. All groups showed a higher response compared to during the extinction phase.
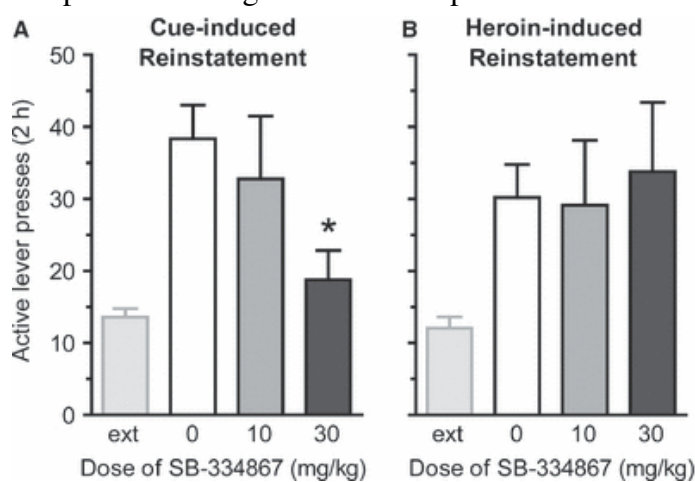
Figure 7.9. Effect of SB-334867 on cue- and prime-induced heroin reinstatement *Rats were pretreated with 10 or 30 mg/kg SB-334867. Number of responses following cue-induced reinstatement(A) versus number of responses following prime-induced reinstatement (B). Only a 30 mg/kg dose of SB decreased responding when reinstatement was cue-induced.*
All groups showed a higher response compared to during extinction phase *: $P<0.05$

Discussion

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SB (30 mg/kg) reduced heroin intake on FR and PR schedules of self-administration. It reduced the breakpoint, but had no effect on time of last infusion. ''The time course data indicate that the SB-334867 effects on PR heroin self-administration cannot be explained by reduced high-effort motivation. Rather, the reductions in PR self-administration appear to parallel the effects of SB-334867 on FR-1 self-administration'' (Smith et al., 2012). Thus SB seemed to have increased the time between the infusions under both schedules of reinforcement, which indicates that SB is acting via the same mechanism in both cases. SB also reduced cue-induced reinstatement, but not prime-induced reinstatement. This has shown to be consistent for other type of drugs as well. The results of this study corroborate with previous studies showing that orexin is involved in reward seeking by conditioned stimuli. Smith concludes the publication with: ''the current studies show that OX1R antagonism reduces opiate self-administration and reinstatement, indicating that the orexin system might be an important target for addiction pharmacotherapies. Our finding that SB-334867 reduced cue-induced reinstatement of heroin seeking supports a general role for orexin signaling in cue-triggered reward seeking''.

In summary, orexin seems to play different roles in stimulant (cocaine) and opiate reinforcement. However, its involvement in reinstatement appears to be the same: orexin is required for cue-induced reinstatement, but not drug-induced reinstatement. Orexins role in opiate addiction lays mainly in reinstating cue-dependent opiate seeking and reinforcing and learning effects of acute opiates, but not for their priming effects.

Orexin and alcohol addiction

Orexin has been demonstrated to play a significant role in ethanol consumption and abuse. However, results have been conflicting and orexins role in ethanol seeking may be more complex than in the other substances addressed. The pharmacological actions of ethanol are more complex (Mahler et al., 2012). Orexin may play different roles in ethanol reward, depending on if its self-administered or experimenter administered, and whether it is administered acutely and chronically.

Orexins involvement in ethanol addiction seems to be different. Whereas with cocaine, nicotine and the opiates, the OX1R seems to play the dominant role, there is evidence that for ethanol, it is the both the OX1R and the OX2R that call the shots (Sakurai et al., 2014).

For ethanol, both receptors seem important

Ethanol consumption is reduced
As with the other substances, there could be circuit specificity in orexins influence on ethanol reward: microinjections of orexin into the LHA and PVN increased, whereas injection into the NAc decreased voluntary ethanol consumption in rats (Schneider et al., 2007). Systematic administration of SB decreased ethanol consumption as well as preference (Moorman et al., 2009). Interestingly, this reduction was primarily expressed in rats that showed high predisposition for ethanol consumption/preference. This indicates orexin may be involved in the propensity to abuse ethanol (Mahler et al., 2012).

Antagonists: (ethanol)

Consumption:↓ (SB)

Conditioned place preference is reduced
For ethanol, orexins role in the rewarding and locomotor-stimulating effects is different (Mahler et al., 2012). SB has been shown to reduce ethanol-induced hyperlocomotion, and reduces ethanol preference in some studies, but it does not in others (Voorhees et al., 2011; Shoblock et al., 2011). In contrast, blocking the OX2R with an antagonist (JNJ-10397049), decreased acquisition, expression and reinstatement of CPP in mice (Shoblock et al., 2011). This suggests that it is the OX2R that plays a more significant role in ethanol preference, but further evidence is needed (Mahler et al., 2012).

CCP :Preference:↓↔ (SB) (JNJ)

Self-administration responding is reduced
Studies on ethanol self-administration also show conflicting results. Some find SB to decrease self-administration (Jupp et al., 2011), where others find no effect (Shoblock et al., 2011). The later study found that blocking the OX2R with JNJ-10397049 (subcutaneous injection, dose dependently) attenuated ethanol self-administration, place preference and reinstatement, whereas SB-408124 (OX1R antagonist) had no effect whatsoever (Shoblock et al., 2011). Shoblock's article will be discussed in box 7.6.

Ethanol-seeking:↓↔ (SB) (JNJ)

Reinstatement is reduced
Furthermore, motivated behaviors like ethanol seeking or context-induced reinstatement of ethanol are also influenced by orexin signaling. SB reduced cue-dependent reinstatement of ethanol seeking (Jupp et al., 2011). Moreover, orexin neurons were activated (Fos expression) during ethanol (beer) seeking (Dayas et al., 2008).

Reinstatement:↓(SB)

Human studies
A very important finding came from a human study by Bayerlein in 2011. They reported that orexin-A levels in blood were associated with withdrawal symptoms in human alcoholics. Orexin A mRNA in blood cells was lower in ''recovered'' (90+ dats abstinent) alcoholics than in those undergoing acute withdrawal. However, OXA expression was negatively correlated with the severity of the physical ethanol withdrawal symptoms (Bayerlein et al., 2011). Other studies reported similar findings, but the relationship between blood and brain orexin levels is not clear enough to conclude anything.

OXA plasma level could be correlated with withdrawal symptoms in human alcoholics

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Selective blockade of the orexin-2 receptor attenuates ethanol self-administration, place preference, and reinstatement

(Shoblock et al., 2011)

Most of the studies prior to the one by Shoblock had focused mainly on elucidating the function of the OX1R. Due to the previous lack of publicity concerning the OX2R in addiction, the recent study wanted to investigate the effects of a ''novel by them described'' OX2R antagonist, JNJ-10397049 on ethanol addiction. The aim of the study was to test JNJ-10397049 in the oral ethanol self-administration and conditioned place preference for rats and mice respectively, furthermore they examined reinstatement of CPP in mice. They also tested SB-408124 (SB-4), a 1-SORA.

Methods

Male DBA/2 mice were used for the CPP studies, male Wistar rats were used for the self-administration and withdrawal studies. Subjects were housed on a normal light-dark cycle with ad libitum food and water.

Experiment 1: Self-administration

The self-administration experiments were conducted in operant chambers, with two levers, two lights and two liquid hoppers. Sessions started with illumination of the left or right light (that became the active lever) and lasted for 30 minutes or until 100 saccharin rewards were delivered. All rats were trained to lever press under a fixed-ratio (FR-1), each press resulting in the delivery of 0.1 ml solution of saccharin (0.1% v/v). When the number of deliveries was higher than 50, the FR changed to 2, with an additional 50 deliveries, the FR increased to 3 (3 presses resulted in 1 delivery). When the number of saccharin deliveries was stable for three sessions (did not change more than 20%), the experiment began: half of the rats were switched to a mixed solution containing saccharin (0.1% v/v) and ethanol (8% v/v) and upon reaching stability, the solution switched to only ethanol (8% v/v). The remaining half stayed on the saccharin (0.1% v/v). Tests began when responding was stable. Pretreatment with JNJ-10397049 (1, 3, 10 mg/kg) was given 15 minutes prior to the tests, pretreatment with SB-408124 (3, 10, 10 mg/kg) minutes.
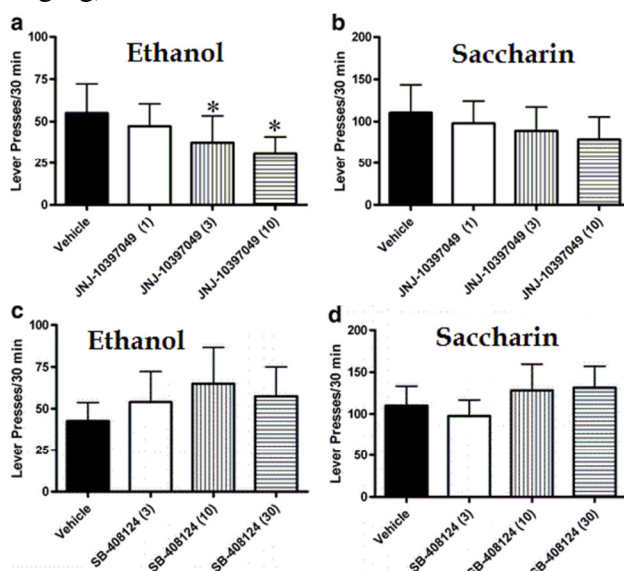
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Figure 7.10. Effects of OX1R and OX2R antagonists on ethanol-seeking behavior. *Ethanol seeking measured as number of lever presses (a). JNJ-10397049 (2-SORA; 3 and 10 mg/kg) significantly reduced number of responses compared to vehicle and had no effect of responding for saccharin. SB-408124 (1-SORA) did not have an effect on both ethanol and saccharin responding. *:P<0.05 Adapted from Smith et al., 2012*

Compared to the findings for other drugs, it was the OX2R antagonist that seemed primarily involved in reducing responses during ethanol self-administration (figure 7.10a). JNJ significantly (and dose-dependently) reduced ethanol-seeking (number of lever presses) and not saccharin seeking. The OX1R SB-408124 did not affect ethanol seeking.

Experiment 2: Conditioned Place Preference +cues
The CPP apparatus consisted of two chambers, one paired with the drug (contained specific light -cow print- and odor cues), the other neutral. On day 1, animals were placed in the apparatus for 15 minutes with free access to both chambers for habituation. The following 11 days (day 2-11) rats were injected with vehicle (water; 12.5ml/kg) and confined to the cow print chamber or injected with ethanol (2 g/kg ip) and confined to the opposite chamber, for 5 minutes. Pretreatment with JNJ-10397049 (10 mg/kg subcutaneous), SB-408124 (30 mg/kg sc) or vehicle was injected 1 hour before the ethanol conditioning (the vehicle group only received vehicle pretreatment). Locomotor activity was recorded to determine the effects of JNJ or SB-4 on ethanol-induced hyperactivity. Day 13 was the day of testing: rats were placed into the apparatus, which was drug free, for 15 minutes, with free access to both chambers. Time spent in each chamber was recorded as a test for CPP.

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Animals pretreated with vehicle or SB-4 significantly displayed a place preference when conditioned to ethanol. Animals pretreated with JNJ did not show this preference (figure 7.11a). JNJ-10397049 was conditioned by itself using the same procedure, to determine if the effects of JNJ-10397049 on ethanol CPP were due to intrinsic aversive properties (Shoblock et al., 2011). JNJ-10397049 did not differ from its vehicle in terms of %time spent in the drug-paired chamber (figure 7.11b). This indicates that it JNJ is void of motivational properties per se. Furthermore, in the animals pretreated with SB-4 and vehicle, locomotor activity increased when conditioned with ethanol. JNJ did not differ from vehicle in terms of locomotor activity, and thus reduced ethanol-induced hyper locomotion (figure 7.11c).
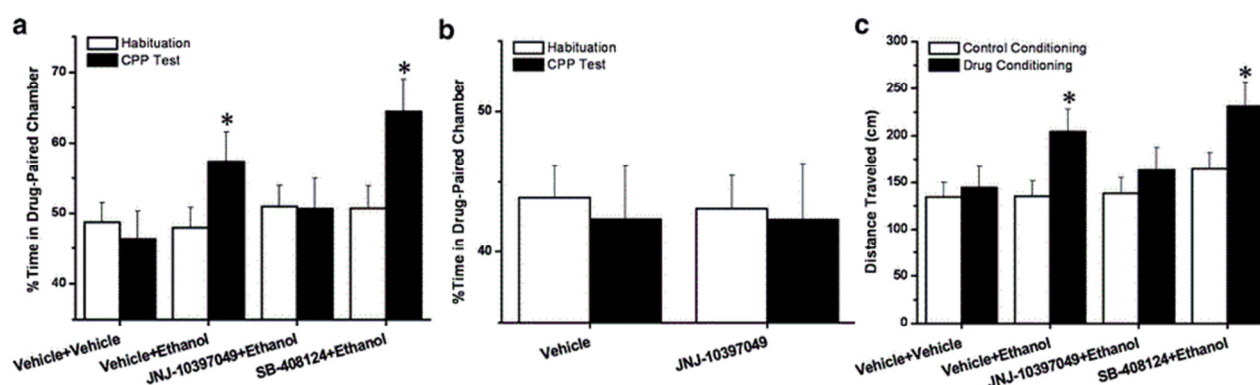
Figure 7.11. Effects of OX1R and OX2R antagonists on ethanol conditioned place preference. *Preference is measured as % time in drug-paired chamber. JNJ (10 mg/kg) significantly reduced preference compared to vehicle and SB-4 (30 mg/kg) administration (a). JNJ did indeed not differ in preference compared to vehicle treatment (b). JNJ also significantly reduced ethanol-induced hyper locomotion. *P<0.05*

Experiment 3: Reinstatement of ethanol CPP + cues
A separate group of animals, previously exposed to experiment 2 continued with testing. They got free access to both chambers without vehicle or ethanol injections for 30 minutes to extinguish CPP. When extinguished, animals were assigned to three treatment groups, balanced for time spent in the drug-paired chamber during habituation, the CPP test and the last extinction day. The animals were either treated with JNJ (10 mg/kg) or vehicle 1 hour before received a priming injection of ethanol (1 g/kg) and were then placed in the apparatus for 15 minutes for another CPP tests.

JNJ pretreatment significantly attenuated prime-induced reinstatement (figure 7.12). Animals pretreated with JNJ failed to reinstate following a priming injection compared to the vehicle group.
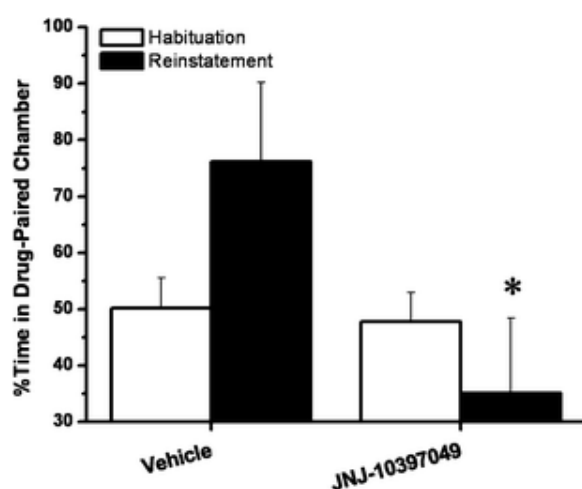
Figure 7.12. Effects of OX2R antagonist on prime-induced reinstatement of ethanol-seeking.
*Preference is measured as % time in drug-paired chamber. JNJ (10 mg/kg) significantly attenuated reinstatement of place preference compared to vehicle pretreatment. *:P<0.05*

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Discussion
JNJ significantly reduced ethanol seeking, ethanol place preference and prime-induced reinstatement of ethanol preference. Blockage of the OX1R with SB-408124 failed to reduce this. Shoblocks starts the discussion with stating ''our results demonstrate for the first time that orexin-2 receptors are involved in mediating the reinforcing effects of alcohol, and that blockade of orexin-2 receptors attenuate alcohol reward'' (Shoblock et al., 2011). However, involvement of the OX1R cannot be precluded. They do argue for a role of the OX1R in ethanol addiction. Recent studies have pointed at the OX1Rs involvement so it is clear this receptor is important. What is interesting about Shoblocks findings is that it is not only the OX1R that plays a role in (ethanol) addiction, the OX2R has to be kept into mind.

Both the OX1R and
OX2R are implicated in
ethanol addiction

In summary, although the specifics are still somewhat unclear, there is a definite role of orexin in ethanol addiction. As for the other substances, orexin is required for cue-induced ethanol seeking, and the OX1R is predominantly required for ethanol self-administration. Both receptors appear to be important in ethanol ''addiction'', but it is, as with the other drugs, the OX1R that is more dominantly required for cue-induced ethanol seeking.

A summary: Orexins role in addiction

In summary, for cocaine and amphetamine, orexin seems to play a role in sensitization and drug-seeking motivation (especially when triggered by external stimuli, such as cues, context or stressors). Orexin doesn't seem to be involved in the reinforcing or priming properties of cocaine.

For nicotine orexin is mostly involved in primary reinforcement and withdrawal, but apparently not in stress-induced reinstatement.

Orexins role in opiate addiction appears to be in cue-driven drug seeking, it may influence the rewarding/reinforcing properties of the opiates itself, and it also mediates withdrawal.

As with the other substances, orexin's role in ethanol addiction again seems to mediate seeking behavior, but also self-administration.

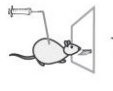Concluding, orexins major role appears to be in modulating high-motivated reward seeking, especially when this seeking is triggered by external stimuli (Mahler et al., 2012). However, orexins role seems to vary depending on the drug. It has been suggested that this is due to the fact that the drugs influence different mechanisms by which they increase forebrain dopamine release (which is believed to modulate motivated behavior, see chapter 6). It has been shown that nicotine, opiates and ethanol all increase dopamine by acting within the VTA, where they modulate glutamate and/or GABA inputs and cause increased dopamine firing (Cami et al., 2003), whereas cocaine and the amphetamines increase dopamine primarily by their actions on the terminal to increase synaptic levels of dopamine instead of increasing the firing rate of these neurons (Aston-Jones et al., 2010;Mahler et al., 2012). It is therefore important to keep the neural circuitry that orexin acts upon in mind.

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	Cocaine	Nicotine	Opiates	Ethanol
Acute withdrawal		↓	↓	
Sensitization	↓			
Conditioned place preference	↓ / No effect		↓	No effect
Self-administration — Fixed ratio	No effect	↓	↓	↓
Self-administration — Progressive ratio	↓	↓	↓	↓
Cue-induced reinstatement	↓		↓	↓
Stress-induced reinstatement	↓	No effect		↓
Prime-induced reinstatement	No effect		No effect	

Figure7.12: Effects of OX1R antagonist on different addiction models testing for different substances
Blocking the OX1R reduces cocaine sensitization, conditioned place preference, self-administration, cue-and stress- induced reinstatement, but not prime-induced reinstatement. Nicotine withdrawal, CCP, self-administration and cue-induced reinstatement are also reduced. For the opiates, OX1R antagonists reduce self-administration, CPP and cue- and stress-induced reinstatement. The antagonists effects for ethanol are the same as those for the opiates, apart from CPP, which showed no effect. Adapted from Mahler et al, 2012

CHAPTER 8
Clinical implications

The previous chapters emphasized orexin's and its antagonist's influence on the reward system and on the different models of addiction for varying substances. The big question remains however: *could administration with orexin's antagonists become a treatment for addiction?* The studies and evidence in the previous chapter inclines that maybe it could. Antagonists appear to reduce seeking behavior, self-administration, preference and overall thus motivation to obtain the drug. However, there are two orexin receptors, and many kinds of antagonists. SB is by far the most investigated one, but there are more. Moreover, are there known side-effects? Which other antagonists could be used? Are only OX1R antagonists favorable, or can we use OX2R antagonists? What about dual antagonists, that block both receptors? Furthermore, is everything about orexins role within the reward system or even within the periphery understood? And most importantly, is there really enough clinical evidence to come to a definite conclusion for this thesis' question? To answer these questions, the current chapter will first briefly address some challenges that have to be overcome or better understood, followed by an overview of the current position of antagonistic research on orexins receptors.

Orexin is an extensive molecule and its signaling remains only partially understood

Orexin: Reward VS Arousal?
Orexin does not only influence the reward system, it has many other functions, both centrally and peripherally (Chapter 4). One of its major functions lays within modulating arousal. The question if orexin's function in these two is separate or independent remains. Even though it has been shown that there is functional dichotomy between the both receptors (and where the orexin neurons project to) and the OX1R seems have the upper hand in regulating orexins function on reward, whereas the OX2R appears more dominant in orexin's arousal/stress stimulating role, arousal and reward could be functionally linked, since rewarding stimuli are often arousing and arousing stimuli can be rewarding. If this is the case, more research is needed to filter out possible alternatives. Furthermore, although the distribution of both receptors has (in part) been brought to card, the explicit function of either one still remains partially unclear.

> Orexin's function in reward and arousal could be linked

Which brain circuitries are involved?
The VTA appears to be the dominant target for orexins function on the reward system, but other studies found roles for orexin in the NAc, PVN and insular cortex as well. However, there might be more areas involved in orexin's role within the reward center. It should not be forgotten that orexin neurons project throughout the central nervous system and its receptors have been found in the

> There might be more areas involved in mediating orexin's function in the reward system

periphery. Its many different functions have briefly been discusses in chapter 4, from thereon out it should be clear that pointing orexin in a certain direction or function is not as easy as desired. Orexins functions are widespread, peripherally and centrally. It is therefore of great importance to understand orexins signaling and be well aware of possible side-effects pharmacological treatment could bring with it.

Orexin plays a potential role in learning stimulus-reward association
Orexin also appears to be involved in learning. It influences the plasticity of neural responses in VTA dopamine neurons. Some studies even reveal that the LHA orexin to VTA projection is critical for learning stimulus-reward associations. This could have great implications in drug addiction rehabilitation/prevention. Other questions have emerged: if orexin is involved in learning associations between stimuli and reward, could it be involved in other types of learning? Information about this function is still unclear. However, if orexin really were to play a role in learning the association between cues and rewards, targeting this system could potentially help addicts during rehabilitation processes.

> Orexin appears to be involved in learning

Current position of orexins antagonists

Even though all of these uncertainties remain, researchers (and results) are optimistic. If orexin signaling promotes craving and seeking behavior for different kinds of substances, than targeting this system with pharmacological antagonists seems to be the way to go, at least that has been the thought since Harris' experiment in 2005. Many animal studies have shown that antagonists indeed reduce drug seeking and relapse behavior in addiction models. There are several antagonists under development that target orexin's role in addiction (Ekhtiari, 2016). The most promising are the 1-SORAs, because of the immense preclinical data that has shown that inhibition of the OX1R attenuates the motivation to obtain most drugs. More importantly, when targeting just the OX1R and not the OX2R, side effects that normally come with the OX2R are absent or greatly reduced. This is because the OX2R is more involved in functions of arousal, and treatment with DORAs (OX1R and OX2R antagonists) has been shown to come together with side effects like sleepiness and alternation of vigilance (mediated by the OX2R. However, non-selective antagonists (DORAs) may also have some space in addiction research. There is some controversy in results for the different models and substances of addiction, as addressed in chapter 7. Orexins role in the different substances appears to differ and so do the effects of its antagonists. For example, antagonism of the OX1R in cocaine addiction attenuated the motivation to obtain the substance, antagonism of the OX2R has been shown to lead to sedative effects that (in the case of psychostimulants abuse) are tolerable if not advantageous (Ekhtiari, 2006). However, for other addictive drugs, like opioids or alcohol, DORAs are less likely to be the solution. One of the risks a DORA brings with these drugs (and maybe also with cocaine), is that blockage of the OX2R may enhance the risk of excessive inhibition of the CNS function (think back to the many projections orexin neurons have throughout the brain), by potentiating the depressant properties of the addictive agents (Ekhtiari, 2016). So far only few orexin antagonists have been tested clinically and only one has

> Several antagonists are under development as treatment for addiction

> The OX1R is the primary target
>
> The OX2R also plays a role in some substance disorders so it should not be overlooked

> DORAs can be used to treat some substance addictions but not all

> Clinical studies are still focused on finding treatments for insomnia

> Results with some 1-SORAs are promising and they could soon make their appearance on the market as treatment for addiction

been approved: suvorexant, which is being used for the treatment of insomnia. Unfortunately, other antagonists such as almorexant and filorexant have not made it through clinical testing and they have been dropped. All of these antagonists were supposed to be used to treat insomnia. Fortunately, the search for antagonists has not been ended. Currently, a few selective OX2R antagonists are entering into clinical investigation and some have passed phase I. However, they are all directed toward targeting sleep disorders (Boss et al., 2015). On the other hand, there are a handful of selective OX1R antagonists that are rapidly making their way through preclinical stages and these are the ones that show promising results in treating addiction (Ekhtiari, 2016). Based on the current data, some of these 1-SORAs could soon become available for their first clinical trials (Ekhtiari, 2016).

Could antagonists of the orexin receptors be used as treatment for addiction?

The answer is yes. Although many questions remain, researchers are optimistic. Pre-clinical animal studies show promising results. Orexin antagonists appear to reduce seeking behavior, self-administration, preference and overall thus motivation to obtain the drug. Although orexin's and it antagonist's roles differ a little per substance, administering antagonists (especially OX1R antagonists) has significant effects on all four addressed substance classes: cocaine, nicotine, opiates and ethanol. Even without knowing the precise differences of orexin's function on different substance classes, the current position of the antagonists in addiction research looks promising. It should be taken into account that what kind of antagonist should be used for what substance is something that should be studied more deeply. For some addictions, a 2-SORA or DORA treatment could be advised, but data on these two antagonist groups is still limiting and contradictory. This is why the OX1R should be the primary target, since it, miraculously, doesn't come with (any known) side-effects, like blockage of the OX2R. However, clinical studies should exclude the fact that 1-SORA treatment is not accompanied with side-effects.

Based on the current data the orexin system is indeed a strongly favored target for potential addiction treatment. Not only is it implicated in different addictions (a single treatment method could potentially be used to target more than one addiction), the system is well studied and quite well understood. Pre-clinical studies show promising results and perhaps the time has come for the clinical testing of orexin antagonists as a target for addiction treatment.

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