INVESTIGATIONS INTO THE ROLE OF OREXIN (HYPOCRETIN) AND DYNORPHIN IN DRUG SEEKING, REINFORCEMENT, AND WITHDRAWAL

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ABSTRACT

Psychostimulant dependence remains a major health and economic problem, leading to premature death and costing $181 billion annually in health care, crime, and lost productivity costs. Currently, no pharmacotherapies are available to effectively treat psychostimulant dependence. Psychostimulants cause changes in neural circuits involved in reward and affect, but addiction neurocircuitry is incompletely understood and new targets for therapeutic intervention are needed. Lateral hypothalamic orexins (hypocretins) have been shown to have functional roles in arousal, reward processing, attention, motivation, and impulsivity. The opioid peptide dynorphin, co-localized with orexin, has critical roles in producing negative affective emotion states through interactions with, among others, stress circuitry. Orexin-dynorphin neurons project to neural substrates governing positive and negative motivated behavior, including the bed nucleus of the stria terminalis (BNST), amygdala, locus coeruleus and ventral tegmental area (VTA). Orexin and dynorphin modulate post-synaptic membrane activity through opposing signaling mechanisms; while orexins bind to predominantly excitatory orexin-1 and -2 G-protein coupled receptors, dynorphins bind to predominantly inhibitory G-protein coupled kappa opioid receptors (KORs). Multiple psychopathologies, including anxiety and substance abuse disorders, are characterized in part by alterations in orexin-dynorphin signaling. While these peptides have been shown to co-localize within single presynaptic vesicles and exert opposing effects on post-synaptic membrane potentials, the utility of producing oppositely-behaving peptides and the implications on psychopathologies remains unknown. The present studies were conducted to explore the role of orexin and dynorphin activity in cocaine's rewarding effects as well as the negative effects of withdrawal.

To accomplish this, we measured 1. Effects of orexin and cocaine administration on impulsive behaviors that increase the likelihood of psychostimulant addiction, using 5-choice serial reaction time task in concert with systemic and site directed pharmacology.

2. Effects of orexin and dynorphin on motivation for cocaine administration and intracranial self-stimulation. Using immunohistochemistry, ultrasonic vocalizations, and fast scan cyclic voltammetry we explored possible dopaminergic mechanisms of orexin and dynorphin contributions to reward. Lastly 3. Effects of orexin, dynorphin and chronic cocaine on withdrawal-induced anhedonia using intracranial self-stimulation, elevated plus maze, and correlations with immunohistochemistry and plasma corticosterone levels to explore further mechanisms.

The results of this dissertation support our hypothesis that orexin receptor activity contributes to cocaine-induced impulsivity, motivation to self-administer cocaine and the reinforcing effects of psychostimulants. Dynorphin activity contributes to anhedonia and anxiety seen during drug abstinence after chronic exposure. Orexin and dynorphin exert these effects, in part, by modulating activity of dopaminergic neurons projecting from the ventral tegmental area to the nucleus accumbens.

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LIST OF ABBREVIATIONS

5-choice serial reaction time task – 5-CSRTT;
Adenylyl Cyclase – AC;
Adrenocorticotropic hormone – ACTH;
Analysis of variance – ANOVA;
Area under curve – AUC;
Attention deficit hyperactivity disorder – ADHD;
Bed Nucleus of the Stria Terminalis – BNST;
Conditioned Place Aversion – CPA;
Conditioned Place Preference – CPP;
Corticotropin Releasing Hormone – CRH;
Cyclic Adenosine Monophosphate - cAMP;
Dopamine – DA;
Dopamine Transporter – DAT;
Delay Discounting – DD;
Dimethyl sulfoxide – DMSO;
Elevated Plus Maze – EPM;
Fast Scan Cyclic Voltammetry – FSCV;
Federal Drug Association – FDA;
G-Protein Couple Receptor – GPCR;
Gamma-Aminobutyric acid - GABA
Immunohistochemistry - IHC
Intracranial Self-Stimulation – ICSS;
Intravenous Self-Administration – IVSA
Kappa Opioid receptor – KOR;
Lateral hypothalamic area – LHA;
Locus Coeruleus – LC
Medial Prefrontal Cortex – mPFC;
Medium Spiny Neuron – MSN;
N-methly-D-aspartate – NMDA;
Nucleus accumbens – NAc;
Orexin receptor 1 – OX1R;
Orexin receptor 2 – OX2R;
Paraventricular Nucleus – PVN;
Phosphate-buffered saline – PBS;
Progressive Ratio – PR;
Protein Kinase A- PKA;
Protein Kinase C – PKC;
Phospholimase C – PLC;
Post-Traumatic Stress Disorder – PTSD
Raphe Nuclei - RN
Tyrosine hydroxylase – TH;
Ultra-Sonic Vocalizations - USV
Ventral tegmental area – VTA.

INTRODUCTION

'Why do people do what they do?' is a question of great importance and of even greater mystery. The philosophy of mind and how it interacts with brain and body has been argued for centuries, from Plato to Descartes. By the early 20th Century, psychologists had discarded metaphysical explanations and sought to link behavior more systematically to concrete environmental and biological factors. Work examining how physiology evokes emotional states highlighted the importance of different brain regions such as the hypothalamus in producing complex emotions and the corresponding behavioral outputs. American physiologist Walter Cannon's work also emphasizes that to accomplish this task, brain regions do not function individually and instead interact with each other. For example, based on observations of thalamic tumors, lesions and anesthesia; the *thalamic theory* attributes the thalamic connection with the cortex contributes to 'emotional' reactions (Cannon 1931). It is these complex interactions between brain regions and behaviors that this dissertation hopes to illuminate.

The discovery of orexin (hypocretins) has been an important step in understanding how distinct neural circuitries interact to govern emotional and physiologic states, and subsequently, behavior. Orexins are present almost exclusively in the hypothalamus with strong excitatory projections to the mesolimbic dopamine pathway, widely accepted as one of the most critical circuits involved in motivation and reinforcement (Di Chiara & Imperato, 1988; Marcus et al., 2001; Peyron et al., 1998). The mesolimbic dopamine pathway is a key player in the pathophysiology of drug abuse and the mechanism of "hijacking" the reward pathways. However, negative-reinforcement models and the idea of a "dark side of drug addiction" continue to gather support, positing that it is not just the rewarding properties of drug of abuse that drives drug seeking behavior but the desire to

avoid the negative consequences of withdrawal, like dysphoria and anhedonia. These states are mediated in part by kappa opioid receptors of their endogenous ligands, dynorphins, which also are thought to play a critical role in the behavioral stress-response that can contribute to relapse in humans, and drug-taking in animal models of addiction. Evidence suggests that inhibitory activity on dopaminergic neurons is at least in part responsible for some negative phenotypes associated with dynorphin (Margolis, Hjelmstad, Bonci, & Fields, 2003). New work in the last decade has shown a relationship between these seemingly opposite peptides. In neurons of the hypothalamus, they are integrally connected, even co-localized within the same presynaptic vesicles in almost all orexin neurons.

The purpose of this dissertation is to forge a link between the positive and negative states that drive cocaine seeking and relapse, specifically the mechanism by which orexin and dynorphin contribute to reward and withdrawal. Much of the preclinical addiction literature on orexin or dynorphin is devoted to one of the neuropeptides. However, despite their presynaptic co-localization, little work has been done to demonstrate the intricacies of their interactions. This dissertation seeks to fill this gap in our knowledge by: 1. determining the role of orexin and dynorphin receptor activity in drug seeking and altering brain reward thresholds following cocaine use and withdrawal; 2. Measuring the effects of orexin and dynorphin activity in anatomical regions governing cocaine abuse during reward and withdrawal (VTA); 3. Pharmacologically manipulating orexin and dynorphin receptor activity in order to attenuate both cocaine reward and withdrawal.

Together these experiments describe new mechanisms in the pathophysiology of addiction that may help to formulate the novel treatments for cocaine abuse.

CHAPTER 1

MOTIVATED BEHAVIOR AND PSYCHOSTIMULANT ADDICTION

Although the most tangible manifestations of the basal ganglia effects on psychomotor behavior may be in disorders like Parkinson's and Huntington's disease, the role of basal ganglia is much more expansive (DeLong, 1990; Niv & Rivlin-Etzion, 2007; Obeso et al., 2000). Patients with Parkinson's disease may present with manifestations of depression or difficultly initiating motivation and goal achievement, while lesions in the caudate nucleus or globus pallidus may hamper the ability of patients to initiate simple everyday behaviors despite being physically capable (Caplan et al., 1990; Pluck & Brown, 2002). This chapter will briefly highlight the role of the basal ganglia and its relationships with motivated behavior, dopamine, and drugs of abuse. The focus will be to demonstrate that the NAc is a major locus for actions of DA projections from VTA on behavioral activation/output and rodent models of addiction.

Role of Basal Ganglia in Motivated Behavior

The basal ganglia entail the striatum, pallidum, substantia nigra (SN) and subthalamic nucleus (STN). These components are situated within many of the corticofugal fibers and directly below the cerebral cortex, placing the basal ganglia in a privileged location to interface between the cortex and lower order brain regions (Parent & Hazrati, 1995). Further subdivisions relevant to this dissertation include the nucleus accumbens within the ventral striatum, and the ventral area of the pallidum which make up part of the "limbic system". It is important to note that the anatomical boundaries of some structures'

subdivisions—like those of the nucleus accumbens—are nominal, widely contested and thus not universally espoused.

The basal ganglia convey information directly or indirectly from the cortex to the striatum into the globus pallidus, substantia nigra, and from there, into the thalamus before the cortex is again stimulated, to ultimately recruit groups of skeletal muscle. Information processing is hypothesized to flow as separate, parallel circuits from different cortical areas into the striatum, known as 'parallel processing'. However, 'information funneling' posits that cortical information to the striatum converges in the striatum. Although the process has been found to be more complex than either of these early hypotheses, the direction of information "flow" is nevertheless an important first step of the cortico-basal ganglia loop (Alexander & Crutcher, 1990; Percheron & Filion, 1991). Almost all areas of the cortex project to the striatal areas with especially strong connections from the sensorimotor cortex. Although there is debate as to the exact organization of cortico-striatal projections [see early work by (Kemp & Powell, 1970; Selemon & Goldman-Rakic, 1985)] the large difference in the greater number of neurons in the cortex compared to their

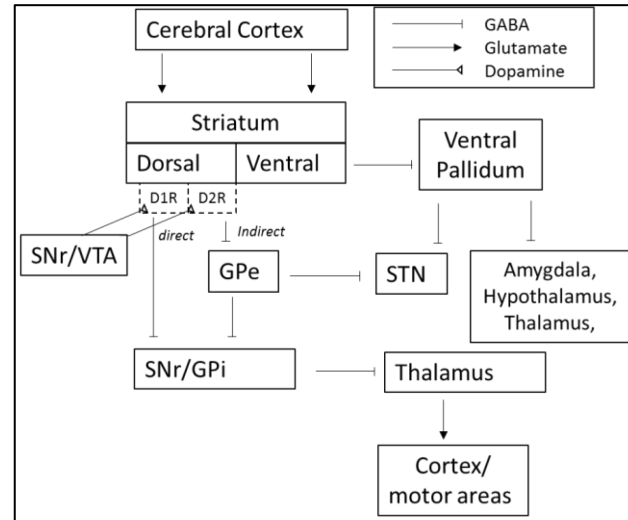


Figure 1. Detailed Basal ganglia circuits for reward-oriented learned behavior. Direct, indirect and reward-related pathways. The striatum receives inputs from the cerebral cortex. D1R-expressing neurons in the striatum connect to SNr/GPi directly (direct pathway). D_2 Rexpressing neurons connect to SNr/GPi indirectly through GPe and STN (indirect pathway). Dopaminergic neurons in SNc/VTA innervate the striatum. Ventral striatum disinhibits important reward related brain regions. STN subthalamic nucleus, GPe external segment of the globus pallidus, GPi/SNr internal segment of the globus pallidus, SNc substantia nigra pars compacta, VTA ventral tegmental area. (Kim & Hikosaka, 2015; Marchand, 2010)

targets in the striatum is indicative of a great deal of convergence. Multiple cortical neurons communicating with fewer striatal neurons allows a degree of 'decision making' in the early stages of the cortico-striatal-thalamo-cortical loop.

The large majority of projection neurons within the striatum have multiple dendrites densely populated with spines that are connected to a medium sized cell body; hence the nomenclature medium spiny neurons (MSN) (Wilson & Groves, 1980). MSNs utilize the inhibitor neurotransmitter γ-aminobutyric acid (GABA) which allows the flexibility to inhibit or disinhibit activity based on cortical and thalamic inputs. MSN are subject to manipulation extrinsically from glutamate and dopamine as well as acetylcholine and GABA released from interneurons.

As depicted in **Figure 1,** the striatum output of GABAergic MSN projects to both segments of the pallidum, the external and internal globus pallidum (GPe and GPi) as well as to the midbrain dopamine nuclei (Haber, Fudge, & McFarland, 2000). Both structures further relay to specific thalamic nuclei and back to the cortex to coordinate motor movement as well as more complex reward related behaviors. The additional GPi efferents to the lateral habenula as well as SNr projections to the pedunculopontine tegmental nuclei support the role of the cortico-basal ganglia circuitry in governing rewarding behavior (Parent & Hazrati, 1995). The continuous inhibitory GABAergic output from the SNr and GPi must be strengthened or weakened to produce the appropriate level of kinesis. The direct pathway involves a weakening (or a disinhibition) of this signal as dopamine receptor 1 (D_1R) in the striatum inhibit GABAergic projections from the SNr/Gpi to the thalamus and corresponding motor areas. Additionally, the indirect pathway is responsible for

strengthening the inhibitory signal as dopamine receptor 2 (D_2R) activates striatal GABA inhibition onto the GPe. The GPe therefore exerts less inhibitory control over the SNr/GPi which consequently releases a greater amount of GABAergic signaling to the thalamic and motor projections, thus causing akinesia (Hikosaka, Takikawa, & Kawagoe, 2000; Y. Smith, Bevan, Shink, & Bolam, 1998).

Activity within the direct pathway correlates heavily to rewarding behaviors while not surprisingly, the indirect pathway is primarily utilized to suppress nonrewarding actions. Together the direct and indirect pathways help to initiate motor behavior towards more rewarding stimuli, allowing animals to select the appropriate behavior (Frank, 2005; Hong & Hikosaka, 2011). Strong dopaminergic projections from ventral tegmental area (VTA) and SNc to the striatum tune the basal ganglia to reward and facilitate a selection process of movement towards reward (direct pathways D_1R) and away from non-reward (indirect pathway D_2R)(Ikemoto, 2007; Kim & Hikosaka, 2015). Although this introduction to basal ganglia may seem far afield, subsequent chapters will cover the role of orexin and dynorphin peptides in contributing to basal ganglia inputs. The focus of this dissertation is the role of orexin and dynorphin on motivated behavior and thus the complexities of the downstream circuitry is relevant.

Habit learning vs action selection

Habit learning: Exactly how the basal ganglia “decides” which action has been heavily debated and discussed. Graybiel and colleagues argue for a school of thought known as ‘habit learning’ within the basal ganglia. Here, habits are slowly acquired as a consequence of stimulus-response learning (Graybiel, 1998). The dorsal striatum has long

been implicated in learning and memory due to disrupted memory in caudo-putamen lesions in rodent models (McDonald & White, 1994). Real-time functional imaging techniques such as positive emission topography (PET) scans allow scientist to see the basal ganglia's involvement in not just optimization and selection [see (Mink & Thach, 1993)] but also learning tasks that require access to memories. Graybiel argues that the basal ganglia alters its action and decision making circuitry when a behavior is being learned (between repeated pairing of stimulus and response) and when a behavior is being expressed (Graybiel, 1995).

As mentioned earlier the striatum is a convergence point for many neurons of the cortex, with as many as thousands of inputs onto the dendrites of a single striatal neuron. This convergence gives the basal ganglia an ability to optimize signal-to-noise ratios; only firing for action when there is a sufficient collection of input. Adding to this plasticity, multiple distinct areas of the cortex project to overlapping areas within the striatum; however, the connection from what cortical to what striatal area are capable of changing. Altering these converging neocortical inputs to the MSNs of the striatum provides a perfect platform to allow synaptic plasticity and facilitate habit learning in response to stimulus. With the added potency of Hebbian synapsing, long term potentiation and depression, small changes in neuronal mapping at this level of the basal ganglia could drastically alter input-output dynamics.

Recent work has built upon these ideas using tools like optogenetic suppression of prefrontal cortical activity to block habits that were formed in animals that were over-trained on a T-maze (turning right or left following a cue to receive a food reward.) (K.

S. Smith & Graybiel, 2013). This suggests that even in deep-seated behaviors, there is a cortical component to the circuit that is still weighing in on the reward value of even highly automated habits. Further work on cell-assemblies in the striatum that receive strongest input from midbrain dopamine neurons (commonly referred to as striosomes) explores decision making where animals were allowed to choose a reward based on the "cost" of a series of mildly annoying stimuli. These studies demonstrated an increase in activity in the projection to striosomes during decisions making regardless of if the animal choose to accept the reward at cost OR reject it (Amemori & Graybiel, 2012; Graybiel, 2016). Although the exact mechanisms of habit learning and decisions making are not well understood, our knowledge of the complexity and elegance of the circuitry is continuing to evolve.

Action Selection: Redgrave et al. sought to explore the computation roles of the basal ganglia positing an essential hypothesis that "[the role of] the basal ganglia is to select some actions/motor programmes at the expense of others" (Redgrave, Prescott, & Gurney, 1999). The basal ganglia may be responsible for solving problems where limited resources are available to satiate multiple sensorimotor system inputs and demands. A simplistic selection problem example may go like this: 'an animal's hypothalamus detects low blood glucose and signals to the basal ganglia the need to forage for food. At the same time the suprachiasmatic nucleus and other regions involved in the circadian cycle is signaling that it is night time and the animal should remain dormant.' These actions are mutually exclusive, and many believe it is the role of basal ganglia to balance the intrinsic and extrinsic factors to select the most appropriate action.

Dr. Jonathan Mink takes the theory of action selection further and begins to propose a mechanism of the basal ganglia control. Acting as an inhibitor to "brake" motor pattern generators (MPG) for actions that are deemed less important while disinhibiting MPGs of movements that are selected for (Mink, 1996). Governance of the indirect and direct pathways of the basal ganglia allow this brain region to select which actions can be selected from the numerous inputs that compete for an organisms' limited time and energy (Groenewegen, 2003). More recent work focuses on the basal ganglia connectivity through cortical and subcortical parallel loops. Human brain activity while selecting sequential actions from memory demonstrates a role of these basal ganglia 'distributed processing modules' selecting different kinds of actions (Houk et al., 2007). Future studies are still necessary to elucidate the exact workings of habit learning, action selection and other mechanisms of decisions making. An understanding of basal ganglia movement and decision-making mechanisms is important for interpreting and treating disease states that involve maladaptive motivational behavior.

Role of Midbrain Dopamine in Motivated Behavior and Drug Abuse

For decades dopamine was hypothesized to modulate plasticity in the cortico-striatal pathway to impart reward-learning and action selection (Beninger, 1983; Wickens, Horvitz, Costa, & Killcross, 2007). A great deal of electrophysiologic and selective dopaminergic pharmacological experiments further support this hypothesis contributing to modern views on dopamine's role in early stage learning and the selection of the environmentally appropriate reward-seeking action (Cheer et al., 2007; Reynolds & Wickens, 2002). As previously mentioned, this selection of the motor action occurs

primarily in the basal ganglia. However, the focus of this chapter will be on the strong inputs from the nucleus accumbens to influence decision making.

In the early 1980's neurons of the nucleus accumbens were hypothesized to play a role in a 'limbic-motor interface'. This is based on early electrophysiological recordings and behavioral data showing limbic projections to the VTA, projections from the VTA to the nucleus accumbens, and projections from the NAc to the globus pallidus that regulated spontaneous motor activity (Mogenson, Jones, & Yim, 1980). Work demonstrating a role of the dopaminergic and noradrenergic system contributing to positive reinforcement using intracranial self-stimulation and pharmacological tools such as amphetamines contributed greatly to our current understanding of midbrain dopamine in motivated behavior (Phillips & Fibiger, 1973; Ungerstedt, Herrera-Marschitz, & Zetterstrom, 1982). The early understanding that loss of dopamine-producing neurons in the substantia nigra was responsible for the motor problems related to Parkinson's Disease helped accelerated interest and finance into the field. Nicola et al. proposed a simplified model where nucleus accumbens projections and dopamine contribute to reward-related actions in a similar way as previously discussed with dorsal striatum projections to the globus pallidum directly and indirectly in the basal ganglia. This approach would help the basal ganglia motor selections maximize actions that are rewarding to the animal (Nicola, 2007). The dorsal striatum correlates environmental cues and motor actions. Strong electrophysiological support comes from the fact that many dorsal striatal projections neurons exhibit characteristics that are necessary in stimulus-action associations such as increasing activity when a stimulus is present and consequently encoding some sort of action information in response to the stimuli (Apicella, 2002; Schultz, 1998).

However, the remainder of this section will focus on not the dorsal but the ventral striatum and how this is involved in natural and drug-induced reward seeking. Dopamine (DA) is a catecholamine produced in the substantia nigra and ventral tegmental area and acts at a number of regions where it controls movement as well as motivated and emotional behavior. Dopamine interacts with a number of G-protein coupled receptors expressed in the nucleus accumbens (as well as other brain regions). The five dopamine receptor subtypes fall into two broad categories; D_1-like and D_2-like. D_1-like receptors consist of excitatory D_1 and D_5 receptors; increasing secondary messengers like cyclic adenosine monophosphate (cAMP). D_2-like receptors consist of inhibitory D_2, D_3, and D_4 receptors and largely decrease intracellular cAMP levels and tend to be found presynaptically (Suzuki, Hurd, Sokoloff, Schwartz, & Sedvall, 1998). Both the D_1 and D_2-like receptors are found primarily on GABAergic medium spiny neurons (MSNs) within the nucleus accumbens (NAc).

The nucleus accumbens consists of two distinct subregions known as the core and the shell. The NAc core is predictably the interior portion and consists primarily of MSNs that possess D_1 or D_2-like receptors. These neurons are responsible for motor functioning in pursuit of rewards and positive reinforcers. This function requires neurons with significant dendritic spines that project to brain regions involved with motor activity such as the aforementioned substantia nigra and globus pallidus (Shirayama & Chaki, 2006). The shell consists of the outer region surrounding the core and is similarly made of MSN that possess D_1 or D_2-like receptors. However, many MSNs within the shell express both D_1 and D_2-mRNA (Nishi, Kuroiwa, & Shuto, 2011). Further differences include a less branched and dendritically rich neuronal morphology compared to the core. The shell is

responsible for the hedonic response to rewards and some motivation for reinforcing stimuli. This explains projections to areas involved in reward such as the VTA, hypothalamus and ventral pallidum (Meredith, Pennartz, & Groenewegen, 1993; Saddoris, Cacciapaglia, Wightman, & Carelli, 2015).

Electrophysiologic evidence from the midbrain dopamine neurons of monkeys have demonstrated a role of dopamine neurons in the prediction of reward and learning. Activity in VTA dopaminergic neurons is increased in the presence of rewarding stimuli, especially when it is unexpected while the same neurons presented with suppressed activity when expected rewards were omitted (Schultz, 1998). Different midbrain neuronal activity is thus involved in learning and reward. Dopamine neurons are further involved in accounting for the amount of reward, risk involved and reward type in the decision making process (Lak, Stauffer, & Schultz, 2014). These "economic" decisions were further elucidated by measuring neuronal activity during optogenetic stimulation of dopamine terminals in the NAc. Animals were presented with cues for different amounts of rewards (sugar pellets) with different delays. Dopamine release increased with increased predictive reward and decreased with increased delays. Optogenetic manipulation was able to disrupt delayed choices (Saddoris, Sugam, et al., 2015). These experiments represent only a fraction of available work suggesting that mesolimbic dopamine neuron activity is largely responsible for motivation to seek reinforcing stimulus while also contributing to decision making about the value of a reward (Bickel et al., 2017).

Substance abuse is an insidious disease effecting 23.5 million people in the United States alone. Tobacco, alcohol, and illicit drugs are responsible for exacting more than $700 billion annually in health care, crime, and lost productivity costs (Harwood et al., 1998; Holland & Mushinski, 1999). However, there are currently no FDA-approved treatments available for treating stimulant abuse. Cocaine elicits its rewarding effects primarily by antagonizing the activity of the dopamine transporter (DAT). The hindrance of synaptic dopamine reuptake by DAT increases levels of dopamine within the synaptic cleft leading to increased dopamine available to interact with dopamine receptors post

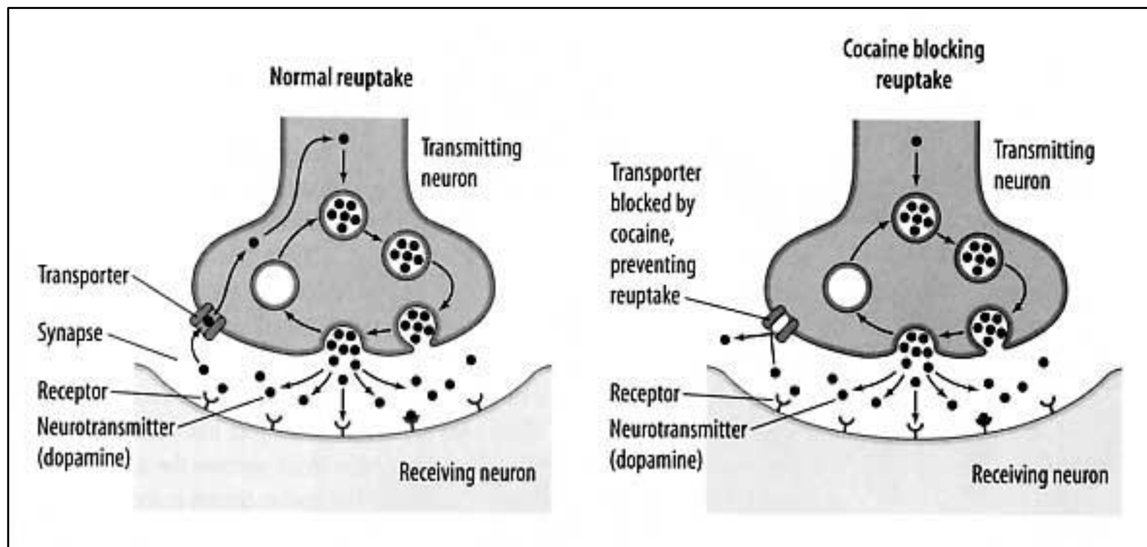


Figure 2. Cocaine Mechanism of action. Demonstrating dopamine transporter (DAT) reuptake inhibition in the synaptic cleft during acute cocaine use (Abadinsky, 1997).

synaptically **(Figure 2.)** (Galloway, 1988). The postsynaptic D_1-like dopamine G-coupled protein receptors signal a secondary messenger pathways such as adenylyl cyclase to cyclic AMP, PKA and eventually CREB (Anderson & Pierce, 2005). Increased activation in neurons especially within the nucleus accumbens and the prefrontal cortex manifests as a euphoric and rewarding experience. However, after repeated cocaine

exposure neurophysiologic adaptations within the NAc and other brain structures may begin to decrease the subjective reward during acute use. Although acute injections of cocaine have been shown to potentiate glutamatergic signaling in VTA dopamine neurons (Borgland, Taha, Sarti, Fields, & Bonci, 2006) there is extensive inhibitory changes within the VTA that decreases cellular firing (Niehaus, Murali, & Kauer, 2010). Chronic cocaine injections continue to decrease VTA dopamine neuronal activity after the dissipation of acute effects (Liu, Pu, & Poo, 2005). Decreased activity within the VTA-NAc pathway using dopamine antagonists has been shown to increase depressive-like behaviors as early as 1982 (Wise, 1982). More recently, decreases in the VTA-NAc pathway activity increased immobility in the forced swim test and anhedonia in other behavioral models of depression and reward (Mague et al., 2003; Todtenkopf, Marcus, Portoghese, & Carlezon, 2004). Cocaine exposure activates additional circuits such as GABA neuron projections from the lateral habenula to the VTA further enhancing inhibition of DA neurons (Jhou et al., 2013). Eventually drug-induced changes in neuronal physiology of this VTA-NAc pathway leads to withdrawal symptoms during abstinence (Luscher, 2016). It has been demonstrated that during cocaine abstinence the decreased dopamine levels in the mesolimbic dopamine pathway, notably the nucleus accumbens, contributes to the withdrawal symptomology (Weiss, Markou, Lorang, & Koob, 1992). However, to explain cocaine reward and withdrawal phenotypes solely based on dopamine activity in the nucleus accumbens would be a gross oversimplification. Multiple neurotransmitter systems in different brain regions effect an animal physiology through the different stages of drug abuse. This dissertation focuses on inputs from the lateral hypothalamus orexin-dynorphin neurons to the dopaminergic

neurons of the ventral tegmental area and subsequent projections to the nucleus accumbens (Marcus et al., 2001; Peyron et al., 1998).

CHAPTER 2

THE LATERAL HYPOTHALAMUS AND OREXIN-PRODUCING NEURONS

Orexin and Dynorphin peptide and receptors

The biologically active orexin A and orexin B peptides are encoded by the hypocretin neuropeptide precursor gene (HCRT) located on human chromosome 17q21 (Sakurai et al., 1998; Sakurai et al., 1999). The gene product, prepro-orexin, is subsequently cleaved by proteolytic prohormone convertase to produce the 33-amino acid peptide (~3.5kDa): orexin A, and the 28-amino acid peptide (~2.9kDa): orexin B (Sakurai et al., 1999). Importantly, these peptides are well conserved across mammalian species with significant homology between mouse and human peptides. Orexin A binds to both G protein coupled receptors (GPCRs) orexin receptor 1 and 2 (OxR1 and OxR2) while orexin B binds preferentially to orexin receptor 2 (Langmead et al., 2004). These GPCRs are each coded by a different gene (HCRTR1 and HCRTR2 located on chromosome 1 and 6, respectively) to produce two excitatory receptors OxR1 and OxR2 (Spinazzi, Andreis, Rossi, & Nussdorfer, 2006). OxR1 and OxR2 activation increases intracellular calcium concentrations in conjunction with coupling to guanine nucleotide-binding proteins G_q and G_s to activate enzymes such as adenylyl cyclase (AC) and phospholipase C (PLC) (Uramura et al., 2001). Adenylyl cyclase further catalyzes cyclic AMP and protein kinases A (PKA) activation while PLC can influence further calcium release as well as activation of protein kinase C (PKC). Together PKA and PKC can increase nuclear transcription factor cAMP response element binding protein (CREB) and extracellular signal-related kinase 1 and 2 (ERK1/2). These signaling cascades are altered during exposure to orexin peptide and result in increased cellular activity in HEK293 cells

through DNA transcription (Spinazzi et al., 2006; Tang et al., 2008; Xu, Yang, Ward, Gao, & Liu, 2013).

The precursor protein prodynorphin is coded by the PDYN gene on chromosome 20 (Horikawa et al., 1983). Once transcribed and translated prodynorphin is cleaved by proprotein convertase 2 to give rise to several biologically active opioid peptides such as dynorphin A, dynorphin B, "big dynorphin" (an unsuccessfully cleaved prodynorphin) and neo-endorphin (Day et al., 1998). Dynorphin A binds primarily to the GPCR, kappa opioid receptor (KOR). The inhibitory KOR signals through guanine nucleotide-binding protein G_i as well as β-arrestin recruitment. Different ligands for the KOR produce unique signaling cascades. These ligands utilize differing G_i activities compared to β-arrestin recruitment. G_i signaling decreases cyclic AMP levels and behaviorally decreases nociception in animals while β arrestin alters P38 Mitogen-activated protein kinase and may be important in dysphoria. Dynorphin A activates KOR G_i signaling approximately 34 times more the βarrestin pathway (White et al., 2014).

Discovery of Orexin and Dynorphin Peptides

The orexin (or hypocretin) peptide was discovered simultaneously by two different groups within months of each other, and is responsible for the conflicting nomenclature. De Lecea et al. named the peptide hypocretin for its structural similarity to the secretin hormones, and because it is located in the hypothalamus- "hypo" "cretin" (de Lecea, 1998). Sakurai et al. dubbed the peptide orexin referring to the Greek word "orexis", or appetite, having observed that exogenous orexin increases food consumption when given centrally to rats.

De Lecea, Kilduff, Sutcliffe and colleagues of the Scripps Research Institute used directional tag PCR to "subtract" the mRNA that coded for protein within the hypothalamus. Of these clones one was selected that was exclusive to the posterior hypothalamus. This clone codes for two peptides (hypocretin 1 and 2) and was thus named Hcrt. De Lecea and colleagues used *in situ* hybridization to show Hcrt mRNA in the lateral hypothalamus that agreed with immunohistochemical labeling of the hypocretin peptide and the hypocretin-positive cell projections. Lastly, De Lecea applied hypocretin to cultured hypothalamic neurons and saw increased electrophysiological activity measured as postsynaptic events in rat hypothalamic neurons (de Lecea et al., 1998).

Dr. Takeshi Sakurai, Masashi Yanagisawa and colleagues of Howard Hughes Medical Institute employed binding assays of orphan GPCRs to identify the orexin peptide. Known DNA sequences suggested that there were many GPCRs without a known ligand that would activate it. Sakurai and colleagues began by creating 50+ cell lines that each expressed one of the known GPCRs. Subsequently each cell line (and receptor) was inundated with tissue extracts and the second messenger activity was recorded. When activity was detected from an extraction at a specific cell line (but not in other unrelated cell lines) the extract was purified though high performance liquid chromatography. The purified peptides were analyzed using mass spectroscopy and sequencing analysis and found to be the 33-amino acid orexin-A and 28 amino acid orexin-B. With the peptides' sequences, Sakurai isolated the cDNA by RT-PCR and deduced the structure of the precursor prepro-orexin. Deducing the orexin sequences allowed synthesis of radiolabeled orexins to probe cell lines for the human orexin receptors. Together these

sequences also allowed the researchers to use northern blot analysis to localize orexin receptor transcription to the lateral hypothalamus (Sakurai et al., 1998).

Enkephalins were discovered much earlier in the 1970's when Dr. Hughes, Morris and colleagues analyzed previously discovered endogenous substances within the brain that activated opiate receptors. Using a novel radioligand binding assay and mass spectroscopy two peptide sequences were discovered and then synthesized. These newly discovered enkephalins were isolated from pituitary extracts and were found to stimulate opiate activity that was distinct from previously discovered opioids. This peptide was later found to bind to the kappa opioid receptor and named dynorphin-A (Chavkin, James, & Goldstein, 1982; Hughes et al., 1975). Like many discoveries of the era, a separate laboratory, Dr. Hisayuki Matsuo's, was also working on similar peptide identification in parallel. Dr. Matsuo's team was utilizing hypothalamic tissue from pigs to isolate the precursor protein that is cleaved to dynorphin-A and dynorphin-B (Chavkin, 2013; Kangawa & Matsuo, 1979). At this point neuropeptide identification was increasing due to better available technology and cDNA cloning and sequencing was completed for the precursor of the dynorphin family, prepro-dynorphin (Kakidani et al., 1982).

Anatomy, and Projections

Orexins are neuropeptides produced almost exclusively in the lateral hypothalamic area (LHA) [in rat brain: AP: -1.8-3.9 mm from bregma] (Peyron et al., 1998; Swanson, Sanchez-Watts, & Watts, 2005) where they mediate many motivated behaviors. Seminal work mapping the circuitry of the orexin neurons was completed by Christelle Peyron, Thomas Kilduff and colleagues using immunohistochemistry with novel antibodies targeting orexin A peptides in Wistar rats. These experiments measured fiber and cell

body density throughout the entire adult brain, including areas of high innervation that are relevant to this dissertation such as the lateral hypothalamus (cell bodies), and the VTA (projections) (Peyron et al., 1998). The results indicated that orexin is found only in neurons of the hypothalamus and that this expression pattern is well-conserved across species. Three years later, Jacob Marcus, Joel Elmquist and colleagues mapped relative densities of orexin *receptor* mRNA expression in the rat brain using *in situ* hybridization. Although the orexin 1 and orexin 2 receptors have slightly different expression; OxR1 is more present in the locus coeruleus and amygdala while OxR2 is expressed more in the paraventricular nucleus of the hypothalamus, however, they are generally expressed together in most brain regions when present. Again, these results highlight both receptors in the VTA and BNST as well as a number of other nuclei that contribute to motivated behaviors such as reward, feeding, circadian rhythms, and stress (Marcus et al., 2001). *In situ* hybridization demonstrates expression of KOR mRNA in a wide range of CNS regions. Areas like the VTA, extended amygdala and BNST share KOR mRNA expression with the orexin receptors while areas such as the cortex, hippocampus, and nucleus accumbens express only KOR mRNA mRNA (Mansour, Khachaturian, Lewis, Akil, & Watson, 1988). The pattern of expression correlates well with the many stress behaviors that dynorphin has exhibited a roll in producing. The overlapping KOR and OxR expression in areas and distinct expression in others is not surprising. As will be discussed later, the large majority (~94%) of orexin neurons contain dynorphin, however, there are many dynorphin producing neurons that do not produce, contain or release orexin peptide (Chou et al., 2001).

Orexin-dynorphin producing neurons, orexin receptors and kappa opioid receptors within the ventral tegmental area: After the establishing the basic pattern of orexin peptide and receptor expression, further work has begun to highlight the functional complexity of the system. Orexin-dynorphin-producing neurons project to the VTA and increase neuronal activity through multiple mechanisms. Excitatory neurotransmitter such as glutamate may be localized within the same neuron as orexin and dynorphin presynaptically as well as interact with N-methyl-D-aspartate (NMDA) receptors found on dopaminergic neurons (Chergui et al., 1993; Rosin, Weston, Sevigny, Stornetta, & Guyenet, 2003). Additionally, the presence of orexin receptors on glutamatergic interneurons in this complex tripartite synapse may be responsible for further exciting VTA dopamine producing neurons (Y. Li, Gao, Sakurai, & van den Pol, 2002). Lastly and most directly, excitatory Gq coupled orexin receptors can increase dopaminergic neueron activity via their presence on post synaptic neurons (Korotkova, Sergeeva, Eriksson, Haas, & Brown, 2003).The presence of orexin receptors on dopaminergic cell bodies has been shown by single cell PCR performed on neurons from VTA in slice or culture. (Borgland et al., 2006; Korotkova et al., 2003; Muschamp et al., 2014). See **Figure 3.**

In addition to orexin receptors, neurons within the VTA also express KORs as demonstrated in electrophysiological experiments in which dopamine producing neurons were inhibited by KOR agonists (Margolis et al., 2003). Additional evidence supporting inhibition of VTA dopamine neurons by KOR can be gleaned from a series of experiments using DA neuron-specific KOR conditional KO (cKO) mice that did not exhibit the same KOR agonist-induced conditioned place aversion and reduced dopamine

release as wild type mice. However, these phenotypes were rescued when KOR was delivered into the VTA in an adeno-associated viral gene construct, suggesting the presence of KOR on VTA dopaminergic neurons is responsible for kappa-mediated aversion (Shippenberg, 2013; Ehrich, 2015). Together these mechanisms allot orexin and dynorphin peptides a significant amount of control to increase or suppress activity within the dopaminergic neurons of the VTA.

Orexin-dynorphin producing neurons, orexin receptors and kappa opioid receptors within the bed nucleus of the stria terminalis: Studies of orexin-dynorphin projections to the VTA continues to be fruitful (e.g. Baimel et al. 2017), but there have also been recent advances in understanding orexin-dynorphin neurons and their links to stress circuitry and the bed nucleus of the stria terminalis (BNST). The BNST is a brain region heavily involved in stress and anxiogenic responses to threats. The paraventricular nucleus (PVN) synthesizes corticotropin-releasing hormone (CRH) which acts upon the anterior pituitary to increases adrenocorticotropin (ACTH) release. ACTH subsequently increases cortisol release from the adrenal cortex which is responsible for many phenotypes of a stress response including increased blood pressure and the sympathetic nervous system activation (Herman et al., 2003). The amygdala has been well established in contributing to the HPA stress axis and dysregulation of amygdala activity is involved in post-traumatic stress disorder (PTSD), depression and other stress-related disorders. With afferent connection to the amygdala and efferent projections from the PVN, the BNST is well positioned to regulate the hypothalamic–pituitary–adrenal (HPA) axis (Choi et al., 2007).

It has been demonstrated that orexin imparts an anxiogenic effects in social interaction and elevated plus maze paradigms after direct injection into the BSNT (containing a large concentration of CRF neurons) via glutamatergic NMDA receptors (Lungwitz et al., 2012; Winsky-Sommerer et al., 2004). The stimulation of CRF could be contributing to anxiogenesis for a number of reasons including increasing activity within the hypothalamic-pituitary-adrenal stress axis (Al-Barazanji, 2001). Additionally, a complex feedback system allows CRF in the hypothalamus to depolarize orexin-dynorphin neurons due to the presence of CRF receptors on orexin neurons (Winsky-Sommerer et al., 2004). Interestingly, the presence of KOR on GABA producing interneurons of the BNST may disinhibit CRF producing neurons and subsequent anxiety (Li et al., 2012). This dissertation focuses mainly on the outcomes of orexin and dynorphin peptides opposing one another's actions in the VTA dopaminergic neurons. However, the presence of KOR on GABA producing inter neurons in the BNST and OxR on CRF producing neurons in the BNST raises the possibility that dynorphin activity disinhibits CRF neuronal activity while and orexin potentiates CRF neuronal activity. In this instance the orexin and dynorphin activity within a discrete brain regions such as the BNST would have a cooperative effect (C. Li et al., 2012). This circuitry is highlighted and simplified in **Figure 3a** followed by the VTA synaptic circuitry diagram in **Figure 3b.**

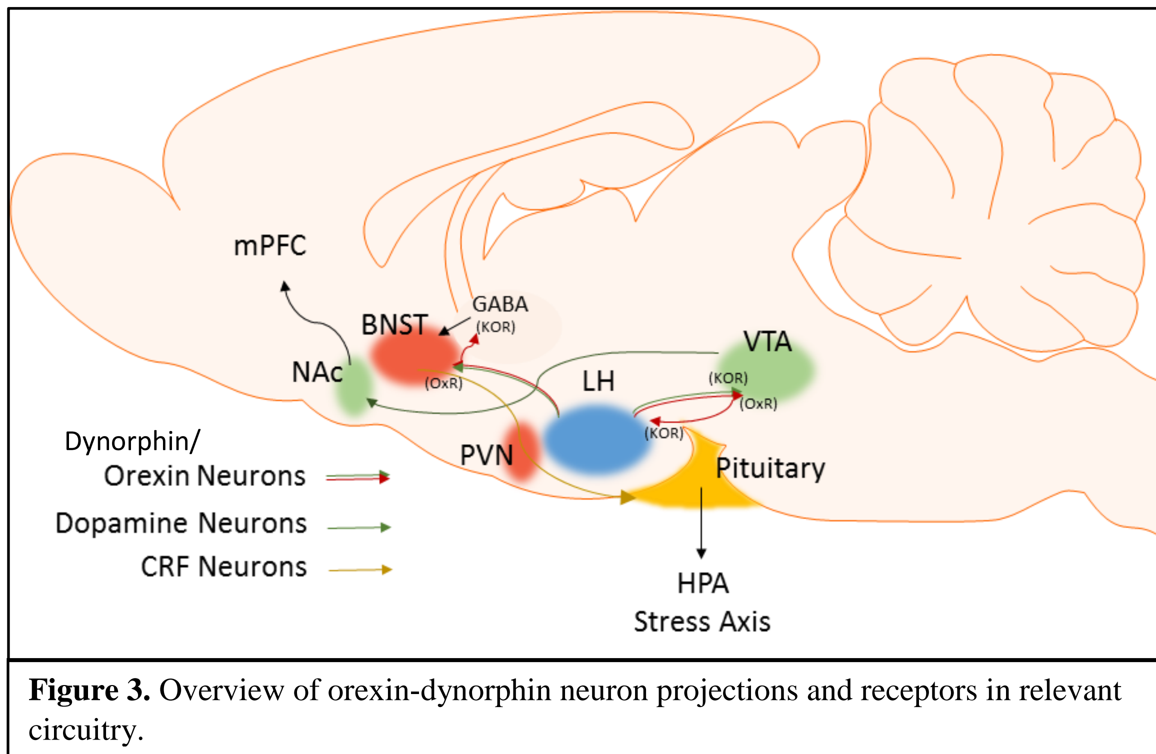
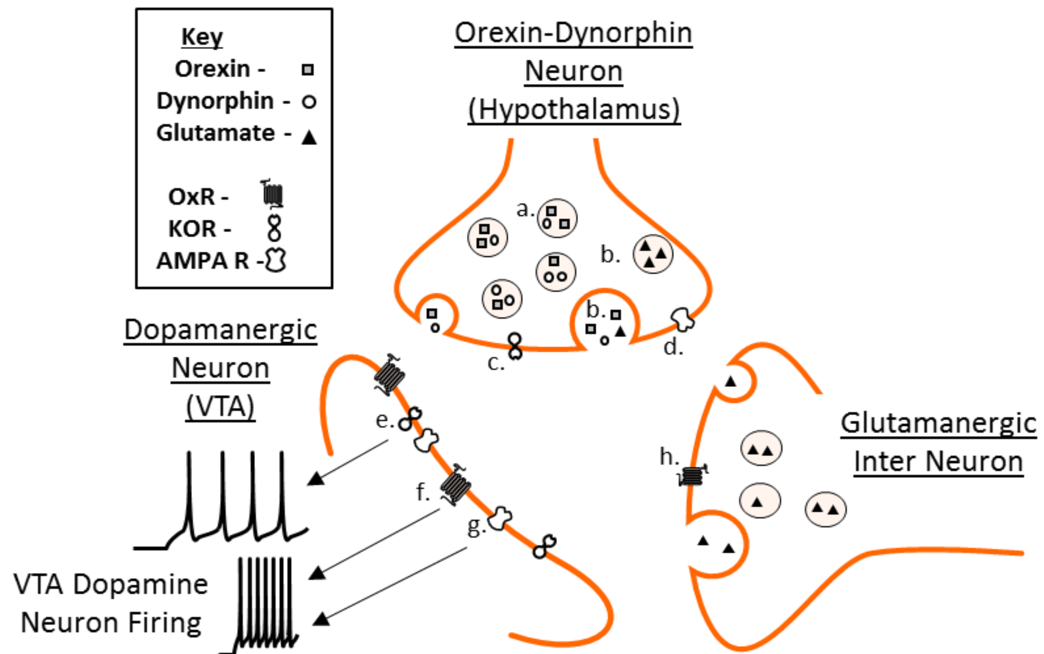


Figure 3. Overview of orexin-dynorphin neuron projections and receptors in relevant circuitry.



	Aspect of Synapse	Reference
a.	Orexin Dynorphin colocalization	(Muschamp et al., 2014)
b.	Orexin glutamatergic tone	(Rosin et al., 2003)
c.	KOR on orexin-dynorphin producing neuron	(Y. Li & van den Pol, 2006)
d.	Glutamatergic R on orexin-dynorphin producing neuron	(Y. Li et al., 2002)
e.	KOR on dopaminergic neuron	(Chefer et al., 2013; Margolis et al., 2003)
f.	OxR on dopaminergic neuron	(Korotkova et al., 2003)
g.	Glutamate receptor on dopaminergic neuron	(Chergui et al., 1993)
h.	OxR on glutamatergic interneuron	(Y. Li et al., 2002)

Figure 3, continued. Tripartite synapse between, orexin-dynorphin, glutamate and dopaminergic neurons in the VTA

Physiologic effect of Orexin and Dynorphin in Other Projections

Locus coeruleus (LC): Orexin-dynorphin producing neurons project widely to brain structures that in many cases express both KOR, and orexin receptors (Mansour et al., 1994; Marcus et al., 2001). The LC is an important structure for stimulating arousal and its norepinephrine-producing neurons have long been known for their 'wake-active' pattern of firing. In the LC, the actions of orexin on these neurons has been demonstrated by electrophysiological experiments where the peptide strongly increases noradrenergic cell firing as well as relevant behavioral correlates like arousal, stress and motor activity. Most dramatically, literature suggests a mechanism for orexin activity contributing to cataplexy in orexin-deficient narcoleptic patients (and rodent models), as the LC has long been implicated in cataplexy and maintenance of postural tone in skeletal muscle (Mahlios, De la Herran-Arita, & Mignot, 2013). This manifests as cataplectic attacks in patients suffering from narcolepsy. Orexin A peptide injection into the LC increased arousal (measured as a sleep stage determined by EEG), grooming behavior and locomotor activity. This alteration in behavioral states may be due to an increase in norepinephrine which in turn promotes cortical activity associated with waking/arousal. Additionally, neuromodulatory effects of orexin on prolactin, growth hormone levels and increasing corticosterone are associated with HPA activation and arousal (Hagan et al., 1999).

Work to assess the role of dynorphin circuitry in the LC has shown it contributes to models of stress-induced relapse. When the KOR selective agonist U50,488 is infused into the LC of animals with previously extinguished cocaine conditioned place preference, the preference is reinstated. These effects were significantly attenuated when

the KOR antagonist NorBNI was injected into the LC. This effect was further elucidated by injecting viral vectors expressing KOR in KOR knockout mice, in which reinstatement was rescued (Al-Hasani, McCall, Foshage, & Bruchas, 2013). Although this thesis work does not specifically address the role of orexin and dynorphin activity within the LC, it is important to understand how LC activity effects behavioral phenotypes and how these behaviors may be effected by systemic pharmacological tools.

Raphe Nuclei: Orexin-dynorphin producing neurons project strongly to the raphe nucleus where *in situ* hybridization data demonstrate KOR and Ox1/Ox2 receptors are also present (Mansour et al., 1994; Marcus et al., 2001). *In vitro* Orexin A peptide excites the dorsal raphe serotonergic neurons post synaptically (deduced from the evidence that the inward current is not blocked by the sodium channel blocker tetrodotoxin). Additionally, there is strong immunohistochemical evidence supporting orexin containing axons in close proximity to serotonergic neurons (R. J. Liu, van den Pol, & Aghajanian, 2002). Similar to the LC, the RN innervates structures important for governing circadian cycle and arousal, such as the suprachiasmatic nuclei. Reverse microdialysis of orexin A and B infusion into the RN increases serotonin in the dorsal raphe nucleus (orexin A and B) and median raphe nucleus (orexin B only). The ability of orexin to regulate serotonin release in a site specific manner implies a role in regulating activities like sleep-wake cycles (Tao et al., 2006). Orexin producing neurons have interesting characteristics in which local glutamatergic neurons can regulate orexin activity by glutamate release presynaptically, allowing orexin to play a role in the hypothalamic arousal system without direct activity of orexin on orexin neurons (Li et al., 2002). Similar neuropeptides such as ghrelin

injected into the RN increase feeding and anxiety, however little work has been done with the direct behavioral effects of orexin peptides in the RN (Carlini et al., 2004). Similar to the LC experiments, selective KOR agonist, U50,488, injected into the RN caused conditioned place aversion (CPA). Injection of the KOR antagonist, NorBNI, into the RN significantly attenuated this CPA. KOR KO mice did not develop aversion however injecting viral vectors expressing KOR in KOR knockout mice restored CPA (Land et al., 2009). This helps to demonstrate that activity in the RN is important for rewarding behaviors such as CPP as well as general arousal. Orexin and dynorphin activity in this brain region should be accounted for when discussing holistic roles for the neuropeptides.

Hippocampus: The hippocampus is involved in encoding, interpreting, and recalling contextual representations of reward. Orexin-dynorphin producing neurons project widely to brain areas. Although there are few KOR and, Ox1/Ox2 receptors present in within the hippocampus, previous literature suggest orexin and dynorphin in the hippocampus is still important to certain behaviors (Mansour et al., 1988; Marcus et al., 2001). Orexin A peptide applied to the Schaffer collaterals in adult mice caused a slow onset long-term potentiation (LTP) in the hippocampal synaptic transmission (Selbach et al., 2004). When orexin A peptide was administered intraventricularly to animals before a forced swim test, those animals exhibited less immobility compared to controls (suggesting an antidepressant effect) without effecting spontaneous motor activity. This behavior was correlated to increased 5-bromo-2'-deoxyuridine incorporation (a measure of cellular neurogenesis) in the dentate gyrus 4 days after orexin administration. A

selective Ox1R antagonist, SB334867 mitigates both the anti-depressive and neurogenic effects of the orexin peptide (Ito et al., 2008).

Consistent with its inhibitory effects in other neuron types and structures, activation of the KOR by dynorphin has been demonstrated to reduce excitatory postsynaptic currents (EPSC) within the dentate gyrus. Further experiments demonstrate dynorphin blocks LTP induction and hypothesize this is due to retrograde inhibitory neurotransmission (providing negative feedback to the presynaptic terminal). Dynorphin blocks the induction of LTP by inhibiting glutamate release in the dentate gyrus (Wagner, Terman, & Chavkin, 1993). Age-related increases in dynorphin A peptide in the hippocampus can decrease cognition and spatial learning in the Morris water maze tasks (Jiang, Owyang, Hong, & Gallagher, 1989). Because the hippocampus is heavily involved in memory, including encoding, interpreting, and recalling reward memories, orexin and dynorphin influences are relevant to additive behaviors.

Prefrontal cortex: Orexin-dynorphin producing neurons project to the prefrontal cortex where few KOR, orexin 1 receptors and no orexin 2 receptors are present (Mansour et al., 1988; Marcus et al., 2001). The medial prefrontal cortex (mPFC) is an important brain region contributing glutamatergic inputs to dopaminergic terminals of the nucleus accumbens (Karreman & Moghaddam, 1996). This innervation places the mPFC as a main player in governing reward seeking, impulse control and drug addiction as well as higher order cognitive processes (Lambe & Aghajanian, 2003; Lambe, Olausson, Horst, Taylor, & Aghajanian, 2005). Orexin A peptide delivered directly to the mPFC was found to increase both firing rates and bursting in the dopamine neurons. The authors

hypothesize that orexin regulates mPFC excitatory glutamatergic control of dopamine neurons to the NAc in order to enhance the selectivity of dopamine neurons in response to different environmental contexts (Aston-Jones et al., 2010). Decreases in orexin levels within the mPFC are shown following social defeat models and can be correlated to depressive phenotypes such as decreased sexual pursuit and increased immobility in the forced swim test (Nocjar, Zhang, Feng, & Panksepp, 2012). KOR generally exerts negative effects, which was demonstrated in an experiment where the intra-medial frontal cortex injections of the KOR agonist U50,488 caused significant CPA (Bals-Kubik, Ableitner, Herz, & Shippenberg, 1993).

All together, these patterns of anatomical projections highlights the ubiquity of the orexin-dynorphin circuitry, in important 'limbic' structures known to regulate emotion, and suggests a circuit for regulating affective behavior. Many of these structures participate in a common set of behavioral outputs centered on arousal, motivation, reward and movement. The orexin-dynorphin system within the mesolimbic dopamine pathway is important for regulating reward seeking behaviors, with further fine-tuning and plasticity through other projections such as the LC, hippocampus, mPFC and others.

Dynamic interplay between Orexin and Dynorphin

Orexin neuron projections from the lateral hypothalamic area (LHA) (Peyron et al., 1998) mediate many motivated behaviors. Orexin is involved in arousal (Adamantidis, Zhang, Aravanis, Deisseroth, & de Lecea, 2007) and sexual motivation (Muschamp, Dominguez, Sato, Shen, & Hull, 2007) and is critical for integrating information about rewarding stimuli such as food (Borgland et al., 2009; Sharf et al., 2010) and drugs of abuse

(Borgland, Taha, Sarti, Fields, & Bonci, 2006; Harris, Wimmer, & Aston-Jones, 2005). In contrast, the opioid peptide dynorphin has been found to underlie negative affective behavioral phenotypes. It has been demonstrated that dynorphin activity attenuates the rewarding effects of drugs of abuse by decreasing activity in the mesocorticolimbic dopamine system (Ehrich et al., 2015; Margolis et al., 2003). As previously mentioned, orexin binds to the excitatory orexin receptors (Ox1R and Ox2R) in many brain regions involved in reward such as the ventral tegmental area (VTA) and dorsal raphe (Marcus et al., 2001). Dynorphin elicits its actions though the inhibitory kappa opioid receptor (KOR) leading to dysphoria, anxiogenesis and depressive-like effects in humans and rodents in many areas including the VTA and BNST (Bals-Kubik, Ableitner, Herz, & Shippenberg, 1993; Pfeiffer, Brantl, Herz, & Emrich, 1986). These behavioral effects can be partially attributed to expression of OXRs and KORs in the mesolimbic dopamine system, specifically VTA dopamine neurons. However, much of the interest in studying orexin and dynorphin peptide activity derives from the fact that they are co-localized within the same presynaptic vesicles of nearly all orexin-dynorphin producing neurons. The evolutionary explanations or the biological function of this co-localization is not fully understood. However, the breadth of addictive behaviors these peptides are involved with provides excellent rational to study orexin and dynorphin relations. There is a great amount of literature supporting neurotransmitters that are co-released from the same presynaptic vesicle, for example, GABA co-localized in some dopaminergic synapses or CRF and vasopressin in neurosecretory vesicles (Stensrud, Puchades, & Gundersen, 2014; Whitnall, Mezey, & Gainer, 1985). However, there are very few examples of opposing neurotransmitters being released at the same time (Devoto, Flore,

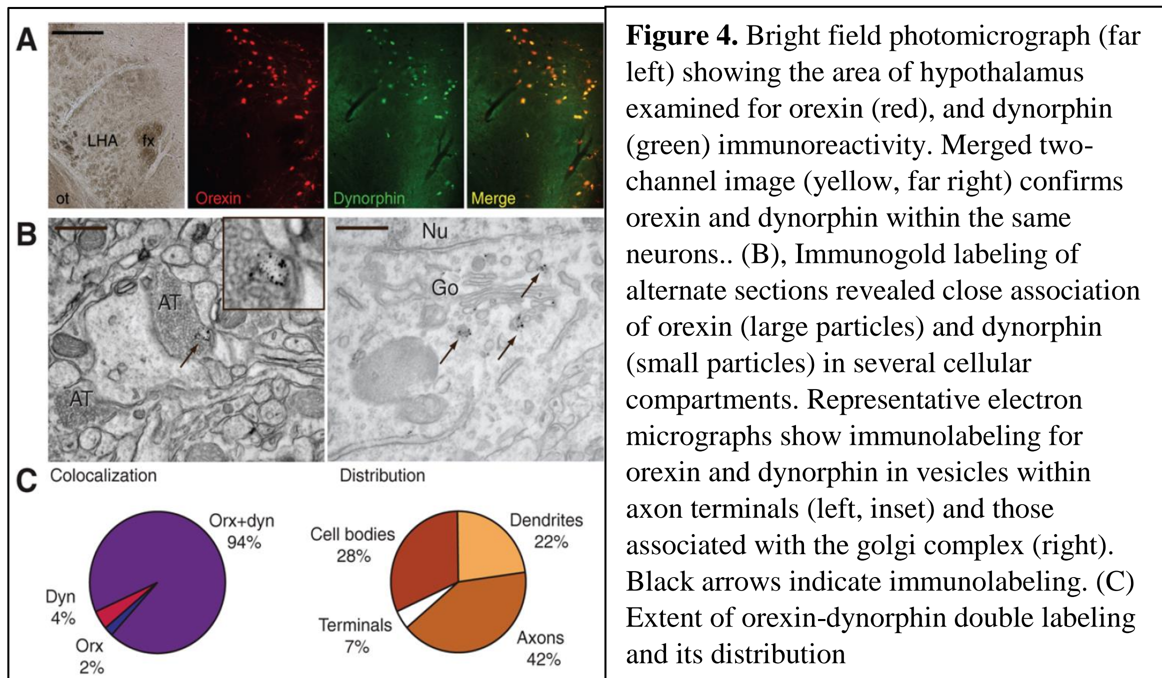


Figure 4. Bright field photomicrograph (far left) showing the area of hypothalamus examined for orexin (red), and dynorphin (green) immunoreactivity. Merged two-channel image (yellow, far right) confirms orexin and dynorphin within the same neurons.. (B), Immunogold labeling of alternate sections revealed close association of orexin (large particles) and dynorphin (small particles) in several cellular compartments. Representative electron micrographs show immunolabeling for orexin and dynorphin in vesicles within axon terminals (left, inset) and those associated with the golgi complex (right). Black arrows indicate immunolabeling. (C) Extent of orexin-dynorphin double labeling and its distribution

Pani, & Gessa, 2001; El Mestikawy, Wallen-Mackenzie, Fortin, Descarries, & Trudeau, 2011; Pernow, 1988). While ostensibly serving opposite functional roles, nearly all orexinergic neurons in LHA contain dynorphin. Using in situ hybridization. Chou et al. found nearly all neurons expressing prepro-orexin mRNA also expressed prodynorphin mRNA. Additionally, immunohistochemical analysis revealed orexin and dynorphin peptides were colocalized in the lateral hypothalamus of 94% of neurons examined (Chou et al., 2001). Further co-localization was recently demonstrated with higher spatial resolution by the Muschamp et al. , using electron microscopy to reveal a co-localization of orexin and dynorphin not just in the same neurons but also within the same vesicles for nearly all orexinergic neurons (**Figure 4**) (Muschamp et al., 2014). The functional significance of these anatomical observations was shown in experiments where electrical stimulation of GFP labeled orexin neurons in hypothalamic slices evoke both orexin and dynorphin release. Predictably, stimulation with the released peptides had opposite effects on post-synaptic neuronal excitability (Y. Li & van den Pol, 2006). To add to the

complexity of this bivalent activity, both orexin and dynorphin may alter GABA interneurons in certain brain regions, thus maybe not always be opposing each other with regard to ultimate effects on principal (projection) neurons (C. Li et al., 2012; Liu, van den Pol, & Aghajanian, 2002). The potential utility of having two opposing peptides released simultaneously is not fully understood. Insight into the behavioral implications of the orexin-dynorphin system will prove novel and therapeutically beneficial.

To investigate the behavioral relevance of these observations Muschamp et al showed that intracranial self-stimulation and brain reward thresholds demonstrated decreasing orexin signaling increased anhedonia while concurrent blockade of orexin and dynorphin allows baseline reward threshold to return to normal. NorBNI alone does not produce reinforcement, implying that the kappa system can be activated to increase negative emotion or inhibited to decrease negative emotion but cannot be inhibited to produce reward (**Figure 5**). This work is one of the first to touch upon the importance concomitant OX1R and KOR signaling in behavioral measures (Muschamp et al., 2014).

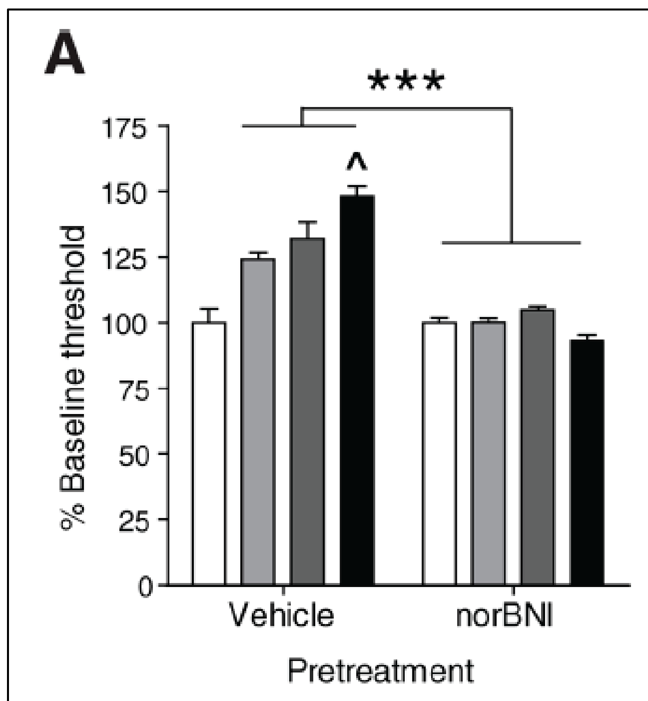


Figure 5. Brain reward threshold-elevating effects of orexin blockade are reversed by dynorphin blockade (previous work by J. Muschamp.) Blockade of orexin signaling elevates reward thresholds in the ICSS test, indicating decreased reward. This effect is blocked by pretreatment with the KOR antagonist.

Figure 5. Orexin receptor 1 may contribute to positive emotions while the KOR contributes to negative affect. Together blockade returns the animals to baseline or

"normal" levels. A rewarding and anhedonic affect together would appear as baseline, however antagonism of KOR alone is not rewarding and thus the mechanism by which norBNI attenuates orexin receptor-induced anhedonia is intriguing. Further evidence illuminating orexin and dynorphin effects on the reward system can be inferred from electrophysiology data in which VTA dopamine neurons were treated with orexin to increase firing rate and treated with dynorphin to decrease firing rates. As expected neurons that were treated with both responded close to baseline. Although there seems to be neurons that are preferentially inhibited by dynorphin (orexin + dynorphin fire rate < baseline) or neurons that are preferentially excited by orexin (orexin + dynorphin fire rate > baseline) (Y. Li & van den Pol, 2006; Muschamp et al., 2014). Future work is beginning elucidate the exact roles of preferentially inhibited or excited dopamine neurons in the VTA and the projections of such neurons (Baimel, Lau, Qiao, & Borgland, 2017).

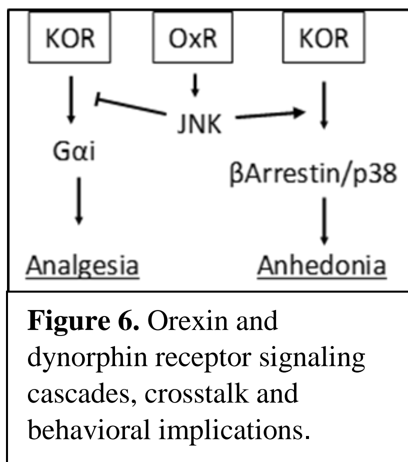


Figure 6. Orexin and dynorphin receptor signaling cascades, crosstalk and behavioral implications.

In vitro work by Patricia McDonald and colleagues has recently demonstrated that orexin 1 receptors modulate the function of KORs through a JNK-dependent pathway **Figure 6**. (Robinson & McDonald, 2015). Western blots revealed that KOR activation increased p38 and β-arrestin signaling and decreased Gαi signaling after OxR activation; potentially leading to KOR-mediated reduction in anhedonia and anxiety. Earlier work demonstrates that dynorphin alone activates Gαi signaling preferentially over p38 signaling (Bruchas, 2010). There are significant gaps in the literature as no behavioral studies have been

conducted to link orexin and dynorphin second messenger signaling cross-talk to cocaine reward. This work is important for parsing another layer of complexity, and illustrates the orexin-dynorphin system is more complicated than a simple “stop” and “go” signal.

CHAPTER 3

ROLE OREXIN AND DYNORPHIN IN DRUG REWARD AND WITHDRAWAL

Importance of Orexin and Dynorphin in motivated behavior

As noted above, recent work has begun to examine the relationship between peptide stoichiometric activity and affective behavior. Previous literature demonstrates decreased peptide levels of orexin and dynorphin within the hypothalamus correlated with decreased sexual pursuit, latency to immobility in forced swim, and increased time in frozen crouched position after chronic social defeat. (Lutter et al., 2008; Nocjar et al., 2012). This presents implications for the peptides in stress depression and other psychiatric disorders such as drug abuse. Although there are a number of brain region specific outcomes following OxR and KOR activation, orexin generally contributes to reward and arousal while increased KOR activity is hypothesized to increase the activity of the hypothalamic-pituitary-adrenal stress axis and contribute to negative affective states (Wee & Koob, 2010). Because drug abuse is driven by the euphoria of drug reward and the desire to avoid negative withdrawal symptoms, this chapter will focus on different behavioral models of cocaine seeking and reward as well as stress-induced reinstatements and anhedonia (Koob, 1996, 2009).

Preclinical studies have used various behavioral paradigms to model cocaine addiction. The following tables highlight the use of rodent models to define the relationships between orexin transmission and reinforcing properties of cocaine. In addition to models of reward and anhedonia, self-administration, conditioned place preference, and

behavioral sensitization paradigms have been used to demonstrate a role of orexin or KOR activity in cocaine seeking and motivation **See Table 1 and Table 2** respectively**.** More literature is available on orexin pharmacology and cocaine seeking, while the majority of dynorphin-related pharmacology focuses on reinstatement paradigms. This is reflected in Table 1 & 2 as well as in our hypothesis that orexin is more prevalent in drug taking and reward, while dynorphin is involved in the negative hedonic states during drug abstinence.

Orexin and Dynorphin in Drug Abuse

Self-Administration/CPP (Table 1 &2): Pharmacologically inhibiting OxRs during drug use may reduce acute reward and reduce drug seeking. Early success decreasing cocaine reinstatement laid the foundation for further studies on the role of orexin and drug seeking (Harris, 2005). Orexin activity increases cocaine self-administration while pharmacologically decreasing activity decreases motivation to self-administer cocaine as seen with progressive ratio self-administration tasks (Brodnik, Bernstein, Prince, & España, 2015; Gentile, Simmons, Barker, et al., 2017). To further explore relevant circuitry, orexin peptide was infused into the VTA of rodents immediately preceding self-administration and successfully increased cocaine infusions and breakpoints (España et al., 2010). The effects of clinically available dual orexin receptor antagonist at decreasing cocaine taking and subjective reward will be addressed in chapter 5 of this dissertation (Gentile, Simmons, Barker, et al., 2017). The body of work on orexin receptor antagonists (systemically and in a site directed manner) decreasing drug seeking

highlights the importance of orexin in the mesolimbic dopamine pathways in contributing to drug abuse.

Other self-administration models seek to model clinical drug relapse using reinstatement paradigm. Orexin 1 receptor antagonist, SB334867, has established a role in reducing many reinstatement models of cocaine abuse. SB334867 blocks stress-induced, cue-induced and even cocaine-induced reinstatement (Martin-Fardon & Weiss, 2014; R. J. Smith, See, & Aston-Jones, 2009; L. Zhou et al., 2012). Similar to cocaine itself, orexin-A administered into the VTA causes animals to reinstate self-administration of cocaine after responses had previously been extinguished (Harris, Wimmer, & Aston-Jones, 2005; Wang, You, & Wise, 2009). Reinstatement models expand the role orexin plays not just in cocaine reward seeking but also in stress and memory circuitry that contribute to reinstatement.

Following data demonstrating an increase in orexin producing neuron activity during morphine conditioning and reinstatement, Aston-Jones and colleagues showed that intra VTA injections of orexin reinstated an extinguished place preference for morphine. The ability of orexin peptide directly injected into the VTA to produce drug seeking behaviors and reinstates conditioned place preference compliments self-administration studies and further strengthens the role of orexin activity in the reinforcing properties of drugs of abuse (Harris et al., 2005; Lungwitz et al., 2012; Mahler, Smith, & Aston-Jones, 2013; Wang et al., 2009). To examine neuromodulatory changes that orexin activity may be responsible for; Borgland used electrophysiology and an orexin peptide injection into the

VTA to demonstrate that orexin is necessary for the synaptic plasticity that is required for locomotor sensitization to cocaine (Borgland et al., 2006).

Part of the additional circuitry can be explored in work demonstrating region specific activities of orexin receptors in the BNST. Orexin A peptide injection directly into the BNST decreases social interactions and time spent on the open arms of an elevated plus maze – indicative of an anxiogenic phenotype (Lungwitz et al., 2012). OxR1 signaling in the BNST enhanced alcohol seeking in rats (Ubaldi et al., 2016). These results may partially explain the roles of orexin receptors outside of the VTA in the negative hedonics of drug abuse.

Evidence suggests strong influences from dynorphin and the KOR in underlying the negative affective behavioral phenotype of addiction (Shippenberg, Zapata, & Chefer, 2007). Antagonism of the KOR system has been shown to help alleviate depression, anhedonia and cocaine seeking in both human and rodent populations (Chartoff et al., 2012; E. Ehrich et al., 2015; Mooney et al., 2013). Pharmacologically decreasing KOR activity can reduce reinstatement of cocaine seeking after acute stressors such as the force swim test, foot shock and food deprivation (Graziane, Polter, Briand, Pierce, & Kauer, 2013; Polter et al., 2014). Long acting KOR antagonists such as NorBNI decrease cocaine-induced conditioned place preferences (CPP) after acute stressors but generally not after a drug prime (Jackson, McLaughlin, Carroll, & Damaj, 2013; McLaughlin, Marton-Popovici, & Chavkin, 2003). The ability of KOR antagonists to decrease stressful events from reinstating CPP or self-administration while unable to decrease cocaine-induced cues from reinstating CPP or self-administration suggests a strong role

for KOR activity in the ‘dark side’ of drug abuse and little in the acute reinforcement (Bruchas, Land, & Chavkin, 2010). Elegant studies altering expression of KOR on VTA dopamine neurons suggest that U50,488 conditioned place aversion in mice is due to KOR activity within the mesolimbic reward pathway (Chefer et al., 2013)

Table 1. Select Findings of Orexin Pharmacology on Behaviors Associated with Rodent Cocaine Self-Administration and Reinstatement of Cocaine Seeking

Paradigm	**Cocaine Dose (mg/kg/inf)**	**Dependent Measure**	**Pretreatment**		**Effect Relative to Vehicle**	**Reference(s)**
			Compound	*Dose*		
			Self-administration			
PR	0.50	Breakpoint	SB-334867	10.0 mg/kg	↓**	Borgland et al. (2009)
DT	1.50	Infusions (per 6 h)	SB-334867	7.5 mg/kg	↓**	España et al. (2010)
				15.0 mg/kg	↓**	
				30.0 mg/kg	↓**	
PR	0.75	Breakpoint		7.5 mg/kg	↓*	
				15.0 mg/kg	↓**	
				30.0 mg/kg	↓**	
				10.0 nmol (intra-VTA, unilateral)	–	
				10.0 nmol (intra-VTA, bilateral)	↓**	
FR 1	0.75	Infusions (per 1 h)	Orexin-A	0.5 nmol (icv, unilateral)	–	España et al. (2011)
	1.50	Infusions (per 6 h)			↑*[a]	
PR	0.75	Breakpoint		0.5 nmol (intra-VTA, bilateral)	↑*	
VR 1-3	0.50	# of infusions (Day 1)	SB-334867	10.0 mg/kg	–	Hutcheson et al. (2011)
				30.0 mg/kg	↓**	
		# of infusions (Day 4)		10.0 mg/kg	↓**	
				30.0 mg/kg	↓**	
FR 5	0.30	# of infusions	SB-334867	3.0 µg (intra-VTA, bilateral)	↓***	Muschamp et al. (2014)
PR	0.75	Breakpoint	SB-334867	7.5 mg/kg	–	Prince et al. (2015); Brodnik et al. (2015)
				15.0 mg/kg	↓*	
				30.0 mg/kg	↓*	
			4PT	7.5 mg/kg	–	
				15.0 mg/kg	–	
				30.0 mg/kg	–	
			Almorexant	25.0 mg/kg	–	
				50.0 mg/kg	↓*	
				100.0 mg/kg	↓*	

Table 1, continued.

Paradigm	Cocaine Dose (mg/kg/inf)	Dependent Measure	Pretreatment		Effect Relative to Vehicle	Reference
			Compound	Dose		
Reinstatement						
Stress-induced reinstatement	N/A	Lever presses (per 2 h)	SB-334867	15 mg/kg	–	Boutrel et al. (2005)
				30 mg/kg	↓*	
Cued reinstatement	N/A	Lever presses (per 2 h)	SB-334867	10 mg/kg	–	Smith et al. (2009, 2010)
				20 mg/kg	↓*	
				30 mg/kg	↓**	
			4PT	10 mg/kg	–	
				30 mg/kg	–	
Cued reinstatement	N/A	Lever presses	SB-334867	10.0 mg/kg	–	Zhou et al. (2012)[b]
				20.0 mg/kg	–	
				30.0 mg/kg	↓*	
Stress-induced reinstatement					↓*	
Cocaine-induced reinstatement					–	
Cocaine 1 cued reinstatement					↓*	
Cued reinstatement	N/A	Lever presses (per 2 h)	SB-334867	1.0 mmol (intra-	↓***	Mahler et al. (2013)
Cued reinstatement	N/A	Lever presses (per 1 h)	SB-334867	1.0 mg/kg	–	Martin-Fardon and Weiss (2014)
				3.0 mg/kg	↓***	
				10.0 mg/kg	↓***	
Cued reinstatement	N/A	Demand elasticity(α)	SB-334867	30 mg/kg	↑*	Bentzley and Aston-Jones (2015)
		Consumption (Q_1)			–	

*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001.

Table 2 Select Findings of Dynorphin Pharmacology on Behaviors Associated with Rodent Addiction-like behaviors.

Model	Paradigm	Treatment	Drug	Result	Reference
	(Reinstatement)	**(Antagonists)**			
Self-Ad	Food deprived Stress	norBNI	Heroin	↓ lever press	(Sedki et al., 2015)
Self-Ad	Footshock induced	RTI-194	Cocaine	↓ lever press	(Beardsley, Pollard, Howard, & Carroll, 2010)
Self-Ad	Footshock induced	JDTic	Cocaine	↓ lever press	(Beardsley, Howard, Shelton, & Carroll, 2005)
Self-Ad	Drug induced	JDTic	Cocaine	-	(Beardsley et al., 2005)
CPP	Forced Swim Stress Induced	norBNI	Cocaine	↓	(McLaughlin et al., 2003)
CPP	Forced Swim Stress Induced	Zyklophin	Cocaine	↓	(Aldrich, Patkar, & McLaughlin, 2009)
CPP	Social Defeat (potentiation)	NorBNI	Cocaine	↓ time	(McLaughlin, Land, Li, Pintar, & Chavkin, 2006)
CPP	Footshock induced	NorBNI	Nicotine	↓	(Jackson et al., 2013)
CPP	Drug primed	NorBNI	Nicotine	-	(Jackson et al., 2013)
Sensitization	Food deprivation	NorBNI	Cocaine	↓ sens.	(Allen, Zhou, & Leri, 2013)
		(Agonists)			
Self-Ad	Treatment induced	spiradoline	Cocaine	↑	(Valdez, Platt, Rowlett, Ruedi-Bettschen, & Spealman, 2007)
CPP	Treatment induced	U50,488	Cocaine	↑	(Al-Hasani et al., 2013)

Intracranial Self-Stimulation (Table 3): Using electrical stimulation of the medial forebrain bundle during intracranial self-stimulation (ICSS), the effects of orexin, dynorphin and drugs of abuse on reward thresholds has been partially elucidated. A great deal of work has utilized ICSS as a behavioral model of reward and anhedonia. How an animal's responses to the changes in electrical stimulation can be interpreted as increasing positive or negative reinforcement or affect (R. A. Wise, 1996). Psychostimulant administration has long been shown to increase dopamine release in the mesolimbic reward pathway and decrease brain reward thresholds. Conversely, acute withdrawal has been shown to decrease dopamine release and increase brain reward thresholds (interpreted as anhedonia/withdrawal) (A. M. Barr, Markou, & Phillips, 2002; Shippenberg et al., 2007; Weiss et al., 1992; R. A. Wise & Munn, 1995). **See Table 3** for ICSS pharmacology review.

Systemic blockade of orexin 1 receptors with SB33867 attenuated cocaine-induced reward in ICSS. This suggested that orexin contributed to the regulation and reward-enhancing effects of cocaine (Hollander, Pham, Fowler, & Kenny, 2012). However, the same treatment, SB33867, also produced anhedonia in animals that were given the OxR antagonist alone (Muschamp et al., 2014). Furthermore, studies on the role of orexin in reward processing have produced mixed findings. Boutrel et al. demonstrated increased brain reward thresholds (anhedonia) following intracerebroventricular orexin-A administration, possibly due to activating receptor populations that might not typically be activated together (Boutrel et al., 2005). It is possible the anhedonic effects of ICV orexin arise from the BNST or amygdala, and occlude orexin in the VTA. Further work needs

to be conducted to examine the different roles of orexin receptors in specific brain regions on brain reward thresholds.

In an attempt to mimic the effect of dynorphin kappa activation by U69,593 has been demonstrated to be responsible for negative affect seen in rodent models of ICSS. However, U69,593 can be used to block the cocaine-induced reward in the same paradigm (Chefer et al., 2013; Tomasiewicz, Todtenkopf, Chartoff, Cohen, & Carlezon, 2008). Together this suggests that KOR activity elevates rewards thresholds both in the context of reward or from baseline. Conversely, KOR antagonists attenuated cocaine withdrawal-induced anhedonia but produced no rewarding properties alone (Chartoff et al., 2012). KOR antagonists are promising, as they can alleviate negative affect during psychostimulant abstinence, but possess little abuse potential.

Few studies have explored the regional distribution of orexin and dynorphin receptor activities together during drug use. Available ICSS data highlighting the roles of orexin activity in cocaine reward and KOR activity in withdrawal-induced anhedonia provides strong incentive to utilize orexin and dynorphin activity to alter bivalent mood states such as drug abuse.

Table 3. Select Findings of Orexin and dynorphin Pharmacology on ICSS Behaviors Associated with Rodent Drug Reward and Withdrawal

Reward Threshold taken during:	Treatment:	Reward Threshold Change from Baseline:	References
Control (saline)	Vehicle	Same as Baseline	
Control (saline)	Kappa Agonist	Elevated	(Tomasiewicz et al., 2008)
Control (saline)	Orexin Antagonist	Elevated	Gentile 2017 Submitted
Control (saline)	Kappa Antagonist	Same as Baseline	(Chartoff et al., 2012)
Drug Use	Vehicle	Decreased	(Hollander et al., 2012)
Drug Use	Kappa Agonist	Same as Baseline	(Tomasiewicz et al., 2008)
Drug Use	Orexin Antagonist	Same as Baseline	(Hollander et al., 2012)
Drug Withdrawal (20 hours)	Vehicle	Elevated	(Chartoff et al., 2012)
Drug Withdrawal (20 hours)	Kappa antagonist	Same as Baseline	(Chartoff et al., 2012)
Drug Withdrawal (20 hours)	Orexin antagonist	Elevated	Gentile 2017 Submitted
Control (saline)	Kappa Antagonist + Orexin Antagonist	Same as Baseline	(Muschamp et al., 2014)
Drug Use	Kappa Antagonist + Orexin Antagonist	Same as Baseline	Gentile 2017 Submitted
Drug Withdrawal (20 hours)	Kappa Antagonist + Orexin Antagonist	Same as Baseline	Gentile 2017 Submitted

Mesolimbic Dopamine Measurements: The behavioral effect of orexin and dynorphin on drug seeking, reward and withdrawal behavior are thought to arise in part from actions of

the peptides on DA transmission in structures like the VTA and NAc. Orexin-A plays in important role in regulating the mesolimbic dopamine pathway, which is a major circuit involved in processing reward from drugs of abuse. Injections of orexin-A into the (VTA) alone and in concert with psychostimulants, increase extracellular levels of transmitters associated with reward such as dopamine and glutamate (Calipari & España, 2012; Wang et al., 2009). Consistent with earlier microdialysis studies (Narita et al., 2006) fast scan cyclic voltammetry (FSCV) has demonstrated an increase in NAc dopamine during cocaine self-administration and significantly decreased dopamine levels when animals are pretreated with an orexin antagonist (Prince, Rau, Yorgason, & España, 2015). Orexin peptide increases phasic dopamine in the NAc shell while orexin antagonist, SB334867, blocks this increase (Patyal, Woo, & Borgland, 2012). Orexin activity on the VTA→NAc pathway plays an important role in dopamine reward processing (España et al., 2010; Prince, Rau, Yorgason, & España, 2015). This role was demonstrated after orexin knockout mice possess aberrant condition place preference and dopamine signaling (Shaw et al., 2016). Although it should be noted that seemingly opposite effects have been found with varying region selectivity. An intraventricular injection of orexin A has been shown to increase plasma ACTH and activate the HPA axis (Al-Barazanji, Wilson, Baker, Jessop, & Harbuz, 2001). Together, this work highlights the role of dopamine levels in the VTA and NAc and provides a molecular mechanism in which orexin activity contributes to the aforementioned self-administration, CPP and ICSS behaviors.

Early *in vivo* microdialysis experiments originally indicated that KOR agonists inhibited dopamine release in the NAc (Di Chiara & Imperato, 1988). Similarly, KOR agonist,

U69,593 decreases dopamine release in NAc tissue slices (Ehrich et al., 2015). Pharmacologically activating KORs reduces dopamine transmission that's associated with behavioral activation and reinforcement within the mesolimbic dopamine pathway and decreases acute cocaine reward (Bruchas et al., 2010; Gerfen, McGinty, & Young, 1991; Zhang, Butelman, Schlussman, Ho, & Kreek, 2004). KOR stimulation in the VTA imposes inhibitory effects demonstrated *ex vivo* by a decrease in dopaminergic cell firing (Margolis et al., 2003; Polter et al., 2017). This has been further strengthened with techniques such as FSCV that possess higher spatial and temporal resolution. KOR conditional knockouts in dopaminergic neurons block U69,593 inhibition of dopamine signaling (Ehrich et al., 2015) while activation of KOR by salvinorin A elicits depressive-like effects due largely to decreases in phasic dopamine release in the NAc (Ebner, Roitman, Potter, Rachlin, & Chartoff, 2010).

Kappa activation via U50,488 administration mimics behavioral and dopaminergic characteristics of chronic ethanol exposed animals. Chronic ethanol exposure and U50,488 treatment caused increased anxiety and decreased accumbal dopamine release (Rose et al., 2016). Dynorphin activity in discrete brain regions such as the caudate putamen has been shown to decrease cocaine evoked NAc dopamine and attenuate cocaine-induced locomotion and conditioned place preference (Zhang et al., 2004). Further contributing to behavior, prepro-dynorphin mRNA expression increases after acute cocaine administration and an increase in dynorphin has been noted to induce reinstatement of drug-seeking behavior. (Spangler et al., 1997; Y. Zhou, Leri, Grella, Aldrich, & Kreek, 2013). KORs on dopamine cell bodies of the mesolimbic pathway are in part responsible for these aversion-driven behaviors (Chefer et al., 2013). Antagonists

of the KOR help improve aberrant mood states by blocking the signaling and hypodopaminergic effects of the endogenous ligand dynorphin (Bruchas et al., 2010). Work with KOR in the mesolimbic dopamine pathway complements the previous orexin section and demonstrates the opposing effects of OxR and KOR on evoked dopamine release in the ventral striatum.

Together the available literature highlights a strong role for orexin in the reinforcing properties of cocaine, while KOR activity is heavily involved in the stress induced aspects of cocaine relapse. Site-directed pharmacology suggests the general rewarding and antirewarding effects of orexin and dynorphin occurs, at least in part, in the VTA and mesolimbic dopamine pathway. Microdialysis and FSCV literature supports that much of the reinforcing effects and behavior is orexin and dopamine driven while the KOR activity seems to contribute to hypodopaminergic states that result is anhedonia. Overall, this chapter supports the idea the stoichiometric ratios of orexin-dynorphin activity could be altered in reward centers of the brain to attenuate maladaptive behaviors common to cocaine abuse, such as drug seeking and reinstatement.

Suvorexant and LY2456302 as Pharmacologic Tools

Throughout this dissertation suvorexant and LY2456302 are used as the primary pharmacological antagonists for the orexin and kappa opioid receptors, respectively, for a number of reasons. Suvorexant is a dual orexin receptor antagonist with high affinity for human hOxR1 (.055nM) and hOxR2 (0.35nM), (Winrow et al., 2011). Suvorexant was approved by the Food and Drug Administration (FDA) on August 13, 2014 as Belsomra

for the indication of insomnia. Having already passed the arduous FDA phase I and II clinical trials for safety and efficacy suvorexant provides a novel and therapeutically relevant pharmacological tool that could theoretically be re-purposed for additional indications such as cocaine reducing cocaine cravings or cocaine use (NCT02785406. https://ClinicalTrials.gov/show/NCT02785406: Last updated: May 24, 2016).

LY2456302 was developed by Eli Lilly as a short-acting kappa opioid receptor antagonist. Unlike commonly used KOR antagonists like JD-Tic and NorBNI, which have duration of action that can last months, LY2456302 has an approximately 48-hour half-life (Munro et al., 2012; Rorick-Kehn et al., 2014). The significant reduction in duration of action allows us to conduct within-subjects designs, dose responses, and appropriate Latin Squares. LY2456302 is also selective for KOR over Mu and Delta receptors up to 30 mg/kg orally in male Sprague-Dawley rats. In February 2015 Cerecor Inc. acquired rights to develop and commercialize LY2456302 as CERC-501. Currently LY2456302 (CERC-501) is in phase II clinical trials for treatment-resistant depression as well as phase II trials for smoking cessation (Naganawa et al., 2016). Together the pharmacokinetics and translational utility makes LY2456302 and suvorexant valuable pharmacologic tools.

SPECIFIC AIMS

The dissertation sought to examine the role of the orexin-dynorphin system in a systemic and brain structure-specific manner during the acute reinforcing effects following cocaine administration as well as during aversive negative states. This dissertation further delineated how orexin-dynorphin transmission regulates cocaine-associated changes in mesolimbic (i.e. from VTA→NAc) dopamine function. Finally, this dissertation determined potential functional contributions of how OxR/KOR mediates NAc dopamine *in vitro* and examined how this impacts cocaine-associated positive affect. Collectively, this disseration refined our understanding of the mechanisms that coordinate orexin-dynorphin interactions *in vivo,* and explored potential utility of blocking co-transmission in specific structures.

Aim 1: Determine the role of orexin and kappa opioid receptors in altering brain reward thresholds following chronic cocaine use and during withdrawal

Intracranial self-stimulation, self-administration and 5-CSRTT were be used to measure the effects of systemic orexin or dynorphin receptor activity on baseline mood states as well as cocaine reward and cocaine in rodent models of drug related behaviors. *We hypothesized that OxR signaling underlies reward following acute cocaine, and that KOR signaling contributes towards anhedonia. OxR antagonism (via suvorexant) will normalize decreases in reward thresholds following acute cocaine, whereas KOR antagonism (via LY-2456302) will reduce suvorexant-associated elevations in reward thresholds.*

Aim 2: Determine structure-specific roles of VTA Orexin-Dynorphin in mediating brain reward thresholds following acute cocaine use.

Intracranial self-stimulation and 5-CSRTT were be used to measure the effects of altered orexin or dynorphin transmission within the VTA on baseline mood states as well as cocaine reward and impulsivity in animals. *We hypothesized that OxR signaling underlies reward following acute cocaine, and that KOR signaling contributes towards anhedonia. Intra VTA OxR antagonism (via suvorexant) will normalize decreases in reward thresholds following acute cocaine, whereas KOR antagonism (via LY-2456302) will reduce suvorexant-associated elevations in reward thresholds.*

Aim 3: Examine effects of local Orexin-Dynorphin receptor activity on NAc DA in vivo following acute cocaine.

Using OxR and KOR antagonists as in Aim 1&2, this Aim measured electrically-evoked extracellular NAc DA levels via fast-scan cyclic voltammetry. Using this technique, we determined how local OxR and KOR modulate elevations in NAc DA following acute cocaine. *We hypothesized that OxR antagonist (suvorexant) will diminish elevations in NAc DA following acute cocaine. Conversely, KOR antagonist (LY-2456302) will restore otherwise reduced NAc DA levels during anhedonia.*

CHAPTER 4

OREXIN ROLE IN DRUG-INDUCED IMPULSIVITY

Introduction to Orexin contributions to impulsive actions.

While the anatomy of orexin neurons suggests that orexin transmission is involved in impulsive behavior, only a few studies have addressed the question of orexin in this behavior, and a clear picture has not yet emerged (Boschen, Fadel, & Burk, 2009; Lambe et al., 2005; Muschamp et al., 2014). Defined as a tendency to engage in behaviors without forethought (Evenden, 1999), impulsivity is apparent across psychiatric diagnostic categories and can lead to poorer outcomes in patients with attention deficit hyperactive disorder (ADHD), bipolar disorder, and substance use disorders (de Wit, 2009; Kessler et al., 2014; Lopez-Torrecillas, Perales, Nieto-Ruiz, & Verdejo-Garcia, 2014; Swann, 2010; Vall et al., 2015). The putative relationship between motivated behavior and orexin transmission supports the idea that targeting orexin receptors for therapeutic intervention. The recent approval for clinical use of the OX_1R/OX_2R antagonist suvorexant in the treatment of insomnia raises the question of whether this compound may also be valuable in treating psychiatric disorders characterized by high levels of impulsivity (Khoo & Brown, 2014; Roecker, Cox, & Coleman, 2016; Scammell & Winrow, 2011). The present studies sought to assess the potential therapeutic utility of suvorexant as well as subtype-selective OXR antagonists in a rodent model of impulsivity, the five-choice serial reaction time (5-CSRTT) (Winstanley, Eagle, & Robbins, 2006). A previous study convincingly demonstrates significant attenuation of motor impulsivity following pre-treatment with a selective OX_1R antagonist (Muschamp et al., 2014). To gain insight into the functional neuroanatomy that may underlie effects

of OXR blockade on impulsive behaviors, we performed direct infusions of suvorexant into the VTA, a DA-rich principal target of orexin afferents (Fadel & Deutch, 2002; Peyron et al., 1998). In addition, we assessed how orexin receptor blockade altered Fos immunoreactivity within DA-producing neurons of VTA and further how suvorexant influenced orexin-and cocaine-evoked calcium transients within VTA neurons *in vitro*.

Methods and Materials utilized for chapter 4 experiments.

Animals. Adult male Sprague-Dawley rats (Charles River) were pair-housed and food-restricted to 85% of their free-fed bodyweight. All animals were acclimated to the vivarium for at least one week before beginning experiments. All experimental procedures were approved by Temple University's Institutional Animal Care and Use Committee.

Drugs. For all experiments, suvorexant (Selleckchem; Munich, Germany), SB334867, and TCS-OX2-29 (provided by Dr. Yanan Zhang, Research Triangle Institute, NC) were dissolved in dimethyl sulfoxide (DMSO) through ultrasonication, and administered at 0.3 mL/kg (i.p), or 200 nL when bilaterally injected into VTA (100 nL/hemisphere). Cocaine hydrochloride (Sigma Chemical Company, St. Louis, MO) was dissolved in 0.9% saline and administered at 1.0 mL/kg (i.p.). Orexin A was purchased from American Peptide Inc. (Sunnyvale, CA).

Surgical Procedure. General anesthesia was induced in rats and maintained with 2-5% isoflurane mixed in oxygen (flow rate: 1.5 L/min). Rats were implanted with bilateral stainless steel guide cannulae (22-gauge, 8.5 mm in length) into VTA [from bregma in mm: AP -5.4; ML ±2.1 at 10° angle; DV -6.8]. Stainless steel stylets (28-gauge, 8.5 mm in length) were used in guide cannulae to maintain their patency. When ready for VTA-

directed injections, internal cannulae (28-gauge, 9.5 mm in length) were used for injections (Plastic One, Roanoke, VA).

Experimental procedures.

Experiment 4.1 & 4.2: 5-Choice Serial Reaction Time Task (5-CSRTT).

Rats were trained in computer-controlled operant chambers housed inside ventilated,

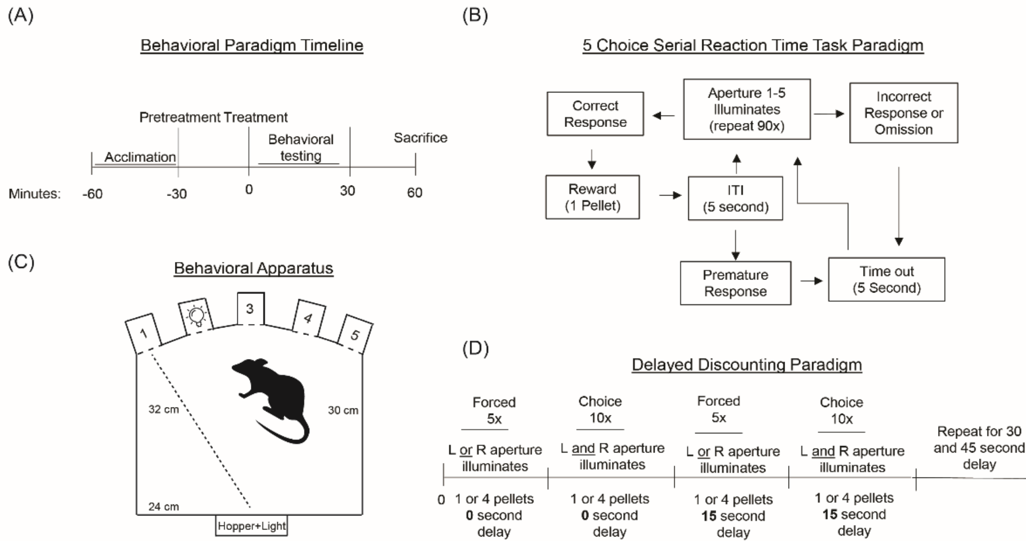


Figure 7. Behavioral testing designs (A, B, D) and apparatus (C) used in Experiments 1 and 2.

sound-attenuating cabinets (Med Associates; St. Albans, VT). The chambers were fitted with five internally-illuminated apertures monitored by infrared detectors. The opposite wall contained an illuminated food hopper equipped with an infrared detector (**Figure 7**). Rats were trained 7 d/wk for ninety trials or for thirty minutes and received either a sucrose pellet (45 mg; Bio-Serv; Flemington, NJ) for a correct response (poking illuminated aperture) or a “time-out” period in which the house light was turned off after an incorrect, premature, or omitted response. After rats could respond with greater than 70% correct responses and fewer than 15% responses omitted across four consecutive daily sessions, drug treatment sessions proceeded. For drug treatment sessions,

suvorexant or TCS-OX2-29 (0, 3, 10 or 30 mg/kg) was administered 30 minutes before testing and, in subsequent sessions, cocaine (0 or 3 mg/kg) was additionally administered 5 minutes before the start of 5-CSRTT testing. For direct site injections, suvorexant (3 μg/hemisphere) was administered via 28-gauge injector cannulae attached to Hamilton syringes (Model 7101, Hamilton) using an automatic infusion pump (PHD 2000, Harvard Apparatus) at a rate of 100 nL/min. To confirm injection site, tissue was harvested from rats after behavioral tests and tyrosine hydroxylase (TH) was detected by fluorescent labeling and used to verify targeting within the DA-producing population of VTA

Experiment 4.3: DA VTA immunohistochemistry.

To assess effects of OXR blockade on neuronal activation of DA-producing neurons in VTA, a behaviorally and pharmacologically naïve cohort of rats were administered suvorexant (0 or 30 mg/kg, i.p.) 30 minutes prior to cocaine (0 or 3 mg/kg) in their home cage. Ninety minutes following cocaine, rats were deeply anesthetized with phenytoin phenobarbital cocktail (120 mg/kg, i.p.) and perfused with ~100 mL cold phosphate-buffered saline (PBS; 0.1M, pH 7.4) followed by ~300 mL 4% paraformaldehyde (PFA). Brains were post-fixed for in 4° C PFA for 24 hours followed by 72 hours in 4° C 30% sucrose and finally flash-frozen in 2-methyl butane chilled on dry ice. Brains were subsequently sectioned using a cryostat, and coronal sections (40 μm thickness), containing the entire VTA (from bregma: AP -5.0 mm to -6.5 mm; *Brain Maps III* atlas available online: http://larrywswanson.com/) were collected.

For immunolabeling, tissue sections were washed in 0.1 M PBS, blocked in 5% donkey serum in PBS with 0.3% Triton X-100 (PBS+) and incubated in primary antibody solution for 72 hours at 4° C (rabbit anti-cFos [1:1,000; SC-52, Santa Cruz] in 1.5%

donkey serum in PBS+). Sections were then incubated in secondary antibody solution for 3 hours (donkey anti-rabbit 555 [1:400; AlexaFluor] in 1.5% donkey serum in PBS+). Tissue sections underwent repeated immunolabeling procedures as above using rabbit anti-tyrosine hydroxylase (TH) [1:1,000; AB-152, Millipore] primary antibody and donkey anti-rabbit 488 [1:400; AlexaFluor] secondary antibody.

Tissue was mounted on glass slides, dried for 24 hours and coverslipped with antifade reagent [Life Technologies]. Fluorescent images were taken with Coolsnap EZ CCD camera (Photometrics; Tucson, AZ) connected to an Eclipse 80*i* microscope (Nikon Instruments, Inc.; Melville, NY). Capture parameters used to acquire fluorescent images were identical across all subjects and quantified using ImageJ software (National Institutes of Health; Bethesda, MD).

All TH-immunoreactive neurons and Fos-immunoreactive nuclei that were visible in every third tissue section collected were counted by an experimenter blind to treatment conditions using an Eclipse 80*i* upright fluorescent microscope (Nikon) under 10x objective. Three sections were averaged together for counting. The percentage of Fos^+-TH^+ neurons was calculated by: $[(TH^+\text{-}Fos^+) / (\text{total } TH^+) * 100]$.

Experiment 4.4: Calcium Imaging

Neuronal cell culture. Neurons from the VTA were dissociated from neonatal (1-2 day old) Sprague Dawley rats (Ace Animal Inc., Boyertown, PA) of both sexes as previously described. Newborn rats were decapitated, the brains removed surgically and immersed in ice-cold Hanks balanced salt solution (HBSS) (Mediatech; Herndon, VA). The VTA was subjected to enzymatic digestion (papain, 37° C) followed by mechanical trituration

in presence of total medium – Neurobasal A (Invitrogen; Carlsbad, CA) containing 1% GlutaMax (Invitrogen), 2% penicillin-streptomycin-amphotericin B solution (Mediatech) and 10% fetal bovine serum. Cells were cultured on round 25 mm glass coverslips coated with poly-L-lysine (Sigma) in six-well plates. Cultures were maintained at 37° C in a humidified atmosphere with 5% CO_2. The mitotic inhibitor cytosine β-arabinofuranoside (1 μM) (Sigma) was added to the culture the third day to inhibit glial cell proliferation. Cells were used after 5 days in culture.

Calcium imaging. $[Ca^{2+}]_i$ was measured as previously described (Arslan et al., 2000; Barr et al., 2015). Cells were incubated with 5 μM fura-2 AM (Invitrogen) in HBSS at room temperature for 45 min in the dark, washed three times with dye-free HBSS, and then incubated for another 45 min to allow for complete de-esterification of the dye. Coverslips (25 mm diameter) were subsequently mounted in an open bath chamber (RP-40LP, Warner Instruments; Hamden, CT) on the stage of an inverted microscope Nikon Eclipse TiE (Nikon Inc.; Melville, NY) equipped with a Perfect Focus System and a Photometrics CoolSnap HQ2 CCD camera (Photometrics; Tucson, AZ). During the experiments, the Perfect Focus System was activated. Fura-2 AM fluorescence (emission = 510 nm), following alternate excitation at 340 and 380 nm, was acquired at a frequency of 0.25 Hz. Images were acquired and analyzed using NIS-Elements AR 3.1 software (Nikon Inc.). After appropriate calibration with ionomycin and $CaCl_2$, and Ca^{2+} free and EGTA, respectively, the ratio of the fluorescence signals (340/380 nm) was converted to Ca^{2+} concentrations. In Ca^{2+}-free experiments, $CaCl_2$ was omitted. Calcium transients from VTA neurons were measured following application of orexin (10 nM), orexin with cocaine (10 μM) in 0.9 % saline, and orexin with cocaine and suvorexant (1 μM).

Statistical analyses. For 5-CSRTT, one-way repeated measures ANOVA with Bonferroni-corrected contrasts against vehicle-treated control groups were used to compare number of premature responses, omissions, latency to retrieve sucrose reward, and accuracy [# Correct / (# Correct + # Incorrect)] as measures of impulsivity. Due to the repeated measures design the authors did not assume sphericity and used the Geisser-Greenhouse correction. For delay discounting, a two-way ANOVA with Bonferroni-corrected post-hoc tests were used to compare the number of large rewards chosen at each delay time block with multiple doses of suvorexant or TCS-OX-229. For immunohistochemical analyses, a one-way ANOVA was used to examine mean estimated proportion of TH^+/Fos^+ cells as well as total Fos^+ cells between groups. Calcium imaging data were analyzed using Bonferroni-corrected ANOVA comparing testing groups of experimental interest (orexin only, cocaine + orexin + suvorexant) against the orexin + cocaine group.

Results from chapter 4 experiments.

*Experiment 4.1A: Suvorexant attenuates motor impulsivity (**Figure 8**).*

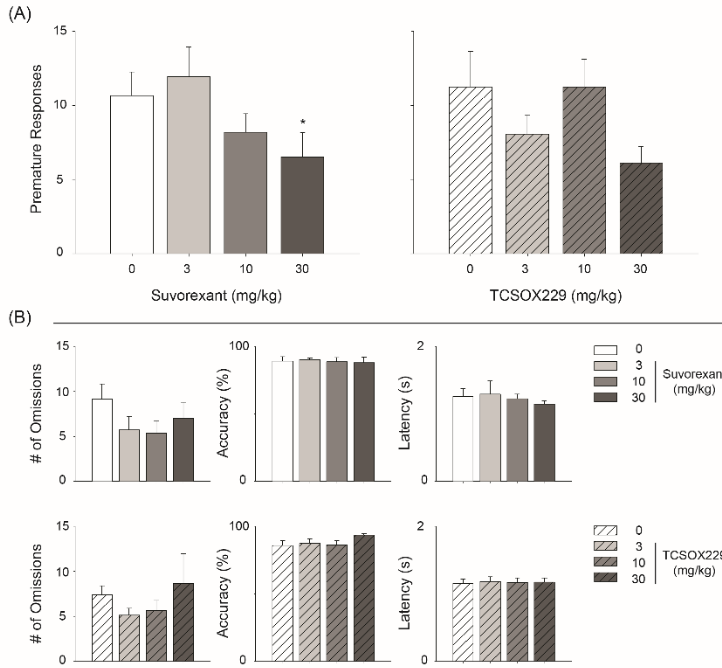
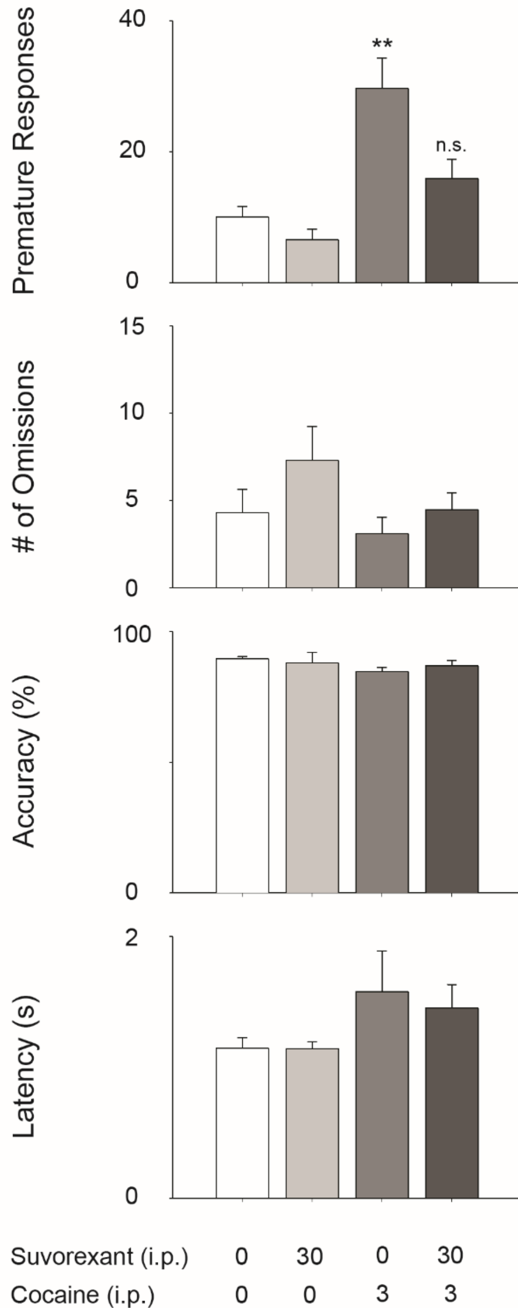


Figure 8. (A) Effects of suvorexant and TCS-OX2-29 on number of premature responses in 5-CSRTT. (B) Effects of suvorexant and TCS-OX2-29 on number of omissions, % accuracy and latency to retrieve sucrose reward are also depicted. Data are mean ± S.E.M. (n = 15-16 rats per group). * p < 0.05 compared to the respective 0 mg/kg group.

Results from the present study revealed a significant main effect of suvorexant on premature responses in 5-CSRTT [$F(2.8, 39.9) = 2.90$, $p < 0.05$]. Planned contrasts against the vehicle-treated control group found that the 30 mg/kg suvorexant was effective in reducing premature responses [$p < 0.05$]. In a separate cohort of rats trained for 5 d/wk, a significant effect of suvorexant (30 mg/kg, i.p.) on reducing premature responses relative to vehicle pre-treatment was additionally found. No significant effect of TCS-OX2-29 on premature responses [$F(2.0, 29.2) = 2.38$, n.s.], omissions [$F(2.1, 28.8) = 2.68$, n.s.], latency to retrieve sucrose reward [$F(1.7, 24.4) = 1.42$, n.s.] or accuracy [$F(1.7, 22.8) = 0.29$, n.s.] were found. These results support the conclusion that suvorexant has a selective effect on motor impulsivity without interfering with the motivation to consume sucrose rewards.

*Experiment 4.2: Suvorexant attenuates cocaine-induced motor impulsivity by systemic and intra-VTA injection (**Figures 9,10**).*



A main effect of drug group (vehicle-saline, suvorexant-saline, vehicle-cocaine, suvorexant-cocaine) on premature responses was found [$F(1.67, 23.4) = 10.88, p < 0.001$]. Planned contrasts against vehicle-saline control group revealed that cocaine increased premature responding [$p < 0.01$], but that suvorexant pretreatment normalized cocaine-elicited premature responses to control levels. Neither cocaine nor suvorexant, alone or in combination, had significant effect on omissions [F (3, 55) = 1.52, n.s.], latency to retrieve sucrose reward [$F(1.9, 26.6) = 1.02$, n.s.] or accuracy [$F(1.7, 23.42) = 0.67$, n.s.].

Figure 9. Effects of suvorexant on cocaine-evoked premature responses, number of omissions, % accuracy and latency to retrieve sucrose reward in 5-CSRTT. (n = 15 rats per group). Data are mean ± S.E.M. ** p < 0.01 compared to Veh-Sal group.

A main effect of drug group on premature responses was additionally observed when suvorexant (3 µg/hemisphere) was infused into bilateral VTA [F (1.8, 11.1) = 9.99, $p < 0.01$]. Post-hoc tests revealed that cocaine increased premature responding relative to the vehicle-saline control group [$p < 0.05$], while suvorexant pretreatment normalized cocaine-elicited premature responses to control levels. Neither cocaine nor suvorexant, either alone or in combination, had any significant effect on omissions [F(1.9, 11.23) =

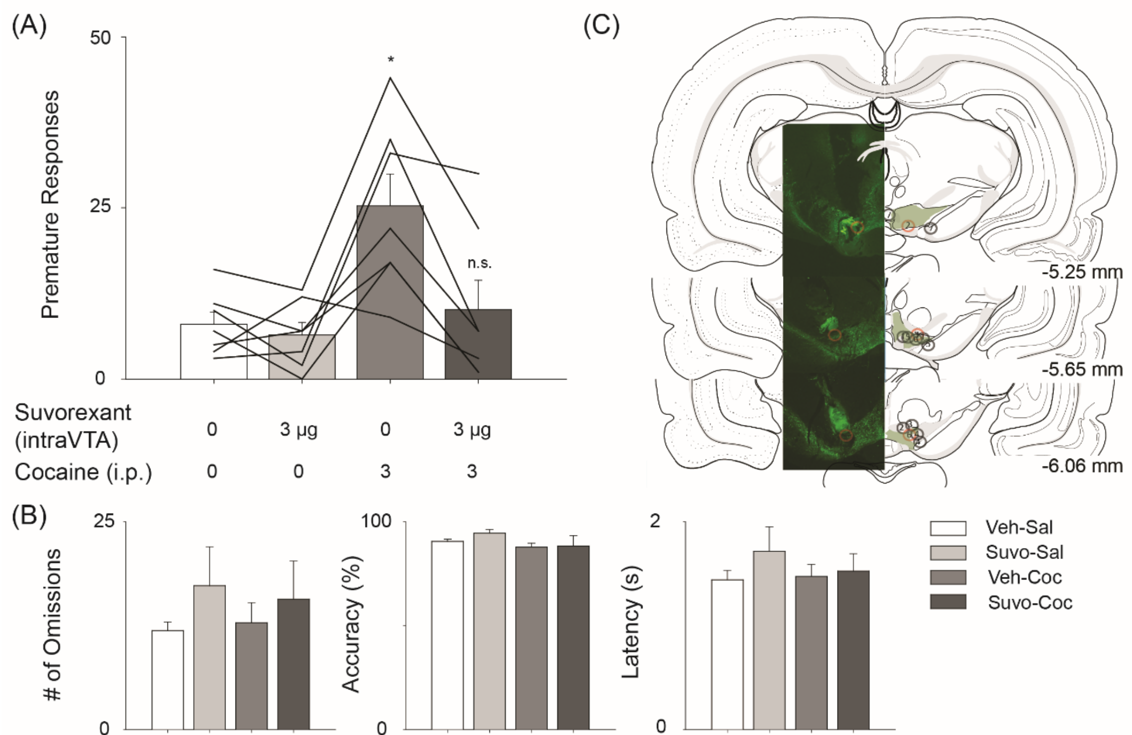


Figure 10. Effects of site-directed suvorexant in the VTA on (A) cocaine-evoked premature responses, (B) number of omissions, % accuracy and latency to retrieve sucrose reward in 5-CSRTT. (n=7 rats per group). (C) Atlas images of cannula placements. Cannula tracks of animals 1-7 are shown as circles within TH^+ neurons of the ventral tegmental area. Atlas images taken from Brain Maps III, available online: http://larrywswanson.com/?page_id=164. Solid lines showing individual animal responses across sessions. Data are mean ± S.E.M. * $p < 0.05$ compared to Vehicle-Saline group.

0.58, n.s.], latency to retrieve sucrose reward [$F(1.2, 6.95) = 0.76$, n.s.] or accuracy [$F(1.5, 9.13) = 1.61$, n.s.].

*Experiment 4.3: Neither cocaine nor suvorexant, alone or in combination, alter Fos-immunoreactivity within DA-producing VTA neurons (**Figure 11**).*

A one-way ANOVA examining TH^+-Fos^+ neurons in VTA revealed no effect of drug group (vehicle-saline, suvorexant-saline, vehicle-cocaine, suvorexant-cocaine) [$F(3, 40) = 0.76$, $p > 0.05$]. Additionally, no differences in total Fos^+ neurons in VTA were observed between drug groups [$F(3, 40) = 0.62$, $p > 0.05$]. Finally, no difference in Fos^+ nuclei of non-TH cytosols was found.

*Experiment 4.4: Orexin A elevates intracellular calcium additively with cocaine in a suvorexant-sensitive manner within VTA neurons in vitro (**Figure 11**).*

Cocaine (10 μM) did not elicit an increase in $[Ca^{2+}]_i$ within primary VTA neurons *in vitro* [$\Delta[Ca^{2+}]_i = 9 \pm 0.6$ nM; area under curve (AUC) of Ca^{2+} response = 8.53 ± 0.7 nM]. Application of orexin A (10 nM) elevated $[Ca^{2+}]_i$ of primary VTA neurons by 381 ± 3.7 nM and AUC of 154.75 ± 2.9 nM. Combined administration of cocaine (10 μM) 10 min prior to orexin A produced a potentiated increase in $[Ca^{2+}]_i$ [$\Delta[Ca^{2+}]_i = 544 \pm 4.6$ nM; AUC = 304.3 ± 3.8 Nm, $p < 0.001$]. The effect of cocaine and orexin A was completely abrogated by pretreatment with suvorexant (1 μM, 10 min): $\Delta[Ca^{2+}]_i = 12 \pm 0.8$ nM and AUC = 29.24 ± 1.1 nM Amplitude: [$F(3, 20) = 3914$, $p < 0.001$]; AUC: [$F(3, 20) = 9655$, $p < 0.001$].

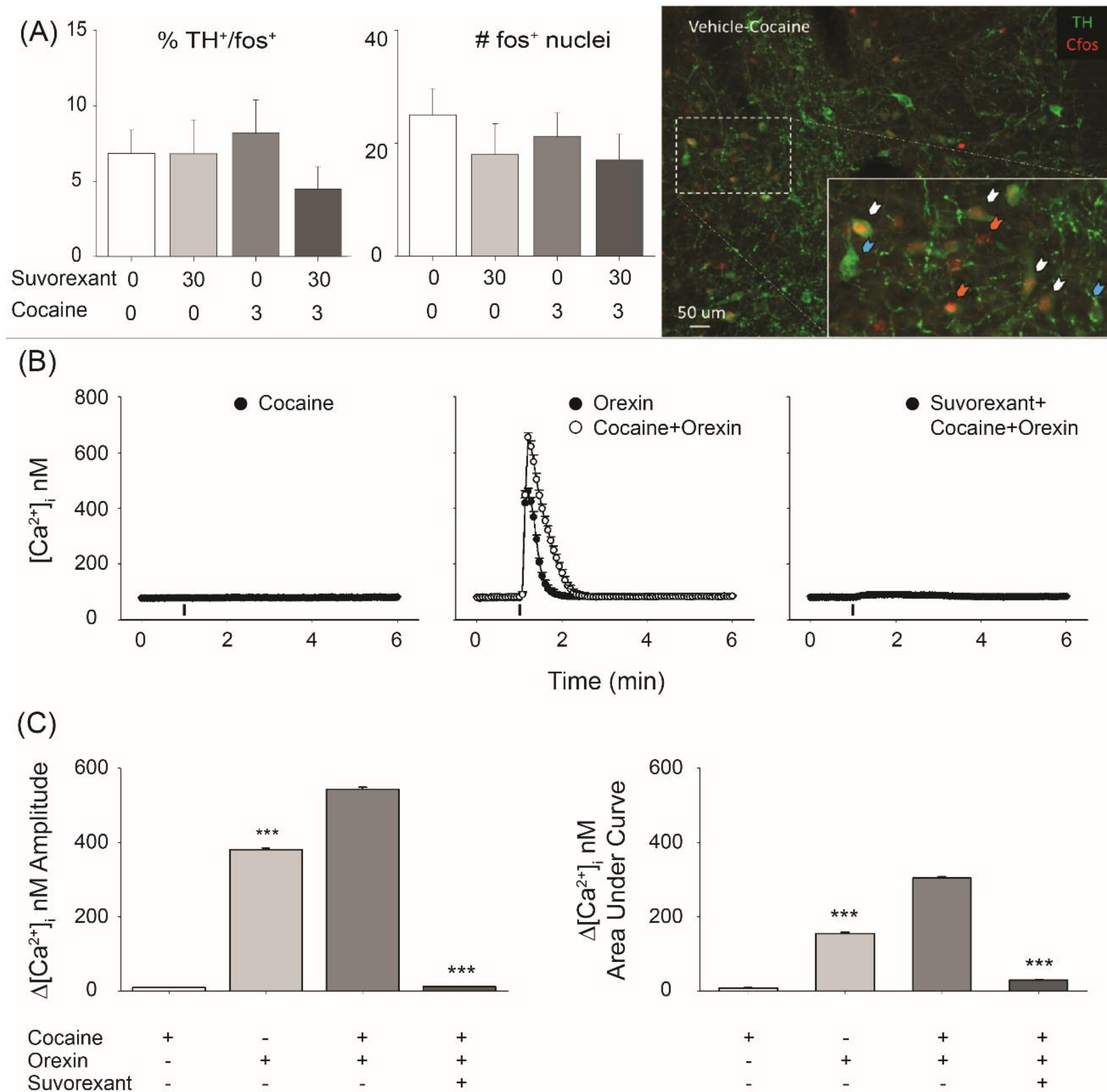


Figure 11. Effects of suvorexant on Fos-immunoreactivity within ventral tegmental area DA producing neurons and cocaine- and orexin-evoked calcium transients within VTA neurons in vitro. (A) % of total TH^+ neurons expressing $cFos^+$, average total TH^--$cFos^+$ counts within primary VTA tissue slices and representative photomicrograph of TH^+-$cFos^+$ neurons [white arrows] TH^+-Fos^- [blue arrows] TH^--Fos^+ [orange arrows] in vehicle-pretreated rats. Data in (A) are mean ± S.E.M. Under 10x magnification (n = 4-6 rats per group). (B) Tracings of VTA Ca^{2+} responses elicited by different cocaine, orexin A, and suvorexant treatments. (C) Comparison of amplitudes and AUCs of Ca^{2+} responses. Data are expressed as mean ± S.E.M. (n = 11 – 17 cells per group). ***$p < 0.0001$ compared to cocaine + orexin calcium transients.

Discussion of orexin role in cocaine-induced impulsivity.

We found that the dual orexin receptor antagonist suvorexant attenuated premature responses in the 5-CSRTT, a rodent test of motor impulsivity (Bari, Dalley, & Robbins,

2008; Winstanley et al., 2006). Future work will focus on further clarifying the role, if any, of OX_2R in impulsivity (Hirose et al., 2003). The reductions in premature responding produced by suvorexant are similar to those we have demonstrated using the OX_1R-selective antagonist SB334867 (Muschamp et al., 2014; Porter et al., 2001; Smart et al., 2001), suggesting the effect is mediated by this receptor. These findings are consistent with a dichotomous view of orexin receptor function where OX_1R regulates motivated behavior and OX_2R sleep homeostasis (Carter, de Lecea, & Adamantidis, 2013; España, Melchior, Roberts, & Jones, 2011; Willie et al., 2003). Importantly and despite the clinical use of suvorexant to treat insomnia (Roecker et al., 2016), reductions in impulsive-like behavior were not due to somnolence or non-specific locomotor decrements. For instance, we observed no increase in the number of trials omitted, subjects' latency to retrieve sucrose rewards, or accuracy even during high-dose drug trials. This is seemingly at odds with reports that reflect decreases in open-field ambulation and increased sleep following orexin receptor blockade (Bonaventure et al., 2015; Letavic et al., 2015; Rodgers et al., 2001). The effects we see may be due to higher level of engaging sensory stimulation present in the 5-CSRTT compared to the environment present during tests of open field ambulation. Moreover, in humans and rodents, dual orexin receptor antagonists act primarily to prevent awakening (i.e. reduced wake after sleep onset) rather than to induce sleep (i.e. modest effect on sleep latency; Brisbare-Roch et al., 2007). Together, these results suggest that basal orexin tone at OX_1Rs facilitates prepotent locomotor responses and may mediate impulsivity which itself is characteristic of substance use disorders (reviewed in Alcaraz-Iborra & Cubero, 2015). The decrease in motor impulsivity in the high-dose suvorexant group (30 mg/kg,

i.p.) is modest, and we suspected this to be a product of a ‘floor effect’ in which baseline (vehicle-pretreatment) premature responses were low. To examine this possibility, we trained a separate cohort of rats with a 5 d/wk regimen to induce higher baseline responding. Indeed, we observed a relatively greater reduction in premature responses between vehicle- and suvorexant-pretreated groups under these training conditions. Since decreases in premature responding following suvorexant can be observed, mitigating a hyper-impulsive state provides adjunctive clinical utility for psychiatric conditions including ADHD and substance use disorders. Accordingly, we then tested the extent to which suvorexant could attenuate premature responses evoked by a moderate dose of cocaine. As expected, cocaine alone produced a two-fold increase in premature responses. This effect was mitigated by suvorexant pretreatment while measures of response capacity (i.e. omissions, latency, accuracy) again remained unchanged. Taken together, these results support possible clinical utility of orexin receptor antagonists in treating psychiatric disorders characterized by significant trait impulsivity or for transient impulsive behavior engendered by cocaine intoxication. Strong support from previous literature implicating the mesolimbic DA pathway in impulsivity coupled with the presence of orexin receptors in the VTA lead us to hypothesize this as an important locus for orexin and cocaine-induced impulsivity (Buckholtz et al., 2010; Jentsch & Taylor, 1999; Martin & Potts, 2004; Robbins, 2002). In agreement with this hypothesis, systemic cocaine again increased motor impulsivity, while suvorexant infused directly into the VTA attenuated this effect.

In light of past literature showing direct excitatory effects of orexins on activity of VTA DA neurons (Korotkova et al., 2003), we sought to quantify differences in Fos

immunoreactivity (ir) within tyrosine hydroxylase-positive (TH^+) neurons of the VTA after rats were treated with suvorexant alone or in combination with cocaine. Contrary to our expectations, we observed no change in Fos-ir in TH^+ neurons of rats in any treatment group. We also saw no increase in Fos-ir nuclei (TH^-) in VTA. Though many authors have reported effects of cocaine or other rewards on Fos-ir in VTA DA neurons, our results are more consistent with reports where robust manipulations (i.e. rewarding electrical stimulation of afferents to VTA) that evoke marked increases in Fos-ir, fail to do so in TH^+ neurons of VTA (Hunt & McGregor, 1998; Ishida et al., 2001). It is then plausible that among the many identified immediate early genes expressed by neurons, *Cfos* may not be a reliable marker of neuronal activation in some experimental settings. With this possibility in mind, we next used a related measure (i.e. changes in intracellular calcium) to explore the effect of orexin A, suvorexant, or cocaine on VTA neuronal activity. Applied together, we saw that cocaine appears to potentiate the effects of orexin A on intracellular calcium transients in a manner consistent with prior electrophysiology studies examining influence of orexin on VTA DA neurons (Borgland et al., 2006; Korotkova et al., 2003). Notably, we also found that increases in calcium transients produced by orexin A with or without cocaine were abolished by suvorexant. While we did not examine effects of suvorexant alone *in vitro*, we would anticipate a low response comparable to transients observed with cocaine alone. We previously reported that pharmacologically relevant *in vivo* cocaine dosages resulting in low µM cocaine concentrations in the striatum can amplify IP_3 signaling in medium spiny neurons of the NAc (J. L. Barr et al., 2015). Both OX_1R and OX_2R also use G_q signaling to generate IP_3. Our results suggest analogous signal transduction occurs in orexin-sensitive VTA

neurons. Based on existing *in vivo* neurochemistry data, these events can be expected to increase the probability of VTA neuronal burst firing and can in turn elevate levels of synaptic DA in NAc (España et al., 2011; Overton & Clark, 1997; Prince, Rau, Yorgason, & España, 2015). Prior central infusion experiments show that pharmacologically-evoked DA release in the NAc results in greater premature responses in the 5-CSRTT (Robbins, 2002).

The modest effects of orexin receptor antagonists on premature responses in the 5-CSRTT but not on delay discounting suggests a preferential action on motor impulsivity and the capacity to inhibit a prepotent response. The reasons for this are not clear but are consistent with data that describe an important role for the orexin system in motor output. For instance, central infusion of orexin peptides increases ambulation that is mediated in part by DA transmission (Nakamura et al., 2000; Thorpe & Kotz, 2005). Electrophysiological recordings of identified orexin neurons in awake, freely moving rats also show that firing rates are highest during active waking, particularly exploratory behavior, and lowest during sleep (Mileykovskiy, Kiyashchenko, & Siegel, 2005). The successful performance of the 5-CSRTT observed here can be readily be described as 'active waking'. We hypothesize that this behavior is typically accompanied by increased orexin transmission. Together our findings suggest that this pattern of neuronal activity can cause decrements in response inhibition. Accordingly, it provides a rationale for further study of suvorexant and similar drugs in treating psychopathologies characterized by impulsive-like behaviors including substance use disorders and ADHD.

This chapter was reproduced with permission from Nature Publishing group from the manuscript (Gentile, Simmons, Watson, et al., 2017):

Effects of Suvorexant, a Dual Orexin/Hypocretin Receptor Antagonist, on Impulsive Behavior Associated with Cocaine. Gentile TA, Simmons SJ, Watson MN, Connelly KL, Brailoiu E, Zhang Y, Muschamp JW. Neuropsychopharmacology. 2017 Jul 25. doi: 10.1038/npp.2017.158.

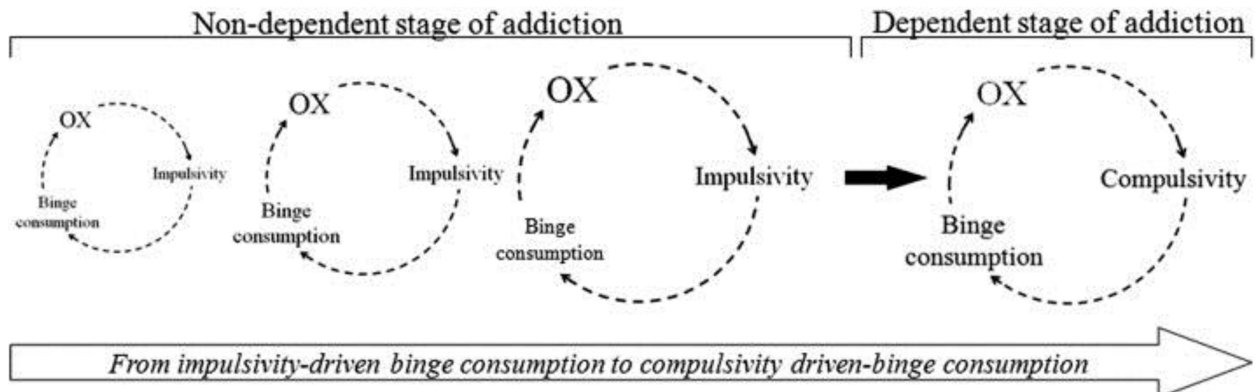


Figure 12. Orexin activity drives impulsive behaviors that contribute to drug consumption. This cycle becomes an aberrant feedback loop which develops into compulsive drug seeking behavior

Impulsive action to compulsive action and addiction

The cyclic process of orexin activity, impulsivity and drug seeking steered the research from Chapter 4 (impulsivity) to chapter 5 (cocaine seeking). As previously mentioned, research suggests a role of orexin peptide in cocaine conditioned place preference, sensitization, and excitatory currents in dopaminergic neurons as well as compulsive feeding (Borgland et al., 2006; Gozzi et al., 2011; Merlo Pich & Melotto, 2014). Alcaraz-Iborraa and Cubero made a case for the strong connection between the role of orexin in these rewarding and compulsive behaviors and the aforementioned impulse control **Figure 12** (Alcaraz-Iborra & Cubero, 2015).

CHAPTER 5

OREXIN ROLE IN PROGRESSIVE RATIO COCAINE SELF-ADMINISTRATION

Introduction to the role of orexin in cocaine seeking and reward.

Psychostimulants such as cocaine cause rapid and long-lasting changes in brain reward circuitry principally by elevating synaptic levels of DA (Heal, Gosden, & Smith, 2014). Cocaine administration transiently causes euphoria and subjective positive affect. It is generally believed that the positive affective state following cocaine administration functions as a reward signal which is conducted in part through mesolimbic DA transmission afferents within the VTA. These neurons transmit DA to post-synaptic targets within ventral striatum. Pharmacological interventions that work to reduce positive affect following cocaine use may prove useful by reducing the rewarding value of the drug.

As discussed in previous chapters, dual orexin receptor antagonists also reduce cocaine-seeking behavior in part through decreasing cocaine-evoked elevations in ventral striatal DA (Prince, Rau, Yorgason, & España, 2015). Moreover, signaling via OX_1Rs appears to selectively modulate motivation for high-incentive rewards, such as cocaine and high-fat food but not normal chow (Borgland et al., 2009). Recently, selective OX_2R antagonism was shown to reduce escalation in self-administered heroin under extended-access conditions (Schmeichel et al., 2015). Orexin transmission thus appears to mediate aspects of the rewarding and reinforcing properties of abused drugs through both receptor subtypes, and antagonists reliably reduce the rewarding properties of cocaine in preclinical models. Converging anatomical and functional reports highlight the

significance of orexin transmission within the mesolimbic reward circuit for addiction-related psychiatric disorders (Calipari & España, 2012). Orexins provide both direct and feed-forward excitatory input to dopaminergic neurons of the VTA (Fadel & Deutch, 2002; Korotkova et al., 2003; Muschamp et al., 2014). Intra-VTA application of the orexin-A increases cocaine-seeking and enhances cocaine-evoked increases in DA transmission to ventral striatal targets (España et al., 2010).

The goal of the present study was to evaluate the therapeutic potential of suvorexant, a clinically-available dual orexin receptor antagonist, in rodent models of cocaine use disorder. Specifically, we used a self-administration model to study the effects of suvorexant on cocaine-seeking. We further assessed effects of suvorexant on conditioned cocaine reward and on hedonic processing of cocaine using conditioned place preference (CPP) and through recording positively-valenced 50-kHz ultrasonic vocalizations (USVs), respectively. Next, we measured the effects of suvorexant on cocaine-induced locomotor activity. Finally, we performed *in vivo* fast scan cyclic voltammetry to assess effects of systemic suvorexant on cocaine-evoked elevations of ventral striatal DA.

Materials and Methods utilized for experiments in chapter 5.

Animals. Adult male Sprague-Dawley rats (Charles River; Horsham, PA, USA) arrived at Temple University's vivarium, were pair-housed and given food and water *ad libitum*. Rats acclimated to the vivarium for at least one week before beginning experiments. For Experiment 1, rats were singly-housed following jugular vein catheterization surgery and were placed on a reverse 12-hour:12-hour light:dark cycle with lights turning off at 9:00 AM. For Experiment 2, rats were pair-housed throughout the experiment. For Experiment 3, mice (Charles River; Horsham, PA, USA) were used and were housed in cages of 2-5

mice per cage until surgery and were provided food and water *ad libitum*. All experimental procedures were approved by Institutional Animal Care and Use Committees of Temple University and Drexel University.

Drugs. For all Experiments, suvorexant (Selleckchem; Munich, Germany) was dissolved in 100% dimethyl sulfoxide (DMSO) through vortexing and ultrasonication and was administered at a fixed volume (100 µL, i.p.). For all Experiments, cocaine hydrochloride (Sigma; St. Louis, MO, USA) was dissolved in 0.9% physiological saline.

Experimental Procedures.

Experiment 5.1: Cocaine Self-Administration.

For jugular vein catheterization surgery to permit cocaine self-administration in Experiment 1, rats were anesthetized with isoflurane gas (5% induction, 2-3% maintenance) and an aseptic environment was maintained throughout. Once under surgical anesthesia, a permanent indwelling catheter (Camcaths; Cambridgeshire, United Kingdom) was implanted in the right jugular vein of the rat and was connected to a stainless steel exit port of the rat's mid-scapular region. Incisions were closed with 9 mm wound clips and treated post-operatively with antibiotic ointment. Rats recovered from surgery for 5-7 days before beginning cocaine self-administration training.

For all self-administration sessions, rats were moved to sound-attenuating, ventilated behavioral chambers (MED Associates; St. Albans, VT, USA) after receiving a 100 µL experimenter-administered infusion of heparinized saline to aide in maintaining catheter patency. Rats were weighed daily and connected to polyurethane tubing via the stainless steel exit port, which was enclosed within a metal spring leash, attached to a fluid swivel and connected to a syringe pump for drug delivery. Cocaine infusion duration was

adjusted each session based on rat bodyweight, averaging around 3 seconds, and dose was maintained at ~0.36mg/kg/infusion for all infusions. For fixed-ratio (FR) reinforcement, session duration was 2-hours or 60 infusions, and the inter-trial interval (ITI) was 20-seconds. For progressive-ratio (PR) reinforcement, session duration was 4-hours, 60 infusions or an absence of responding for 90 minutes, and the ITI was 30-seconds. Rats began cocaine self-administration training on a FR-1 schedule of reinforcement, and criterion for advancement was ≥ 20 infusions for 3 consecutive sessions. Rats were then moved to a PR schedule of reinforcement, where infusions were rewarded upon performing an incrementing number of lever-press operant responses for subsequent infusions (e.g., 1, 1, 2, 4, 6, 9, 12, 15…), and criterion for advancement was 3 consecutive sessions where the number of infusions was maintained within ± 1 infusion relative to prior-day performance. Rats were then moved to the treatment phase of self-administration where a 3-day repeating block of sessions consisting of (1) FR-1, (2) PR without suvorexant pretreatment, and (3) PR with suvorexant pretreatment was used. A summary timeline of events can be found in **Figure 13.**

Experiment 5.2: Conditioned Place Preference (CPP), 50-kHz Ultrasonic Vocalizations (USVs), and Locomotor Activity.

For CPP, a constructed two-chamber apparatus with visually- and tactilely-distinguished contexts, separated by a removable partition, was used following a biased, forced-choice design. Rats (n=8/group) were first allowed to freely shuttle between the two contexts during a 30-minute pre-test to assess natural preference, and time on each context was recorded. Eight daily, 30-minute conditioning trials proceeded. Rats were pre-treated

with either suvorexant (30 mg/kg, i.p.) or vehicle followed by injections of either cocaine (10 mg/kg, i.p.) or saline vehicle and placed in Context A (non-preferred; 4 trials) or Context B (preferred; 4 trials), respectively. Lastly, rats were given a post-test for 30 minutes and time spent on each Context was recorded. Pre- and post-test times were used to calculate CPP core.

For USV recording, a condenser microphone (CM16/CMPA; Avisoft Bioacoustics; Berlin, Germany) was suspended above each of two distinguished contexts and recorded 50-kHz USVs during first conditioning trials in Context A and Context B of CPP (described above; n=6-8/group). Audio was sampled at 192-kHz (16-bits) from an amplification unit (UltraSoundGate 116H; Avisoft Bioacoustics; Berlin, Germany) and was processed by recording software (Avisoft Bioacoustics; Berlin, Germany) on an IBM laptop. Recorded ".wav" files were analyzed offline using either RavenPro software (Cornell Lab of Ornithology, Bioacoustics Research Program; Ithaca, NY, USA) or from an XBAT/MATLAB-based auto-detection program (Barker, Herrera, & West, 2014) for generating 50-kHz USV count data.

For the locomotor activity assay, rats were placed individually into activity chambers and allowed to acclimate for 30 minutes. Activity was measured as beam breaks collected in 5-minute time bins for 180 minutes. Baseline activity was recorded for 30 minutes, followed by suvorexant pretreatment (30.0 mg/kg, i.p.) or vehicle (0.1 mL, i.p.) and a subsequent activity recording for 30 minutes. An acute cocaine (10.0 mg/kg, i.p.) or saline (1.0 mL/kg, i.p.) injection was administered and activity recorded for the following 120 minutes. Digiscan DMicro system (Accuscan, Inc.; Columbus, OH, USA) measured

ambulatory activity as consecutive beam breaks resulting from horizontal movement and non-ambulatory activity as repetitive-beam breaks.

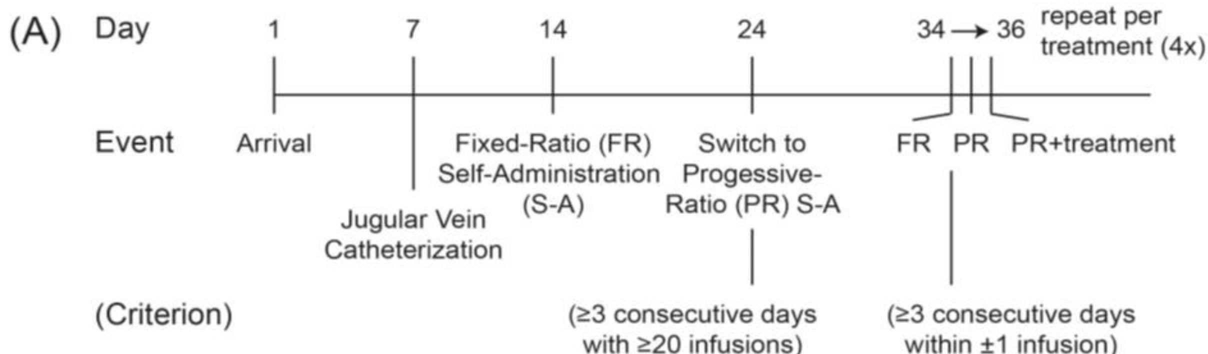


Figure 13. Timeline of events to train rats in cocaine self-administration.

Experiment 5.3: *In Vivo* Fast-Scan Cyclic Voltammetry in Ventral Striatum

Mice for *in vivo* voltammetry (n=5/group) were anesthetized with isoflurane, placed into a stereotaxic apparatus and subsequently implanted with a carbon fiber microelectrode aimed at the ventral striatum (AP: +1.3, ML: +1.3, DV: -4.5) and a Ag/AgCl reference electrode placed in contralateral cortex. A bipolar stimulating electrode (Plastics One; Roanoke, VA, USA) aimed at the VTA (AP: -3.0, ML: +1.1, DV: -4.0) was lowered in 100 µM increments until a 0.5-s, 60-Hz monophasic (4 ms; 250 µA) stimulation train elicited a robust DA response. Stimulation trains were delivered every 5 mins for at least 30 mins until DA release reached stability (three consecutive collections within 10%). Once stability was achieved, mice were injected with suvorexant (30mg/kg, i.p.) and subsequent changes in DA release recorded for 30 mins. Following the last collection, mice were injected with 10 mg/kg cocaine i.p. (6 mg/ml) and the change in DA release was recorded for at least 60 mins.

For data acquisition, the electrode potential was linearly scanned (-0.4 to 1.2 V and back to -0.4 V vs Ag/AgCl) and cyclic voltammograms were recorded at the carbon fiber electrode every 100 ms with a scan rate of 400 V/s using a voltammeter/amperometer

(Chem-Clamp, Dagan Corporation; Minneapolis, MN, USA). The magnitude of electrically-evoked DA release was monitored. DA overflow curves were analyzed as previously described for DA release (peak concentrations of DA) and uptake (*tau*) using Demon Voltammetry and Analysis software written in Labview language (National Instruments; Austin, TX, USA; Yorgason, España, & Jones, 2011).

Statistical Analyses.

For self-administration data, Infusions and Correct Responses were expressed as % Baseline relative to prior-day performance. One-way repeated-measures ANOVAs were used with Drug Treatment as the within-subjects factor (0, 3, 10, 30 mg/kg of suvorexant). Bonferonni-corrected contrast analyses proceeded for all suvorexant-pretreated groups (3, 10, 30 mg/kg) against vehicle-pretreated (0 mg/kg) control data. For CPP data, CPP Score was expressed as % Baseline relative to pre-test time (s) in Context B. A one-way ANOVA was used with Treatment Group as the between-groups factor (Veh-Sal, Suvo-Sal, Veh-Coc, Suvo-Coc), and Bonferonni-corrected contrasts were conducted against the Veh-Sal control group.

For USV analyses, standardized change scores ("Δ USV Score") were used to normalize data for parametric assessment and to assess changes in 50-kHz USVs following initial exposure to cocaine or saline using the formula [(B-A)/(A+B)]. For between-groups analysis, independent samples t-tests were conducted against "0"—the point of no-change. For within-session analyses, a two-way mixed ANOVA examining Treatment Group by Time was conducted.

For locomotor activity, a between-groups one-way ANOVA was used to analyze total activity during minutes 0 to 120 (post-cocaine). Tukey's HSD post-hoc tests were used

for pairwise comparisons, and an additional series of independent samples Bonferroni-corrected t-tests were used to examine locomotor differences within each 5-minute time bin during the pre-drug phase (-30 to 0 minutes).

For voltammetry analyses, % Dopamine was expressed as % Baseline relative to evoked dopamine release from VTA stimulation (recorded every 5 minutes for 30 minutes prior to drug pretreatment). Two-way ANOVAs were used examining % Dopamine with Treatment Group and Time as factors in 5-minute bins for time prior to or following acute cocaine administration.

For contrasts on self-administration, CPP and USV data, Type I error rate (α) within each family of comparisons was maintained at 0.05.

Results of experiments in chapter 5.

*Experiment 5.1: Suvorexant Decreases Cocaine-Seeking (**Figure 14**).*

Repeated-measures ANOVAs examining Infusions [$F(3, 8) = 2.286$, n.s.] or Correct Responses [$F(3, 8) = 1.679$, n.s.] by Drug Treatment were not significant although contrast analyses revealed that the high dose of suvorexant (30.0 mg/kg) caused a significant reduction in Infusions earned relative to prior-day performance [$|t(11)| =$

2.892, $p < 0.05$] and had a minor effect at reducing the number of Correct Responses [$|t(11)| = 2.583$, $p = 0.075$].

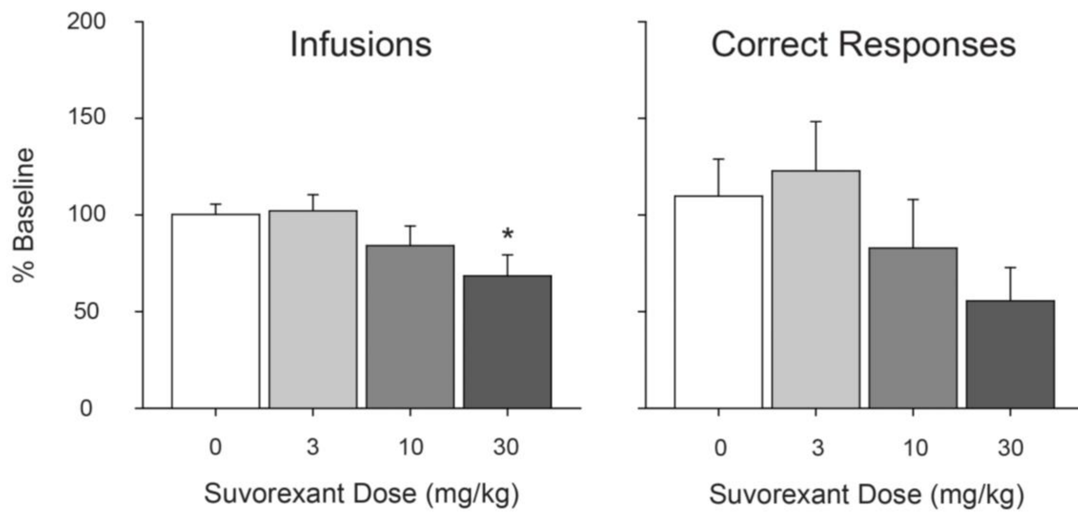


Figure 14. Infusions and correct responses relative to prior-day baseline performance. * p < 0.05 relative to vehicle-pretreated control data. Data are presented as mean ± S.E.M. n=12.

*Experiment 5.2: Suvorexant Prevents Conditioned Cocaine Reward and Attenuates Initial Hedonic Reactivity to Cocaine (**Figures 15 A and B**).*

A between-groups one-way ANOVA examining CPP Score revealed a significant main effect of Treatment Group [$F(3, 28) = 3.635$, $p < 0.05$]. Contrast analyses revealed that CPP Score from the Veh-Coc group was significantly greater than the Veh-Sal control group [$t(14) = 2.600$, $p < 0.05$], but that CPP Score from the Suvo-Coc group was not different from the Veh-Sal control group [$t(14) = 1.662$, n.s.].

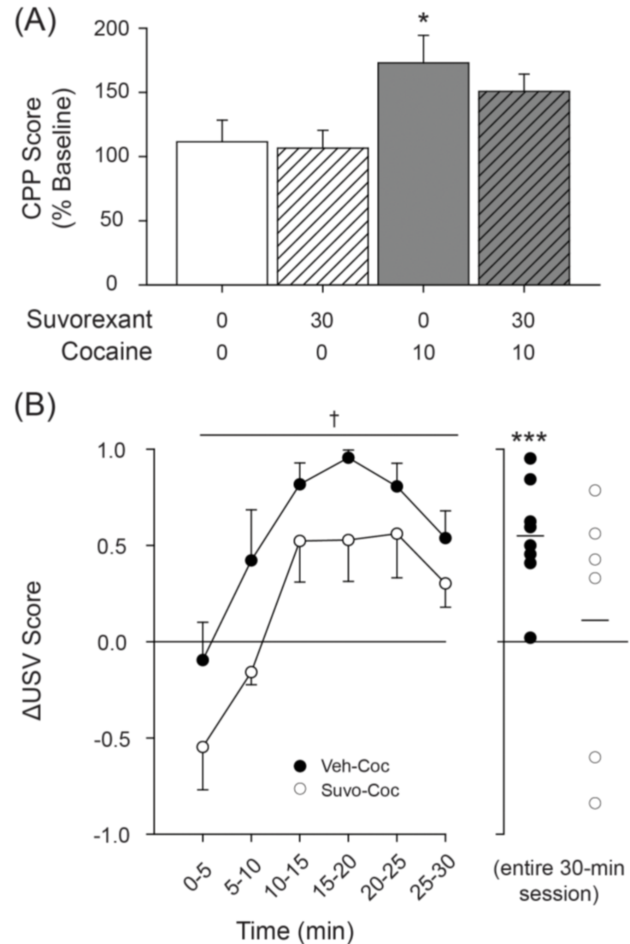


Figure 15. Effects of suvorexant on (A) CPP Score and (B) Δ USV Score. † p = 0.053 main effect of Drug Type. *** p < 0.001 compared to "0" in (B). Data in (A) are presented as mean ± S.E.M. n=6-8/group.

One-sample t-tests examining Δ USV Score found that positive affective reactivity to cocaine was significant for the vehicle-pretreated group [$t(7) = 5.461$, $p < 0.001$], but not for the suvorexant-pretreated group [$t(5) = 0.408$, n.s.] versus "0"—the point of no-change—when examining USVs across the entire 30-minute session. For within-session analyses, a two-way mixed model ANOVA examining Δ USV Score did not find a significant interaction between Treatment Group and Time but did find a significant main effect of Time [F(5,

60) = 11.415, $p < 0.001$] and a marginally-significant main effect of Treatment Group [$F(1, 12) = 4.600$, $p = 0.053$]. No differences between Veh-Coc and Suvo-Coc groups were found when examining 50-kHz USV duration ($M_{\text{Veh-Coc}} = 57.5$ ms, $M_{\text{Suvo-Coc}} = 59.4$ ms) or bandwidth ($M_{\text{Veh-Coc}} = 45.0$ kHz, $M_{\text{Suvo-Coc}} = 34.5$ kHz) following systemic cocaine injection.

A one-way ANOVA examining activity counts during minutes 0 to 120 revealed a significant main effect of Treatment Group [$F(2, 72) = 11.640$, $p < 0.001$]. Post-hoc tests revealed significantly greater activity from minutes 0 to 120 in cocaine-treated groups irrespective of pretreatment compared to the vehicle-pretreated, acute saline-treated group [Veh-Coc: $p < 0.001$; Suvo-Coc: $p < 0.001$]. T-tests within each 5-minute bin

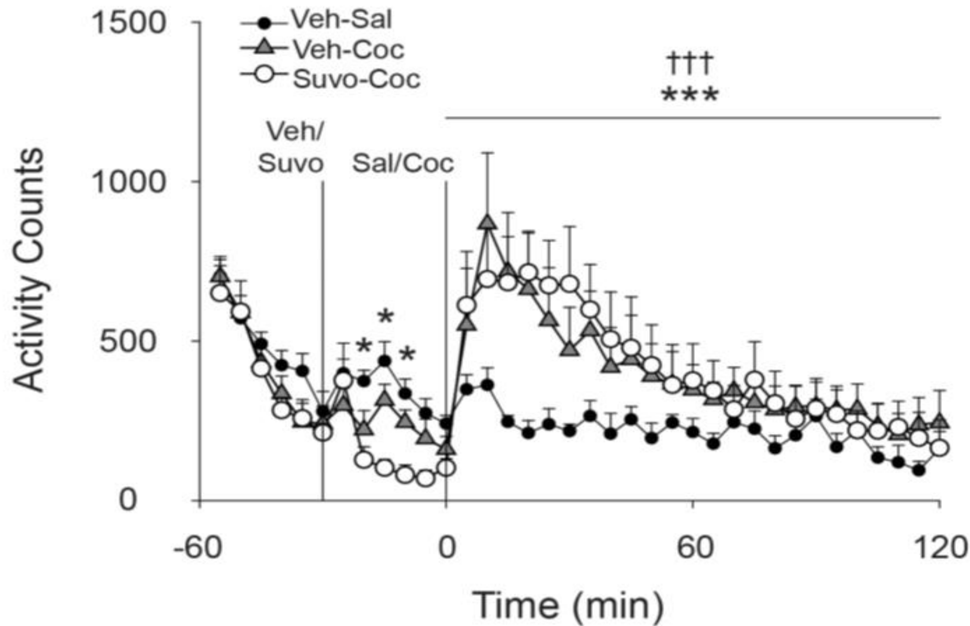


Figure 16. Effects of suvorexant on cocaine-induced locomotor activity expressed as total ambulatory activity. Solid vertical line indicates pretreatment with either suvorexant (30 mg/kg) or vehicle at -30 minutes. Solid vertical line indicates acute treatment with either cocaine (10 mg/kg) or vehicle at 0 minutes. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ indicates activity count difference between Veh-Sal and Suvo-Coc groups. † $p < 0.05$, †† $p < 0.01$, ††† $p < 0.001$ indicates activity count difference between Veh-Sal and Veh-Coc groups. Data are presented as mean ± S.E.M. n=8/group.

revealed significantly lower activity at minutes -20,-15 and -10 [all $p < 0.05$] in the Suvo-Coc animals relative to Veh-Sal control animals.

*Experiment 5.3: Suvorexant Reduces Cocaine-Induced Elevations in Ventral Striatal Dopamine (**Figure 17**).*

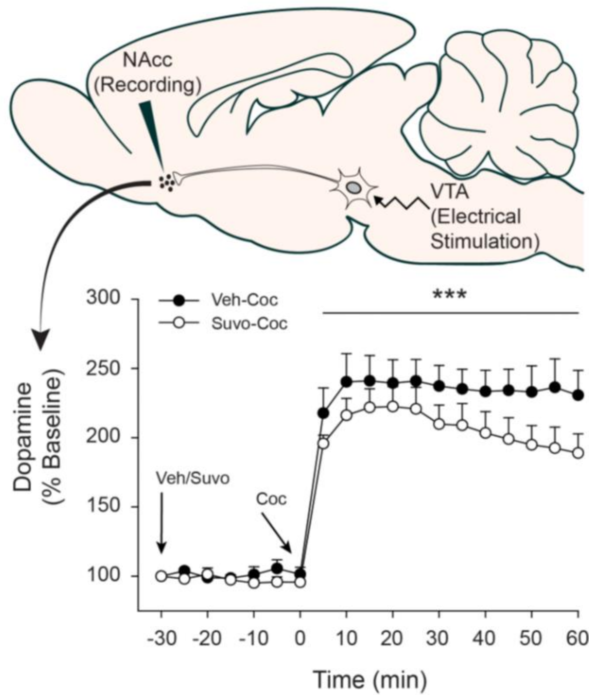


Figure 17. **Dopamine levels** (% Baseline) in ventral striatum between Veh-Coc and Suvo-Coc groups. Arrows indicate points of vehicle/suvorexant injection (-30 min; 30 mg/kg, i.p.) and cocaine injection (0 min; 10 mg/kg, i.p.). *** p < 0.001 relative to Veh-Coc group. Data are presented as mean ± S.E.M. n=5/group.

For time bins prior to acute cocaine injection (-30 to 0 minutes), a two-way ANOVA examining % Dopamine revealed a non-significant interaction [$F(5, 48) = 0.598$, n.s.], a non-significant main effect of Time [$F(5, 48) = 0.242$, n.s.] and a trend towards significance in main effect of Treatment Group [$F(1, 48) = 3.726$, $p = 0.06$]. No significant pairwise comparisons were found. Following acute cocaine injection (0 to 60 minutes), a two-way ANOVA examining % Dopamine revealed neither a significant interaction between Treatment Group and Time [$F(11, 96) = 0.167$, n.s.] nor a main effect of Time [$F(11, 96) = 0.597$, n.s.]. There was, however, a significant main effect of Treatment Group indicating greater % Dopamine in the Veh-Coc group compared to Suvo-Coc group [$F(1, 96) = 20.072$, $p < 0.001$].

Although not shown, there were no significant effects of suvorexant on cocaine's propensity to block the dopamine transporter (tau) were observed [main effect of Treatment Group during the post-cocaine phase: $F(1, 96) = 0.902$, n.s.].

Discussion of orexin activity in cocaine seeking and reward.

Results from the present study suggest that suvorexant, a clinically-available dual orexin receptor antagonist (DORA), reduces cocaine-seeking in a preclinical cocaine self-administration model (**Figure 14**). These results corroborate recent reports showing that either single or dual orexin receptor antagonism reduces cocaine-seeking using progressive-ratio schedule of reinforcement (Brodnik et al., 2015; Prince, Rau, Yorgason, & España, 2015). Moreover, intra-VTA OX_1R antagonism reduces cocaine infusions during self-administration in rats (España et al., 2010; Muschamp et al., 2014), suggesting that orexinergic transmission to and signaling within the VTA is critical for the reinforcing properties of cocaine. While studies have shown that orexins can induce food consummatory behavior (Sakurai et al., 1998), signaling via OX_1Rs preferentially modulates motivation to consume highly-salient rewards (Borgland et al., 2009; Martin-Fardon & Weiss, 2014). Furthermore, significant effects of orexin receptor blockade on drug infusions are usually observed when rodents are placed on a progressive-ratio schedule of reinforcement and not under fixed-ratio access conditions (e.g. España et al., 2010; Hutcheson et al., 2011; Mahler et al., 2013; Smith et al., 2009). A separate line of evidence reveals a critical role for signaling via OX_2Rs in mediating escalation of drug-taking in self-administering rats using an extended-access design (Schmeichel et al., 2015). OX_2Rs additionally potentiate VTA neuronal activity by enhancing pre-synaptic

glutamate transmission (Borgland, Storm, & Bonci, 2008). Orexins normally excite VTA DA neurons through somatodendritic OX_1Rs (Korotkova et al., 2003) as well as by heteroceptors on glutamatergic terminals in VTA (Borgland et al., 2006). Mechanistically, our data support that suvorexant may occlude cocaine-evoked striatal DA by reducing excitability of VTA DA neurons. Notably, OX_1Rs in NAcc positively regulate phasic DA release when tested *ex vivo*, but our study revealed a selective effect of systemic suvorexant on cocaine-evoked DA changes and not on NAcc DA dynamics before cocaine injection (Patyal et al., 2012). Results from the present study add to a growing literature detailing receptor-specific aspects of targeting OX_1Rs and OX_2Rs alone or in combination (Khoo & Brown, 2014).

Results from the present study further suggest that suvorexant modestly attenuates cocaine CPP (**Figure 15A**). An earlier report demonstrated that systemic OX_1R antagonism significantly attenuated morphine CPP (Harris et al., 2005), while other studies find that systemic OX_1R antagonism attenuates expression of amphetamine CPP and reduces motivation for conditioned cocaine reinforcement (Hutcheson et al., 2011; Shaw et al., 2016). CPP assesses reward following passive contextual conditioning, and thus orexin transmission in rats may only be modestly recruited for development of conditioned reward. Additionally, orexin peptide levels in plasma and cerebrospinal fluid are highest during active hours (i.e. when in dark lighting) and lowest during inactive hours (Blouin et al., 2013; Kiyashchenko et al., 2002; Lee, Hassani, & Jones, 2005; Mileykovskiy et al., 2005). As CPP training and testing took place during the rats' inactive hours, effects of orexin transmission blockade may have been relatively weak, compared to transmission blockade during active hours.

Ultrasonic vocalizations (USVs) have been used in preclinical models of substance use disorders to characterize changes in affective processing upon drug administration as well as during withdrawal and relapse (Barker, Simmons, et al., 2014; Barker, Simmons, & West, 2015; Knutson, Burgdorf, & Panksepp, 2002). Experimenter- and self-administered cocaine robustly evoke 50-kHz USVs in rats (Barker et al., 2010; Maier, Abdalla, Ahrens, Schallert, & Duvauchelle, 2012; Mu et al., 2009; Williams & Undieh, 2010). Further, while 50-kHz USVs elicited following cocaine injection belong to fixed-frequency, frequency-modulated and "trill" categories, 22-kHz USVs associated with negative affective states are typically monotonic and thus classified predominantly as fixed-frequency USVs. Results from the present study suggest that DORAs are capable of reducing affective reactivity to cocaine as indicated by an augmentation of Δ USV Score (**Figure 15B**) *In vivo* voltammetry data from previous investigations (Prince, Rau, Yorgason, & España, 2015) as well as the present study (**Figure 17**) find that single or dual orexin receptor antagonists reduce cocaine-evoked elevations in ventral striatal DA. A separate line of evidence finds that 50-kHz USVs following cocaine injection are modulated in part by mesolimbic DA transmission. Systemic or direct blockade of DA signaling in the nucleus accumbens reduces 50-kHz USVs elicited by psychostimulant injection (Thompson, Leonard, & Brudzynski, 2006; Williams & Undieh, 2010; Wright, Dobosiewicz, & Clarke, 2013). Furthermore, systemic OX_1R antagonism decreases reward sensitivity in mice performing an operant task for brain stimulation reward in an intracranial self-stimulation paradigm. This may indicate that blocking orexin signaling pharmacologically alters hedonic and motivational state (Muschamp et al., 2014). Results from the present study support the idea that dopaminergic signaling along the mesolimbic

pathway is critical for positive affect associated with cocaine administration, and that blockade of orexin transmission decreases activity of this pathway and subjective experience following cocaine.

Suvorexant contributes to somnolence and typically decreases locomotor activity in animals (Winrow et al., 2011). Results from the present study support that suvorexant reduces activity but likely does not interfere with behavioral task performance when an operandum is employed (i.e., during cocaine self-administration). It is possible that suvorexant-associated sedation contributed to the observed reduction in infusions earned during progressive-ratio cocaine self-administration, but data reveal that all but one suvorexant-pretreated rat did indeed self-administer cocaine. Notably, systemic cocaine elicited comparable hyperlocomotor responses irrespective of drug pretreatment (**Figure 16**). Taken together, it is unlikely that the reported decrease in motivation to self-administer cocaine was due to suvorexant-induced hypolocomotor effects.

Together, our results support the possibility that DORAs may promote abstinence from drug taking in human cocaine users. We demonstrate that suvorexant attenuates both the hedonic as well as the motivational properties associated with cocaine use. These effects are likely due to a reduction in cocaine-evoked forebrain DA transmission by suvorexant. It should be noted that suvorexant is currently used as a sleep aid and thus, if investigated clinically for treating substance use disorders, may elicit sedation that could interfere with normal waking hours. Future studies are needed to characterize the effectiveness of DORAs more thoroughly in preclinical models of addiction relapse and to determine circuits responsible for the effects of suvorexant on motivation versus affective processing.

This was reproduced with permission from John Wiley and Sons from the manuscript (Gentile, Simmons, Barker, et al., 2017):

Suvorexant, an orexin/hypocretin receptor antagonist, attenuates motivational and hedonic properties of cocaine. Gentile TA, Simmons SJ, Barker DJ, Shaw JK, España RA, Muschamp JW. Addict Biol. 2017 Apr 17. doi: 10.1111/adb.12507.

CHAPTER 6

ROLE OF OREXIN AND DYNORPHIN RECEPTOR ACTIVITY DURING COCAINE USE AND ABSTINENCE

Introduction to the role of orexin and dynorphin in cocaine reinforcement and abstinence

As previously mentioned in chapters 2&3, behavioral effects of orexin and dynorphin transmission appear thus as functionally divergent within the context of reward-seeking but they share overlapping expression of receptors within reward-regulating circuits (Bruijnzeel, 2009; Chavkin et al., 1982). OxR and KOR activity imparts opposing physiological effects on putative DA-producing VTA neurons *ex vivo* which may account for divergent behaviors emerging during canonical "reward" and "withdrawal" phases observed in addiction models (Korotkova et al., 2003; Margolis et al., 2003). Muschamp and colleagues (2014) initially revealed that systemic KOR antagonism normalizes anti-reward effects associated with systemic OxR antagonism and proposed functional crosstalk *in vivo* between co-transmitted orexin and dynorphin worth more extensive exploration.

Clinical observations and theory alike support the notion that, while acute euphoric actions of psychostimulants promote initial use, compulsions directed to avoid negative outcomes associated with craving and withdrawal predominate thereafter and propagate addiction (Koob, 1996). A sizeable literature has addressed the role of orexin transmission on reward as well as KOR activity on withdrawal, however there is little to show for the complex relationship of co-localized orexin/dynorphin on bivalent mood states (Chartoff et al., 2012; España et al., 2010; Gentile, Simmons, Barker, et al., 2017; Hollander et al., 2012; Muschamp et al., 2014). In an effort to achieve highest

translational potential, the present report utilized FDA-approved OxR antagonist suvorexant (Belsomra) and the relatively short-acting KOR antagonist LY2456302, which has entered phase II clinical trial for treatment-resistant depression and smoking cessation (CERC-501, Cerecor Inc.) (Eisenhower, Koronkowski, & Marcum, 2016; W. Li et al., 2016; Winrow et al., 2011).

The present study sought to utilize the combination of OxR and KOR blockage in order to mitigate acute cocaine reward and attenuate cocaine withdrawal-induced anhedonia in the intracranial self-stimulation paradigm. These experiments logically build on behavioral work demonstrating KOR antagonism can mitigate the anti-reward properties of OxR blockade (Muschamp et al., 2014). We further utilized direct site injections, immunohistochemistry and fast scan cyclic voltammetry within the mesolimbic dopamine pathway to elucidate a circuit and molecular mechanisms that underlay the bivalent behaviors.

Materials and Methods utilized for experiments in chapter 6.

Animals. Mice (Jackson Laboratory; Bar Harbor, ME, USA) were housed in cages of 2-5 mice per cage until surgery and were provided food and water *ad libitum*. Mice were singly housed and allowed to recover for 5 days after surgery. All experimental procedures were approved by Institutional Animal Care and Use Committees of Temple University and Drexel University. Adult male Sprague-Dawley rats (Charles River; Horsham, PA, USA) arrived at Temple University or Drexel University's vivarium, were pair-housed and given food and water *ad libitum*. Rats acclimated to the vivarium on a reverse 12-hour:12-hour light:dark cycle with lights turning off at 7:00 AM for at least one week before beginning experiments.

Drugs. For all intra-VTA experiments, suvorexant (Selleckchem; Munich, Germany) and LY2456302 (NIDA drug supply; Bethesda, MD, USA) was dissolved in 100% dimethyl sulfoxide (DMSO) through vortexing and ultrasonication and was administered at a fixed volume (200 nL, intra-VTA.). For systemic injections Suvorexant and LY2456302 was dissolved in 10% dimethyl sulfoxide (DMSO) 90% physiological saline and injected at a volume of 4ml/kg. For all Experiments, cocaine hydrochloride (Sigma; St. Louis, MO, USA) was dissolved in 0.9% physiological saline.

<u>Experiment 6.1&3: Intracranial self-stimulation (ICSS).</u>

For electrode and cannula placement surgery to permit intracranial self-stimulation; mice were anesthetized with isoflurane gas (5% induction, 2-3% maintenance) and an aseptic environment was maintained throughout. Once under surgical anesthesia, mice were stereotactically implanted in the medial forebrain bundle (from bregma: AP:-1.9, ML: +0.8, DV: -4.8 mm) with a monopolar stimulating electrode (PlasticsOne, Roanoke, VA) as well as a 26G internal cannula (PlasticsOne, Roanoke, VA) implanted in the ventral tegmental area (from bregma: AP:-3.0, ML: -0.4, DV: -4.6 mm). After recovery, mice were trained to respond to electrical stimulation using computer-controlled operant chambers housed in ventilated, sound-attenuating cabinets (Med Associates; St. Albans, VT). Mice were trained in daily sessions at the lowest current that would sustain responding at a rate of approximately one response per sec ± 10%. This current was maintained through testing in which mice were responded for stimulation frequencies (200-32 Hz) in descending order for 50 seconds each. Authors computed reward threshold for each subject using a least-square line of best fit analysis. Drug testing began when reward thresholds reached stable performance criteria (± 10% for 3 consecutive

days). For systemic drug treatment sessions, doses of suvorexant (0, 3, 10, or 30 mg/kg, IP) were administered 45 minutes after beginning the task and 30 minutes prior to cocaine (0, or 10, mg/kg, IP), LY2456302 (0, or 1 mg/kg, IP) was given 3 hours prior to beginning the task due to a longer onset of action. For intra VTA drug treatment sessions, doses of Suvorexant (0, 0.3, 1, or 3 µg/side, intra-VTA) or, LY2456302 (0, or .1 µg/side, intra-VTA) were administered 45 minutes after beginning the task and 30 minutes prior to cocaine (0, or 10, mg/kg, IP). Thresholds were quantified from performance during 15-min test sessions. For drug trial percent baseline was computed as: (threshold after cocaine/threshold before treatment)*100 see Muschamp et al., 2014.

For withdrawal experiments doses of suvorexant (0, or 10 mg/kg, IP) were administered 15 minutes before the task, LY2456302 (0, or 1 mg/kg, IP) was given 3 hours prior to beginning the task due to a longer onset of action. Cocaine (0, or 10, mg/kg, IP) was given once per hour for 3 hours after the ICSS paradigm was over (20 hours before the next ICSS session). A percent baseline was the average of 5 days before chronic cocaine was administered. Percent baseline change was then computed as (mean threshold for day x/baseline threshold)*100 similar to (Chartoff et al., 2012).

Experiment 6.2: *In Vivo* Fast-Scan Cyclic Voltammetry in Ventral Striatum

Rats for *in vivo* voltammetry were anesthetized with isoflurane, placed into a stereotaxic apparatus and subsequently implanted with a carbon fiber microelectrode aimed at the ventral striatum (AP: +1.3, ML: +1.3, DV: -6.5) and a Ag/AgCl reference electrode placed in contralateral cortex. A bipolar stimulating electrode (Plastics One; Roanoke, VA, USA) aimed at the VTA (AP: -5.2, ML: +1.1, DV: -7.0) was lowered in 100 µM increments until a 0.5-s, 60-Hz monophasic (4 ms; 250 µA) stimulation train elicited a

robust DA response. Stimulation trains were delivered every 5 mins for at least 30 mins until DA release reached stability (three consecutive collections within 10%). Once stability was achieved, rats were injected with suvorexant (0,1ug, intra-VTA) and LY2456302 (0, 0.1ug, intra-VTA) and subsequent changes in DA release recorded for 30 mins. Following the last collection, animals were injected with 1.5 mg/kg cocaine i.v. and the change in DA release was recorded for at least 60 mins.

For data acquisition, the electrode potential was linearly scanned (-0.4 to 1.2 V and back to -0.4 V vs Ag/AgCl) and cyclic voltammograms were recorded at the carbon fiber electrode every 100 ms with a scan rate of 400 V/s using a voltammeter/amperometer (Chem-Clamp, Dagan Corporation; Minneapolis, MN, USA). The magnitude of electrically-evoked DA release was monitored. DA overflow curves were analyzed as previously described for DA release (peak concentrations of DA) and uptake (*tau*) using Demon Voltammetry and Analysis software written in Labview language (National Instruments; Austin, TX, USA; Yorgason et al., 2011).

Experiment 6.3: Elevated Plus maze (EPM)

Separate cohorts of rats were administered cocaine (0 or 10mg/kg, i.p.) 3 times a day, one hour apart, for 14 days, 48 hours prior to undergoing EPM procedure. Animals were given Suvorexant (0 or 30 mg/kg, i.p.) 30 minute prior and/or LY2456302 (0 or 10mg/kg, i.p.) 3 hours prior to being place on the elevated plus maze. The EPM apparatus contains four perpendicular equal-sized runways (19” by 4”) that are elevated 20” off the ground. Two of the arms are enclosed by walls (13” high) on three sides (“closed arm”), while the other two arms do not have walls (“open arm”). Rats were tested using low lighting with the open arm illuminated to 20 lx, while the center was set to 10 lx. Arm entry was

considered when a rat's center (head, shoulder, and two front limbs) moved into the arm. The apparatus was cleaned with 70% ethanol and allowed to dry between each animal. Rats were placed in the center of the maze facing an open arm and allowed to ambulate for 10 min. The session was recorded, using AnyMaze software (Wood Dale, IL) number of entries and time on the open and closed arms was recorded. The amount of time spent on the open arms was determined as a percentage of the 600 total seconds.

Experiment 6.4: BNST cFos, VTA DA immunohistochemistry and CORT ELISA.

Forty five minutes following EPM, rats were deeply anesthetized with phenytoin phenobarbital cocktail (120 mg/kg, i.p.) and perfused with ~100 mL cold phosphate-buffered saline (PBS; 0.1M, pH 7.4) followed by ~300 mL 4% paraformaldehyde (PFA). Brains were post-fixed for 24 hours in 4° C PFA followed by 72 hours in 4° C 30% sucrose and finally flash-frozen in 2-methyl butane chilled on dry ice. Subsequently, brains sectioned using a cryostat, and coronal sections (40 μm), containing the entire BNST (from bregma: AP -0.11 mm to -0.83 mm) and VTA (from bregma: AP -5.0 mm to -6.5 mm; *Brain Maps III* atlas available online: http://larrywswanson.com/) were collected.

For immunolabeling, tissue sections were washed in 0.1 M PBS, blocked in 5% donkey serum in PBS with 0.3% Triton X-100 (PBS+) for one hour and incubated in primary antibody solution at 4° C for 72 hours (rabbit anti-cFos [1:1,000; SC-52, Santa Cruz] in 2.0% donkey serum in PBS+). Subsequently, sections were incubated in secondary antibody solution for 3 hours (donkey anti-rabbit 555 [1:400; AlexaFluor] in 1.5% donkey serum in PBS+). VTA Tissue sections underwent repeated immunolabeling procedures as above using rabbit anti-tyrosine hydroxylase (TH) [1:1,000; AB-152,

Millipore] primary antibody and donkey anti-rabbit 488 [1:400; AlexaFluor] secondary antibody.

All TH-immunoreactive neurons and Fos-immunoreactive nuclei that were visible in every third tissue section collected were counted by an experimenter blinded to treatment conditions using an Eclipse 80*i* upright fluorescent microscope (Nikon) under 10x objective magnification. All sections were averaged together for counting. The number of Fos^{+}-TH^{+} neurons was calculated by: [# $TH^{+}Fos^{+}$ cells per slice counted*25 slices total]. The Fos^{+} cells of the BNST were counted and the average number per section was recorder.

Before perfusion, blood samples were collected in 3 ml tubes with ethylenediaminetetraacetic acid (EDTA) and aprotinin and centrifuged at 12,000 RPM for 20 minutes at 4°C. The supernatant was collected and analyzed using a competitive immunoassay of corticosterone using polyclonal antibodies to corticosterone from Enzo Life Sciences (Farmingdale, NY). Briefly, samples, an alkaline phosphatase molecule covalently attached to corticosterone and antibody were added in duplicate to the 96 well plate and incubated at RT for 2 hours. The wells were emptied and washed 3 times with PBS. A p-nitrophenyl phosphate substrate was added to the wells and allowed to incubate at RT for 1 hour. 1N HCL is added to arrest the color change and the ELISA is read for optical density at 405 nm. The optical density is analyzed using 4 parameter logistic curve fitting based on the standard curve.

Statistical Analyses.

For acute intracranial self-stimulation (ICSS) data, % baselines were expressed as % baseline relative to prior trial performance. One-way ANOVAs were used with drug

treatment as the within-subjects factor. Contrast analyses proceeded using Dunnett's multiple comparisons of all treatment-pretreated against vehicle-pretreated (0 mg/kg) control data. For withdrawal-ICSS data, a two-way ANOVA was used to analyze brain reward threshold during 20 hour forced abstinence. Dunnett's multiple comparison post-hoc tests were used to examine baseline differences within each 2-day time bin. For voltammetry analyses, % Dopamine was expressed as % Baseline relative to evoked dopamine release from VTA stimulation (recorded every 5 minutes for 30 minutes prior to drug pretreatment). Two-way ANOVAs were used examining % Dopamine with Treatment Group and Time as factors in 5-minute bins for time prior to or following acute cocaine administration. For contrast analyses on self-administration, CPP and USV data, Bonferroni corrections were applied and Type I error rate (α) within each family of comparisons was maintained at 0.05. For immunohistochemical analyses, a one-way ANOVA was used to examine mean estimated number of TH^+/Fos^+ cells as well as total Fos^+ cells between treatment groups.

Results of experiments in chapter 6.

*Experiment 6.1: Systemic suvorexant mitigates cocaine-induced reward alone and in combination with LY2456302 (**Figure 18**).*

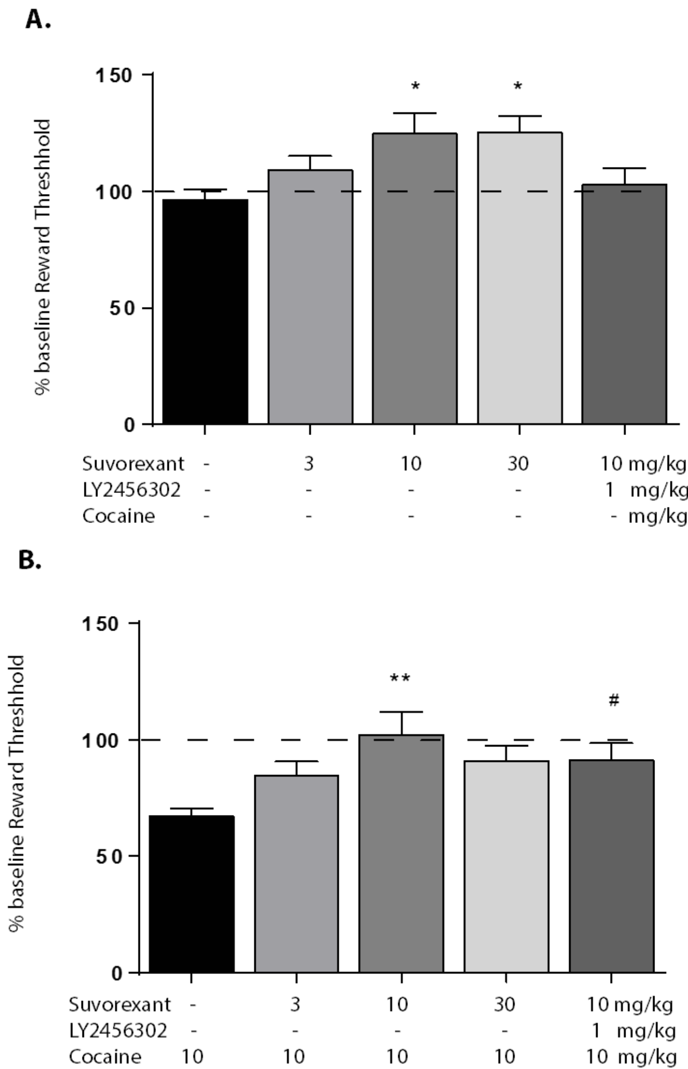


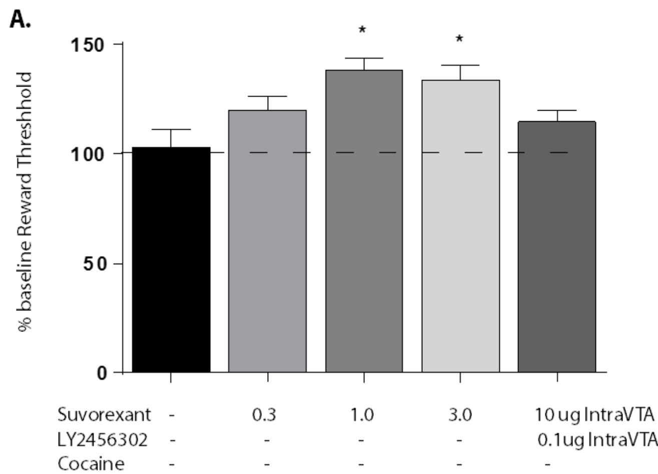
Figure 18. Effects of systemic suvorexant and LY2456302 on (A.) baseline threshold alone (B.) cocaine-induced decreases in baseline threshold. Data presented as mean % of baseline responses ± *S.E.M*. in ICSS (n=11-14/cohort).

Results from the present study revealed a significant main effect of drug group on attenuating changes in baseline threshold after cocaine utilizing a ordinary measures one way ANOVA [$F(4,561) = 3.32$, $p < 0.05$]. Dunnett's multiple comparisons test against the vehicle-treated control group reveal that 10mg/kg Suvorexant was effective in mitigating % baseline threshold [$p < 0.01$] while Ly2456302 pretreatment was also effective in attenuating cocaine-induced decreases in % baseline threshold (n=11-14/cohort) [$p \leq 0.051$] . Additionally, the present study revealed a significant main effect of Suvorexant dose on baseline threshold alone utilizing an ordinary measures one way

ANOVA $F(4, 48) = 3.59$, $p < 0.05$]. Dunnett's multiple comparisons test against the vehicle-treated control group reveal that 10 and 30mg/kg suvorexant increased % baseline threshold [$p < 0.05$] while pretreatment with LY2456302 does not elevate % baseline threshold [$p>0.05$] (n=9-11/cohort). Lastly, no differences were found in % maximum rate for the suvorexant, cocaine cohort [$F(4, 56) = 0.514$, $p > 0.05$] or suvorexant dose response [$F(4, 47) = 0.916$, $p < 0.05$] to the relevant vehicle cohorts.

*Intra-VTA suvorexant elevates brain reward thresholds (and modestly normalizes cocaine-induced decreases in brain reward thresholds) (**Figure 19**).*

Similar to Experiment 1, results from the present study revealed a significant main effect of Suvorexant dose on altering baseline thresholds alone using a repeated measures one way ANOVA [$F(2.5, 24.8) = 4.63$, $p < 0.05$]. Dunnett's multiple comparisons test against the vehicle-treated control group reveal that 1 or 3 µg/side Suvorexant was effective in mitigating % baseline threshold [$p < 0.05$] (n=11/cohort).



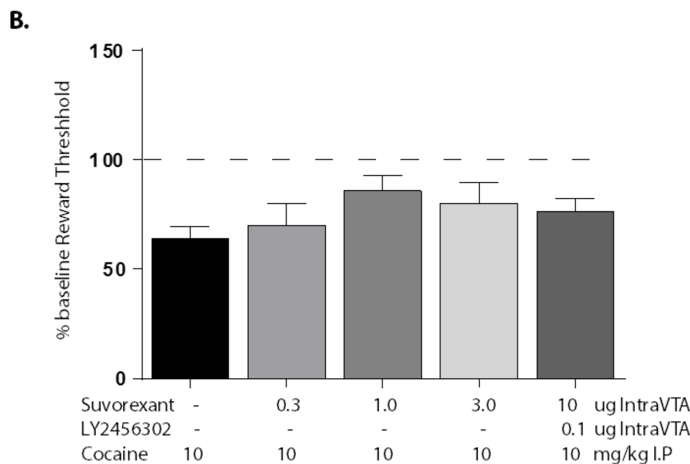


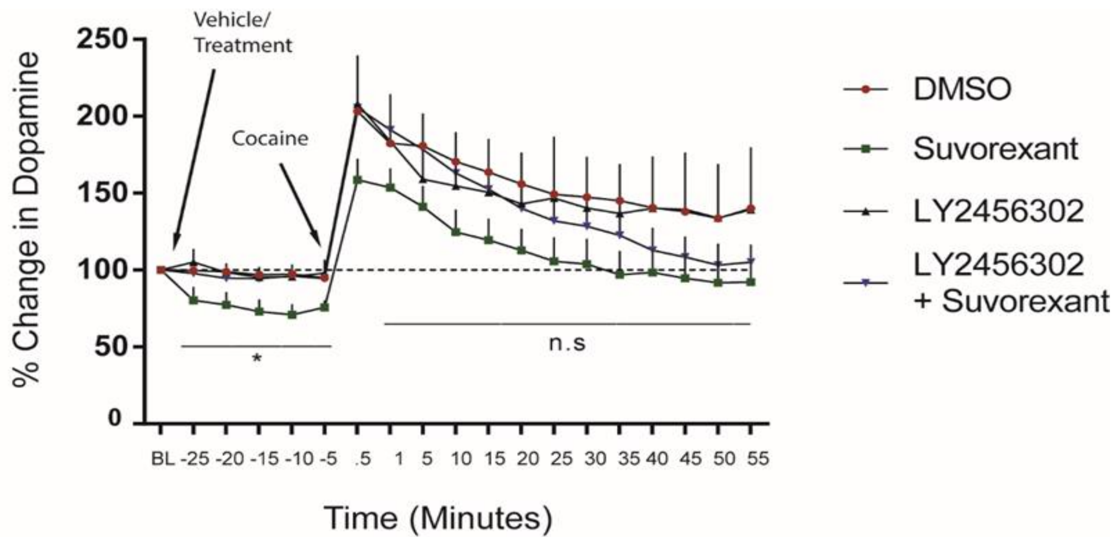
Figure 19. Effects of site-directed suvorexant and LY2456302 in the VTA on (A.) Changes in baseline threshold alone. (B.) cocaine-induced decreases in baseline threshold. Data presented as mean % of baseline responses ± *S.E.M.* in ICSS (n=11/cohort).

However, the present study did not reveal a significant main effect of Suvorexant dose on mitigating cocaine induced decreases in baseline threshold alone utilizing an ordinary measures one way ANOVA $F(3.1, 31.2) = 2.1$, $p > 0.05$]. (n=11)

Lastly, no differences were found in % maximum rate for the suvorexant, cocaine cohort [$F(2.5, 27.5) = 0.92$, $p > 0.05$] or suvorexant dose response ([$F(2.5, 27.5) = 0.28$, $p > 0.05$] to the relevant vehicle cohorts.

*Experiment 6.2. Suvorexant decreases VTA-stimulated dopamine in the nucleus accumbens, but does not attenuate cocaine-induced elevations (**Figure 20**).*

The results from a 2-way RM ANOVA demonstrate a significant main effect of treatment alone on percentage dopamine change from baseline [$F(3,19)=6.46$ $p<0.01$]. Additionally, there is not an effect of time, and no interaction [$F(4,76)=2.12$ $p>0.05$] [$F(12,76)=0.41$ $p>0.05$]. Dunnett's correction for multiple comparisons post hoc revealed only suvorexant was significantly different from vehicle controls ($p<0.05$) Furethermore, results from a 2-way RM ANOVA demonstrate a significant main effect of treatment alone on amount of dopamine release from baseline [$F(3,19)=6.25$ $p<0.01$]. Additionally, there is an effect of time [$F(4,76)=2.54$ $p<0.05$] and no interaction [$F(12,76)=0.62$ $p>0.05$]. Dunnett's correction for multiple comparisons post hoc revealed only suvorexant was significantly different from vehicle controls ($p<0.01$). There were no significant differences among treatments on percentage dopamine change or amount of dopamine release after acute cocaine administration [$F(3,18)=2.55$ $p>0.05$], [$F(3,18)=2.42$ $p>0.05$].



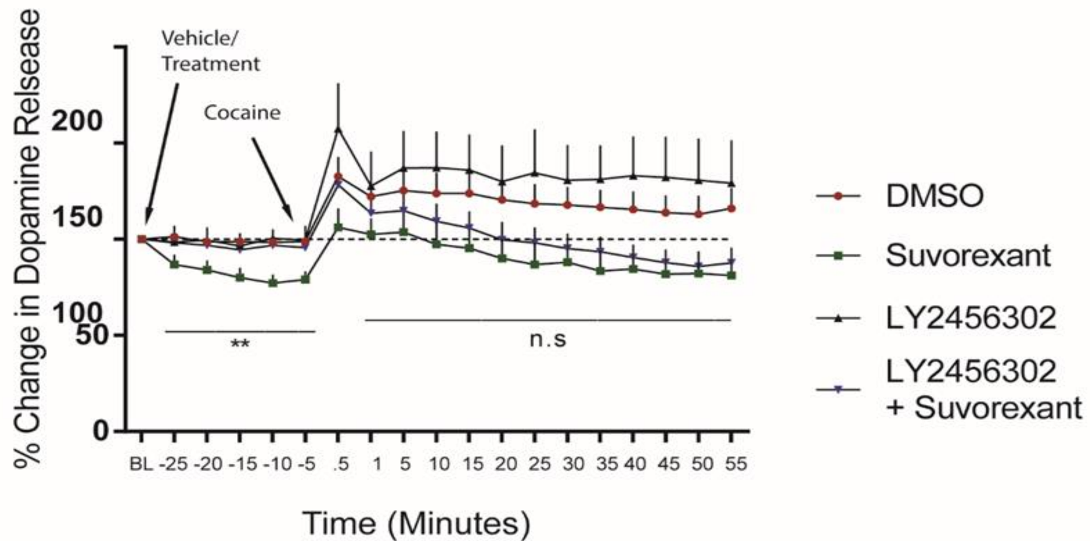
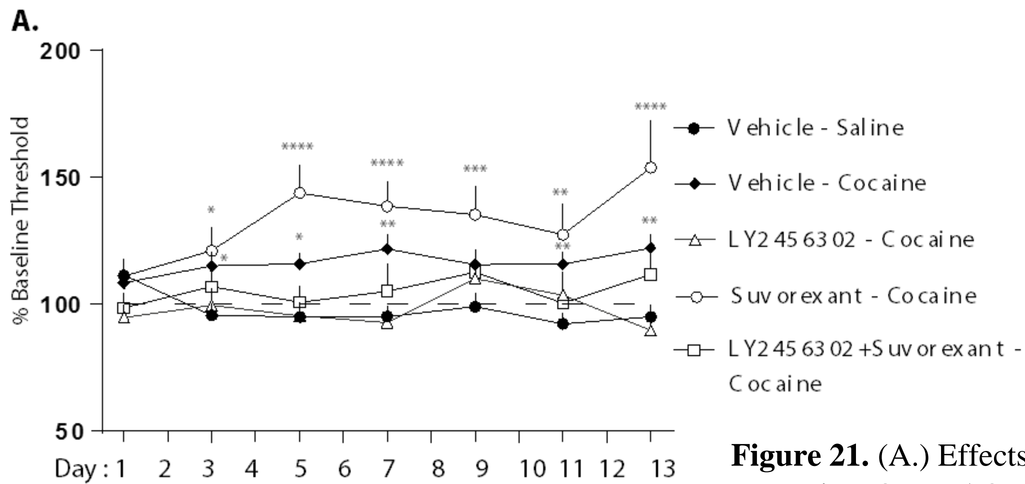


Figure 20. Effects of Electrically stimulated dopamine in the NaC with suvorexant and LY2456302 pretreatment in the VTA on % changes in baseline DA and % changes in baseline DA release. Data presented as mean % of baseline responses ± *S.E.M*. in FSCV (n=5-8/cohort).

Experiment 6.3: LY2346302 mitigates cocaine withdrawal-induced increases in baseline reward threshold alone and in combination with suvorexant ***(Figure 21.)***

Results suggest a significant main effect of treatment on baseline reward thresholds during acute cocaine withdrawal utilizing a 2 way ANOVA [$F(4,531)=30.55$ $p<0.0001$] however no significant effects over time [$F(6,531)=1.29$ $p>0.05$] or interaction

[F(24,531)=1.12 p>0.05] were observed (n=6-14). Post hoc Dunnett's multiple comparisons reveal differences in anhedonia after cocaine alone during days 3,4,5,6,7,8,11,12,13,14 and in suvorexant pretreatment at every day after day 2 [p<0.05]. The present study revealed a main effect of treatment and time on the % maximum rate during withdrawal utilizing a 2 way ANOVA [F(4,531)=7.78 p<0.0001], [F(6,531)=3.44 p<0.05] however no significant effect on interaction [F(24,531)=1.46 p>0.05]. Post hoc Dunnett's multiple comparisons reveal differences in vehicle, suvorexant and suvorexant-LY2456302 groups compared to saline control, at days 7 and 8. Additionally, an ordinary one way ANOVA reveals a main effect in changes in % time in open arms [F(4,35)=2.63 p≤0.05]. Post hoc Dunnett's multiple comparisons reveal differences in vehicle-cocaine and suvorexant-cocaine groups compared to vehicle-saline control (p<0.05) (n=5-10/cohort).



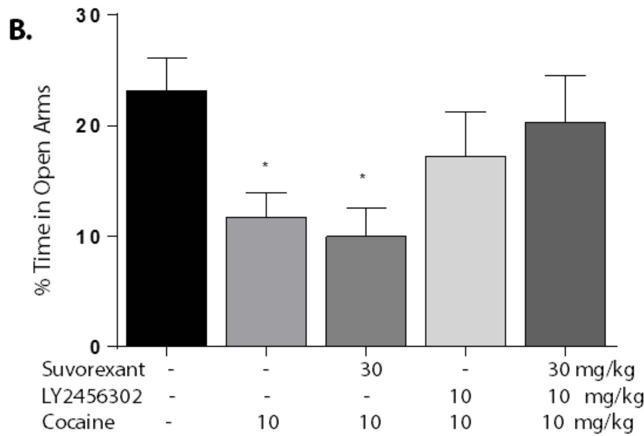


Figure 21. (A.) Effects of systemic KOR and OxR antagonists on ICSS thresholds following 20 hr cocaine abstinence and Data presented as 2-day mean of baseline responses ± *S.E.M*. in ICSS. (n=6-14/cohort). (B.) Effects of systemic suvorexant and LY2456302 on elevated plus maze Data presented as mean % time spent in open arms ± *S.E.M*, n=6-10/cohort.

Experiment 6.4: Acute withdrawal decreases transcriptional activity in VTA dopaminergic neurons and increases activity in the BNST, however, has no effect on HPA CORT levels ***(figure 22).***

A one way ANOVA demonstrates significant differences of treatment on # of TH^+cFos^+ neurons F(2,19)=8.06 p<0.01]. Post hoc Tukey multiple comparisons show both saline-saline and LY2456302-cocaine cohorts are different compared to saline-cocaine cohorts (p<0.05). A one way ANVOA suggests significant differences in BNST % $cFos^+$ cells [F(2,18)=12.75 p<0.01]. Post hoc Dunnett's multiple comparisons reveal differences in both vehicle-cocaine (p<0.01) and LY2456302-cocaine (p<0.05) compared to vehicle-saline control. Further analysis reveals no significant correlation between EPM time in open arms and # of $cfos^+$ cells in the BNST using a linear regression (p>0.05). However, results do not demonstrate a significant effect of drug pretreatment altering cocaine induced changes in plasma CORT levels using a one way ANOVA [F(4,24)=1.80 p>0.05].

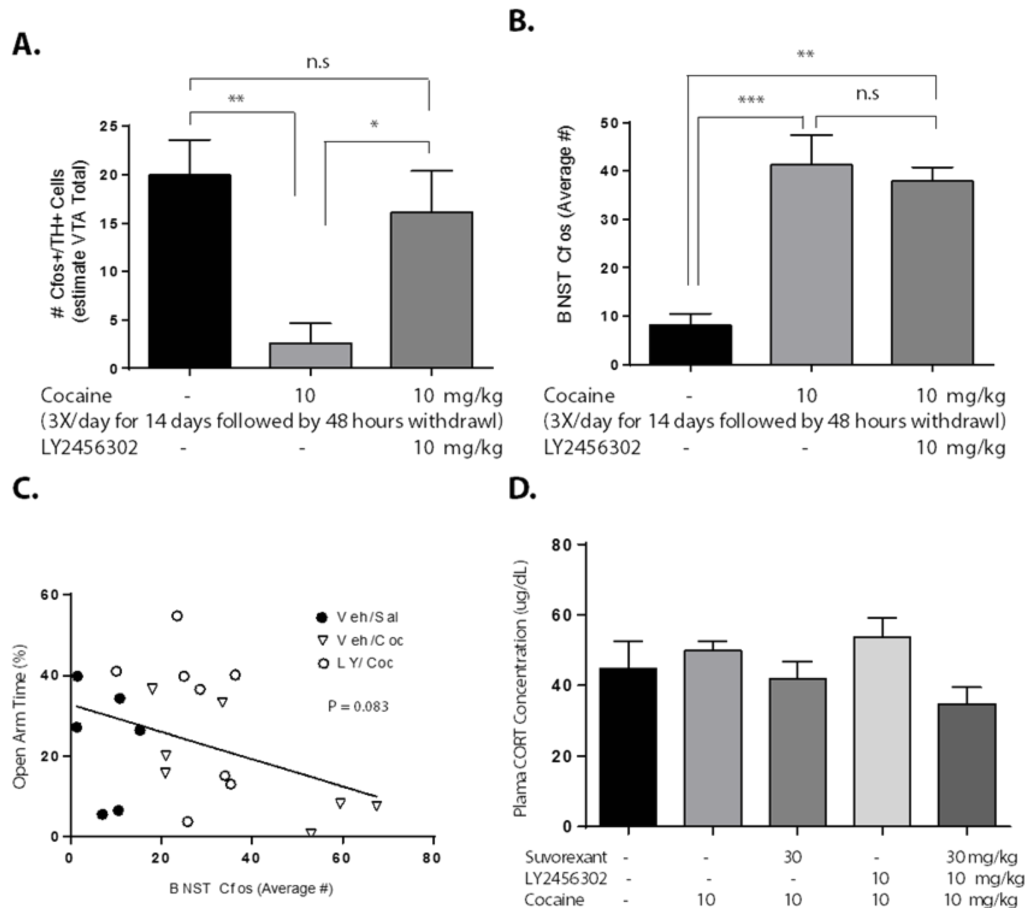


Figure 22. Effects of systemic suvorexant and LY2456302 on (A.) VTA #cFos$^+$TH$^+$ neuron activity (B.) # of BNST cFos activity (C.) Correlation of EPM open arm time and BNST cFos activity (D.) Plasma CORT concentration during cocaine withdrawal. All data presented as means ± *S.E.M*. (n=5-8/cohort)

Experiment 5: LY2346302 has no effect on cocaine-induced rewards in baseline threshold

The present study did not reveal a significant main effect of treatment dose on potentiating cocaine-induced decreases in baseline threshold utilizing an ordinary measures one way ANOVA $F(4, 23) = 0.37$, $p > 0.05$] (n=5-6/cohort). Additionally, the study did not reveal a significant main effect of LY2456302 dose alone in altering baseline threshold utilizing an ordinary measures one way ANOVA $F(4, 31) = 0.57$, $p > 0.05$] (n=5-8/cohort).

Discussion of orexin and dynorphin activity in cocaine reinforcement and abstinence.

We report here that the dual orexin receptor antagonist suvorexant counters the reward-facilitating effects of cocaine as demonstrated by normalization of thresholds during intracranial electrical self-stimulation. Previous literature supports the role of suvorexant in attenuating cocaine-induced reward, impulsivity and ultrasonic vocalizations (Gentile, Simmons, Barker, et al., 2017; Gentile, Simmons, Watson, et al., 2017; Simmons et al., 2017). Prior work demonstrated that blockade of OxR1 using the research-only agent SB334867 can attenuate the reinforcing effects of cocaine while OxR1 knockout mice have a decreased motivation for cocaine seeking (Bernstein, Badve, Barson, Bass, & España, 2017; Hollander et al., 2012). In the present study, we additionally find that systemic blockade of both Ox1R and Ox2R using suvorexant can produce an anhedonic-like effect as interpreted from dose-related elevations in reward

thresholds compared to within-session baseline rates (Wise, 1996). The observed dose-related anhedonic effects of suvorexant indicates a potential problem with translational appeal of the compound, but is not inconsistent with reported adverse effects from clinical trials (Kuriyama & Tabata, 2016). In effort to normalize suvorexant-induced suppression of reward function, we pharmacologically suppressed transmission of dynorphin—a co-localized peptide with suspected anti-reward function. Prior work showed that norBNI, a long-acting KOR antagonist, normalized reward-suppressing effects of SB334867 in electrically self-stimulating mice (Muschamp et al., 2014). In our study, pre-treatment with LY2456302, a relatively shorter-acting KOR antagonist in trial for smoking cessation, effectively normalized the reward-suppressing effect following suvorexant injection. The combination of suvorexant and LY2456302 did not alter reward thresholds relative to within-session baseline rates, and LY2456302 alone did not appreciably facilitate self-stimulation. Notably, LY2456302 pretreatment permitted the therapeutically-favorable effect of suvorexant in normalizing cocaine-associated reward facilitation. Thus, the reward-suppressing effect of suvorexant alone can be normalized by pretreatment with LY2456302 yet the ability of suvorexant to normalize cocaine-associated threshold changes is maintained in the state of systemic KOR blockade (i.e. with LY2456302 on-board).

In attempt to interrogate the underlying neurobiology of orexin-dynorphin functional opposition, we employed a site-directed pharmacological approach within VTA. A collection of previous reports shows that orexin transmission within VTA contributes to behaviors associated with reward-related pathology including cocaine-associated impulsivity and reinforcement and additionally modulates physiology of

putative dopamine-producing cells (Borgland et al., 2006; España et al., 2011; España et al., 2010; Gentile, Simmons, Watson, et al., 2017). Our results demonstrate a dose-related aversion (threshold elevation) to intra-VTA suvorexant compared to vehicle-injected control rates. Intra-VTA suvorexant modestly normalized reward-facilitating effects following acute cocaine injection. It remains possible that only a trend was observed as intra-VTA injections were unilateral (contralateral to the stimulating electrode) due to technical constraints—namely, inability to place bilateral guide cannulae and a stimulating electrode with grounding wire on a mouse skull. In a prior report, intra-VTA SB334867 showed laterality-dependent effects on cocaine reinforcement with bilateral injections producing greater reductions in motivated cocaine-taking (España et al., 2010).

We next measured electrically-evoked dopamine levels in the NAc following intravenous cocaine and site-directed pharmacological agent pretreatments. Unilateral intra-VTA suvorexant produced a significant decrease in stimulated DA levels, and this effect was normalized following treatment with intra-VTA LY2456302. These data support the hypothesis that suvorexant-induced anhedonia as observed from electrical self-stimulation experiments may be due in part to a suppressed DA release, and that KOR blockade can normalize suvorexant-induced suppression of ventral striatal DA release. Intra-VTA suvorexant modestly normalized cocaine-evoked elevations in ventral striatal DA. Thus, additional circuits beyond the canonical reward-regulating mesolimbic DA pathway likely contribute to the behavioral and neurobiological effects following systemic cocaine injection, and site-direct pharmacology using agents that target orexin-dynorphin transmission can further disentangle functional structures.

Psychostimulant abuse can initiate "positive" reward experience but is theorized to persist, cyclically, in an effort to escape negative effects of withdrawal (Koob, 1996, 2009). We modeled withdrawal-associated anhedonia by testing mice in daily sessions 20 hours following a prior-day cocaine injection regimen (10 mg/kg/inj, 3 inj/d, 1 hr apart) as utilized in prior work (Chartoff et al., 2012). After establishing withdrawal-associated elevations in reward thresholds (anhedonia), we found that pretreatment with LY2456302 dose-dependently normalizes reward function, supporting prior work using norBNI (Chartoff et al., 2012). Additionally, suvorexant was found to exacerbate threshold elevations during withdrawal which was not altogether surprising considering our earlier experiments showed suvorexant-induced anhedonia. Lastly, the combination of suvorexant and LY2456302 that did not alter reward thresholds, but mitigated cocaine-associated reward was efficacious in attenuating withdrawal-associated threshold elevations. To complement these results, we used an identical design but assessed effects of pharmacological agents on anxiety-like behavior using the elevated plus maze (EPM) test. LY2456302, alone or in combination with suvorexant, normalized withdrawal-associated anxiety-like behavior. Recent literature has demonstrated a role for dynorphin in counteracting orexin activity in discrete brain regions likes the paraventricular nucleus and the VTA (Baimel et al., 2017; Matzeu, Kallupi, George, Schweitzer, & Martin-Fardon, 2017) The translatable power of altering the orexin-dynorphin system in bivalent mood states is yet to be fully elucidated. However, the author hypothesize the correct combination of pharmacologically controlled OxR and KOR activity could be used to lessen withdrawal symptoms and consequently relapse while simultaneously preventing

reward from acute drug relapses. Significant future work is required to test this hypothesis, but the preliminary data shown is promising.

To probe cellular substrates of withdrawal-associated reward deficits, we additionally measured neuronal activation of putative DA-producing neurons in VTA using immunolabeling of the immediate early gene cFos. Rats undergoing cocaine withdrawal showed suppressed cFos labeling in VTA relative to saline-treated control rats. Pretreatment with LY2456302 partially restored withdrawal-associated neuronal suppression. Prior work positions suppressed mesolimbic DA transmission (possibly through decreased activity of VTA afferents) as an neurobiological feature of cocaine withdrawal that underlies behavioral disturbances including anhedonia (Weiss et al., 1992). Orexin-dynorphin neurons project to reward areas such as the VTA but also nuclei responsible for the governance of negative affect like the BNST (Marcus et al., 2001; Peyron et al., 1998).

Overall, these data support that aversive effects of orexin receptor blockade can be normalized by suppressing dynorphin transmission while therapeutic benefit remains unchanged. We hypothesize that, at homeostasis, orexins engage mesolimbic DA transmission to support motivated behaviors including those directed at retrieving caloric foods, partners for mating, social interactions while dynorphin acts to regulate orexin transmission in reward-governing structures including the VTA. In pathological states including addiction, dynamic alterations in DA circuit activity usurps the "buffering capacity" of an otherwise regulated orexin-dynorphin system and leads to behavioral disruptions including changes in affect/hedonic state. Through careful titration, the utility of pharmacological agents directed at suppressing orexin-dynorphin activity

carries promise to normalize both the “highs” and “lows” typified in addiction. Substantial work will be required to elucidate further physiologic changes that are occurring during drug abuse that diminished the efficacy of the orexin-dynorphin system to help stabilize affect.

GENERAL DISCUSSION

A General Role for Orexin and Dynorphin in Drug Seeking Behavior

Cocaine addiction is a complex disorder involving many conflicting behavioral inputs; impulsivity vs. planning, motivation vs. anergia, reward vs anhedonia, craving vs. satiety, compulsion vs control and many more. However, there can be only one behavioral outcome at a time. We have so far surveyed the role of orexin and dynorphin in behaviors associated with these psychological constructs, and where possible discussed how pharmacologically altered orexin and dynorphin co-transmission activity could impact them. The orexin-dynorphin neurons appear to do this by integrating information about the body's internal state with regard to primary reinforces or 'drives' and recruiting components of the basal ganglia and cortex to initiate and organize appropriate behavioral outputs. For example, when low blood glucose levels are detected within the hypothalamus, orexin-dynorphin neurons may be activated in order to initiate food seeking behavior. The consumed food may further activate orexin-dynorphin neurons to indicate reward and reinforce subsequent behavior providing a role in behavioral decision making and reward learning (Burdakov, Karnani, & Gonzalez, 2013; Kosse, Gonzalez, & Burdakov, 2015).

Dopamine release by midbrain neurons has long been thought to be involved in motivation and reward, however the release of NAc dopamine is under the control of many forces, including those of the orexin-dynorphin circuit. Orexin-dynorphin neurons affect arousal through locus coeruleus, memory formation and plasticity through the hippocampus, executive function and impulse control through the medial prefrontal cortex, and stress through the amygdala. Orexin-dynorphin neurons appear able to

manage information flow between structures in this network, and this has important behavioral consequences. Perhaps the most studied orexin-dynorphin projections; to the ventral tegmental area, strongly influence motivation, reward and anhedonia.

From the perspective of synaptic physiology, the utility, of co-releasing excitatory (orexin) and inhibitory (dynorphin) peptides is poorly understood, however several hypotheses have been proposed. This physiology may allow for flexibility in arranging different signaling elements in a synapse to produce different outputs from the same set of inputs. Orexin-dynorphin neurons can exert several different permutations of effect that are dependent on the target neuron type, receptor expression and endogenous (synaptic/tonic) neuropeptide activity. For example dynorphin may be more potent at disinhibiting neuropeptide Y (NPY) producing neurons when GABA is present while direct orexin action on NPY neurons would increase activity – together orexin and dynorphin would be able to work synergistically in this instance (Y. Li & van den Pol, 2006). Within the VTA, orexin and dynorphin co-localization may provide a mechanism to buffer the effects of multiple, often spontaneously active, excitatory inputs to dopamine neurons. Neuronal firing occurs after a summation of input charge is sufficient to reach a certain threshold and an action potential occurs. The addition of G_i and G_q active neuropeptides may help to increase the signal-to-noise ratio in the midst of these of

constant fluctuations in ionic elements on dopaminergic dendritic spines. From an engineering point-of-view, three components are necessary to create a system that is both stable and flexible; comparators, regulators and generators. In this hypothetical situation applied to orexin-dynorphin neurons, comparators compute the difference between the current state of arousal and the desired state of arousal. The regulators take this difference and produce a control signal, while the generators compute arousal from this control signal. The produced arousal is integrated back into the comparators error and so on and so forth (see **Figure 23**). Orexin-dynorphin neurons may function as comparators and their VTA projections may function as the regulators and generators (Kosse & Burdakov, 2014). This system handles irrelevant noise (or inputs) onto the generators or regulators well (the feedback loop compensates, and the generator output is appropriate), however disruption onto a solely excitatory orexin neuron would process this new input as a salient set-point and the system would produce the incorrect generator output. To counter this weakness, an inhibitory component from the orexin neuron (such as dynorphin co-localization) could more easily handle aberrant inputs and noise while still keeping the arousal system stable (Kosse & Burdakov, 2014). This feature is important if we view the orexin system as an

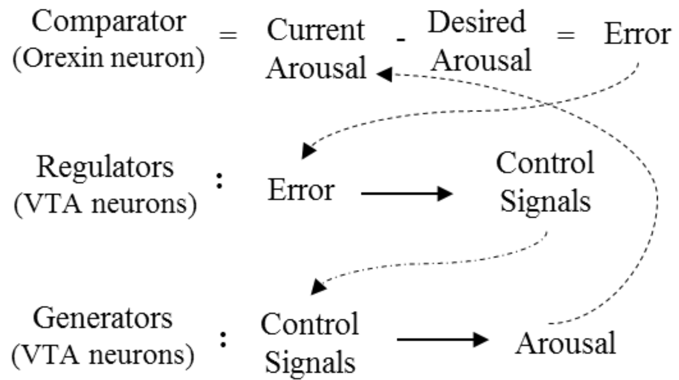


Figure 23. Computational framework for flexible and stable circuit

evolved, flexible feedback loop that solves the problem of noisy inputs to the comparator by expressing dynorphin in those neurons.

We have shown that decreasing orexin receptor activity attenuates reward and dopamine transmission in the NAc while decreasing kappa opioid receptor activity attenuates anhedonia and increases expression of cFos within DA neurons during withdrawal. Interestingly, the reverse does not seem to be true. OxR blockade does not interfere with KOR antagonism decreasing withdrawal (anhedonia) and KOR blockade does not change the negative effects of OxR antagonists on reward. This suggests that the orexin system can increase dopaminergic activity, and altering the OxR/KOR activity ratio in favor of constitutively active KORs (due to endogenous dynorphin) decreases the reward phenotype. Blockade of KORs disinhibits DA activity, however antagonizing an inhibitory receptor cannot cause reward because it does not increase neuronal firing above baseline (a simplistic analogy would involve simply removing one's foot from a break will not accelerate a car). Similarly, when there is hypodopaminergic activity the dynorphin system seems to contribute and altering the OxR/KOR activity ratio in favor of constitutively active OxRs (due to endogenous orexin) decreases withdrawal-induced anhedonia. This dichotomy strengthens the hypothesis that the presence of orexin and dynorphin allows the mesolimbic dopamine system to compensate for transient and extreme changes and influences on the dopaminergic neurons. It is only with exogenous drug rewards (not natural rewards) that these fluctuations become so extreme that they push the reward system past the limits of the orexin-dynorphin buffer. The existence of co-release and dichotomous orexin and dynorphin effects on cell excitability and behavior suggests it is a mechanism for buffering extremes in DA activity and would

work similar to the other physiologic systems like D_1/D_2-like receptors with opposing action in the striatum. Dopamine exerts differing outputs, however this serves an important role in keeping motor functions appropriate that becomes apparent when the lack of dopamine to serve as this buffer manifests in parkinsonian like dyskinesia (Farlow, Pankratz, Wojcieszek, & Foroud, 1993).

Of course, much remains to be answered, for instance, are levels of orexin and dynorphin gene transcription and translation in the cell bodies altered during addiction? Do neurons package and transport differing stoichiometric ratios of the peptides within the vesicles? Are there differences in enzymatic degradation or reuptake of the peptides once released from the neuron? Additionally, the same questions could be posited about the corresponding receptors. Are there changes in the creation, movement, activity and positioning of the OxR and KORs before, during or after periods of drug use and withdrawal? The presented work helps to illuminate a therapeutic value in altering the activity in the orexin-dynorphin system but does not focus on the physiologic changes induced during different stages of drug abuse.

In summary we have shown suvorexant (OxR antagonism) decreases cocaine reinforcement and impulsivity, however this is problematic as orexin receptor blockade seems to cause anhedonia alone. This can be seen using 5 choice serial reaction time task (impulsivity), cocaine self-administration (motivation to seek reward), ultrasonic vocalizations (subjective reward) and intracranial self-stimulation (reward and anhedonia). Further we demonstrated LY2456302 (KOR antagonism) attenuates this suvorexant induced anhedonia but does not interfere with the anti-reinforcement properties of orexin receptor blockade. Conversely LY2456302 mitigates cocaine

withdrawal-induced anhedonia with and without suvorexant. The decreases in withdrawal phenotypes was suggested using ICSS and elevated plus maze behaviors. To define the relevant circuitry, we demonstrated that OxR and KOR in the ventral tegmental area, most likely on dopaminergic nuclei that project to the ventral striatum are largely responsible for these bivalent mood states. We illustrate the likely circuitry using direct site pharmacology, injecting doses of LY2456302 and suvorexant directly into the VTA. In search of a mechanism we examined dopamine levels following multiple combinations of vehicle, suvorexant and LY2456302 before and after cocaine administration in anesthetized rats. Correlating to our behavioral endpoints, OxR blockade decreased VTA-stimulated dopamine in the NAc while KOR antagonism attenuated this decrease but had no effect alone. Although trends existed to suggest suvorexant pretreatment decrease cocaine-induced increases in NAc dopamine, we were unable to correlate OxR blockade decreasing reinforcement and dopamine levels. However, transactional activity in the dopamine producing neurons of the VTA decreases during acute withdrawal and is elevated with a pretreatment of KOR antagonism, suggesting a dopaminergic mechanism for KOR antagonism alleviating withdrawal phenotypes. Together this represents a drug combination that could serve as a therapeutic to “level out” positive and negative affect. Hypothetically a patient could take orexin and kappa opioid receptor antagonists daily because the drug combination itself would not be rewarding (posing little to no abuse potential) but wouldn’t be aversive either. In early stages of psychostimulant abstinence, the combination would help alleviate withdrawal symptoms that theoretically lead to relapse. However, if the patient did relapse the same combination would help to decrease motivation to continue drug seeking during acute

use. This combination represents a novel treatment strategy to influence the reward system during drug abuse in a more natural way and attempts to rescue both overly positive and overly negative mood states. The experiments laid out here offer more than a theoretical framework for psychostimulant addiction but also highlight some of the first literature to examining how the dichotomy of OxR and KOR activity contributes to bivalent mood states systemically and within the VTA. Experiments focusing on the VTA all strengthen the literature on neuropeptides and the governance of mesolimbic dopamine during cocaine abuse.

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