INVOLVEMENT OF THE OREXIN/HYPOCRETIN SYSTEM IN ETHANOL-CONDITIONED BEHAVIOR

By

Charlene M. Voorhees

A THESIS

Presented to the Department of Behavioral Neuroscience

And the Oregon Health & Science University

School of Medicine

in partial fulfillment of

the requirements for degree of

Master of Science

September 2009

School of Medicine

Oregon Health & Science University

CERTIFICATE OF APPROVAL

This is to certify that the Master's thesis of

Charlene M. Voorhees

has been approved

Mentor/Advisor

Member

Member

Member

TABLE OF CONTENTS

List of Abbreviations

αMSH – α-melanocyte stimulating hormone

ANOVA – analysis of variance

BEC – blood ethanol concentration

CPP – conditioned place preference

CS – conditioned stimulus

CRF – corticotropin releasing factor

DMSO – dimethyl sulfoxide

GABA – gamma aminobutyric acid

ICV – intracerebroventricular

IV - intravenous

IP - intraperitoneal

LH – lateral hypothalamus

LTP – long term potentiation

MCH – melanin concentrating hormone

mRNA – messenger ribonucleic acid

NAc – nucleus acumbens

NMDA – N-methyl-D aspartate

NPY – neuropeptide Y

OX1R – orexin 1 receptor

OX2R – orexin 2 receptor

PIT – Pavlovian-instrumental transfer

VTA – ventral tegmental area

List of Figures and Tables

Acknowledgements

This research was supported by NIH-NIAAA grants AA007702 and AA007468, Achievement Rewards for College Scientists (ARCS) Foundation, Inc., and a N.L. Tartar Research Fellowship. I would like to thank Dr. Tara Fidler for assistance in blood sample collection and the Portland Alcohol Research Center Core (Crabbe Laboratory) for assistance with measurement of blood ethanol concentrations in Experiment 3. I would like to thank my mentor Dr. Chris Cunningham for guidance in conducting the experiments in this document and the members of my thesis advisory committee, Dr. Andrey Ryabinin and Dr. K. Matthew Lattal for helpful comments on the experiments and the document. I would also like to thank Dr. Charles Roselli for serving on my oral examination committee. Experiments 1 and 4 were described in a published abstract and presented at the Research Society on Alcoholism Annual Meeting in 2009.

Abstract

Background: Neurons containing the neuropeptide orexin project from the lateral hypothalamus to reward-related areas such as the ventral tegmental area and nucleus accumbens (Harris & Aston-Jones, 2006). Recent studies have highlighted a role for orexin in drug-related behaviors, but investigation of the role of orexin in ethanol-conditioned behaviors is lacking. The present experiments investigate orexin involvement in the acquisition and expression of ethanol conditioned place preference (CPP) using the orexin 1 receptor (OX1R) antagonist SB-334867.

Methods: Effects of SB-334867 on locomotor activity were investigated where animals received 0, 10, 20 or 30 mg/kg IP and activity counts were measured (Experiment 1). SB-334867 was administered during acquisition of ethanol conditioned place preference on trials where DBA/2J mice received 2 g/kg ethanol to determine if orexin signaling is required for the development of ethanol CPP (Experiment 2). Blood ethanol concentrations were also measured from mice receiving SB-334867 or vehicle and 2 g/kg ethanol at 2.5, 10, and 60 min after injection to determine whether SB-334867 altered ethanol pharmacokinetics (Experiment 3). SB-334867 was also given before ethanol-free preference testing in 2 separate experiments to examine the effects of OX1R antagonism on expression of ethanol CPP (Experiments 4 and 5).

Results: SB-334867 did not alter basal locomotor activity (Experiment 1). SB-334867 administered during conditioning did not affect the development of ethanol CPP, but at 30 mg/kg, locomotor stimulation to 2 g/kg ethanol was significantly reduced (Experiment 2). SB-334867 pretreatment before ethanol injection increased BEC at 10 min and 60

min post-injection suggesting that SB-334867 alters ethanol pharmacokinetics (Experiment 3). OX1R antagonism blocked the expression of a weak ethanol CPP disrupted by novel handling and pretreatment injection (Experiment 4), but did not affect expression of a strong CPP when animals were habituated to vehicle pretreatment injections (Experiment 5).

Conclusions: The present studies indicate that blockade of OX1R by systemic administration of SB-334867 does not affect acquisition or expression of ethanol CPP in DBA/2J mice. Other neurotransmitter systems such as dopamine or glutamate may be sufficient to support acquisition and expression of CPP despite alterations in orexin signaling.

Chapter 1: Introduction

Alcohol Use Disorders

The prevalence of alcohol use disorders is a persistent and costly public health issue in the United States (Harwood et al., 2000). Though significant progress has been made in developing treatments for alcoholism, millions of people in the United States are still dependent on or abusing alcohol (NIAAA, 2004). A primary goal of research on alcohol use disorders is to understand the neurobiology of the disease in order to enhance prevention and treatment and to understand the biological processes involved in alcohol related behaviors (NIAAA, 2006). Animal models are well-suited for research on the neurobiological underpinnings of alcohol use disorders, and preclinical data have been used to develop current pharmacological treatments such as naltrexone (O'Malley et al., 1992; O'Malley & Froehlich, 2003; Volpicelli et al., 1992) and acamprosate (Mann et al., 2008; Spanagel & Zieglgansberger, 1997) and new biological targets for the development of future therapeutics (see Heilig & Egli, 2006 for review).

Alcohol drinking produces a multitude of effects including positive effects (euphoria) and negative effects (anxiety, dysphoria), though there are considerable individual differences in the subjective effects of ethanol (Sher et al., 1985). These positive effects may be important in the initial stages of alcohol use, and the negative effects important in the continuation of alcohol seeking and the transition to dependence (Gilpen & Koob, 2008). Using carefully designed studies, one can investigate the underlying neurobiology that contributes to the main components of alcohol abuse and alcoholism including initiation of alcohol use, overconsumption, alcohol-dependence, and relapse (e.g., Koob, 1998; Vengeliene et al., 2008).

The Neurobiology of Ethanol Reward

Animal Models

The animal models of ethanol consumption are fairly straightforward and include free-access two-bottle choice paradigms for measuring ethanol preference and operant self administration procedures in which the animal must emit a response in order to access the drug (Cunningham et al., 2000). The models can be used with outbred animals to investigate individual differences in ethanol consumption, or with animals that have been selectively bred for high or low levels of consumption (see McBride & Li, 1998 for review). Consumption-based models incorporate both the appetitive (or drug-seeking) and consummatory (or drug-taking) phases of use, but it is advantageous to separate these phases in behavioral models to isolate drug-seeking behavior (see Samson & Czachowski, 2003), especially if one is interested in mechanisms underlying relapse to drug seeking.

There are several ways to assess drug-seeking behavior in rodents. Animals that maintain an operant response such as lever pressing resulting in access to (or self-administration of) drug are performing an appetitive or drug-seeking behavior. Measuring the persistence of this behavior in the absence of the drug (in extinction) or after responding has been extinguished (reinstatement test) are common operant methods used to study drug-seeking (see Cunningham et al., 2000 for detailed review).

Another method used in many laboratories is conditioned place preference (CPP), a Pavlovian conditioning procedure where a neutral contextual cue gains motivational value through pairing with the effects of a drug (for review, see Tzschentke, 1998, 2007). CPP is a widely used model for studying the rewarding and aversive effects of drugs

(Tzschentke, 2007). If the drug has rewarding effects, then during a drug-free place preference test, an animal will spend more time in proximity of the cues previously paired with drug than in proximity to cues previously paired with saline (Cunningham et al., 2006). The approach to the cue during the expression test is thought to reflect the conditioned reinforcing properties of the context (Cunningham & Patel, 2007), and thus this model has been used to examine drug-seeking behavior (Cunningham et al., 2000). Whereas self-administration studies are often designed to assess the direct reinforcing effects of a drug, the CPP model can be used in animals to assess processes during drug-conditioning (primary drug effects) or during drug-free CPP expression testing (conditioned reinforcing effects of drugs) (Cunningham et al., 2006).

Neurobiology of Ethanol Reward

Like other drugs of abuse, ethanol is rewarding and accordingly, ethanol affects dopamine signaling in the mesocorticolimbic system (Gonzales et al., 2004; Weiss & Porrino, 2002). The basic circuitry involved in ethanol reward is outlined in Figure 1. It should be noted that the information contained in this figure is not comprehensive and is intended to give a simple overview of the circuit. Ethanol differs from stimulant drugs in directly activating dopamine cells in the ventral tegmental area (VTA) and increasing firing (Brodie et al., 1999; Budygin et al., 2001;Yim & Gonzales, 2000), and as a result increasing extracellular dopamine concentrations in the nucleus accumbens (NAc; Di Chiara et al., 2004). While dopamine signaling is important for ethanol reward (see Gonzales et al., 2004 for a review), dopamine is not the only neurotransmitter involved. Multiple neurotransmitter systems such as opioid, glutamate, gamma aminobutyric acid (GABA), acetylcholine, and neuropeptides participate in processes underlying

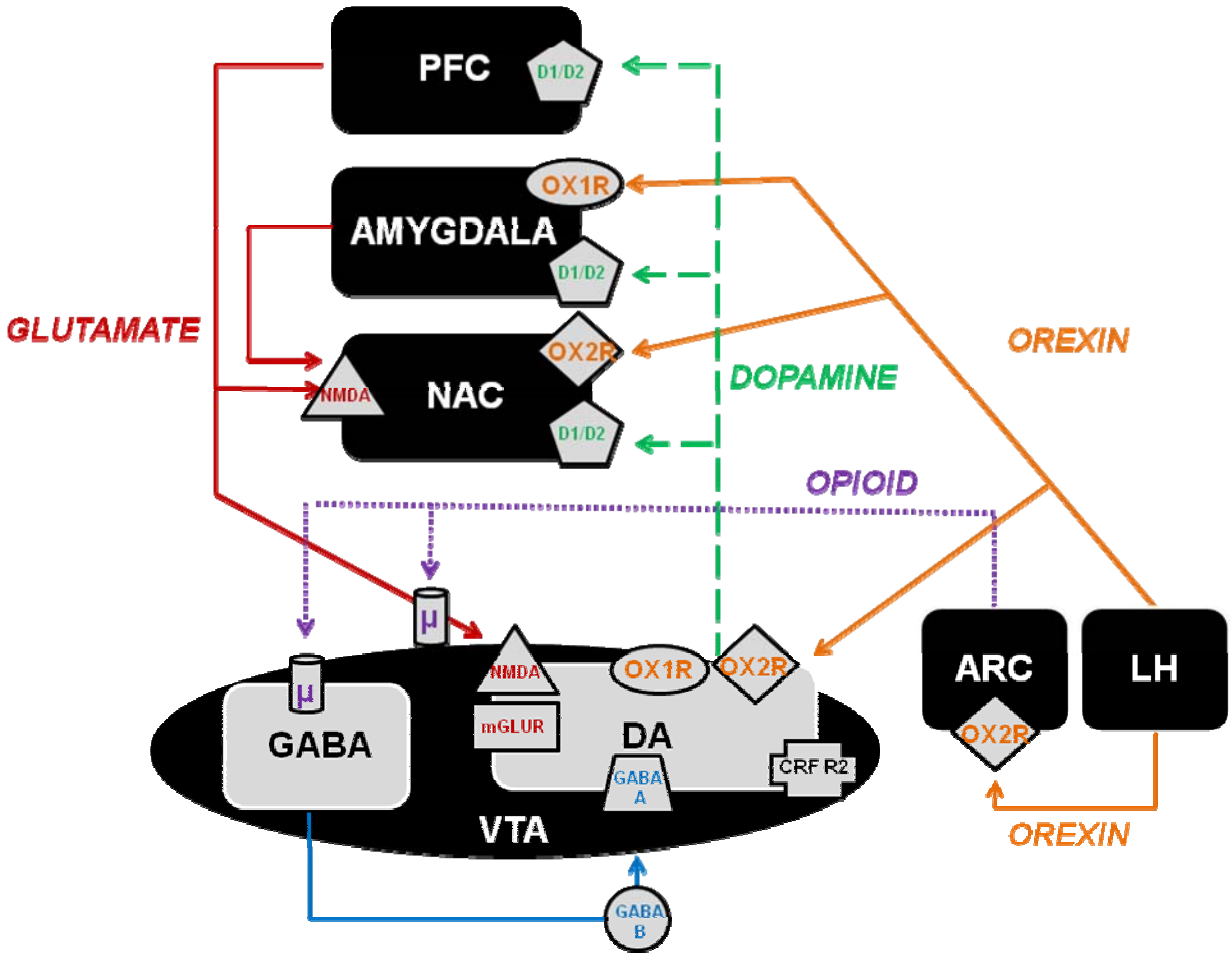

Figure 1. Basic Circuitry of the Mesocorticolimbic System and Interactions with Orexin. This diagram is a basic summary of the important projections, brain regions and receptors relevant for ethanol reward. Orexin projections and interactions with the major neurotransmitter systems are shown. This diagram is not intended to be comprehensive. Receptors presented on projection arrows (e.g., $GABA_B$) are presynaptic. Abbreviations: PFC – prefrontal cortex, NAC- nucleus accumbens, GABA- gamma aminobutyric acid, NMDA - N-methyl-D aspartate, mGLUR – metabotropic glutamate receptor, DA – dopamine, VTA – ventral tegmental area, OXR – orexin receptor, CRF R2 – corticotropin releasing factor receptor 2, ARC – arcuate nucleus, LH – lateral hypothalamus, μ - mu opioid receptor, D1/D2 – dopamine receptor subtypes 1 and 2. This figure was adapted from information in: Bonci & Borgland (2009), Everitt & Wolf (2002), Harris & Aston-Jones (2006), Koob et al. (1998), Marcus et al. (2001), Ohno & Sakurai (2008), Stahl (2008), Swanson (1982), Trivedi et al. (1998), Weiner et al. (1991).

alcohol use and dependence (see Vengeliene et al., 2008 for detailed review) in addition to dopamine. These neurotransmitters have a variety of functions, but also indirectly affect dopamine and glutamate function (Vengeliene et al., 2008) resulting in modulation of reward processing. Receptors for these neurotransmitters and peptides have also been the target of recent pharmacotherapies for alcoholism (see Johnson, 2005 for review).

The dopaminergic system is under the control of excitatory and inhibitory inputs (Kitai et al., 1999). Importantly for modulating ethanol reward, the NAc receives excitatory glutamatergic inputs from cortical regions (Everitt & Wolf, 2002), and therefore glutamate has also been shown to be key modulator of ethanol reward. The NMDA receptor is a primary target for ethanol's actions (Allgaier, 2002), but the effects of glutamate manipulations on the self-administration of ethanol is inconclusive (Venegeliene et al., 2008). NMDA antagonists infused into the NAc have been shown to reduce ethanol self-administration, suggesting that glutamate transmission is important for ethanol's reinforcing effects (e.g., Rassnick et al., 1992). However, other studies have shown no effect of NMDA antagonists on self-administration (e.g., Bienkowski et al., 1999), and it is unclear if the NMDA receptor is directly involved in mediating the reinforcing effects of ethanol (Koob et al., 1998). The metabotropic glutamate receptor 5 (mGluR5), however, has been shown to be important in ethanol consumption and dependence, and mGluR5 antagonists consistently decrease self-administration of ethanol (see Blednov & Harris, 2008 for review).

The mechanism for ethanol's effects is known to involve interactions with the $GABA_A$ receptor (Kumar et al., 2009), and this receptor has been shown to be involved in ethanol dependence (see Enoch, 2008 for review). Early studies supported the

importance of GABA in ethanol dependence by showing that $GABA_A$ antagonists (Boyle et al., 1993; Rassnick et al., 1993) and inverse agonists (Rassnick et al., 1993) decreased self-administration in rats. Disruption (using viral-mediated RNA interference) of specific $GABA_A$ receptor subunits in the NAc also decreases consumption and preference for ethanol (Rewal et al., 2009). GABA has been further implicated in ethanol dependence by findings that GABA receptors are critically involved in ethanol withdrawal, and that neuroactive steroids that act on the GABA receptors are also mediators of ethanol-related behaviors (see Biggio et al., 2007 for review).

In addition to the neurotransmitters discussed above, neuropeptides have been the focus of recent research on ethanol reward. Corticotropin-releasing factor (CRF) antagonists, for example, have been shown to reduce consumption and/or alcohol-seeking behavior in a number of stress or anxiety related animal models (reviewed in Cowen et al., 2004). Urocortin, a member of the CRF peptide family, is also involved in ethanol intake and sensitivity to the effects of ethanol (Ryabinin & Weitemier, 2006). NPY has also been shown to be important for modulating ethanol intake and dependence, though the effects of NPY appear to depend on genetic factors and history of ethanol consumption (see Badia-Elder et al., 2007; Thorsell, 2007 for reviews).

The opioid system is the most widely studied neuropeptide system for a role in ethanol dependence, and has been shown to be critically involved in ethanol reinforcement. Opioids interact with dopaminergic mechanisms in mediating ethanol reward (Weiss & Porrino, 2002). Many studies have shown that ethanol causes the release of β-endorphins when the dose is rewarding (see Cowen et al., 2004 for a review). Accordingly, mu opioid receptor knock-out mice have decreased ethanol consumption in

an operant procedure (Roberts et al., 2000), and administration of opioid antagonists has repeatedly been shown to reduce ethanol drinking in animal models (see Ulm et al., 1995 for review) and humans (see O'Brien et al., 1996 for review).

Overall, literature on the neurobiology of ethanol reward has focused on many different major systems involved in modulating initiation of alcohol use, consumption, and relapse. The neurobiology of ethanol reward is complex and involves interactions among many different neurotransmitters and peptides in processes related to memory, reinforcement, and motivation. Figure 1 summarizes the interaction of neurotransmitter systems and receptors relevant for understanding ethanol reward and how orexin interacts with these systems.

Neurobiology of Ethanol-Conditioned Behavior

Ethanol seeking and consumption in alcoholism is also greatly influenced by the environment. Drug-related stimuli in the environment can gain motivational significance through pairing with the effects of ethanol in a Pavlovian conditioning process, and are involved in craving, maintenance of drug-seeking, and relapse (Stewart et al., 1984; Robinson & Berridge, 1993; Childress et al., 1999, Everitt et al, 2001; Di Ciano & Everitt, 2004). The strong environmental control of drug seeking may explain the high incidence of relapse among alcoholic patients (Cooney et al., 1997), and therefore understanding the neurobiology of conditioned-rewarding effects of ethanol is very important for understanding processes involved in craving and relapse.

Significant progress has been made in understanding drug-seeking behavior using animal models to identify key brain regions and transmitter pathways. Animal models such as the Pavlovian-instrumental transfer (PIT) and stress, cue, context, and drug-

induced reinstatement have been used to investigate ethanol-seeking (Corbit & Janak, 2007, Chaudrhi et al., 2008, see Lê & Shaham, 2002 for review). A PIT paradigm in which ethanol-associated stimuli enhanced self-administration of ethanol was recently developed, and suggests that ethanol-associated cues can motivate ethanol-seeking behavior (Corbit & Janak, 2007). Reinstatement of ethanol-seeking can be triggered by exposure to ethanol, stress, or cues previously paired with ethanol (Lê & Shaham, 2002). These models are used to investigate the neurobiology underlying craving and ethanol-seeking behavior.

Research on the neurobiology of ethanol conditioned-behavior is relatively limited, but many of the same brain regions and neurotransmitter systems implicated in the primary reinforcing effects of ethanol have also been tested for involvement in the conditioned reinforcing effects of ethanol. For example, the mu opioid receptor antagonist naltrexone has been shown to reduce context-induced, drug-induced, or cue-induced relapse to alcohol seeking, suggesting that endogenous opioids mediate ethanol-seeking behavior (Burattini et al., 2006; Dayas et al., 2007; Lê et al., 1999).

The NAc has been shown to play a central role in ethanol-conditioned behavior as well. Reinstatement of lever pressing for ethanol by returning to the self-administration context was significantly attenuated by inactivation of the NAc core but not the shell (Chaudhri et al., 2008). Dayas et al. (2007) reported increased c-Fos (marker of neuronal activity) expression in the NAc, hippocampus, prefrontal cortex, and paraventricular nucleus of the hypothalamus to a previously ethanol-paired stimulus in an operant procedure. Additionally, Hill et al. (2007) identified c-Fos activation in the VTA, dorsomedial hypothalamus, bed nucleus of the stria terminalis, extended amygdala and

hippocampus after exposure to a previously ethanol-paired stimulus in a Pavlovian procedure. These two studies suggest that these brain regions are putatively involved in ethanol-conditioned reward, and future functional studies should further investigate the role of these regions in ethanol-conditioned behavior.

Neurobiology Underlying Expression of Ethanol CPP

Studies from our laboratory using the ethanol CPP model to investigate ethanol-conditioned behavior have focused on neurotransmitter systems often implicated in ethanol reward such as the opioids, dopamine, and glutamate when investigating the neurobiology of ethanol CPP (Bechtholt & Cunningham, 2005; Gremel & Cunningham, 2009). One study found that microinfusion of a nonselective opioid antagonist methylnaloxonium or the $GABA_B$ receptor agonist baclofen into the VTA disrupted expression of ethanol CPP (Bechtholt & Cunningham, 2005). This study suggests that VTA dopamine neurons play an important role in expression of ethanol-conditioned behavior. To elucidate mechanisms of ethanol-conditioned changes in VTA dopamine neuron function, lesion studies and microinfusion studies were performed in the amygdala and NAc because these areas receive dopamine projections from the VTA (Swanson, 1982). The lesion study showed that activation of the amygdala is required for expression of ethanol CPP (Gremel & Cunningham, 2008) and pharmacological microinfusion studies implicated dopamine transmission in the amygdala and glutamate transmission in the NAc (Gremel & Cunningham, 2009).

While it is clear from these studies that VTA, NAc, and amygdala are functionally important regions for expression of ethanol CPP, other transmitters or peptides may be important as modulators of dopamine or glutamate transmission. The neuropeptide

orexin/hypocretin, for example, plays a critical role in VTA neuroplasticity relevant to addiction (Borgland et al., 2006; Aston-Jones et al., 2009), and orexin A or B injected into the VTA has been shown to increase dopamine levels in the NAc (Narita et al., 2006) suggesting that orexin modulates dopamine transmission in the VTA. Orexin may also facilitate excitatory glutamate inputs to the VTA (Aston-Jones et al., 2009). These studies suggest that orexin is a likely candidate for an important role in the expression of ethanol-conditioned behavior through modulation of the transmission of other neurotransmitters.

The Orexins/Hypocretins

The neuropeptides orexin A and B (also called hypocretin 1 and 2) were discovered by 2 independent research groups in 1998 as ligands for two previously orphan G-protein coupled receptors (Sakurai et al., 1998; de Lecea et al., 1998). These peptides are synthesized exclusively in neurons in the lateral hypothalamus region, but orexin projections are quite diffuse covering many regions of the brain and spinal cord (Peyron et al., 1998). The wide distribution of these projections to many different brain regions contributes to the array of physiological function of orexins (Marcus & Elmquist, 2005; Boutrel & De Lecea, 2008).

Orexin Receptors

There are two orexin G-protein coupled receptor subtypes (OX1R and OX2R) (Sakurai et al., 1998). Orexin A has equal affinity for both subtypes while orexin B has approximately 10-fold greater affinity for OX2R (Sakurai et al., 1998). Orexins are generally believed to have excitatory activity in many types of neurons (Sakurai, 2005). For example, dopamine neurons in the VTA and noradrenergic cells in the locus ceruleus

are both activated by orexin (Nakamura et al., 2000; Hagan et al., 1999). The peptides orexin A and orexin B have been used as agonists in many *in vivo* studies, but because orexin B only displays moderate selectivity for OX2R (Sakurai et al., 1998), specific antagonists are very useful pharmacological tools for determining receptor pharmacology (Upton, 2005). There are two OX1R-selective antagonists, SB-334867 and the newer SB-408124, which has slightly enhanced potency and selectivity for OX1R and has been formulated for use as a radioligand (Langmead et al., 2004; Upton, 2005). An OX2R-selective antagonist *N*-acyl 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline was also recently developed (Hirose et al., 2003).

OX1R Antagonist SB-334867

SB-334867 was the first selective non-peptide OX1R antagonist, and is currently one of the most utilized research tools for studying the physiology of the orexin system. The drug was developed by GlaxoSmithKline, and is systemically bioavailable and penetrates the central nervous system (Porter et al., 2001). SB-334867 has been shown to bind to the OX1R with nanomolar affinity and block the OX1-mediated calcium response (Smart et al., 2001). Downstream effects of OX1R blockade have not been well studied, but SB-334867 has been shown to block ERK phosphorylation *in vitro* (Ammoun et al., 2006). The drug has very poor solubility and must be suspended in a solution containing cyclodextrin and/or dimethyl sulfoxide (DMSO) (e.g., Haynes et al., 2000). Micromolar levels are found in brain 30 min after IP injection of 10 mg/kg in rats, and these levels are maintained for 2 hr (Upton, 2005). Another study showed similar results after a 30 mg/kg IP injection in rats with peak brain and plasma concentrations at 30 min and maintenance over 4 hr (Ishii et al., 2005a). This pharmacokinetic profile makes SB-

334867 an excellent research tool for use in behavioral studies.

Orexin Projections and Localization of Receptors in the Brain

As mentioned in previous sections, orexin projections spread widely throughout the brain, and orexin receptor distribution is similarly diffuse. Using in situ hybridization, studies have localized orexin receptor mRNA and found that OX1R and OX2R have different distributions (Hervieu et al., 2001, Marcus et al., 2001; Trivedi et al., 1998). Importantly, for the homeostatic functions proposed at the time, orexin receptor mRNA was found at high levels in the hypothalamus (Trivedi et al., 1998). OX1R mRNA was most abundant in the locus coeruleus, hippocampus, dorsal raphe, and the ventromedial hypothalamic nucleus, while OX2R mRNA was observed in septal, raphe, and hypothalamic nuclei, and the cerebral cortex (Marcus et al., 2001; Trivedi et al., 1998). Important for the proposed role of orexin in drug addiction, OX2R receptors are abundant in the NAc and activation of these receptors has been shown to modulate glutamate and GABA transmission (Martin et al., 2002). OX1R and OX2R receptors are abundant in the VTA and are thought to modulate glutamate transmission from cortical areas and/or modulate dopamine signaling (Aston-Jones et al., 2009; Borgland et al., 2006; Narita et al., 2006).

Orexin Physiology and Pharmacology

The physiology of orexin peptides has been investigated in many animal studies where orexin A or B is injected directly into the brain. Given the wide distribution of orexin fibers in the brain, it is not surprising that these studies have found orexins are involved in a wide variety of functions, including regulation of homeostasis (energy balance and autonomic function) and arousal. Orexins are mediators of feeding, appetite,

arousal, sleep, pain modulation, and neuroendocrine function (see Smart & Jerman, 2002; Upton, 2005 for reviews).

Orexin knockout mice or mice with ablated orexin neurons were found to be hypophagic (Chemelli et al., 1999; Hara et al., 2001). Additionally, Sakurai et al. (1998) described that intracerebroventricular (ICV) administration of orexin A or B dose-dependently increased food intake in rats. Orexin A administration directly into specific brain regions in the hypothalamus, and others areas outside of the hypothalamus such as VTA and NAc also increase food intake (see Kotz, 2006 for review). Orexins were, in fact, originally named for their orexigenic properties (Upton, 2005). The stimulation of feeding is likely OX1R-mediated because treatment with SB-334867 inhibits food intake and increases behavioral satiety (without effects on sedation or illness) (Haynes et al., 2000; Ishii et al., 2004, 2005a, 2005b).

SB-334867 administration alone has also been shown to dose-dependently reduce food intake and even to reduce body weight in rats for at least 24 hours following acute treatment of 10-30 mg/kg (e.g., Ishii et al., 2004). This result and the pharmacological profile of SB-334867 (undetectable in blood or brain after 12 hr) suggest that it may activate signaling events that persist much longer than acute receptor antagonism (Ishii et al., 2004, 2005a; Rogers et al., 2001; Upton, 2005), but the long-term molecular changes resulting from acute blockade of OX1Rs have yet to be determined. These studies suggest that OX1R is important in feeding and energy balance.

Orexin neurons are sensitive to circulating metabolic cues, such as ghrelin, leptin, and extracellular glucose concentrations (Yamanaka et al., 2003). Orexin neurons may be directly activated by these factors or indirectly activated through afferents to the LH

from the arcuate nucleus to influence feeding (Marcus & Elmquist, 2005). In support of this idea, OX1Rs are found on other neurons that contain peptides relevant to feeding such as NPY and melanin-concentrating hormone (MCH) (Backberg et al., 2002), and therefore orexin may interact with multiple hypothalamic systems to modulate feeding.

The most compelling evidence that orexin might be involved in sleep came from the groups that developed the orexin gene knockout and orexin neuron ablated mice. These mice showed a phenotype similar to the sleep disorder narcolepsy, characterized by sudden periods of inactivity in the dark when mice are normally active (Chemelli et al., 1999; Hara et al., 2001). Also, Lin et al. (1999) showed that mutations in the OX2R gene were responsible for this disorder in the canine. In humans, narcolepsy has been shown to be the result of orexin deficiency due to a degeneration of orexin neurons in the hypothalamus (Peyron et al., 2000; Thannickal et al., 2000).

Orexins also have a more general role in arousal. In addition to increasing feeding, ICV administration of orexin A also increases behaviors such as locomotor activity and grooming that characterize a heightened state of arousal (Duxon et al., 2001; Jones et al., 2001). The effects on locomotor activity may be mediated in the NAc because orexin A infusion into the NAc stimulated locomotor activity in rats, an effect blocked by pretreatment infusion of SB-334867 (Thorpe & Kotz, 2005). Orexin neurons are most active during wakefulness (Saper et al., 2005), and supporting this notion, ICV injections of orexin A also increased wakefulness in rats, an effect that is blocked by SB-334867 (Smith et al., 2003). Orexin neurons project to all the major components of the arousal system, and are thought to stabilize the state of arousal by maintaining activity of the monaminergic neurons (see Saper et al., 2005 for review). Early work on the

physiology of orexins has established these peptides as integral to the regulation of appetite, sleep, and arousal.

Orexin and Drug-Related Behaviors

The studies described in the previous sections provided a role for orexin in many different behaviors, and while a large body of research has been done on the role of orexin in feeding and arousal, recent studies have also suggested a role for orexin in behaviors relevant to motivation and reward that may implicate orexin in processes important for the development of drug addiction.

Several neuropeptides have been implicated in drug addiction. For example, CRF, the opioids, and NPY are among the most widely studied. In 2005, however, the first study showed activation of orexin neurons in the lateral hypothalamus (LH) to contextual cues that were previously paired with drugs of abuse or food (Harris et al., 2005). This study led to a recent surge of research on the role of orexin in drug-related behaviors including consumption, sensitization, withdrawal, and drug-seeking. The next sections will summarize the recent literature pertaining to the role of orexin in addiction.

<u>Role of Orexin in Drug Intake</u>

Relatively few studies have investigated involvement of orexin in the consumption of drugs of abuse. However, the small literature suggests a role for orexin in self-administration of several drugs. For example, the intravenous (IV) self-administration of nicotine was significantly lowered by SB-334867 in rats with no effect on responding for food (Hollander et al., 2008). Another study found a significant increase in OX1R mRNA in the arcuate nucleus in animals that had been self-administering nicotine for 4 weeks compared to controls (Corrigall et al., 2009). This

study not only suggests that orexin is important for self-administration of nicotine, but also suggests that interactions between orexin and peptides present in the arcuate nucleus important for appetite such as NPY, may be influenced by drugs of abuse (Corrigall et al., 2009).

Orexin has been shown to be involved in ethanol consumption. Orexin injected into the LH selectively induced ethanol intake rather than food intake in rats (Schneider et al., 2007). Pickering et al. (2007) showed that there was a strong trend for a correlation between levels of OX1R gene expression in the hypothalamus and ethanol consumption in a self administration paradigm in rats. Further supporting a role for OX1R in ethanol consumption, antagonism of OX1Rs with SB-334867 reduced responding for ethanol in an operant self-administration procedure in alcohol preferring (iP) rats (Lawrence et al., 2006). SB-334867 also decreased responding for ethanol but not sucrose in an operant procedure in Long-Evans rats (Richards et al., 2008). SB-334867 has also been shown to decrease preference for alcohol in a two-bottle choice procedure in Sprague-Dawley rats that were labeled "preferrers" based on individual differences in consumption (Moorman & Aston-Jones, 2009). These studies have suggested a role for orexin in the direct reinforcing effects of ethanol.

Orexin does not appear to modulate self-administration of other drugs such as cocaine. When SB-334867 was given to rats showing stable cocaine self-administration, there was no effect on lever pressing for cocaine (Smith et al., 2007, 2009a; Aston-Jones et al., 2009). However, when SB-334867 was given on the first day of extinction (when drug-seeking behavior is particularly high), the drug significantly reduced lever pressing for cocaine (Smith et al., 2007, 2009a; Aston-Jones et al., 2009). The OX2R antagonist

4-pyridylmethyl (S)-tert-leucyl 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline (4PT) also had no effect on stable cocaine self-administration (Smith et al., 2009a). Aston-Jones et al. (2009) suggest that self-administration of cocaine may be independent of orexin modulation because cocaine acts at the dopaminergic terminals in the NAc rather than directly activating VTA dopamine neurons. Other drugs can directly activate VTA dopamine neurons because orexin receptors on the cell body are sensitive to orexin release from en passant fibers (Bonci & Borgland, 2009). Overall, these studies suggest that there are differences between drugs in whether manipulation of orexin signaling alters self-administration.

Orexin and Sensitization

Orexin in the VTA has been shown to be critical for increased sensitivity to cocaine after repeated administration (behavioral sensitization). When SB-334867 was administered either IP or directly into the VTA prior to a sensitization paradigm in rats, sensitization was reduced without any effects on basal locomotor activity (Borgland et al., 2006). In addition, chronic but not acute cocaine exposure up-regulated OX2R protein in the NAc, an effect that persisted 60 days following the last cocaine injection (Zhang et al., 2007). These studies suggest that orexin is involved in neuroadaptations to drugs of abuse. This idea is further supported by the finding that orexin A enhanced NMDA-mediated excitatory post synaptic currents at glutamate synapses onto VTA dopamine neurons, and that administration of an OX1R antagonist blocks plasticity normally induced by cocaine exposure (Borgland et al., 2006; Bonci & Borgland, 2009). An OX1R/OX2R dual orexin receptor antagonist has also recently been shown to reduce sensitization to amphetamine (Winrow et al., 2009). Taken together, these studies

suggest that orexin is involved in synaptic plasticity induced by drugs of abuse, and that this plasticity is required for expression of drug-related behaviors such as sensitization.

Orexin and Morphine Withdrawal

Orexin has been shown to be important for morphine withdrawal in a small literature. Orexin's role in withdrawal of other drugs of abuse has yet to be studied. SB-334867 administered before naloxone blocked precipitated morphine withdrawal in mice and blocked activation of the NAc shell, which is normally activated by withdrawal (Sharf et al., 2008). In addition, c-Fos and the orexin gene are induced in orexin neurons in the LH during morphine withdrawal (Georgescu et al., 2003). In accordance with this study, another study found orexin mRNA in the LH is increased by morphine withdrawal (Zhou et al., 2006). Fos activation in LH orexin neurons was also found to be increased when animals were in protracted morphine withdrawal (i.e., when the preference for morphine would be elevated after a period of forced abstinence), providing further evidence for a role of LH orexin neurons in morpine withdrawal (Aston-Jones et al., 2009). Further study is needed on the withdrawal of other drugs to determine if the importance of orexin neurons in LH generalizes to drugs other than morphine.

Orexin and Drug-Conditioned Behavior

Bilateral lesion of the source of orexin neurons in the LH completely blocks acquisition of morphine CPP (Harris et al., 2007). A disconnection technique further showed that unilateral lesion of the LH and contralateral administration of an OX1R antagonist SB-334867 into the VTA blocked the development of morphine CPP while neither of the treatments on their own had any effect (Harris et al., 2007). Narita et al. (2006) also showed that SB-334867 administered into the VTA blocked the development

of morphine CPP in rats. Since these were CPP acquisition studies, they implicate orexin as being critically involved in learning to associate an environment with drug effects and/or the primary reinforcing effects of morphine. Studies have also linked orexin to expression of drug-conditioned behavior. For example, orexin neurons were shown to be activated in proportion to the magnitude of morphine CPP expressed upon chemical reinstatement of a previously extinguished CPP, and systemic administration of SB-334867 before a place preference test significantly reduced expression of morphine CPP in rats (Harris et al., 2005) suggesting that these neurons are also involved in expression of drug-conditioned reward.

Orexin and Reinstatement of Drug Seeking

The first study implicating orexin in drug-seeking behavior not only found an effect of OX1R antagonist in reducing expression of morphine CPP, but also that chemical stimulation of orexin neurons in the LH or infusion of the orexin A peptide into the VTA reinstated an extinguished morphine CPP (Harris et al., 2005). The authors concluded that activation of orexin neurons in the LH (and presumably subsequent orexin A release in the VTA) triggers relapse to drug-seeking behaviors (Harris et al., 2005).

Orexin seems to be necessary for both cue and stress induced reinstatement. Similar to when orexin A is infused into the VTA, ICV infusion of orexin A can reinstate lever pressing for cocaine in rats (Boutrel et al., 2005). This effect was blocked by administration of a CRF or a noradrenergic antagonist suggesting that orexin may be involved in reinstating drug-seeking behavior through inducing stress (Boutrel et al., 2005). Supporting this explanation, Boutrel et al, (2005) also showed that SB-334867 also blocked reinstatement to cocaine-seeking induced by footshock stress. Similarly, a

recent study by Wang and colleagues (2009) found that orexin A infused into the VTA and footshock stress both reinstated lever pressing for cocaine and caused dopamine and glutamate release in the VTA. The reinstatement of cocaine-seeking by orexin A was blocked by administration of SB-408124 (OX1R antagonist), but not a CRF antagonist. The footshock-induced reinstatement (which was dependent on CRF) was not blocked by SB-408124, suggesting that though CRF and orexin interact in the VTA, they may have independent functions in mediating reinstatement (Wang et al., 2009). For discussion of orexin and CRF circuitry and integration of function in stress systems, see Paneda et al. (2005) and Winsky-Sommerer et al., (2005).

Interestingly, while SB-334867 blocked cue-induced reinstatement of cocaine seeking in an IV operant self administration procedure, it had no effect on cocaine-primed reinstatement (Smith et al., 2007; Aston-Jones et al., 2009). This result is similar to the lack of effect of SB-334867 on cocaine self-administration. The authors concluded that drug-seeking triggered by environmental cues is sensitive to OX1R antagonism, but drug-seeking triggered by cocaine prime or self-administration is not. In agreement with this hypothesis, a recent study showed that SB-334867 blocked cocaine-seeking by re-exposure to a cocaine-paired context after a period of abstinence or extinction (Smith et al., 2009b). Another study supporting this hypothesis showed that 20 and 30 mg/kg SB-334867 reduced cue-induced reinstatement of lever pressing for cocaine, however it notably also reduced inactive lever presses in rats (Smith et al., 2009a). This study also showed that 4PT, an OX2R antagonist, had no effect on cue-induced reinstatement of cocaine seeking (Smith et al., 2009a). In addition to the studies of reinstatement of cocaine-seeking, a dual orexin receptor antagonist also blocked reinstatement of nicotine-

seeking (Winrow et al., 2009). Reinstatement studies have provided strong evidence supporting the idea that orexin signaling is required for drug-seeking behavior.

Ethanol-Seeking Behavior

Only two studies have functionally investigated the role of orexin in ethanol-seeking behavior. Specifically, one study showed that antagonism of OX1Rs abolished olfactory cue-induced reinstatement of lever pressing for ethanol after extinction (Lawrence et al., 2006). The other study found that antagonism of OX1Rs reduced yohimbine-induced reinstatement of ethanol seeking (Richards et al., 2008). Yohimbine, an α_2 receptor antagonist, is a stress-inducing drug (Charney et al., 1983), and thus this study is the first to implicate orexin signaling in stress-induced reinstatement of alcohol seeking. Both studies used operant models, and therefore, further study that isolates ethanol-seeking in a non-consumption-based model would add support to the importance of orexin in ethanol-seeking behavior specifically. Since orexin is a feeding-related peptide and ethanol has caloric value, consumption may confound studying the role of orexin in ethanol-seeking. Thus, the aim of the present studies was to investigate the role of orexin in ethanol-seeking using the ethanol-CPP model. The current understanding of the neurobiology of ethanol CPP also suggests that orexin could be an important modulator of the acquisition and expression of CPP because of its key site of action in the VTA.

Feeding Systems and Ethanol-Related Behaviors

Alcohol use disorders may arise from abnormalities in systems that regulate feeding processes (Thiele et al., 2004), because food and drugs of abuse may activate common neural substrates (Carr et al., 1996; DiLeone et al, 2003). Regulatory systems

for feeding are particularly relevant to studying ethanol consumption because ethanol has caloric value and directly contributes to energy intake (Thiele et al., 2004). Several studies have already implicated feeding systems in drug-related behaviors (see Egli et al., 2003; Thiele et al., 2004). An important example is that food deprivation enhances drug seeking in CPP procedures with stimulant drugs (Bell et al., 1997; Stuber et al., 2002). These studies suggest that similar systems may be activated by food deprivation and drug seeking.

Supporting the idea that feeding systems may be involved in addiction processes, many peptides that are involved in food and water intake are also implicated in ethanol consumption, including galanin, NPY, MCH, α-melanocyte stimulating hormone (αMSH), urocortin, orexin, and the opioids (DiLeone et al., 2003; Egli et al., 2003; Ryabinin & Weitemier, 2006; Schneider at al., 2007; Theile et al., 2004). Study of feeding-related peptides has been very important for progress in alcoholism research. Opioids, for example, have a well-established role in ethanol use disorders and are the target of current pharmacological treatments (i.e., naltrexone). The clinical use of naltrexone was identified based on a large number of preclinical studies (see O'Malley & Froehlich, 2003). The recent studies described in the previous sections have revealed the influence of orexigenic peptides in behaviors relevant to drug addiction and specifically alcoholism. The goal of the present studies is to further investigate the role of orexin in ethanol-conditioned behavior.

Introduction to the Present Experiments

As mentioned previously, orexin is critically involved in ingestive behaviors, and it is advantageous to study its role in ethanol behaviors using a model that does not

involve consumption of ethanol. The present studies used the ethanol CPP model to determine if orexin is involved in the acquisition (Experiment 2) or expression (Experiments 4 and 5) of ethanol CPP using systemic administration of the selective OX1R antagonist SB-334867 in DBA/2J mice. DBA/2J mice were used because a strong ethanol CPP is rapidly conditioned in this strain (Cunningham et al., 2003, 2006). CPP is a good model for interpretation of pharmacological manipulations that may alter effects of ethanol because both aversion and preference can be conditioned, and the dose-effect curve is monophasic as opposed to the biphasic curve often seen in self-administration studies (Bardo & Bevins, 2000). CPP was also chosen because it is rapidly conditioned, and offers experimental control over drug and cue exposure.

CPP acquisition and expression testing manipulations have contributed significantly to our current knowledge about the underlying neurobiology of ethanol-conditioned behaviors (e.g., Boyce-Rustay & Cunningham, 2004; Bechtholt & Cunningham, 2005; Gremel & Cunningham, 2008 and 2009). Therefore, the CPP model is well-suited for studying the role of orexin in the acquisition and expression of ethanol-conditioned behavior. The goal of the acquisition study (Experiment 2) was to determine if orexin antagonism altered either the reinforcing effects of ethanol or the ability of animals to learn the relationship between ethanol exposure and contextual cues, and thus the antagonist was administered before conditioning trials during the acquisition phase of CPP. Because the antagonist was given before ethanol on conditioning trials, it was also necessary to determine if SB-334867 had any effects on ethanol pharmacokinetics (Experiment 3). Effects on ethanol pharmacokinetics could alternatively explain an alteration of acquisition of ethanol CPP. The goal of the CPP expression experiments

(Experiments 4 and 5) was to understand ethanol-conditioned behaviors, and therefore pharmacological manipulations were performed during the ethanol-free expression testing phase to isolate the performance of a conditioned approach response based on learning ethanol-cue associations during conditioning (Cunningham et al., 2006).

Locomotor activity is important for interpreting the results of CPP studies, since the dependent variable in CPP, time spent on previously ethanol-paired floor, depends on the animal's locomotor abilities (see Gremel & Cunningham, 2007). Pharmacological treatments can have effects on locomotor activity that may complicate the interpretation of results when they are used during place preference tests. Therefore, the locomotor effects of SB-334867 were determined at dose ranges and time intervals relevant for the CPP experiments (Experiment 1).

Our hypothesis was based on the studies of morphine CPP described earlier (Harris et al., 2005, 2007; Narita et al., 2006). Because activation of orexin neurons in the LH appears to be important for drug-seeking behaviors, acquisition and expression of ethanol CPP were expected to be attenuated or eliminated due to the blockade of OX1R signaling in brain regions such as the VTA, which has already been shown to be important for expression of ethanol CPP (see Bechtholt & Cunningham, 2005).

Chapter 2: Materials and Methods

Animals

Male DBA/2J mice were purchased from Jackson Laboratory (Davis, CA) and arrived at 6-7 weeks of age. Mice were housed 4 per polycarbonate cage with corncob bedding. The cages were housed in a ventilated Thoren rack. The mice were allowed to acclimate to the housing environment for 2 weeks prior to any experimental procedures. The colony room was kept at 21°C, and on a 12 hr light cycle (lights on 7 am- 7 pm). Laboratory Rodent Diet 5001 (LabDiet, Richmond, Indiana) and water were available continuously in the home cage. All experimental procedures were approved by the Oregon Health & Science University Institutional Animal Care and Use Committee.

Apparatus

The apparatus is also described in detail by Cunningham et al. (2006). Twelve conditioning boxes (30 x 15 x 15 cm) were used and each was enclosed in a light and sound attenuating chamber (55.9 x 40.6 x 45.7 cm, Coulbourn Instruments (Whitehall, PA) Model E10-20) equipped with a fan. All experimental procedures were conducted in dark chambers. The long sides and lid of the conditioning boxes were made of acrylic and the end panels were made of aluminum. Photodetectors and infrared light sources were mounted 2.2 cm above the floor of the conditioning box at 5 cm intervals along the sides of the box. Activity and position of the animals within the conditioning box were measured as beam breaks by the photodetectors and counted by a computer. Interchangeable grid and hole floor halves were used as tactile conditioned stimuli (CSs). During conditioning trials matching floor halves were placed under the conditioning box, and on preference test days, one of each floor type was presented. The grid floor was

made of 2.3 mm stainless steel rods attached 6.4 mm apart on acrylic rails. The hole floor was 16 gauge stainless steel with 6.4 mm diameter round holes on 9.5 mm staggered centers. Previous studies have shown that saline-treated DBA/2J mice show equal preference for each tactile floor cue (Cunningham et al., 2003).

Drugs

20% (v/v) ethanol was prepared from a 95% ethanol stock solution and saline (0.9% NaCl) and injected IP at 12.5 ml/kg. This dose of ethanol has repeatedly been shown to rapidly condition a strong place preference in DBA/2J mice (Cunningham et al., 2006). SB-334867 (Tocris Bioscience, Ellisville, Missouri) was prepared in a stock concentration of 1.5 mg/ml and suspended in 20% hydroxypropol-β-cyclodextrin (w/v) and 1.5% dimethyl sulfoxide (DMSO, v/v) in saline to improve solubility. SB-334867 or vehicle (20% hydroxypropol-β-cyclodextrin (w/v) and 1.5% DMSO (v/v) in saline) was injected IP at a volume of 20 ml/kg unless noted otherwise. SB-334867 was administered at 10, 15, 20, 30, or 40 mg/kg.

Experiment 1: SB-334867 effects on locomotor activity

The effects of SB-334867 on locomotor activity in the DBA/2J strain had not previously been studied. Thus, this experiment investigated the locomotor effects of SB-334867 (0, 10, 20 and 30 mg/kg). Animals were habituated to experimental procedures and injections in one session 3 days before the start of the experiment. During the habituation session, saline was administered IP immediately before a 5 min session in the conditioning apparatus on a white paper floor. During the experimental phase, SB-334867 was administered IP and animals (N=48) were immediately placed into a conditioning chamber on a white paper floor for a 60 min session.

General Behavioral Procedures for CPP Experiments (Experiments 2, 4, and 5)

Habituation

A one-day habituation procedure preceded conditioning in Experiments 2, 4, and 5. The purpose of this session was to adapt the mice to handling and injection procedures. The animals were injected with saline (0.9% NaCl, 12.5 ml/kg) and placed in the conditioning apparatus on a white paper floor for a 5 min session.

Conditioning

An unbiased place conditioning procedure was used (Cunningham et al., 2003). Mice received a 2 g/kg IP injection of ethanol (20% v/v, 12.5 ml/kg) on CS+ days or saline on CS- days immediately before being placed in the conditioning box for a 5 min session. Mice were randomly assigned to two conditioning subgroups (G+ and G-). The G+ subgroup received ethanol before exposure to the matching grid floor halves and saline before exposure to the hole floors. The G- group received the ethanol paired with the hole floor and saline with the grid floor. The order of each type of trial was counterbalanced within subgroups. Conditioning consisted of eight sessions (four CS+ trials and four CS- trials on alternating days). Preference testing (see below) occurred after the second trial and after the fourth trial (Experiment 2 and 5) or following the fourth conditioning trial only (Experiment 4). Animals that received pretreatment injections of vehicle or SB-334867 30 min prior to conditioning trials received these injections in the home cage. This conditioning procedure has been repeatedly shown to induce a strong ethanol CPP in DBA/2J mice in our laboratory, and is described in greater detail by Cunningham et al., (2006).

Preference Test

All animals received a saline injection immediately before being placed in the center of the conditioning apparatus with both floor types present for a 30 min place preference test. Animals that received pretreatment injections of vehicle or SB-334867 30 min prior to preference testing received these injections in the home cage. Activity data (mean counts/min) were also collected during preference tests, and are important for interpretation of CPP data (see Gremel & Cunningham, 2007). When possible, body weights were taken 24 hr after SB-334867 administration to determine whether SB-334867 had an anorectic effect (Ishii et al., 2005a). Experimental procedures occurred during the day, usually several hours before lights-out. Therefore, SB-334867 was not expected to have large or long-lasting effects on normal feeding and drinking.

Experiment 2: SB-334867 effects on CPP acquisition

This experiment was designed to determine if the orexin system is involved in learning to associate environmental stimuli with the effects of ethanol. Conditioning proceeded as described above in *General Behavioral Procedures*, and the OX1R antagonist SB-334867 was administered 30 min before CS+ trials (trials where the animals receive an ethanol injection) at 0, 15, or 30 mg/kg (N=32 per group). All groups received vehicle injections 30 min prior to CS- trials (trials where the animals receive a saline injection). The 30 min pretreatment interval was chosen based on pharmacokinetic studies of 30 mg/kg SB-334867 in rats (Ishii et al., 2005a, Upton, 2005). Mice were tested 48 hr after the second trial and 48 hr after the fourth trial in a 30 min choice place preference test.

Experiment 3: SB-334867 effects on ethanol pharmacokinetics

The results of Experiment 2 showed that SB-334867 treatment at the high dose of 30 mg/kg significantly reduced locomotor stimulation to 2 g/kg ethanol. To address the possibility that SB-334867 altered the absorption or elimination of ethanol, Experiment 3 measured blood ethanol concentrations (BEC) in mice treated with vehicle or 30 mg/kg SB-334867 (n=5-9/group) 30 min before injection of 2 g/kg ethanol. The subjects of Experiment 3 were mice that had previous served as the vehicle control group in Experiment 5. These animals had vehicle (10 injections), saline (4 injections), and ethanol exposure (4 injections, 2 g/kg) 13-14 days prior to the start of Experiment 3, but were SB-334867 naive. Blood samples (20 μl/sample) were collected from the saphenous vein 2.5, 10 and 60 min after ethanol injection in a within subjects design. Samples were analyzed using gas chromatography (Rustay & Crabbe, 2004).

Experiment 4: SB-334867 effects on CPP expression (no vehicle habituation)

This experiment was designed to determine if acute OX1R activation is required for expression of ethanol CPP. Conditioning proceeded as described above in *General Behavioral Procedures*, and, 24 hr after the fourth trial, the OX1R antagonist SB-334867 was administered at 0 (vehicle), 15 or 30 mg/kg (N=64 per group) 30 min before a 30 min choice place preference test. An initial group of 96 animals was used for this experiment; however, because the vehicle group showed a magnitude of CPP that was much weaker than normal, the experiment was repeated in a second replication of 96 animals to ensure the behavior of the vehicle group was not due to sampling error.

Experiment 5: SB-334867 effects on CPP expression (after vehicle habituation)

Due to the apparent disruption in the expression of CPP in the vehicle control group in Experiment 4, Experiment 5 was performed to further investigate the issue using a habituation procedure for the 30 min pretreatment injections. Conditioning proceeded as described above in *General Behavioral Procedures*, except that 30 min before every conditioning trial (CS+ and CS-), animals received an injection of vehicle. The procedure was designed to habituate animals to the 30 min pretreatment injection and to the vehicle solution prior to test day. Preference testing occurred 24 hr after the second trial and 24 hr after the fourth trial. The OX1R antagonist SB-334867 was administered at 0 (vehicle) or 30 mg/kg (N=32 per group) 30 min before the 30 min test after 2 trials. A third group received only a saline injection immediately before the trial (no 30 min pretreatment injection) to determine if the pretreatment injection in the vehicle group was acutely responsible for disruption in place preference. If the habituation procedure during conditioning was successful, the saline and vehicle groups should express the same magnitude of place preference. Since 30 mg/kg had no effect on preference after 2 trials, the dose was increased to 40 mg/kg (by increasing the injection volume to 26 2/3 ml/kg) and given 30 min before the test after 4 trials to the SB-334867 group and to the saline group that was previously SB-334867 naive.

Statistical Analyses

The primary dependent variable on the CPP expression tests was time spent on the grid floor in sec/min. Measurement of the difference in time spent on the grid floor between animals that had ethanol paired with the grid floor (G+) and animals that had saline paired with the grid floor (G-) indexes CPP strength (Cunningham et al., 2003).

Activity data were also collected in counts/min during conditioning and the preference tests and were analyzed using a mixed ANOVA (Group x Time) or a one-way ANOVA (Group). Conditioning activity data were analyzed using a mixed ANOVA (Group x Trial x Trial type). In Experiment 2, conditioning activity data were analyzed using a mixed ANOVA that included minute as an additional within subjects factor. Preference test data were analyzed using a three-way ANOVA (Group x Conditioning Subgroup x Time). For all experiments "group" refers to the dose groups (vehicle, low dose, or high dose), "conditioning subgroup" refers to G+/G- assignment, "trial type" refers to whether saline or ethanol was administered before the trial, and "time" refers to the first 15 min or last 15 min of the preference test. Mean time on the grid floor was compared on the first and second half of the preference test to determine if there were any changes in the strength of CPP expression. When multiple preference tests were conducted (Experiment 2 and 5), a mixed ANOVA (Group x Conditioning Subgroup x Test) was also used to analyze the data across tests. Replication was included as an additional between-subjects factor in the analyses of Experiment 4. The α-level of significance for all analyses was $p=0.05$. All post-hoc analyses were Bonferroni-corrected pairwise comparisons.

Chapter 3: Results

Subject Attrition

Experiment 1 included all animals. In Experiment 2, two animals were excluded because of a procedural error, one animal died, and one animal was excluded because of an injection injury making the total sample size 92 animals for all analyses. In Experiment 4, five animals were removed for procedural errors making the total sample size 187 animals. In Experiment 5, two animals died, two animals were removed due to a procedural error, and one removed for an injection injury making the total sample size for analysis 91 animals.

Experiment 1: SB-334867 effects on locomotor activity

Activity.

Activity counts for each of the 4 dose groups (0, 10, 20, and 30 mg/kg) over the 60 min session are plotted in 5 min time bins in Figure 2. While activity decreased over the course of the session due to habituation to the apparatus, there was no effect of dose on locomotor activity during the 60 min after the first exposure to SB-334867 on the first treatment day. The data were analyzed using dose as a between subjects factor (Dose x Time Bin ANOVA) because all animals were SB-334867 naïve. The main effect of time bin was significant because of the decrease in activity over the session ($F(11,484)=39.9$, $p<0.001$, Figure 1A), but there was no main effect of dose ($F(3,484)=0.5$) or dose x time bin interaction ($F(33,484)=1.2$), confirming that SB-334867 had no acute effect on activity. The results of this study show that SB-334867 treatment did not significantly alter basal locomotor activity, and therefore alterations in activity should not be expected when DBA/2J mice are tested within a 60 min interval in CPP procedures.

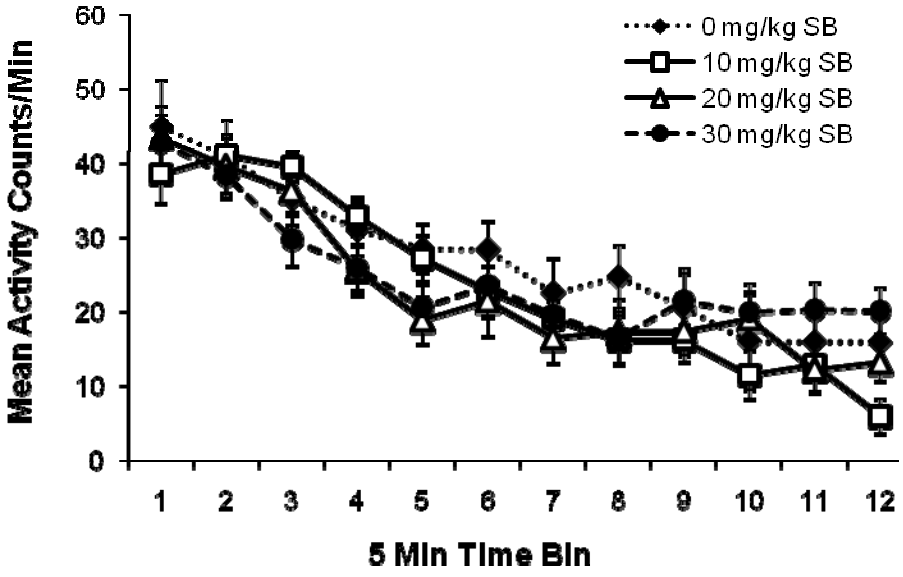

Figure 2. Locomotor Activity in Experiment 1. Mean activity counts per minute in 5 min bins over the first 60 min session. There were no significant differences in locomotor activity.

Body Weight.

SB-334867 treatment had no effect on body weight 24 hr following the first administration in Experiment 1. Animals were weighed on the activity test day where they received a SB-334867 injection (T1) and again 24 hours after testing (T2). There was a difference in baseline body weight on the day the animals were injected (T1; $F(3,44)=5.0$, $p=0.005$, 0 mg/kg 27.9±0.6g, 10 mg/kg 25.9±0.7g, 20 mg/kg 25.6±0.7g, 30 mg/kg 28.4±0.4g) and a significant group difference in the weight change (T2-T1) after 24 hours after injection ($F(3,44)=5.3$, $p=0.003$; 0 mg/kg -0.7±0.2g, 10 mg/kg -0.62±0.11g, 20 mg/kg 0.5±0.4g, 30 mg/kg -0.6±0.2g). This effect was driven by an increase in body weight in the 20 mg/kg group. Upon inspecting the distribution, 3 outliers (all from the 20 mg/kg group) were detected with weight changes greater than 3 standard deviations away from the average weight change across all studies in this document (average change: -0.6 g, SD = 0.8, N = 420). When these outliers were removed, the group effect on baseline weight remained ($F(3,41)=3.9$, $p=0.02$), but there was no longer an effect of drug group on weight change 24 hours following SB-334867 injection ($F(3,41)=1.4$, $p=0.2$, average weight change -0.6±.1g). Overall, these results suggest that SB-334867 does not significantly affect body weight when measured 24 hours after injection.

Experiment 2: SB-334867 effects on acquisition of ethanol CPP

Preference Tests.

The mean time spent on the grid floor for each dose group (0, 15, and 30 mg/kg SB-334867) during a preference test after 2 conditioning trials (left) and after 4 conditioning trials (right) is shown in Figure 3. On both tests, the G+ subgroup spent

more time on the grid floor than the G- subgroup indicating expression of a place preference. The magnitude of place preference was stronger after 4 conditioning trials than after only 2 conditioning trials. However, there was clearly no effect of dose of SB-334867 on magnitude of CPP on any test.

These observations were supported by 2-factor (Group x Conditioning Subgroup) ANOVAs conducted separately for each test, which yielded significant main effects of conditioning subgroup ($F(1,86)=100.2,718$, $p's<0.001$; Figure 3) but no main effect of group ($F(2,86)=0.5,1.1$, $p's=0.6,0.3$) or group x conditioning subgroup interaction ($F(2,86)=1.0,2.0$, $p's=0.4,0.1$). The lack of group x conditioning subgroup interaction on each test indicates that all groups showed a significant place preference of equal magnitude.

To address whether there was a difference in the magnitude of place preference between the test after 2 conditioning trials and the test after 4 trials, a 3 factor (Group x Conditioning Subgroup x Test) ANOVA was conducted. Preference was significantly larger after 4 conditioning trials as indicated by a significant conditioning subgroup x test interaction ($F(1,86)=90.2$, $p<0.001$).

To address any differences in magnitude of preference within each test, separate ANOVAs (Group x Conditioning Subgroup x Time) were also performed on each test. These analyses indicated a significant conditioning subgroup x time interaction on both preference tests ($F(1,86)=3.4, 7.4$, $p=0.07, 0.008$) reflecting stronger preference during the first 15 min of the test. There were no differences in locomotor activity between groups on either test (Table 1).

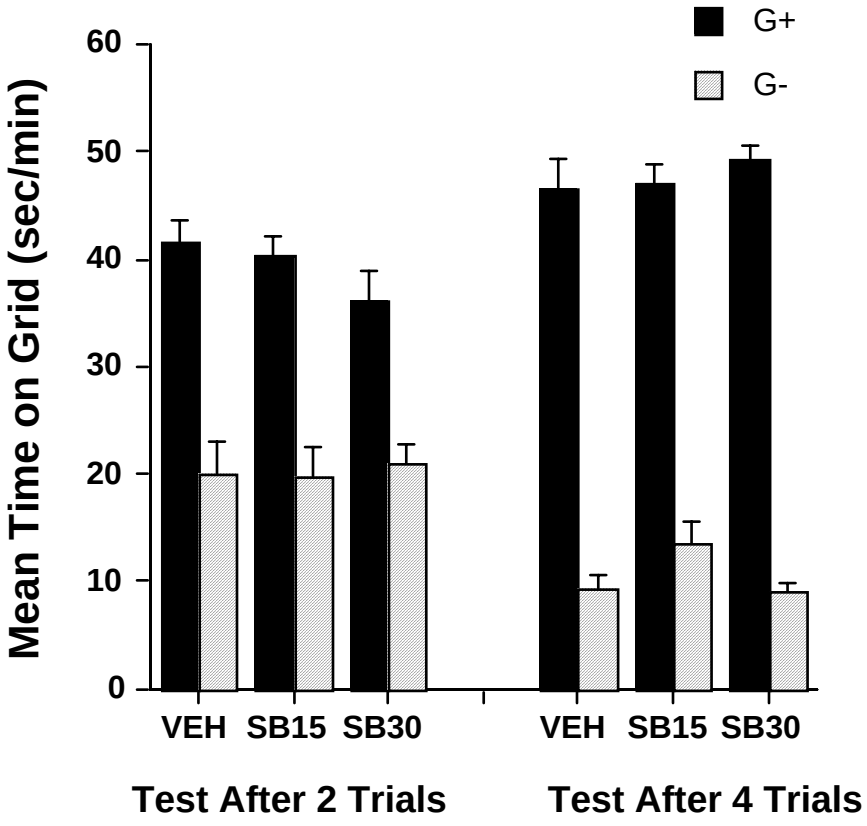

Figure 3. Place Preference Tests in Experiment 2. Mean time of grid floor (sec/min) after 2 trials (left) and 4 trials (right) for the 3 dose groups (N=32/group). Animals received either vehicle, 15 mg/kg, or 30 mg/kg SB-334867 on conditioning trials 30 min prior to 2 g/ethanol injection. There is no difference between groups in the magnitude of CPP expressed one either test (effect of conditioning subgroup, $p<0.001$).

Table 1. Mean Activity Counts per Minute on Preference Tests

Experiment	*Treatment Group*	*Test After 2 Trials Activity (counts/min)*	*Test After 4 Trials Activity (counts/min)*
2	Vehicle	34.8 ± 1.3	30.4 ± 1.5
	SB15	35.9 ± 1.8	32.0 ± 1.4
	SB30	36.1± 1.1	30.3± 1.1
4	Vehicle	NA	31.0 ± 1.1
	SB15	NA	30.3 ± 1.0
	SB30	NA	32.7 ± 1.2
5	Saline (SB40)	34.3 ± 0.9	26.3± 1.5
	Vehicle	31.5 ± 1.0	25.8± 1.3
	SB30 (SB40)	28.2± 0.8*	24.5± 1.4

* $p<0.001$ (vs. saline)

Conditioning Activity.

As can be seen in Figure 4, animals were more active on ethanol trials than on saline trials, confirming the locomotor stimulating effect of 2 g/kg dose ethanol. Pretreatment with SB-334867 dose-dependently reduced the increase in locomotor activity normally produced by ethanol injection, but did not affect basal saline activity. The mean activity counts for each minute of the 5 min ethanol and saline sessions (averaged across trials) are shown in Table 2. The table shows that SB-334867 did not affect ethanol activity during the first 2 min of the ethanol trials. However, the antagonist reduced ethanol-stimulated activity during the last 3 min, indicating that the drug effect developed over the course of the session.

These observations are supported by significant main effects of group ($F(2,89)=4.3$, $p=0.02$), trial ($F(3,356)=14.4$, $p<0.001$), trial type ($F(1,89)=1571$, $p<0.001$), and minute ($F(4,356)=15.9$, $p<0.0001$) in the group x trial x trial type x minute ANOVA. The trial type x group interaction was also significant ($F(2,89)=11.5$, $p<0.001$), reflecting a simple main effect of dose group on the ethanol trials ($F(2,89)=7.7$, $p=0.001$), but not on the saline trials ($F(2,89)=0.8$, $p=0.4$). Overall, average ethanol activity for the 30 mg/kg SB-334867 dose group was significantly lower than the vehicle group (Bonferroni corrected $p<0.001$, Figure 4). The 15 mg/kg group did not differ significantly from either of the other groups (Bonferroni corrected p's=0.07, 0.4). The group x trial type x minute interaction was also significant ($F(8,356)=5.8$, $p<0.001$), because of a group x minute interaction on the ethanol trials ($F(8,356)=8.2$, $p<0.001$), but not the saline trials ($F(8,356)=0.6$, $p=0.8$). Therefore, the relevant post-hoc analyses for each minute are shown in Table 2.

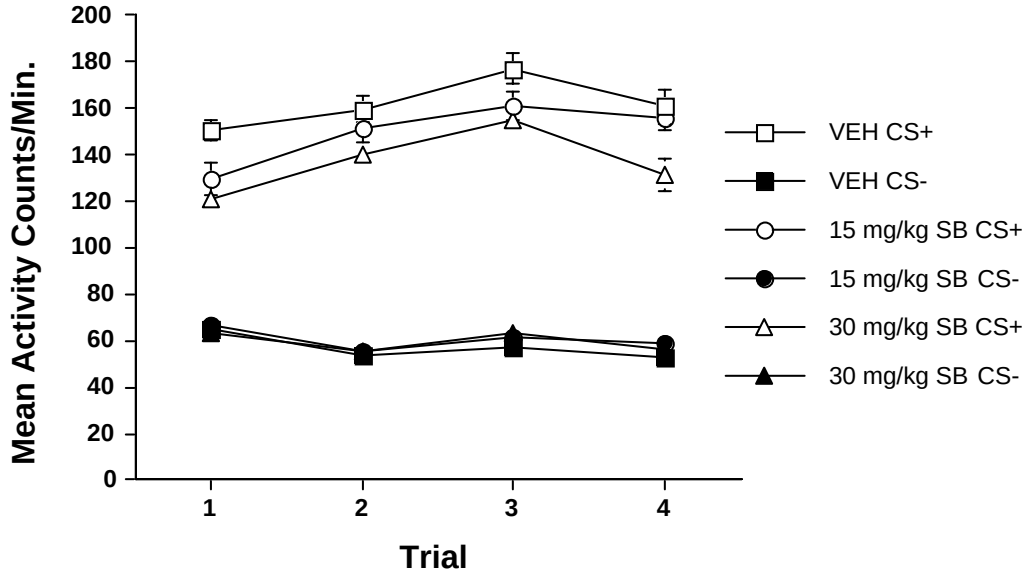

Figure 4. Conditioning Activity in Experiment 2. Mean activity counts/min for saline (CS-) or 2 g/kg ethanol (CS+) trials for the 3 dose groups (N=32/group) that received pretreatment of SB-334867 or vehicle prior to CS+ and vehicle before CS- trials. Activity on the CS+ trials in the SB-334867 (30 mg/kg) group was significantly lower than the vehicle group (p=0.001).

Table 2. Mean Activity Counts per Minute on Conditioning Trials (Experiment 2)

Treatment Group	*Ethanol Min1*	*Ethanol Min2*	*Ethanol Min3*	*Ethanol Min4*	*Ethanol Min5*
Vehicle	124.9±6.2	156.1 ± 7.0	175.4 ± 5.7	179.1±5.2	174.2±4.8
SB15	120.7±5.7	155.6 ± 6.3	164.5 ± 5.0	155.7±4.3*#	151.1±4.5*#
SB30	123.5±5.4	147.8± 5.9	147.7± 5.9*	137.4±4.3*	128.9±5.1*

* $p<0.001$ (vs. vehicle) #$p<0.05$ (vs. SB30)

Treatment Group	*Saline Min1*	*Saline Min2*	*Saline Min3*	*Saline Min4*	*Saline Min5*
Vehicle	90.6±4.4	56.4 ± 1.8	51.5 ± 1.7	46.4±1.5	42.6±1.6
SB15	93.7±2.7	59.8 ± 2.1	55.0 ± 2.0	50.2±1.7	44.5±1.4
SB30	96.1±3.8	59.1±2.2	52.4±2.0	47.1±1.3	43.6±1.4

Body Weight.

SB-334867 treatment also reduced body weight 24 hr after the first exposure in the 30 mg/kg group, suggesting a mild anorectic effect of the initial drug exposure. There were no baseline differences in body weight on the first day of conditioning ($F(2,89)=2.0$, $p=0.1$). Animals were sorted by order of trial type during conditioning for body weight calculations, and the weight change was defined as T2-T1 where T2 was 24 hr following injection. Body weight decreased by 0.5±0.2g in the vehicle group, 0.8±0.08g in the 15 mg/kg group, and 1.0±0.1g in the 30 mg/kg group 24 hr following injection of SB-334867 on the first conditioning trial. There was a significant effect of dose group ($F(2,89)=3.5$, $p=0.04$) where the high dose and vehicle groups were significantly different (Bonferroni $p=0.03$). There was no effect of dose group on change in body weight on any of the last 3 conditioning trials.

In summary, SB-334867 lowered locomotor activation to ethanol at the high dose (30 mg/kg) and significantly reduced body weight 24 hrs following the first treatment, but SB-334867 did not affect the acquisition of ethanol CPP. Thus, these findings indicate that blockade of OX1R does not affect the ability of the animal to associate environmental stimuli with the effects of ethanol and/or that SB-334867 does not alter the primary reinforcing properties of ethanol.

Experiment 3: SB-334867 effects on ethanol pharmacokinetics

Mean blood ethanol concentrations (mg/ml) are shown in Figure 5. The final sample sizes listed in the bars reflect data removal when a sample took more than 1 min to collect or if a sufficient volume could not be collected at a particular time point. Separate one-factor ANOVAs were performed at each time point. The group that was pretreated with 30 mg/kg SB-334867 30 min before receiving ethanol had significantly higher BECs at 10 ($F(1,12)=11.9$, $p=0.005$) and 60 $F(1,12)=8.6$, $p=0.012$) minutes after 2 g/kg IP ethanol injection than the vehicle group. The results of this study suggest that SB-334867 alters ethanol pharmacokinetics, and that this effect may have been responsible for the reduced ethanol-stimulated activity during conditioning trials in Experiment 2.

Experiment 4: SB-334867 effects on expression of ethanol CPP (no vehicle habituation)

Preference Test.

Figure 6 shows the mean time spent on the grid floor (in sec/min) for each group during the first 15 min of the preference test collapsed across both replications of the study. In contrast to Experiment 2, the magnitude of preference was much weaker in Experiment 4. The difference between experiments was particularly evident in the vehicle control group where there appeared to be a general disruption in the magnitude of CPP expressed. This disruption was not due to sampling error, because the effect was present in both replications of the experiment. Moreover, the expression of CPP was not maintained throughout the entire 30 min test (means for the last 15 min were 27.3±1.6 for the G+ group and 30.6±1.9 for the G- group). The observation that CPP was not

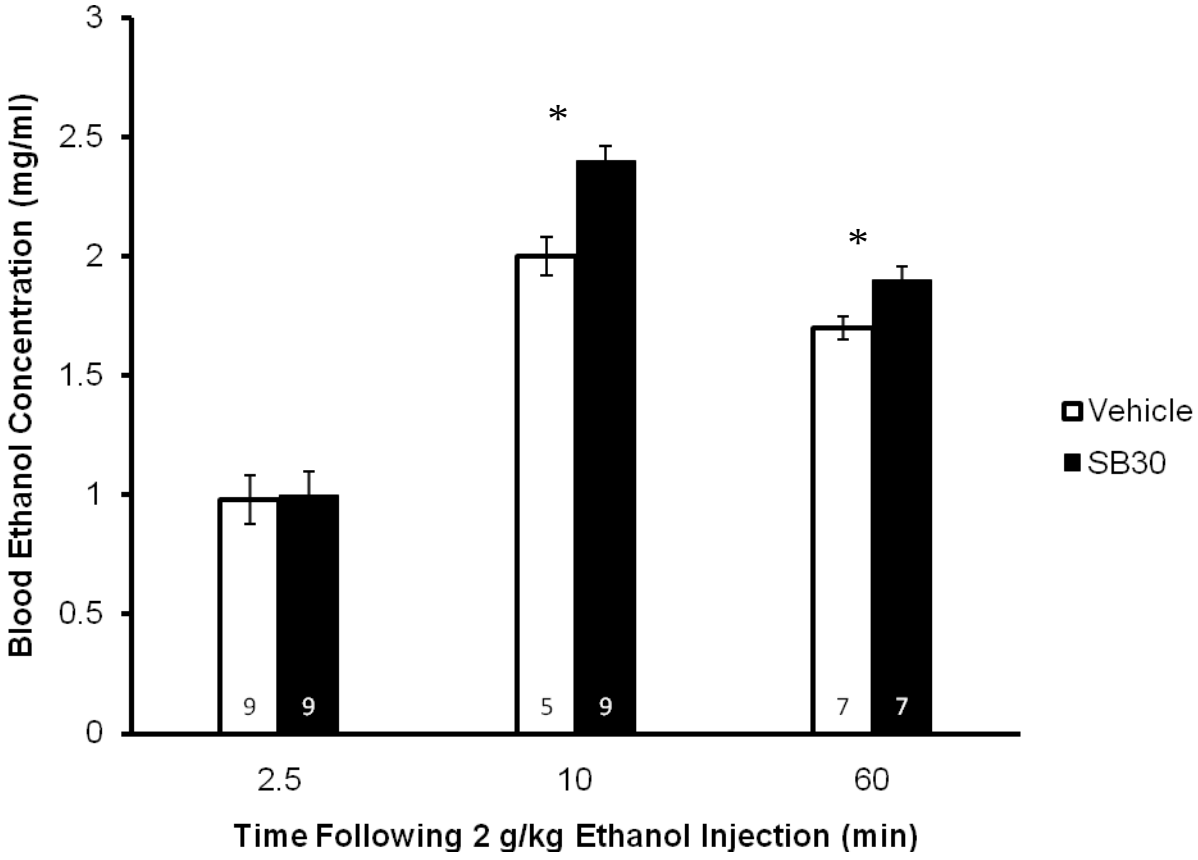

Figure 5. Blood Ethanol Concentrations in Experiment 3. Mice (N=20) were injected with vehicle or 30 mg/kg SB-334867 30 min before injection of 2 g/kg ethanol. Blood samples were taken from the saphenous vein 2.5, 10, and 60 min following injection and analyzed for blood ethanol concentration (BEC) in mg/ml. The group sizes are shown on the bars. SB-334867 group was significantly higher than vehicle at 10 ($p=0.005$) and 60 min ($p=0.01$).

maintained over the 30 min test was supported by a significant conditioning subgroup x time interaction ($F(1,181)=74.1$, $p<0.001$; Group X Conditioning Subgroup X Time ANOVA) where a main effect of conditioning subgroup was present in the first 15 min of the test ($F(1,181)=33.3$, $p<0.001$), but not the last 15 min ($F(1,181)=1.9$, $p=0.2$). Thus, analysis focused on data from the first 15 min.

As can be seen in Figure 6, despite the general disruption of CPP expression, the high dose of SB-334867 reduced CPP in the first 15 min, while the low dose SB-334867 and vehicle groups expressed a significant weak CPP. There appeared to be a difference between the magnitude of preference expressed by the vehicle and 15 mg/kg groups (weak preference) and the 30 mg/kg group (no preference) shown in Figure 6. A 2-factor ANOVA (Group x Conditioning Subgroup) was used to analyze the preference test data. The group x conditioning subgroup interaction is unreliable ($F(2,181)=2.1$, $p=0.1$) for the first 15 min, but there was a significant main effect of conditioning subgroup $F(1,181)=33.3$, $p<0.001$) indicating a significant weak preference overall. Bonferroni-corrected t tests were conducted for the vehicle and drug groups because of the *a priori* interest in comparing the magnitude of preference in the vehicle and drug groups. The effect of conditioning subgroup was significant in the vehicle and 15 mg/kg groups (p's<0.001) indicating significant preference. The effect of conditioning subgroup in the 30 mg/kg group was not significant ($p=0.3$) indicating a blockade of expression of preference. It should be noted that while there was a main effect of replication (Replication x Group x Conditioning Subgroup x Time ANOVA, $F(1,181)=4.5$, $p=0.04$) because animals in the second replication spent slightly more time on the grid floor (means in sec/min 27.7 ± 1.6 and 31.5 ± 1.2), replication did not interact with any of the

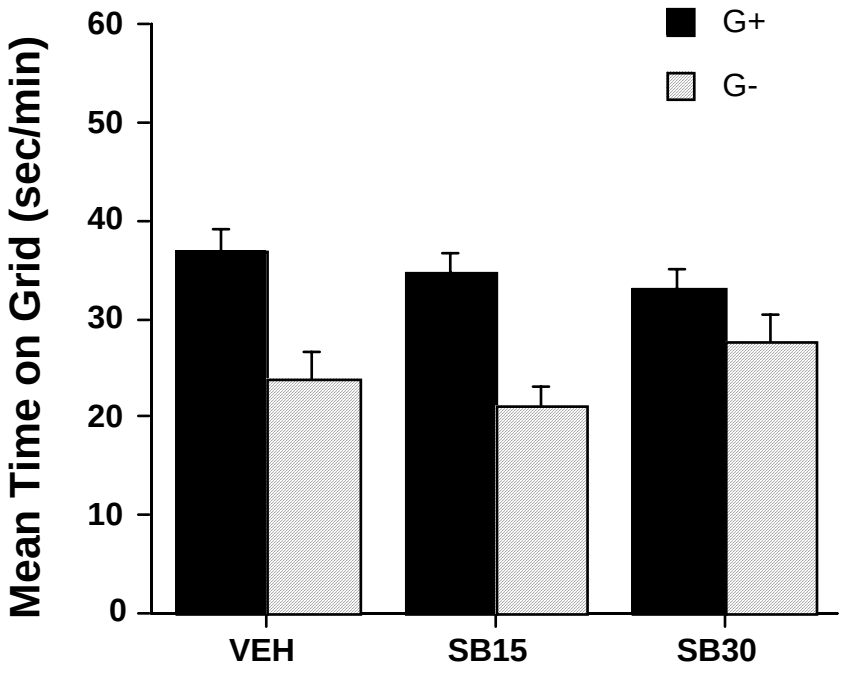

Figure 6. Place Preference Test in Experiment 4. Mean time spent on the grid floor (sec/min) for the first 15 min of the 30 min test in Experiment 4 for vehicle after pretreatment with 15 or 30 mg/kg SB-334867 (N=64/group) 30 min before the test. Vehicle and the 15 mg/kg groups expressed a significant preference ($p<0.001$) while the 30 mg/kg group did not express a preference.

variables so the data for time spent on the grid floor were combined for the analyses described above.

There was a main effect of replication on locomotor activity ($F(1,181)=14.2$, $p<0.001$) because activity was slightly higher overall on the second replication (mean counts/min 36.6±1.1 and 42.8±1.2). However, since the main effect of group was not significant in either replication, the data for the first 15 min were combined for analysis in Table 1.

Conditioning Activity.

On CS+ trials, the average activity was 144.2±6.3 counts/min. On CS- trials the average activity was 55.0±2.0 counts/min. Experiment 4 was completed in 2 replications, and a group x replication x trial x trial type ANOVA was conducted on the conditioning activity data. Analysis yielded the expected main effect of trial type ($F(1,186)=1546$, $p<0.001$) and a trial x trial type interaction ($F(3, 558)=10.5$, $p<0.001$). Though there was a main effect of replication on conditioning activity ($F(1,181)=5.7$, $p=0.02$) because the second replication had slightly higher average activity (mean counts/min 95.9±2.1 and 103.2±2.1), there were no interactions with replication and any variable, so the replications were combined.

Body Weight.

SB-334867 treatment had no effect on body weight 24 hr following the first administration on the preference test. Animals in the first replication only were weighed on the test day where they received a SB-334867 injection (T1) and again 24 hours after testing (T2). There was no difference in baseline body weight on the day the animals were injected (T1; $F(2,91)=0.07$, $p=0.9$, average weight 27.1±0.25g) and no difference in

the weight change (T2-T1) after 24 hours after injection ($F(2,91)=0.6$, $p=0.5$; average weight change -0.64±0.08g).

In summary SB-334867, at a high dose of 30 mg/kg blocked expression of ethanol CPP, but this result was complicated by an unexpectedly low preference in the vehicle control group, an effect that may have been caused by the novelty of receiving a pretreatment injection or the novelty of the vehicle solution. Experiment 5 was designed to further investigate the disruption of CPP by vehicle injection and pretreatment handling to control for these factors.

Experiment 5: SB-334867 effects on expression of ethanol CPP (after vehicle habituation)

Preference Tests.

Figure 7 shows the mean time spent on the grid floor (in sec/min) for all groups on a test after 2 conditioning trials (left) and 4 conditioning trials (right). On the test after 2 conditioning trials, all groups expressed a significant place preference of similar magnitude, indicating no effect of the OX1R antagonist. Importantly, the group that received a vehicle injection 30 min prior to testing and the group that received only a saline injection immediately before testing displayed similar preferences suggesting that the pretreatment habituation procedure during conditioning eliminated any disruption in preference on the expression test due to novelty of the vehicle pretreatment injection. On the test after 4 conditioning trials, both of the groups that received 40 mg/kg SB-334867 showed preferences similar to the vehicle control group, indicating no effect of the drug on expression of CPP.

These observations were supported by 2-factor (Group x Conditioning Subgroup) ANOVAS conducted separately for each preference test, which yielded significant main effects of conditioning subgroup ($F(1,85)=50.9,46.3$ p's<0.001; Figure 7), but no main effect of group ($F(2,85)=0.7, 1.6$, p's$=0.5,0.2$) or group x conditioning subgroup interaction ($F(2,85)=0.3,0.2$, p's$=0.7,0.8$). The lack of significant interaction in either test confirms that SB-334867 did not alter CPP expression after 2 trials or after 4 trials of conditioning.

To address whether there was a difference in the magnitude of place preference between the test after 2 conditioning trials and the test after 4 trials, a 3-factor (Group x Conditioning Subgroup x Test) ANOVA was conducted. There was a small increase in the magnitude of preference after 4 trials of conditioning compared to after 2 trials as evidenced by an unreliable conditioning subgroup x test interaction ($F(1,85)=3.4$, $p=0.07$).

To address any differences in magnitude of preference within each test, separate ANOVAs (Group x Conditioning Subgroup x Time) were also performed on each test. These analyses indicated a significant conditioning subgroup x time interaction on both preference tests ($F(1,85)=14.0$-17.9, p's<0.001) reflecting stronger preference during the first 15 min of the test.

On the preference test after 2 conditioning trials, there was a significant effect of group on locomotor activity ($F(2,88)=10.2$, $p<0.001$), where the 30 mg/kg SB-334867 group was significantly less active than the saline group (Table 1). There was no difference in activity between the SB-334867 group and the vehicle group. These effects were present in the first 15 and last 15 min of the test, so the data was averaged across the

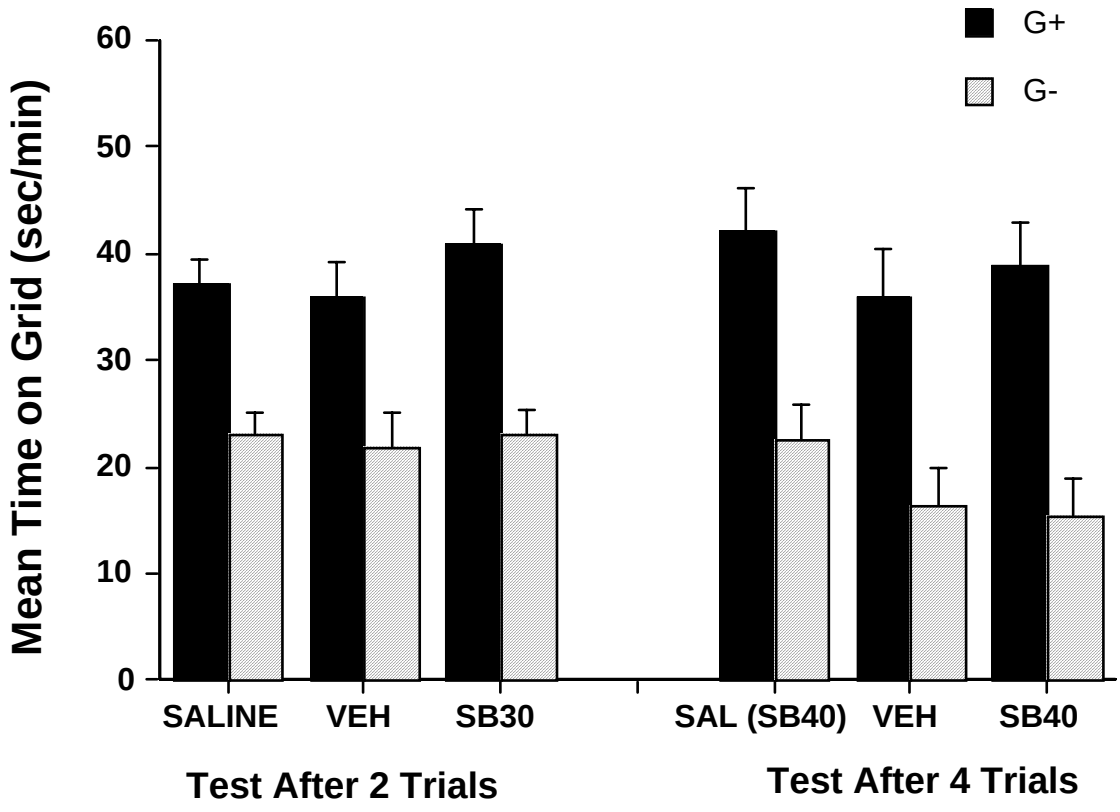

Figure 7. Place Preference Tests in Experiment 5. Mean time spent on the grid floor (sec/min) in Experiment 5 for animals treated with saline (N=32) immediately before or vehicle or 30 mg/kg SB-334867 (N=32/group) 30 min before testing after 2 trials of conditioning (left). After 4 trials of conditioning saline and SB-33867 group (N=64) received 40 mg/kg SB-334867 30 min prior to the test while the vehicle group received vehicle. There was no difference in preference between groups on the test after 2 trials (left) or the test after 4 trials (right)(effect of conditioning subgroup, $p<0.001$).

total 30 min test and presented in Table 1. There were no differences between groups in locomotor activity on the preference test after 4 conditioning trials.

Conditioning Activity.

On CS+ trials, the average activity was 171.6±6.8 counts/min. On CS- trials the average activity was 58.2±2.4 counts/min. Analysis of the conditioning activity (Group x Trial x Trial Type) yielded the expected main effect of trial ($F(3,264)=12.1$, $p<0.001$), trial type ($F(1,88)=1603$, $p<0.001$) and a trial x trial type interaction ($F(3, 264)=24$, $p<0.001$).

Body Weight.

SB-334867 treatment had no effect on body weight 24 hr following administration at 40 mg/kg on the preference test (both groups of animals that received 40 mg/kg were combined, N=61). Animals were weighed on the test day where they received a SB-334867 injection (T1) and again 24 hours after testing (T2). There was no difference in baseline body weight on the day the animals were injected (T1; $F(1,88)=0.2$, $p=0.6$, average weight 23.46±0.22g) and no difference in the weight change (T2-T1) after 24 hours after injection ($F(1,88)=0.5$, $p=0.5$; average weight change -0.48±0.09g).

The results of Experiment 5 suggest that OX1R antagonism during expression testing does not affect expression of CPP when preference is strong and not subjected to disruption as in Experiment 4.

Chapter 4: Discussion

The experiments described here are the first to investigate orexin signaling in ethanol-conditioned behavior using the CPP model. The results suggest that the importance of orexin in morphine-conditioned behaviors (e.g., Harris et al., 2005, 2007) does not generalize to ethanol-conditioned behaviors. Blockade of OX1Rs using the antagonist SB-334867 did not affect the acquisition or expression of ethanol CPP in DBA/2J mice.

CPP Acquisition and Expression Studies

The lack of effect of SB-334867 on acquisition of ethanol CPP (Experiment 2) was unexpected given that studies of morphine CPP in rats have shown that OX1R signaling is required for successful acquisition (Harris et al., 2007; Narita et al., 2006). Since the present study is the first to investigate the acquisition of an ethanol-conditioned behavior, it is unclear whether the effect of SB-334867 on morphine CPP does not generalize to ethanol CPP or whether the difference in species used (rat vs. mouse) is responsible for the null outcome. It is also possible that differences in mechanism of action between ethanol and morphine may underlie differences in the role of orexin in behaviors conditioned by each drug. Morphine is a mu opioid receptor agonist, and thus has specific activity on the endogenous opioid system. In contrast, ethanol has activity on a variety of systems including GABAergic, opioid, and glutamate. Activation of these other systems by ethanol may be sufficient to support the development of ethanol CPP despite blockade of orexin signaling.

It is unlikely that SB-334867 was not active at the dose administered because it had significant effects on locomotor activity and was administered at a similar dose and

pretreatment time interval as another study using C57BL/6J mice that showed an effect of SB-334867 on the expression of precipitated morphine withdrawal (Sharf et al., 2008). There was also a significant effect on body weight at the highest dose in Experiment 2, which further supports that the drug was active.

SB-334867, at the highest dose of 30 mg/kg, reduced expression of a weak CPP disrupted by novel exposure to treatment injections and vehicle solution (Experiment 4). In contrast, the antagonist had no effect on expression of ethanol CPP when animals expressed a strong CPP after habituation to the pretreatment injections of vehicle during training (Experiment 5). The lack of a robust, reliable effect on expression of ethanol CPP was not consistent with the effects of SB-334867 reducing expression of morphine CPP (Harris et al., 2005). It is possible that systemic administration of SB-334867 in our procedure with mice did not produce relevant brain concentrations for full blockade of OX1R signaling and resulting alterations in glutamate or dopamine transmission. While Harris et al. (2005) found a reduction in the strength of CPP using systemic administration, others have shown effects of SB-334867 on drug-related behaviors using infusion directly into the VTA (e.g., Harris et al., 2007; Narita et al., 2006). This explanation, however, is unlikely given the pharmacokinetic profile of SB-334867 in rats resulting in peak brain concentrations at 30 min (Ishii et al., 2005a). A more likely explanation is that orexin signaling is simply not required for expression of ethanol CPP.

Locomotor Activity

SB-334867 did not affect basal locomotor activity (Experiment 1), a result consistent with a study using a similar dose range of SB-334867 in rats (Richards et al., 2008), but inconsistent with a recent study in rats showing an effect of SB-334867 on

horizontal activity but not total distance traveled (Smith et al., 2009a). In the present study, SB-334867 at the highest dose of 30 mg/kg significantly reduced locomotor stimulation to 2 g/kg ethanol without altering the acquisition of ethanol CPP (Experiment 2). This result is generally in agreement with previous studies from our laboratory that have supported a dissociation between ethanol's rewarding and locomotor stimulating effects when an agonist/antagonist pretreatment resulted in a change in ethanol-stimulated activity with no effect on acquisition of CPP (Risinger et al., 1992; Chester & Cunningham, 1999).

Ethanol Pharmacokinetics

SB-334867 administered at 30 mg/kg altered the pharmacokinetics of 2 g/kg ethanol (Experiment 3). Animals that were pretreated with SB-334867 had significantly higher BECs at 10 and 60 min post 2 g/kg ethanol injection. This effect could be due to an alteration in the absorption of ethanol, because increased absorption could account for the higher peak BEC level observed in the present study. However, since only three time points were evaluated, it is possible that the peak BEC occurred before 10 min and that the difference in BEC is related to ethanol metabolism. SB-334867 has been shown to have no effects on blood pressure or heart rate, and no effect on vasodilation in rats (Hirota et al., 2003; Holland et al., 2005). To date, no research has investigated any effects of SB-334967 on ethanol metabolism. Therefore the exact mechanism by which SB-334867 alters ethanol pharmacokinetics is unclear.

Higher BEC in the SB-334867 group is consistent with the behavioral result in Experiment 2 of decreased ethanol-stimulated locomotor activity if one assumes that higher BEC results in the experience of a slightly higher dose. The dose-effect curve for

ethanol-stimulated locomotor activity for DBA/2J mice is an inverted U-shaped curve with a peak at 2 g/kg (Cunningham et al., 1992). Increasing the BEC, which mimics a higher dose, would therefore result in decreased locomotor activity because that higher dose is on the descending portion of the curve. Alterations in ethanol pharmacokinetics that result in increased BEC would not be expected to affect acquisition of CPP, because the dose-effect curve for CPP is monophasic. Moreover, CPP is induced at similar magnitudes for doses ranging from 2 g/kg to 4 g/kg (Groblewski et al., 2007).

In a reference-dose CPP procedure in our laboratory, 2 g/kg ethanol was paired with one tactile cue and 4 g/kg ethanol was paired with another tactile cue in our standard place conditioning procedure. No conditioning resulted from this procedure suggesting that the rewarding effects of 2 g/kg and 4 g/kg ethanol were indistinguishable (Groblewski et al., 2007). The inability of the animals to distinguish between 2 g/kg and higher doses of ethanol may explain the lack of effect on acquisition of CPP in Experiment 2. In addition, SB-334867 likely does not alter the reinforcing properties of ethanol because all groups expressed the same magnitude of preference. The finding that SB-334867 alters ethanol pharmacokinetics, however, might be of relevance to studies of the effect of this drug on ethanol self-administration.

Ethanol Self-Administration and Ethanol-Seeking

Previous studies have found that SB-334867 reduces self-administration of ethanol in rats in operant procedures without affecting responding for water or sucrose (Lawrence et al., 2006; Richards et al., 2008) and reduces preference for ethanol in a two-bottle choice test (Moorman & Aston-Jones, 2009). The results of these experiments may reflect the ability of SB-334867 to increase satiety or alter other feeding-related

processes, but these explanations are unlikely due to the lack of effect of SB-334867 on responding for water and sucrose (Lawrence et al., 2006; Richards et al., 2008). However, the present study suggests that the effects on self-administration could also be due to other factors such as an alteration of ethanol pharmacokinetics by SB-334867 rather than an alteration in the reinforcing properties of ethanol. Animals in self-administration experiments may have been consuming less due to an increase in BEC by SB-334867 because a smaller amount was required to achieve reinforcing effects similar to previously experienced ethanol effects during self-administration sessions.

The results of the present studies are also not in accordance with the reinstatement results of Lawrence et al. (2006). This study found that SB-334867 administered systemically in alcohol-preferring (iP) rats blocked olfactory-cue induced reinstatement and attenuated responding for ethanol under an operant fixed ratio paradigm. To explain the cue-induced reinstatement results, the authors propose that SB-334867 is blocking the ability of endogenous orexin (presumably released in response to the olfactory cue) to activate neural pathways involved in drug-seeking (Lawrence et al., 2006). While a similar process might be expected to occur during expression of ethanol CPP (i.e., orexin release and activation of drug-seeking pathways in response to the tactile floor cue), it is unclear whether exposure to the olfactory cue during reinstatement of ethanol seeking in a consumption-based operant model would recruit the same neural pathways as exposure to a floor cue during a CPP procedure where the ethanol was administered by the experimenter. The present understanding of the underlying neurobiology of ethanol CPP has already highlighted some deviations from the circuitry and neurotransmitters involved in operant drug-seeking models (e.g., Gremel & Cunningham, 2009), but further

study would be needed to determine if there are differences between models specifically related to orexin-facilitation of neurotransmitter release.

Orexin Interactions with Reward Systems

While the mechanism by which orexin antagonism alters CPP induced other drugs such as morphine remains unclear, the VTA is an important component of pathway necessary for expression of drug seeking behavior (Bechtholt & Cunningham, 2005; Narita et al., 2006). Orexin administration into the VTA has been shown to alter dopamine release in the NAc, suggesting that orexin can facilitate activation of mesolimbic dopamine neurons through orexin receptors on the cell body (Narita et al., 2006). While this modulation of dopamine signaling appears to be important for expression of morphine-related behaviors, infusion of the dopamine D1/D2/D3 receptor antagonist flupenthixol into the NAc, however, had no effect on expression of ethanol CPP (Gremel & Cunningham, 2009), suggesting that dopamine release in the NAc is not critical for expression of ethanol CPP. In contrast, infusion of flupenthixol into the amygdala blocked expression of CPP. It is possible that the specific neurocircuitry relevant to expression of ethanol CPP is independent of orexin-facilitation. For example, it is not known whether dopamine levels in amygdala are altered by orexin administration in the VTA. Future studies should investigate the effects of OX1R blockade in the VTA and amygdala on dopamine transmission.

In summary, the present studies suggest that while a previous study specifically showed activation of orexin neurons to an ethanol paired stimulus (Dayas et al., 2008), orexin signaling is not required for acquisition or expression of ethanol CPP. Future experiments should further characterize how OX1R antagonism affects consumption of

ethanol as well as continuing to investigate the role of orexin in ethanol-conditioned behaviors.

Proposed Roles of Orexin in Reward Processing

The current experiments are not in agreement with a recent hypothesis proposed by Aston-Jones et al., (2009) that predicts that drugs of abuse that cause increased dopamine release in the NAc via activation of VTA dopamine neurons are sensitive to orexin facilitation (e.g., ethanol, opiates, or contextual cues associated with drugs of abuse). Aston-Jones and colleagues (2009) further suggest that drugs that act at the dopaminergic terminals to increase dopamine release would not be sensitive to orexin facilitation of signaling (e.g., cocaine, amphetamine). This hypothesis was based in part, on the empirical finding that SB-334867 reduced cue-induced, but not cocaine-primed, reinstatement of cocaine seeking and that SB-334867 did not have any effect on IV self-administration of cocaine (Smith et al., 2007; Aston-Jones et al., 2009).

While the current literature is generally in agreement with this hypothesis, further testing of this idea is required. Prior to the present study, ethanol CPP had not been tested, but according to the hypothesis, ethanol CPP should be disrupted by SB-334867 because expression of ethanol CPP requires opioid and GABA receptor activation in the VTA (Bechtholt & Cunningham, 2005). Studies of ethanol self-administration support this hypothesis because SB-334867 has been shown to reduce self-administration (Lawrence et al., 2006; Richards et al., 2008) and preference for alcohol (Moorman & Aston-Jones, 2009), but this reduction in self-administration may actually be due to other factors such as alterations in ethanol pharmacokinetics as discussed earlier. Opiate self-administration and opiate-primed reinstatement have not currently been tested for

sensitivity to SB-334867, and these studies would add important support for the current hypothesis (Aston-Jones et al, 2009).

Aston-Jones et al., (2009) has argued that this hypothesis predicts that drugs that act through glutamate release into the VTA to motivate behavior would require orexin to facilitate that action. This idea has yet to be fully tested, but one piece of supporting evidence that orexin may modulate glutamate input from prefrontal regions to the VTA comes from a study of medial prefrontal cortex-evoked responses in dopamine neurons in the VTA. Orexin A applied directly to dopamine neurons increase firing and bursting, similar to when orexin A has been applied to VTA slices (Korotkova et al., 2003; Moorman et al., 2007). Orexin A has further been shown to alter (similar proportions of enhancement and reduction) medial prefrontal cortex-evoked responses in the VTA dopamine neurons (Aston-Jones et al., 2009). The ability of orexin to modulate other glutamatergic projections to the VTA, including an input from the LH (Colussi-Mas et al., 2007) has not been tested and would be needed to conclude that orexin's main function in the VTA is to facilitate glutamate-induced activation of dopamine neurons. Additionally, glutamate transmission has not been fully investigated in the ethanol CPP model, and further information on glutamate signaling in hippocampal and cortical regions would be needed to fully evaluate the hypothesis that orexin facilitation of glutamate transmission is involved in ethanol CPP.

Orexin has been shown to mediate plasticity at glutamate synapses in the VTA (Bonci & Borgland, 2009). Orexin A applied directly to the dopamine neuron in the VTA enhanced NMDA receptor-mediated excitatory post synaptic currents and increased the insertion of NMDA receptors to the synapse, an effect that was reduced by SB-

334867 (Borgland et al., 2006). Cocaine-induced plasticity (indicated by AMPA/NMDA receptor ratio at the synapse) was also altered by administration of SB-334867 suggesting that changes induced by cocaine require orexin facilitation (Borgland et al., 2006).

As noted by Bonci & Borgland (2009), interestingly, corticotropin releasing factor (CRF) performs an almost identical function in processes related to plasticity as orexin in the VTA. When CRF is applied to VTA dopamine neurons, NMDA-mediated synaptic transmission is enhanced (Ungless et al., 2003). Elevated synaptic transmission either by orexin or CRF may facilitate long-term potentiation in these synapses (Bonci & Borgland, 2009), and if this is the case, then in the present studies where SB-334867 was administered systemically, CRF may have been able to compensate for reduced orexin facilitation of synaptic transmission by performing the same functions to induce the synaptic plasticity required for either acquisition or expression of ethanol CPP. However, Wang et al. (2009) has recently argued that CRF and orexin have independent functions in modulating stress-induced reinstatement of cocaine seeking. Future studies could address these concerns in studies of ethanol-conditioned behavior by site-specifically administering SB-334867 into VTA or other brain regions to allow for more specific conclusions about the effects of orexin on specific neural substrates.

Summary and Conclusions

The present experiments do not provide strong evidence that orexin is a primary modulator of the acquisition or expression of ethanol-seeking using the CPP model. Systemic administration of SB-334867 did not alter acquisition of CPP, but did significantly reduce ethanol-stimulated locomotor activity and alter BECs 10 and 60 min after ethanol injection. While there was a marginal effect of SB-334867 that blocked expression of a weak ethanol CPP, there was no effect of SB-334867 on a strong CPP with proper habituation to the novelty and handling of pretreatment injections and the vehicle solution. These results suggest that orexin signaling in not essential for acquisition and expression of ethanol CPP. Further studies using different models of ethanol-conditioned behavior should be conducted to confirm these findings.

The results of the present studies suggest that targeting orexin for the development of therapeutics may not be efficacious in treating alcoholism. It should be noted however, that the null results presented in this thesis do not preclude orexin from having effects on ethanol-conditioned behavior in other studies using other models of ethanol-seeking behavior, species, drugs, and/or timing or dose of SB-334867 administration. Orexin likely is involved in ethanol-conditioned behaviors, but further research is needed to understand its importance and mechanism of action in order to indentify targets for novel therapeutics.

Multiple researchers have suggested that orexin receptor antagonists may be useful for treating drug addiction (e.g., Scammel & Saper, 2007; Roecker & Coleman, 2008), but clinical research has largely focused on the use of orexin antagonists for the treatment of insomnia. The dual orexin receptor antagonist Almorexant (ACT-078573)

has shown efficacy in treating insomnia in clinical trials (Bisbore-Roch et al., 2007). Very little information has been released on the efficacy of any of the currently available orexin antagonists in treating drug addiction beyond preclinical animal data, and it is currently not entirely clear from the preclinical research what specific processes in alcoholism orexin might be involved in (i.e., craving, consumption, drug-seeking, etc.). One area of research that has been largely unexplored is the effect of orexin antagonism on extinction of drug-seeking behaviors. Several studies have shown a reduction of expression of drug seeking and relapse after SB-334867 treatment (e.g., Harris et al., 2005; Lawrence et al., 2006), but it is possible that multiple administrations of the drug coupled with extinction training may have long lasting effects on drug-seeking behavior. Addressing these types of questions and many others would result in critical preclinical data that should be assessed before orexin antagonists are considered for clinical trials.

As discussed in the introduction, since orexin has such a wide array of functions, it may be difficult to develop a drug effective for treatment of addiction without general effects on motivation, food intake and appetite, and sleep and arousal. In summary, my data suggest that orexin is not a primary mediator of ethanol-seeking behavior in the ethanol CPP model in mice and much more research must be done to determine if orexin is a viable neurobiological target for the development of therapeutics for alcoholism.

References

Allgaier, C. (2002). Ethanol sensitivity of NMDA receptors. Neurochem Int, 41(6), 377-382.

Ammoun, S. Johansson, L., Ekholm, M.E., Holmqvist, T., Danis, A.S., Korhonen, L., Sergeeva, O.A., Haas, H.L., Akerman, K.E., & Kukkonen, J.P. (2006). OX1 orexin receptors activate extracellular signal-regulated kinase in Chinese hamster ovary cells via multiple mechanisms: the role of Ca2+ influx in OX1 receptor signaling, Mol Endocrinol, 20(1), 80-99.

Aston-Jones, G., Smith, R. J., Moorman, D. E., & Richardson, K. A. (2009). Role of lateral hypothalamic orexin neurons in reward processing and addiction. Neuropharmacology, 56 Suppl 1, 112-121.

Backberg, M., Hervieu, G., Wilson, S., & Meister, B. (2002). Orexin receptor-1 (OX-R1) immunoreactivity in chemically identified neurons of the hypothalamus: focus on orexin targets involved in control of food and water intake. Eur J Neurosci, 15(2), 315-328.

Badia-Elder, N. E., Gilpin, N. W., & Stewart, R. B. (2007). Neuropeptide Y modulation of ethanol intake: effects of ethanol drinking history and genetic background. Peptides, 28(2), 339-344.

Bardo, M. T., & Bevins, R. A. (2000). Conditioned place preference: what does it add to our preclinical understanding of drug reward? Psychopharmacology (Berl), 153(1), 31-43.

Bechtholt, A. J., & Cunningham, C. L. (2005). Ethanol-induced conditioned place preference is expressed through a ventral tegmental area dependent mechanism. Behav Neurosci, 119(1), 213-223.

Bell, S. M., Stewart, R. B., Thompson, S. C., & Meisch, R. A. (1997). Food-deprivation increases cocaine-induced conditioned place preference and locomotor activity in rats. Psychopharmacology (Berl), 131(1), 1-8.

Bienkowski, P., Koros, E., Kostowski, W., & Danysz, W. (1999). Effects of N-methyl-D-aspartate receptor antagonists on reinforced and nonreinforced responding for ethanol in rats. Alcohol, 18(2-3), 131-137.

Biggio, G., Concas, A., Follesa, P., Sanna, E., & Serra, M. (2007). Stress, ethanol, and neuroactive steroids. Pharmacol Ther, 116(1), 140-171.

Blednov, Y.A. & Harris, R.A. (2008). Metabotropic glutamate receptor (mGluR5) regulation of ethanol sedation, dependence and consumption: Relation to acamprosate actions. Int J Neuropsychopharmacol 1:1-19

Bonci, A., & Borgland, S. (2009). Role of orexin/hypocretin and CRF in the formation of drug-dependent synaptic plasticity in the mesolimbic system. Neuropharmacology, 56 Suppl 1, 107-111.

Borgland, S. L., Taha, S. A., Sarti, F., Fields, H. L., & Bonci, A. (2006). Orexin A in the VTA is critical for the induction of synaptic plasticity and behavioral sensitization to cocaine. Neuron, 49(4), 589-601.

Boutrel, B., & de Lecea, L. (2008). Addiction and arousal: the hypocretin connection. Physiol Behav, 93(4-5), 947-951.

Boutrel, B., Kenny, P. J., Specio, S. E., Martin-Fardon, R., Markou, A., Koob, G. F., et al. (2005). Role for hypocretin in mediating stress-induced reinstatement of cocaine-seeking behavior. Proc Natl Acad Sci U S A, 102(52), 19168-19173.

Boyce-Rustay, J. M., & Cunningham, C. L. (2004). The role of NMDA receptor binding sites in ethanol place conditioning. Behav Neurosci, 118(4), 822-834.

Boyle, A. E., Segal, R., Smith, B. R., & Amit, Z. (1993). Bidirectional effects of GABAergic agonists and antagonists on maintenance of voluntary ethanol intake in rats. Pharmacol Biochem Behav, 46(1), 179-182Brisbare-Roch, C., Dingemanse, J., Koberstein, R., Hoever, P., Aissaoui, H., Flores, S., et al. (2007). Promotion of sleep by targeting the orexin system in rats, dogs and humans. Nat Med, 13(2), 150-155.

Brodie, M. S., Pesold, C., & Appel, S. B. (1999). Ethanol directly excites dopaminergic ventral tegmental area reward neurons. Alcohol Clin Exp Res, 23(11), 1848-1852.

Budygin, E. A., Phillips, P. E., Robinson, D. L., Kennedy, A. P., Gainetdinov, R. R., & Wightman, R. M. (2001). Effect of acute ethanol on striatal dopamine neurotransmission in ambulatory rats. J Pharmacol Exp Ther, 297(1), 27-34.

Burattini, C., Gill, T. M., Aicardi, G., & Janak, P. H. (2006). The ethanol self-administration context as a reinstatement cue: acute effects of naltrexone. Neuroscience, 139(3), 877-887.

Carr, K. D. (1996). Feeding, drug abuse, and the sensitization of reward by metabolic need. Neurochem Res, 21(11), 1455-1467.

Charney, D. S., Heninger, G. R., & Redmond, D. E., Jr. (1983). Yohimbine induced anxiety and increased noradrenergic function in humans: effects of diazepam and clonidine. Life Sci, 33(1), 19-29.

Chaudhri, N., Sahuque, L. L., Cone, J. J., & Janak, P. H. (2008). Reinstated ethanol-seeking in rats is modulated by environmental context and requires the nucleus accumbens core. Eur J Neurosci, 28(11), 2288-2298.

Chaudhri, N., Sahuque, L. L., & Janak, P. H. (2008). Context-induced relapse of conditioned behavioral responding to ethanol cues in rats. Biol Psychiatry, 64(3), 203-210.

Chemelli, R. M., Willie, J. T., Sinton, C. M., Elmquist, J. K., Scammell, T., Lee, C., et al. (1999). Narcolepsy in orexin knockout mice: molecular genetics of sleep regulation. Cell, 98(4), 437-451.

Chester, J. A., & Cunningham, C. L. (1999). Baclofen alters ethanol-stimulated activity but not conditioned place preference or taste aversion in mice. Pharmacol Biochem Behav, 63(2), 325-331.

Childress, A. R., Mozley, P. D., McElgin, W., Fitzgerald, J., Reivich, M., & O'Brien, C. P. (1999). Limbic activation during cue-induced cocaine craving. Am J Psychiatry, 156(1), 11-18.

Colussi-Mas, J., Geisler, S., Zimmer, L., Zahm, D. S., & Berod, A. (2007). Activation of afferents to the ventral tegmental area in response to acute amphetamine: a double-labelling study. Eur J Neurosci, 26(4), 1011-1025.

Cooney, N. L., Litt, M. D., Morse, P. A., Bauer, L. O., & Gaupp, L. (1997). Alcohol cue reactivity, negative-mood reactivity, and relapse in treated alcoholic men. J Abnorm Psychol, 106(2), 243-250.

Corbit, L. H., & Janak, P. H. (2007). Ethanol-associated cues produce general pavlovian-instrumental transfer. Alcohol Clin Exp Res, 31(5), 766-774.

Corrigall, W. A. (2009). Hypocretin mechanisms in nicotine addiction: evidence and speculation. Psychopharmacology (Berl).

Cowen, M. S., Chen, F., & Lawrence, A. J. (2004). Neuropeptides: implications for alcoholism. J Neurochem, 89(2), 273-285.

Cunningham, C. L., Ferree, N. K., & Howard, M. A. (2003). Apparatus bias and place conditioning with ethanol in mice. Psychopharmacology (Berl), 170(4), 409-422.

Cunningham, C. L., Fidler, T. L., & Hill, K. G. (2000). Animal models of alcohol's motivational effects. Alcohol Res Health, 24(2), 85-92.

Cunningham, C. L., Gremel, C. M., & Groblewski, P. A. (2006). Drug-induced conditioned place preference and aversion in mice. Nat Protoc, 1(4), 1662-1670.

Cunningham, C. L., Niehus, D. R., Malott, D. H., & Prather, L. K. (1992). Genetic differences in the rewarding and activating effects of morphine and ethanol. Psychopharmacology (Berl), 107(2-3), 385-393.

Cunningham, C. L., & Patel, P. (2007). Rapid induction of Pavlovian approach to an ethanol-paired visual cue in mice. Psychopharmacology (Berl), 192(2), 231-241.

Dayas, C. V., McGranahan, T. M., Martin-Fardon, R., & Weiss, F. (2008). Stimuli linked to ethanol availability activate hypothalamic CART and orexin neurons in a reinstatement model of relapse. Biol Psychiatry, 63(2), 152-157.

de Lecea, L., Jones, B. E., Boutrel, B., Borgland, S. L., Nishino, S., Bubser, M., et al. (2006). Addiction and arousal: alternative roles of hypothalamic peptides. J Neurosci, 26(41), 10372-10375.

de Lecea, L., Kilduff, T. S., Peyron, C., Gao, X., Foye, P. E., Danielson, P. E., et al. (1998). The hypocretins: hypothalamus-specific peptides with neuroexcitatory activity. Proc Natl Acad Sci U S A, 95(1), 322-327.

Di Chiara, G., Bassareo, V., Fenu, S., De Luca, M. A., Spina, L., Cadoni, C., et al. (2004). Dopamine and drug addiction: the nucleus accumbens shell connection. Neuropharmacology, 47 Suppl 1, 227-241.

Di Ciano, P., & Everitt, B. J. (2004). Conditioned reinforcing properties of stimuli paired with self-administered cocaine, heroin or sucrose: implications for the persistence of addictive behaviour. Neuropharmacology, 47 Suppl 1, 202-213.

DiLeone, R. J., Georgescu, D., & Nestler, E. J. (2003). Lateral hypothalamic neuropeptides in reward and drug addiction. Life Sci, 73(6), 759-768.

Duxon, M. S., Stretton, J., Starr, K., Jones, D. N., Holland, V., Riley, G., et al. (2001). Evidence that orexin-A-evoked grooming in the rat is mediated by orexin-1 (OX1) receptors, with downstream 5-HT2C receptor involvement. Psychopharmacology (Berl), 153(2), 203-209.

Egli, M. (2003). Peptides: their role in excess alcohol drinking and their promise as a therapeutic tool. Physiol Behav, 79(1), 89-93.

Enoch, M. A. (2008). The role of GABA(A) receptors in the development of alcoholism. Pharmacol Biochem Behav, 90(1), 95-104.

Everitt, B. J., Dickinson, A., & Robbins, T. W. (2001). The neuropsychological basis of addictive behaviour. Brain Res Brain Res Rev, 36(2-3), 129-138.

Everitt, B.J., & Wolf, M.E. (2002). Psychomotor stimulant addiction: a neural systems perspective, J Neurosci, 22(9), 3312-3320.

Georgescu, D., Zachariou, V., Barrot, M., Mieda, M., Willie, J. T., Eisch, A. J., et al. (2003). Involvement of the lateral hypothalamic peptide orexin in morphine dependence and withdrawal. J Neurosci, 23(8), 3106-3111.

Gilpin, N.W. & Koob, G.F. (2008). Neurobiology of alcohol dependence: focus on motivational mechanisms. Alcoh Res Health, 31(3), 185-195.

Gonzales, R. A., Job, M. O., & Doyon, W. M. (2004). The role of mesolimbic dopamine in the development and maintenance of ethanol reinforcement. Pharmacol Ther, 103(2), 121-146.

Gremel, C. M., & Cunningham, C. L. (2007). Role of test activity in ethanol-induced disruption of place preference expression in mice. Psychopharmacology (Berl), 191(2), 195-202.

Gremel, C. M., & Cunningham, C. L. (2008). Roles of the nucleus accumbens and amygdala in the acquisition and expression of ethanol-conditioned behavior in mice. J Neurosci, 28(5), 1076-1084.

Gremel, C. M., & Cunningham, C. L. (2009). Involvement of amygdala dopamine and nucleus accumbens NMDA receptors in ethanol-seeking behavior in mice. Neuropsychopharmacology, 34(6), 1443-1453.

Groblewski, P. A., Bax, L. S., & Cunningham, C. L. (2008). Reference-dose place conditioning with ethanol in mice: empirical and theoretical analysis. Psychopharmacology (Berl), 201(1), 97-106.

Hagan, J. J., Leslie, R. A., Patel, S., Evans, M. L., Wattam, T. A., Holmes, S., et al. (1999). Orexin A activates locus coeruleus cell firing and increases arousal in the rat. Proc Natl Acad Sci U S A, 96(19), 10911-10916.

Hara, J., Beuckmann, C. T., Nambu, T., Willie, J. T., Chemelli, R. M., Sinton, C. M., et al. (2001). Genetic ablation of orexin neurons in mice results in narcolepsy, hypophagia, and obesity. Neuron, 30(2), 345-354.

Harris, G. C., & Aston-Jones, G. (2006). Arousal and reward: a dichotomy in orexin function. Trends Neurosci, 29(10), 571-577.

Harris, G. C., Wimmer, M., & Aston-Jones, G. (2005). A role for lateral hypothalamic orexin neurons in reward seeking. Nature, 437(7058), 556-559.

Harris, G. C., Wimmer, M., Randall-Thompson, J. F., & Aston-Jones, G. (2007). Lateral hypothalamic orexin neurons are critically involved in learning to associate an environment with morphine reward. Behav Brain Res, 183(1), 43-51.

Harwood, H. *Updating Estimates of the Economic Costs of Alcohol Abuse in the United States: Estimates, Update Methods, and Data*. Report prepared by The Lewin Group for the National Institute on Alcohol Abuse and Alcoholism, 2000. Based on estimates, analyses, and data reported in Harwood, H.; Fountain, D.; and Livermore, G. *The Economic Costs of Alcohol and Drug Abuse in the United States 1992*. Report prepared for the National Institute on Drug Abuse and the National Institute on Alcohol Abuse and Alcoholism, National Institutes of

Health, Department of Health and Human Services. NIH Publication No. 98-4327. Rockville, MD: National Institutes of Health, 1998.

Haynes, A. C., Jackson, B., Chapman, H., Tadayyon, M., Johns, A., Porter, R. A., et al. (2000). A selective orexin-1 receptor antagonist reduces food consumption in male and female rats. Regul Pept, 96(1-2), 45-51.

Heilig, M., & Egli, M. (2006). Pharmacological treatment of alcohol dependence: target symptoms and target mechanisms. Pharmacol Ther, 111(3), 855-876.

Hervieu, G. J., Cluderay, J. E., Harrison, D. C., Roberts, J. C., & Leslie, R. A. (2001). Gene expression and protein distribution of the orexin-1 receptor in the rat brain and spinal cord. Neuroscience, 103(3), 777-797.

Hill, K. G., Ryabinin, A. E., & Cunningham, C. L. (2007). FOS expression induced by an ethanol-paired conditioned stimulus. Pharmacol Biochem Behav, 87(2), 208-221.

Hirose, M., Egashira, S., Goto, Y., Hashihayata, T., Ohtake, N., Iwaasa, H., et al. (2003). N-acyl 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline: the first orexin-2 receptor selective non-peptidic antagonist. Bioorg Med Chem Lett, 13(24), 4497-4499.

Hirota, K., Kushikata, T., Kudo, M., Kudo, T., Smart, D., & Matsuki, A. (2003). Effects of central hypocretin-1 administration on hemodynamic responses in young-adult and middle-aged rats. Brain Res, 981(1-2), 143-150.

Holland, P. R., Akerman, S., & Goadsby, P. J. (2005). Orexin 1 receptor activation attenuates neurogenic dural vasodilation in an animal model of trigeminovascular nociception. J Pharmacol Exp Ther, 315(3), 1380-1385.

Hollander, J. A., Lu, Q., Cameron, M. D., Kamenecka, T. M., & Kenny, P. J. (2008). Insular hypocretin transmission regulates nicotine reward. Proc Natl Acad Sci U S A, 105(49), 19480-19485.

Ishii, Y., Blundell, J. E., Halford, J. C., Upton, N., Porter, R., Johns, A., et al. (2005a). Anorexia and weight loss in male rats 24 h following single dose treatment with orexin-1 receptor antagonist SB-334867. Behav Brain Res, 157(2), 331-341.

Ishii, Y., Blundell, J. E., Halford, J. C., Upton, N., Porter, R., Johns, A., et al. (2004). Differential effects of the selective orexin-1 receptor antagonist SB-334867 and lithium chloride on the behavioural satiety sequence in rats. Physiol Behav, 81(1), 129-140.

Ishii, Y., Blundell, J. E., Halford, J. C., Upton, N., Porter, R., Johns, A., et al. (2005b). Satiety enhancement by selective orexin-1 receptor antagonist SB-334867: influence of test context and profile comparison with CCK-8S. Behav Brain Res, 160(1), 11-24.

Johnson, B. A. (2005). Recent advances in the development of treatments for alcohol and cocaine dependence: focus on topiramate and other modulators of GABA or glutamate function. CNS Drugs, 19(10), 873-896.

Jones, D. N., Gartlon, J., Parker, F., Taylor, S. G., Routledge, C., Hemmati, P., et al. (2001). Effects of centrally administered orexin-B and orexin-A: a role for orexin-1 receptors in orexin-B-induced hyperactivity. Psychopharmacology (Berl), 153(2), 210-218.

Kitai, S. T., Shepard, P. D., Callaway, J. C., & Scroggs, R. (1999). Afferent modulation of dopamine neuron firing patterns. Curr Opin Neurobiol, 9(6), 690-697.

Koob, G. F., Roberts, A. J., Schulteis, G., Parsons, L. H., Heyser, C. J., Hyytia, P., et al. (1998). Neurocircuitry targets in ethanol reward and dependence. Alcohol Clin Exp Res, 22(1), 3-9.

Korotkova, T. M., Sergeeva, O. A., Eriksson, K. S., Haas, H. L., & Brown, R. E. (2003). Excitation of ventral tegmental area dopaminergic and nondopaminergic neurons by orexins/hypocretins. J Neurosci, 23(1), 7-11.

Kotz, C. M. (2006). Integration of feeding and spontaneous physical activity: role for orexin. Physiol Behav, 88(3), 294-301.

Kumar, S., Porcu, P., Werner, D. F., Matthews, D. B., Diaz-Granados, J. L., Helfand, R. S., et al. (2009). The role of GABA(A) receptors in the acute and chronic effects of ethanol: a decade of progress. Psychopharmacology (Berl).

Langmead, C.J., Jerman, J.C., Brough, S.J., Scott, C., Porter, R.A., & Herdon, H.J. (2004) Characterisation of the binding of [^{3}H]-SB-674042, a novel nonpeptide antagonist, to the human orexin-1 receptor. Br.J.Pharmacol. 141, 340

Lawrence, A. J., Cowen, M. S., Yang, H. J., Chen, F., & Oldfield, B. (2006). The orexin system regulates alcohol-seeking in rats. Br J Pharmacol, 148(6), 752-759.

Le, A., & Shaham, Y. (2002). Neurobiology of relapse to alcohol in rats. Pharmacol Ther, 94(1-2), 137-156.

Le, A. D., Poulos, C. X., Harding, S., Watchus, J., Juzytsch, W., & Shaham, Y. (1999). Effects of naltrexone and fluoxetine on alcohol self-administration and reinstatement of alcohol seeking induced by priming injections of alcohol and exposure to stress. Neuropsychopharmacology, 21(3), 435-444.

Lin, L., Faraco, J., Li, R., Kadotani, H., Rogers, W., Lin, X., et al. (1999). The sleep disorder canine narcolepsy is caused by a mutation in the hypocretin (orexin) receptor 2 gene. Cell, 98(3), 365-376.

Mann, K., Kiefer, F., Spanagel, R., & Littleton, J. (2008). Acamprosate: recent findings and future research directions. Alcohol Clin Exp Res, 32(7), 1105-1110.

Marcus, J.N. & Elmquist, J.K. (2005). Orexin projections and localization of orexin receptors. In Hypocretins: Integration of physiological functions. de Lecea & Sutcliffe (Eds). US. Springer.

Marcus, J. N., Aschkenasi, C. J., Lee, C. E., Chemelli, R. M., Saper, C. B., Yanagisawa, M., et al. (2001). Differential expression of orexin receptors 1 and 2 in the rat brain. J Comp Neurol, 435(1), 6-25.

Martin, G., Fabre, V., Siggins, G. R., & de Lecea, L. (2002). Interaction of the hypocretins with neurotransmitters in the nucleus accumbens. Regul Pept, 104(1-3), 111-117.

McBride, W. J., & Li, T. K. (1998). Animal models of alcoholism: neurobiology of high alcohol-drinking behavior in rodents. Crit Rev Neurobiol, 12(4), 339-369.

Mooreman, D.E. & Aston-Jones, G. (2007). Modulation of ventral tegmental area neural responses to prefrontal stimulation by local orexin application in vivo. Neuroscience Meeting Planner Online, Program No. 916.2.

Mooreman, D.E. & Aston-Jones, G. (2009). Orexin-1 receptor antagonism decreases ethanol consumption and preference selectivity in high-ethanol-preferring Spraque-Dawley rats, Alcohol, 379-386.

Nakamura, T., Uramura, K., Nambu, T., Yada, T., Goto, K., Yanagisawa, M., et al. (2000). Orexin-induced hyperlocomotion and stereotypy are mediated by the dopaminergic system. Brain Res, 873(1), 181-187.

Narita, M., Nagumo, Y., Hashimoto, S., Khotib, J., Miyatake, M., Sakurai, T., et al. (2006). Direct involvement of orexinergic systems in the activation of the mesolimbic dopamine pathway and related behaviors induced by morphine. J Neurosci, 26(2), 398-405.

NIAAA Mission Statement. (2006). Retrieved July 20, 2009, from http://www.niaaa.nih.gov/AboutNIAAA/OrganizationalInformation/Mission.htm

NIAAA (2004, June 10). Alcohol abuse increases, dependence decline across decade: Young adult minorities emerge as high risk subgroups. [press release]. Retrieved July 20, 2009, from http://www.niaaa.nih.gov/NewsEvents/NewsRelease/NESARCNews.htm

O'Brien, C. P., Volpicelli, L. A., & Volpicelli, J. R. (1996). Naltrexone in the treatment of alcoholism: a clinical review. Alcohol, 13(1), 35-39.

Ohno, K., & Sakurai, T. (2008). Orexin neuronal circuitry: role in the regulation of sleep and wakefulness. Front Neuroendocrinol, 29(1), 70-87.

O'Malley, S. S., & Froehlich, J. C. (2003). Advances in the use of naltrexone: an integration of preclinical and clinical findings. Recent Dev Alcohol, 16, 217-245.

O'Malley, S. S., Jaffe, A. J., Chang, G., Schottenfeld, R. S., Meyer, R. E., & Rounsaville, B. (1992). Naltrexone and coping skills therapy for alcohol dependence. A controlled study. Arch Gen Psychiatry, 49(11), 881-887.

Paneda, C., Winsky-Sommerer, R., Boutrel, B., & de Lecea, L. (2005). The corticotropin-releasing factor-hypocretin connection: implications in stress response and addiction. Drug News Perspect, 18(4), 250-255.

Peyron, C., Faraco, J., Rogers, W., Ripley, B., Overeem, S., Charnay, Y., et al. (2000). A mutation in a case of early onset narcolepsy and a generalized absence of hypocretin peptides in human narcoleptic brains. Nat Med, 6(9), 991-997.

Peyron, C., Tighe, D. K., van den Pol, A. N., de Lecea, L., Heller, H. C., Sutcliffe, J. G., et al. (1998). Neurons containing hypocretin (orexin) project to multiple neuronal systems. J Neurosci, 18(23), 9996-10015.

Pickering, C., Avesson, L., Liljequist, S., Lindblom, J., & Schioth, H. B. (2007). The role of hypothalamic peptide gene expression in alcohol self-administration behavior. Peptides, 28(12), 2361-2371.

Porter, R. A., Chan, W. N., Coulton, S., Johns, A., Hadley, M. S., Widdowson, K., et al. (2001). 1,3-Biarylureas as selective non-peptide antagonists of the orexin-1 receptor. Bioorg Med Chem Lett, 11(14), 1907-1910.

Rassnick, S., D'Amico, E., Riley, E., & Koob, G. F. (1993). GABA antagonist and benzodiazepine partial inverse agonist reduce motivated responding for ethanol. Alcohol Clin Exp Res, 17(1), 124-130.

Rassnick, S., Pulvirenti, L., & Koob, G. F. (1992). Oral ethanol self-administration in rats is reduced by the administration of dopamine and glutamate receptor antagonists into the nucleus accumbens. Psychopharmacology (Berl), 109(1-2), 92-98.

Rewal, M., Jurd, R., Gill, T. M., He, D. Y., Ron, D., & Janak, P. H. (2009). Alpha4-containing GABAA receptors in the nucleus accumbens mediate moderate intake of alcohol. J Neurosci, 29(2), 543-549.

Richards, J. K., Simms, J. A., Steensland, P., Taha, S. A., Borgland, S. L., Bonci, A., et al. (2008). Inhibition of orexin-1/hypocretin-1 receptors inhibits yohimbine-induced reinstatement of ethanol and sucrose seeking in Long-Evans rats. Psychopharmacology (Berl), 199(1), 109-117.

Risinger, F. O., Dickinson, S. D., & Cunningham, C. L. (1992). Haloperidol reduces ethanol-induced motor activity stimulation but not conditioned place preference. Psychopharmacology (Berl), 107(2-3), 453-456.

Roberts, A. J., McDonald, J. S., Heyser, C. J., Kieffer, B. L., Matthes, H. W., Koob, G. F., et al. (2000). mu-Opioid receptor knockout mice do not self-administer alcohol. J Pharmacol Exp Ther, 293(3), 1002-1008.

Robinson, T. E., & Berridge, K. C. (1993). The neural basis of drug craving: an incentive-sensitization theory of addiction. Brain Res Brain Res Rev, 18(3), 247-291.

Rodgers, R. J., Halford, J. C., Nunes de Souza, R. L., Canto de Souza, A. L., Piper, D. C., Arch, J. R., et al. (2001). SB-334867, a selective orexin-1 receptor antagonist, enhances behavioural satiety and blocks the hyperphagic effect of orexin-A in rats. Eur J Neurosci, 13(7), 1444-1452.

Roecker, A. J., & Coleman, P. J. (2008). Orexin receptor antagonists: medicinal chemistry and therapeutic potential. Curr Top Med Chem, 8(11), 977-987.

Rustay, N. R., & Crabbe, J. C. (2004). Genetic analysis of rapid tolerance to ethanol's incoordinating effects in mice: inbred strains and artificial selection. Behav Genet, 34(4), 441-451.

Ryabinin, A. E., & Weitemier, A. Z. (2006). The urocortin 1 neurocircuit: ethanol-sensitivity and potential involvement in alcohol consumption. Brain Res Rev, 52(2), 368-380.

Sakurai, T. (2005). Orexin and orexin receptors. In Hypocretins: Integration of physiological functions. de Lecea & Sutcliffe (Eds). US. Springer.

Sakurai, T., Amemiya, A., Ishii, M., Matsuzaki, I., Chemelli, R. M., Tanaka, H., et al. (1998). Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. Cell, 92(4), 573-585.

Samson, H. H., & Czachowski, C. L. (2003). Behavioral measures of alcohol self-administration and intake control: rodent models. Int Rev Neurobiol, 54, 107-143.

Saper, C.B., Scammel, T.E. & Lu, J. (2005). Hypothalamic regulation of sleep and circadian rhythms, Nature, 437, 1257-1263.

Scammell, T. E., & Saper, C. B. (2007). Orexins: looking forward to sleep, back at addiction. Nat Med, 13(2), 126-128.

Schneider, E. R., Rada, P., Darby, R. D., Leibowitz, S. F., & Hoebel, B. G. (2007). Orexigenic peptides and alcohol intake: differential effects of orexin, galanin, and ghrelin. Alcohol Clin Exp Res, 31(11), 1858-1865.

Sharf, R., Sarhan, M., & Dileone, R. J. (2008). Orexin mediates the expression of precipitated morphine withdrawal and concurrent activation of the nucleus accumbens shell. Biol Psychiatry, 64(3), 175-183.

Sher, K. J. (1985). Subjective effects of alcohol: the influence of setting and individual differences in alcohol expectancies. J Stud Alcohol, 46(2), 137-146.

Smart, D., & Jerman, J. (2002). The physiology and pharmacology of the orexins. Pharmacol Ther, 94(1-2), 51-61.

Smart, D., Sabido-David, C., Brough, S. J., Jewitt, F., Johns, A., Porter, R. A., et al. (2001). SB-334867-A: the first selective orexin-1 receptor antagonist. Br J Pharmacol, 132(6), 1179-1182.

Smith, M. I., Piper, D. C., Duxon, M. S., & Upton, N. (2003). Evidence implicating a role for orexin-1 receptor modulation of paradoxical sleep in the rat. Neurosci Lett, 341(3), 256-258.

Smith, R.J., See, R., Aston-Jones, G. (2007). The orexin-1 receptor antagonist SB-334867 blocks cue-induced reinstatement of cocaine-seeking. Neuroscience Meeting Planner Online, Program No. 916.1.

Smith, R.J., See, R.E., & Aston-Jones, G. (2009a). Orexin/hypocretin signaling at the orexin 1 receptor regulates cue-elicited cocaine seeking. Eur J Neurosci, 30, 493-503.

Smith, R. J., Tahsili-Fahadan, P., & Aston-Jones, G. (2009b). Orexin/hypocretin is necessary for context-driven cocaine-seeking. Neuropharmacology (in press).

Spanagel, R., & Zieglgansberger, W. (1997). Anti-craving compounds for ethanol: new pharmacological tools to study addictive processes. Trends Pharmacol Sci, 18(2), 54-59.

Stahl, S. M. (2008). Essential Psychopharmacology Online. Retrieved August 17, 2009 from

http://stahlonline.cambridge.org/essential_chapter.jsf?page=chapter19_3.htm&name=Chapter%2019&title=Alcohol

Stewart, J., De Wit, H. & Eikelboom, R. (1984). Role of unconditioned and conditioned drug effects in the self-administration of opiates and stimulants. Psychol. Rev. 91, 251–268.

Stuber, G. D., Evans, S. B., Higgins, M. S., Pu, Y., & Figlewicz, D. P. (2002). Food restriction modulates amphetamine-conditioned place preference and nucleus accumbens dopamine release in the rat. Synapse, 46(2), 83-90.

Swanson LW (1982). The projections of the ventral tegmental area and adjacent regions: A combined fluorescent retrograde tracer and immunofluorescence study in the rat. Brain Research Bulletin 9: 321–353.

Thannickal, T. C., Moore, R. Y., Nienhuis, R., Ramanathan, L., Gulyani, S., Aldrich, M., et al. (2000). Reduced number of hypocretin neurons in human narcolepsy. Neuron, 27(3), 469-474.

Thiele, T. E., Stewart, R. B., Badia-Elder, N. E., Geary, N., Massi, M., Leibowitz, S. F., et al. (2004). Overlapping peptide control of alcohol self-administration and feeding. Alcohol Clin Exp Res, 28(2), 288-294.

Thorpe, A. J. & Kotz, C. M. (2005). Orexin A in the nucleus accumbens stimulates feeding and locomotor activity. Brain Res 1050(1-2), 156-162.

Thorsell, A. (2007). Neuropeptide Y (NPY) in alcohol intake and dependence. Peptides, 28(2), 480-483.

Trivedi, P., Yu, H., MacNeil, D. J., Van der Ploeg, L. H., & Guan, X. M. (1998). Distribution of orexin receptor mRNA in the rat brain. FEBS Lett, 438(1-2), 71-75.

Tzschentke, T. M. (1998). Measuring reward with the conditioned place preference paradigm: a comprehensive review of drug effects, recent progress and new issues. Prog Neurobiol, 56(6), 613-672.

Tzschentke, T. M. (2007). Measuring reward with the conditioned place preference (CPP) paradigm: update of the last decade. Addict Biol, 12(3-4), 227-462.

Ulm, R. R., Volpicelli, J. R., & Volpicelli, L. A. (1995). Opiates and alcohol self-administration in animals. J Clin Psychiatry, 56 Suppl 7, 5-14.

Ungless, M. A., Singh, V., Crowder, T. L., Yaka, R., Ron, D., & Bonci, A. (2003). Corticotropin-releasing factor requires CRF binding protein to potentiate NMDA receptors via CRF receptor 2 in dopamine neurons. Neuron, 39(3), 401-407.

Upton, N. (2005). *In vivo* pharmacology of orexin (hypocretin) receptors. In Hypocretins: Integration of physiological functions. de Lecea & Sutcliffe (Eds). US. Springer.

Vengeliene, V., Bilbao, A., Molander, A., & Spanagel, R. (2008). Neuropharmacology of alcohol addiction. Br J Pharmacol, 154(2), 299-315.

Volpicelli, J. R., Alterman, A. I., Hayashida, M., & O'Brien, C. P. (1992). Naltrexone in the treatment of alcohol dependence. Arch Gen Psychiatry, 49(11), 876-880.

Wang, B., You, Z. B., & Wise, R. A. (2009). Reinstatement of cocaine seeking by hypocretin (orexin) in the ventral tegmental area: independence from the local corticotropin-releasing factor network. Biol Psychiatry, 65(10), 857-862.

Weiner, D.M., Levey, A.I., Sunahara, K.K., Niznik, H.B., O'Dowd, B.F., Seeman, P., & Brann, M.P. (1991). D1 and D2 dopamine receptor mRNA in rat brain, Proc Nat Acad Sci, 88(5), 1859-1863.

Weiss, F., & Porrino, L. J. (2002). Behavioral neurobiology of alcohol addiction: recent advances and challenges. J Neurosci, 22(9), 3332-3337.

Winsky-Sommerer, R., Boutrel, B., & de Lecea, L. (2005). Stress and arousal: the corticotrophin-releasing factor/hypocretin circuitry. Mol Neurobiol, 32(3), 285-294.

Winrow, C.J., Tanis, K.Q., Reiss, D.R., Rigby, A.M., Uslaner, J.M., Uebele, V.N., Doran, S.M., Fox, S.V., Garson, S.L., Levine, D.M., Rocecher, A.J., Coleman, P.J., Koblan, K.S., Renger, J.J. (2009). Orexin receptor antagonistm prevents transcriptional and behavioral plasticity from stimulant exposure, Neuropsychopharm, doi:10.1016/j.neuropharm.2009.07.008.

Yamanaka, A., Beuckmann, C. T., Willie, J. T., Hara, J., Tsujino, N., Mieda, M., et al. (2003). Hypothalamic orexin neurons regulate arousal according to energy balance in mice. Neuron, 38(5), 701-713.

Yim, H. J., & Gonzales, R. A. (2000). Ethanol-induced increases in dopamine extracellular concentration in rat nucleus accumbens are accounted for by increased release and not uptake inhibition. Alcohol, 22(2), 107-115.

Zhang, G. C., Mao, L. M., Liu, X. Y., & Wang, J. Q. (2007). Long-lasting up-regulation of orexin receptor type 2 protein levels in the rat nucleus accumbens after chronic cocaine administration. J Neurochem, 103(1), 400-407.

Zhou, Y., Bendor, J., Hofmann, L., Randesi, M., Ho, A., & Kreek, M. J. (2006). Mu opioid receptor and orexin/hypocretin mRNA levels in the lateral hypothalamus and striatum are enhanced by morphine withdrawal. J Endocrinol, 191(1), 137-145.