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The Role of Melanin-Concentrating Hormone in the Motivation to Consume Alcohol

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by

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Abstract

Alcohol is a complex substance that acts as a reward, an anxiolytic and a macronutrient. Consequently, alcohol consumption is believed to be influenced by neuronal systems that regulate reward, anxiety and energy balance. A better understanding of these systems will lead to novel targets for the development of pharmacological treatments for alcoholism. The melanin-concentrating hormone (MCH) system has qualities that make it a likely modulator of alcohol drinking. Best known as a signal of negative energy balance, MCH is also implicated in the regulation of reward and anxiety. Therefore, the purpose of this dissertation was to test the hypothesis that MCH signaling enhances the motivation to drink alcohol. Initial studies found that MCH administered into the 3rd cerebral ventricle of rats increased alcohol consumption at a dose that had no effect on anxiety-like behavior. MCH also increased food, water and sucrose/quinine intake. These initial studies along with the finding that MCH increased operant responding for alcohol suggested that the ability of MCH to augment alcohol intake was due to its effect on reward and/or energy balance as opposed to anxiety or fluid balance. Although it was apparent that pharmacological administration of MCH augmented alcohol drinking, it was not clear if the endogenous MCH system was involved. Antagonism of the MCH receptor effectively reduced chow intake, but had no impact on alcohol or sucrose/quinine consumption in the rodent model. On the other hand, MCH receptor 1 knock-out mice (MCHR1 KO) consumed more alcohol than wildtype mice. The peculiar phenotype of these mice (i.e., hyperphagia and increased dopamine receptor sensitivity) may be responsible for the effect of genotype on alcohol drinking. In conclusion, the research reported in this dissertation supports a novel

function for MCH in the regulation of alcohol intake. No definite conclusion could be made from these results regarding the impact of endogenous MCH signaling on alcohol intake. Thus, future studies should focus on the role of MCHR1 signaling in discrete brain regions to clarify the involvement of endogenous MCH signaling in the motivation to drink alcohol.

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LIST OF ABBREVIATIONS

α-MSH	alpha-melanocyte-stimulating hormone
AA	ALKO Alcohol strain
ACTH	adrenocorticotropin hormone
AGRP	agouti-related peptide
AII	angiotensin II
ALC	alcohol
ANA	AKLO Non-Alcohol strain
ANOVA	analysis of variance
AT-1	angiotensin II receptor-1
AT-2	angiotensin II receptor-2
ATP	adenosine triphosphate
BAL	blood alcohol level
CCK	cholecystokinin
CNS	central nervous system
CRH	corticotropin-releasing factor
CRH	corticotropin-releasing hormone
CRHR1	corticotropin-releasing hormone receptor 1
CRHR2	corticotropin-releasing hormone receptor 2
DOR	dynorphin opioid receptor
EPM	elevated plus maze
FR	fixed ratio
GABA	gamma-aminobutyric acid
HAD	high alcohol drinking strain
HAP	high alcohol preferring strain
HET	heterozygote
i3vt	intra-3rd-cerebroventricular
KO	knock-out
KOR	kappa opioid receptor
LAD	low alcohol drinking strain

LAP	low alcohol preferring strain
Lv	lateral ventricle
MC	melanocortin
MC3R	melanocortin receptor 3
MC4R	melanocortin receptor 4
MCH	melanin-concentrating hormone
MCHR1	melanin-concentrating hormone receptor 1
N/ORQ	nociceptin/orphanin FQ
NAc	nucleus accumbens
NMDA	N-methyl D-aspartate glutamate receptor
NOP	opioid-like receptor 1
NP	non-preferring strain
NPY	neuropeptide Y
P	preferring strain
POMC	proopiomelanocortin
PR	progressive ratio
PVN	paraventricular nucleus
pVTA	posterior ventral tegmental area
SEM	standard error of the mean
sNP	Sardinian non-preferring strain
sP	Sardinian preferring strain
SUC	sucrose
UChA	University of Chile A strain
UChB	University of Chile B strain
w/v	weight per volume
WT	wildtype

CHAPTER 1

Introduction and General Background

Melanin-concentrating hormone: A novel regulator of alcohol drinking

Alcohol and alcoholism

Ethyl alcohol, or alcohol as it is commonly known, is a simple molecule composed of two carbons and a hydroxyl group (OH) produced by fermentation when yeast metabolizes sugar in an anaerobic environment. Because of its polar structure it is miscible in both water and fat, and thus diffuses easily across biological membranes, including the blood brain barrier. Alcohol has many effects on the central nervous system (CNS), but is generally classified as a depressant. Historically it was believed that the effects of alcohol on the CNS were solely the result of an increase in membrane permeability (Deitrich et al., 1989); however, it is now thought that the drug can also impact the CNS via direct interaction with proteins (Peoples et al., 1996). Alcohol has been produced and consumed by humans for a variety of purposes (e.g., religious ceremonies, hygiene and as a dietary component) since before recorded time (Hanson, 1995). Today, however, it is most commonly thought of as a psychoactive drug with abuse potential.

Alcohol dependence, or alcoholism, has been clinically defined as a disease where the following symptoms are present: tolerance to the effects of alcohol, impaired control over drinking, and continued drinking despite adverse consequences (Association, 1994). In the US, alcohol is the 3rd major non-genetic cause of death, behind tobacco and obesity (Mokdad et al., 2004), and the annual cost to society is estimated to be almost 185 billion dollars (Harwood et al., 1998). Ten years ago the only treatment options available for alcoholism were psychosocial therapy and/or disulfiram (Antabuse) (Swift, 1999). Psychosocial therapy is effective in reducing alcohol consumption in the short

term, but relapse is high (Finney et al., 1996). Disulfiram is an aversive agent that causes an accumulation of acetaldehyde, an alcohol metabolite, which deters alcohol ingestion by causing tachycardia, flushing, dyspnea, nausea, and vomiting (Swift, 1999). Because of its nature, compliance is low with this drug and its use neither improves the rate of continuous abstinence, nor delays the resumption of drinking (Fuller et al., 1986).

The past 10 years have witnessed an increased interest in developing drug therapies to treat alcohol dependence (Swift, 1999). Naltrexone, an opioid antagonist, reduces alcohol craving and alcohol-induced euphoria, and decreases relapse rate in humans (O'Brien et al., 1996). Other pharmacological therapies that are currently being assessed for the treatment of alcohol dependence include acamprosate, an n-methyl d-aspartate (NMDA) glutamate receptor modulator, tiapride, a dopamine receptor antagonist, and various compounds already approved for the treatment of anxiety and depression (Swift, 1999). Because alcoholism is a complex condition with multiple genetic and environmental causes, it is unlikely that one therapy will suffice in treating all patients. Therefore, exploring novel pharmacological targets is an important strategy.

Rodent models of alcohol intake and alcoholism

Rodent models have contributed vastly to understanding the underlying mechanisms responsible for alcohol use disorders (Weiss and Porrino, 2002). Although rats are commonly used in alcohol consumption studies, they do not voluntarily consume pharmacologically relevant doses of alcohol. Withstanding the use of food and/or water deprivation, two common approaches have been taken to develop a rat model of alcohol

intake. First, out-bred rat strains (e.g., Long Evans, Sprague-Dawley or Wistar) can be trained to drink alcohol using a sucrose-fading procedure (Grant and Samson, 1985), where a sweet sucrose solution is gradually adulterated with alcohol and then the sucrose is decrementally removed. This technique overrides the initial aversion to the bitter taste of alcohol and in analogous to the way an alcohol naïve person initially prefers a sweetened alcoholic drink. In this model the rat is usually given limited access to alcohol in its home cage or an operant chamber. Moderate alcohol intake is obtained (i.e., 0.5-1.0 g/kg body weight per session) (Grant and Samson, 1985; Samson, 1986) with blood alcohol levels ranging between 5-50 mg/dl (van Erp and Miczek, 1997).

The second approach in developing a rat model of alcohol drinking has been selective breeding for both high and low alcohol preference. There are currently five pairs of alcohol-preferring and non-preferring lines in existence: ALKO alcohol and ALKO non-alcohol (AA/ANA) lines (Eriksson, 1968), University of Chile A and B (UChA/UChB) lines (Mardones and Segovia-Riquelme, 1983), Sardinian alcohol-preferring and non-preferring (sP/sNP) lines (Colombo et al., 1995), Indiana alcohol preferring and non-preferring (P/NP) lines (Lumeng, 1977), and Indiana high and low alcohol drinking (HAD/LAD) lines (Li et al., 1993; Murphy et al., 2002). The selection criteria for each of these lines was not uniform, but in general the upper and lower extremes of daily 10% alcohol intake in the original out-bred population were selected (Murphy et al., 2002). These "high preference" selectively breed lines are useful because they consume large amounts of alcohol without training (5-10 g/kg body weight/ day) (Murphy et al., 2002). In addition, investigation of the genetic differences between the

high and low alcohol preferring strains is believed to be an important model for uncovering genetic predispositions to alcoholism human population (Arlinde et al., 2004; Caberlotto et al., 2001; Hwang et al., 2004; Hwang et al., 1999; Pandey et al., 2005).

Mouse models of alcohol intake are similar to the selected rat lines in that inbred strains have differing alcohol preferences, with some mice consuming large quantities of alcohol voluntarily (Rodgers and McClearn, 1962). For example, when given a choice between water and 10% alcohol, the C57BL/6 strain of mice prefer 10% alcohol to water, while the DBA strain almost completely avoid 10% alcohol (Rodgers and McClearn, 1962). On average, the male C57BL/6 mouse will drink 10 g/kg body weight 10% alcohol per day (Blednov et al., 2005). In addition to these innately differing inbred strains, selective breeding has been used to create a line of mice with diverging alcohol preference (i.e., high alcohol preferring mice (HAP) and low alcohol preferring mice (LAP)) (Grahame et al., 1999). The differences in alcohol preference in the inbred strains as well as the HAP and LAP mice is believed to be useful for locating and investigating genetic factors that might predispose alcohol dependence in humans (Bachtell et al., 2003; Hungund and Basavarajappa, 2000; Ng et al., 1994; Ng et al., 1996). In addition, many studies have taken advantage of genetically modified mice to investigate the role of a specific protein in alcohol consumption (Maul et al., 2005; Navarro et al., 2005; Roberts et al., 2000b; Sillaber et al., 2002; Thiele et al., 1998; Thiele et al., 2000).

The above models are useful in understanding the motivation to consume alcohol, but they do not entirely mimic the human situation of alcoholism. The animals are not considered "dependent" on the alcohol because if the alcohol is taken away they will not suffer severe physical withdrawal symptoms. Therefore, a separate model is used to investigate alcohol drinking in "dependent" animals. The first step in developing a model of alcohol dependence is to force a rat or mouse into very high levels of alcohol intoxication, the point being to mimic the blood alcohol levels that are observed in human alcoholic (around 200 mg/dl body weight). There are numerous ways to accomplish this level of intoxication in an animal. Commonly animals are provided an alcohol-adulterated liquid diet as their sole source of calories (DeCarli and Lieber, 1967). In addition, alcohol-vapor has been used to induce high levels of alcohol intoxication in rodents (Ferko and Bobyock, 1977). Both of these procedures result in "dependent" models because when the alcohol is removed the rodents suffer severe physical withdrawal symptoms (Becker and Hale, 1993; Macey et al., 1996). In addition, they exhibit heightened levels of anxiety (Kliethermes, 2005) and voluntarily drink more alcohol than non-exposed pair-fed controls (Roberts et al., 2000a; Valdez and Koob, 2004). This increase in alcohol intake has been demonstrated to occur even after the physical withdrawal signs have subsided, indicating the importance of the psychological withdrawal symptoms in the motivation to consume alcohol (Roberts et al., 2000a).

Neurobiological mechanisms underlying alcohol consumption

Decades of research have sought to understand the neurobiological mechanisms underlying the motivation to drink alcohol. Although there is speculation that fermenting

fruit influenced modern man's metabolism of alcohol (Dudley, 2002), it is not probable that the brain evolved a specific mechanism to regulate alcohol intake, per se. Instead, it is more likely that neuronal systems that evolved to regulate fundamental physiological and behavioral processes are involved in the modulation of alcohol consumption. Four systems in particular have been implicated in the motivation to drink alcohol. First, neuronal circuits that are responsible for motivating approach and consumption of natural rewards (e.g., food, sex, and water) are artificially activated by alcohol (Wise, 1996). Consequently, alcohol intake is reinforced because it is perceived as a salient and rewarding substance to be desired and consumed. Second, as a source of calories, alcohol consumption can be reinforced by systems that regulate energy balance (Thiele et al., 2004b). Third, because alcohol is consumed orally in water, alcohol drinking could potentially be reinforced for the fluid it provides. Finally, alcohol intake can be negatively reinforced via the drug's anxiolytic properties, at least in stressful environments (Conger, 1956). Hence, systems that evolved to regulate anxiety, reward, energy and/or fluid balance may all influence the drive to consume alcohol.

Alcohol as a reward

Although it is common knowledge that alcohol is a rewarding substance, the definition of reward and exactly what the word should encompass is currently a matter of debate (Berridge and Robinson, 2003; Schultz, 2006; Wise, 2004). For the purpose of this dissertation, the term reward will be defined as a stimulus that induces approach, and for which an animal will work (i.e., a reinforcer). The mesolimbic dopamine system has long been thought to be the universal signal for reward in the brain (Wise, 1978). Today

it is still viewed as an important and possibly necessary component of reward processing (Wise, 2004). The dopamine theory of reward is largely based on the fact that a common denominator of natural rewards and abused drugs is their ability to activate the mesolimbic dopamine system, leading to an increase in extracellular dopamine in the nucleus accumbens (NAc) (Wise, 2002).

There is good evidence that alcohol increases mesolimbic dopamine neurotransmission. Dopamine release in the NAc is augmented by peripheral (Di Chiara and Imperato, 1986) or central (Yoshimoto et al., 1992) administration of alcohol as well as oral self-administration (Weiss et al., 1993) in rodents. Increased dopamine release in the NAc is also reported in humans following alcohol intake (Boileau et al., 2003). The effect of alcohol on dopamine release is believe to be carried out by dopamine-containing neurons in the posterior ventral tegmental area (pVTA) (Di Chiara, 1997; McBride et al., 1999). Alcohol could activate pVTA dopamine neurons directly by enhancing the excitatory effects of serotonin on the serotonin 1B (Yan et al., 2005) and 3 receptors (Campbell et al., 1996). In addition, an indirect activation of pVTA dopamine neurons could occur via the release of inhibition from gamma-aminobutyric acid (GABA) interneurons. Alcohol is reported to inhibit GABA interneurons by potentiating the action of GABA on $GABA_A$ receptors (Harris et al., 1995; Melis et al., 2002) and/or impeding the action of glutamate on NMDA receptors (Lovinger et al., 1989; Weight et al., 1991).

The importance of mesolimbic dopaminergic transmission in the rewarding properties of alcohol is substantiated through pharmacological studies in animal models of alcohol self-administration. Blocking alcohol-induced increases in dopaminergic signaling with dopamine receptor antagonists reduces alcohol self-administration in rodent models (Samson et al., 1992). In addition, preventing the activation of pVTA dopamine neurons with serotonin-3 receptor antagonists (Rodd-Henricks et al., 2003) or $GABA^A$ receptor antagonists (Rassnick et al., 1993a) also reduces alcohol self-administration in rodents. Further, Acamprosate (calcium-acetyl homotaurinate), which has been proposed as a pharmacological treatment to attenuate relapse in human alcoholics, might act by reducing the rewarding properties of alcohol. Although the specific mechanism of action of the drug is not clear, it is believed to interact with both GABAergic and glutamatergic neurotransmission in the brain (Dahchour and De Witte, 2000). Acamprosate has been reported to delay and suppress alcohol-induced dopamine release in the NAc of rodents (Olive et al., 2002b). Further, it was found to reduce alcohol self-administration (Czachowski et al., 2001) and inhibit the development of a conditioned place preference to alcohol in rodents (Bachteler et al., 2005). These data support the idea that its efficacy in the treatment of alcoholism is at least partly dependent on its ability to reduce alcohol reward.

Alcohol as an anxiolytic

Anxiety has long been implicated in the etiology of alcohol dependence (Jellinek, 1952). In 1956, Conger presented the tension reduction hypothesis to explain the relationship among stress, anxiety and the motivation to drink alcohol. He proposed that

alcohol intake is negatively reinforced by the anxiolytic or fear-reducing properties of alcohol during stressful situations (Conger, 1956). Although there has been some debate over the effectiveness of alcohol as an anxiolytic (Cappell and Herman, 1972), there are numerous reports of alcohol's ability to reduce anxiety-like behavior in rodent models (Blanchard et al., 1993; Coop et al., 1990; Dudek et al., 1986; Moraes Ferreira and Morato, 1997). Further, compounds that are known to reduce anxiety have been reported to reduce alcohol consumption in rodent models of voluntary alcohol intake (Badia-Elder et al., 2003; Badia-Elder et al., 2001) and alcohol dependence (Thorsell et al., 2005a; Thorsell et al., 2005b).

The impact of alcohol on anxiety in humans is less clear. Epidemiological data support a link between stress, anxiety and the tendency to abuse, or be dependent on alcohol. On average, alcoholics experience more stressful life events than non-alcoholics (Gorman and Brown, 1992; Linsky et al., 1985), and alcoholism is often found co-morbid with anxiety disorders (Bowen et al., 1984; Merikangas et al., 1998; Swendsen et al., 1998; Weiss and Rosenberg, 1985). However, unambiguously determining whether the anxiety disorder is the primary illness is difficult. Although a few retrospective studies have reported that certain types of anxiety disorders do precede the development of alcoholism (Merikangas et al., 1998; Swendsen et al., 1998), it has also been argued that anxiety, which is a common symptom of alcohol withdrawal, is simply a result, and not a cause, of the alcohol dependence (Anthenelli and Schuckit, 1993; Schuckit and Hesselbrock, 1994).

Regardless of whether anxiety is a cause or result of alcoholism, it is considered a powerful trigger for relapse in abstaining alcoholics (Kushner et al., 2005). Brain regions regulating anxious states are thought to be altered following withdrawal from drugs of abuse leading to a characteristic anxious state that accompanies drug-seeking behavior (Aston-Jones and Harris, 2004; Weiss et al., 2001). Numerous interconnected limbic (e.g., amygdala, bed nucleus of the stria terminalis and hippocampus) and cortical structures (e.g., medial prefrontal cortex) are proposed to be important in the regulation of anxious states in general (Millan, 2003). During alcohol withdrawal, the extended amygdala (i.e., bed nucleus of the stria terminalis, central and medial amygdala, shell of the NAc) is thought to be the region that mediates alcohol withdrawal-induced anxiety (Koob, 2003). Increased corticotropin-releasing factor (CRH) and reduced neuropeptide Y signaling in the extended amygdala are associated with alcohol dependence, and are believed to be the cause of the increased anxiety during alcohol withdrawal (Slawecki et al., 1999; Valdez and Koob, 2004) (see chapter 2). Further, there is some evidence that genetic factors leading to imbalances in NPY signaling within the amygdala could lead to a predisposition for a high level of anxiety, resulting in an innately higher motivation to consume alcohol and develop dependence (Pandey et al., 2003a; Pandey et al., 2005).

Alcohol as a macronutrient

Alcohol is unique among drugs of abuse because it is a usable source of energy (i.e., calories) for the body. The metabolism of alcohol is carried out by two enzymes: alcohol dehydrogenase and aldehyde dehydrogenase. These reduction reactions provide energy for the cell by forming of six adenosine triphosphate (ATP) molecules per alcohol

molecule. Further, the end product of alcohol metabolism, acetate, can be converted to acetyl-CoA, which can then enter the citric acid cycle providing more energy for the cell (i.e., a total of 11 ATP / alcohol molecule) (Antoshechkin, 2002). As a macronutrient, alcohol is estimated to have 7 kCal/g, making it more energy dense than pure sugar or protein (4 kCal/g), and only slightly less than fat (9 kCal/g) (Atwater and Benedict, 1993). Importantly it has been demonstrated that the calories provided by alcohol can significantly contribute to energy intake in rodents and humans (Buemann and Astrup, 2001; Richter, 1953). In addition to these caloric properties, studies in humans indicate that alcohol can stimulate appetite, and at moderate doses puts the body in a state of positive energy balance (Caton et al., 2004; Caton et al., 2005; Yeomans et al., 2003). Because it contains energy, is administered orally, and acts as an appetite stimulant, alcohol consumption is likely to be influenced by systems that evolved to regulate food intake and energy expenditure. In support of this hypothesis many of the systems involved in energy homeostasis are disrupted during chronic alcohol consumption and in rodent models of high alcohol preference (Kim et al., 2005; Kinoshita et al., 2000; Kraus et al., 2004; Lindblom et al., 2002; Suzuki et al., 2004). In addition, some peptides involved in the regulation of energy homeostasis also modulate alcohol consumption (see chapter 2). These peptidergic systems represent potential targets for the development of pharmacological therapies for alcohol dependence.

Melanin-Concentrating Hormone (MCH)

Because of its involvement in reward, anxiety, energy and fluid balance, we believed that MCH would regulate alcohol intake, and therefore possibly represent a

target system for the development of pharmacological treatments for alcoholism. MCH was first isolated in the salmon pituitary, and was originally named for its ability to induce aggregation of melanocytes to lighten skin tone (Kawauchi et al., 1983). Although MCH is not involved in the regulation of skin color in mammals, the amino-acid structure of the peptide is well-conserved across species suggesting an importance in basic physiological and behavioral regulation. The cyclic neuropeptide is produced solely by magnocellular neurons in the lateral hypothalamus and zona incerta within the CNS of mammals (Pissios and Maratos-Flier, 2003). Two G-coupled protein receptors have been identified which bind with the peptide: MCH receptor 1 and 2 (MCHR1 and MCHR2). MCHR2 expression is limited to primates and some carnivorous species (e.g., dogs), while MCHR1 has been found in every mammalian species explored to date (Tan et al., 2002). MCHR1 is expressed through out the CNS, from the brain stem to the cortex. The extensive expression of MCHR1 and the far-reaching monosynaptic projections of MCH neurons indicate that the peptide is a major signal in the CNS with a broad functional repertoire.

MCH and energy balance

MCH was first implicated in the regulation of energy balance in 1996 when the peptide's mRNA was found to be over-expressed in an obese mouse model (i.e., ob/ob mice lacking the ability to make the anorectic cytokine leptin) (Qu et al., 1996). In addition, this report found that fasting increased MCH mRNA expression, and administration of the peptide in the lateral cerebral ventricle increased food intake (Qu et al., 1996). Following this initial finding, numerous studies have supported the idea that

MCH is a crucial endogenous signal in the regulation of energy balance. Chronic central infusion of MCH promotes obesity on chow and high fat diet (Gomori et al., 2003). The weight gain promoted by MCH is thought to be due to both an increase in food intake and a reduction in energy expenditure (Ito et al., 2003). Additional evidence of MCH's role in energy balance and weight gain was found in genetic mouse models. Transgenic mice that over-express MCH in the lateral hypothalamus eat more and gain more weight than wildtype mice (Ludwig et al., 2001). On the other hand, mice lacking the ability to produce MCH are lean and hypophagic (Shimada et al., 1998). Finally, pharmacological antagonists to the MCHR1 potently decrease food intake and body weight in rats (Borowsky et al., 2002; Kowalski et al., 2004). All of these data demonstrate that MCH is an important signal of negative energy balance in the CNS.

There is one reproducible finding that is counterintuitive to MCH's accepted role in energy balance regulation. Mice that lack the MCHR1 (i.e., MCHR1 KO mice) are lean and resistant to diet-induced obesity, but hyperphagic (Chen et al., 2002; Marsh et al., 2002). As MCH is thought to be a signal of negative energy balance, it was surprising that MCHR1 KO mice would eat more than their wildtype counterparts. Because the MCHR1 KO mice have an elevated metabolic rate (Astrand et al., 2004; Chen et al., 2002; Marsh et al., 2002) and activity level compared to wildtype mice (Astrand et al., 2004; Marsh et al., 2002; Zhou et al., 2005), it has been proposed that these mice are eating more merely to counteract a hyperactive metabolism (Marsh et al., 2002). In other words, they have to eat more just to maintain a lean body weight. An additional factor that might be involved in the hyperphagic phenotype of these mice is the

finding that they have increased binding sensitivity of the D1 and D2 dopamine receptors in the mesolimbic dopamine pathway (Smith et al., 2005b). Therefore, the MCHR1 mice might be inclined toward an increase in goal-directed behaviors in general. As these specific mice are utilized in experiments within this dissertation, it is important to keep their peculiar phenotype in mind.

MCH and anxiety

MCH is also thought to be involved in anxiety, although the data thus far have been somewhat confusing and the exact role MCH plays in anxiety is to date unclear. There are a few studies that suggest that MCH acts as an anxiolytic. In male rats MCH has been reported to reduce anxiety-like behavior when administered into the third-cerebral ventricle, amygdala or hippocampus (Carlini et al., 2006; Kela et al., 2003; Monzon and De Barioglio, 1999; Monzon et al., 2001). On the other hand, many studies suggest that the peptide has an anxiogenic effect. MCH was reported to increase anxiety when administered into the medial preoptic area of female rats (Gonzalez et al., 1996) and in the lateral cerebral ventricle of mice (Smith et al., 2005a). In addition, MCHR1 antagonists reduce anxiety-like behavior in a variety of animal models (Borowsky et al., 2002; Chaki et al., 2005; Smith et al., 2005a), and the MCHR1 KO mouse exhibits less anxiety-like behavior than it's wildtype littermate (Roy et al., 2006). A tentative conclusion of these data is that the role of MCH in anxiety is region-specific. More studies are necessary to elucidate the importance of MCH in the regulation of anxiety.

MCH and reward

The possibility that MCH might regulate reward has been proposed (DiLeone et al., 2003) because of the anatomical position of the MCH-producing neurons (i.e., the lateral hypothalamus) and MCH receptors (e.g., the shell of the NAc) (DiLeone et al., 2003). The reciprocal neuronal circuit between the lateral hypothalamus and the NAc is likely to regulate reward behavior in general, and it is logical that this pathway would be involved in reward-mediated ingestive behaviors, like alcohol intake. There is evidence that MCH is involved in food intake mediated by this neural circuit. MCH administered directly into the shell of the NAc increased food intake, while blockade of MCHR1 signaling in the same region reduced feeding (Georgescu et al., 2005). Further, MCH might modulate reward by impacting dopaminergic transmission. MCHR1 KO mice have increased D1 and D2 binding in the NAc (Smith et al., 2005b). However, it is not clear why a lack of MCH signaling leads to an increase in dopaminergic sensitivity in the NAc. The authors of this study suggested that MCH might normally be inhibitory on dopaminergic signaling. Another possibility is that MCH and dopamine are parallel signals of reward and in the absence of MCH, dopamine must compensate. The findings that MCH signaling in the NAc regulates feeding as well as dopaminergic sensitivity encourage further exploration of the role of the MCH in reward and motivation.

Purpose and Specific Aims

Initially, it was hypothesized that MCH signaling in the central nervous system would reduce anxiety, and thereby decrease the drive to drink alcohol. This idea was tested by administering MCH into the brain of rodents that were voluntarily consuming

alcohol (Chapter 3). Contrary to the hypothesis, following the administration of MCH the rats consumed more alcohol than the saline control. Further, the dose of MCH that increased alcohol intake had no impact on anxiety-like behavior measured in a rodent model. Consequently, the remainder of the research in this dissertation was guided by the hypothesis that endogenous MCH signaling augments the motivation to drink alcohol.

CHAPTER 2

The role of other neuropeptides

in the motivation to consume alcohol

Introduction

For a long time studies investigating the underlying neuronal circuitry responsible for regulating alcohol intake focused on the role of the classic neurotransmitters (e.g., dopamine, serotonin and glutamate) (Nevo and Hamon, 1995). More recently, with the availability of pharmacological tools and genetic animal models, an increasing amount of effort has gone into exploring the role of neuropeptidergic systems in the regulation of alcohol consumption. The purpose of this chapter is to summarize the recent literature regarding this topic. The regulation of alcohol intake by each neuropeptidergic system will be discussed in the context of the peptide's known involvement in reward, anxiety, fluid and /or energy balance. The findings of these studies are relevant to this dissertation because they provide insight into the mechanisms that underlie the regulation of alcohol drinking by the MCH system.

Corticotropin-releasing hormone system

Corticotropin-releasing hormone (CRH, a.k.a. corticotropin-releasing factor) is critical for regulating the physiological and behavioral responses to stress (i.e., a real or inferred threat to homeostatic balance) (McEwen and Stellar, 1993; Schulkin et al., 1998). The classic example of this is the endocrine response to stress in which paraventricular CRH acts in the pituitary to stimulate the release of adrenocorticoreleasing hormone (ACTH), among other proopiomelanocortin (POMC) gene-derived peptides. ACTH then acts on the adrenal glands to release glucocorticoids to deal with the stressor (Taylor and Fishman, 1988). Other important roles of CRH in the stress response include activating the sympathetic nervous system (Valentino et al.,

1983) and coordinating various behavioral responses (Koob and Heinrichs, 1999; Menzaghi et al., 1993). A couple behaviors impacted by CRH that are pertinent to this review are anxiety and energy balance (Mastorakos and Zapanti, 2004; Reul and Holsboer, 2002). Central administration of CRH is anxiogenic (Koob and Thatcher-Britton, 1985) and reduces food intake (Arase et al., 1988; Bell et al., 1998). CRH binds with two receptor subtypes to carry out these actions: CRH receptor 1 (CRHR1) and 2 (CRHR2). However, CRH binds with much greater affinity to the CRHR1 (Lovenberg et al., 1995).

Most of the studies exploring the role of CRH in the motivation to consume alcohol have focused on the withdrawal period or abstinence after long-term alcohol exposure. Abstaining alcoholics suffer high rates of recidivism. This is thought to be at least partly due to the long-lasting increase in anxiety, and the deregulation of mood that occur when an alcoholic quits drinking (Weiss et al., 2001). Because of the ability of CRH to increase anxiety in general, it has been implicated as a neurobiological factor underlying alcohol withdrawal-induced anxiety. Antagonism of CRHR1 in the brain has been demonstrated in to attenuate anxiety-related behavior (Baldwin et al., 1991; Knapp et al., 2004; Menzaghi et al., 1994; Overstreet et al., 2004; Overstreet et al., 2005; Valdez et al., 2002; Valdez et al., 2003b) and alcohol intake (Le et al., 2000; Valdez et al., 2002) following alcohol withdrawal. Hence, these studies suggest that endogenous CRH signaling mediates alcohol-withdrawal-induced anxiety, and thereby increases alcohol consumption during the withdrawal period.

CRH and its receptors are widely distributed throughout the central nervous system (CNS), but only a subset of receptors are believed to be important in mediating alcohol withdrawal-induced anxiety. Many reports have pointed to the involvement of amygdalar CRH signaling. In an animal model of alcohol dependence, CRH immunoreactivity is reported to be increased in the amygdala 6-8 hours after alcohol removal (Merlo Pich et al., 1995). Expression levels of CRH remain elevated for up to 6 weeks (Weiss et al., 2001). Blocking this elevation in CRH by administering a CRHR1 antagonist into the central nucleus of the amygdala attenuates anxiety-like behavior resulting from alcohol withdrawal (Rassnick et al., 1993b). Dysregulation of amygdalar CRH signaling has been associated with an alcohol preference phenotype. It was reported that the Sardinian preferring (sP) rat has increased levels of extracellular CRH in the central nucleus of the amygdala and increased anxiety levels as compared to the Sardinian non-preferring (sNP) (Richter et al., 2000) Another CRH-containing brain region that may be involved in the anxiogenic effect of alcohol withdrawal is the bed nucleus of the stria terminalus. CRH protein is reportedly increased in this area following alcohol withdrawal. Further, this effect is reversed with alcohol consumption. However, because there was no pair-fed control in this study, it is difficult to know if the changes in CRH were the result of alcohol withdrawal per se or energy status (Olive et al., 2002a). More work is necessary to pinpoint the brain regions important in the CRH-mediated anxiety following alcohol withdrawal.

A logical prediction that can be made based on the finding that CRH antagonists reduce alcohol intake in alcohol dependent models is that the administration of CRH

itself will increase alcohol consumption, by increasing anxiety. However, it has been repeatedly demonstrated that increased levels of CRH reduce alcohol intake. CRH administered into the 3rd cerebral ventricle (i3vt) of rats receiving limited daily 1-hr access to 8 % alcohol, dose-dependently reduced alcohol and food intake (Bell et al., 1998). This finding was replicated in a different strain of rat, given a comparable dose of CRH into the lateral ventricle (Lv) (Thorsell et al., 2005b). Further, studies in mice are consistent with an inverse relationship between CRH and the motivation to consume alcohol. Mice lacking CRH drink more alcohol (Olive et al., 2003) and CRH over-expressing mice drink less alcohol than their respective wildtype controls (Palmer et al., 2004). Therefore, voluntary alcohol consumption in a non-stressed or anxiety-free state is apparently reduced by central CRH activity.

It is critical to reconcile these two seemingly incompatible conclusions, that blocking endogenous CRH activity during alcohol withdrawal reduces alcohol intake, while increasing CRH levels in a freely drinking animal does the same. A likely explanation is that the inconsistent findings are reflective of the differences between the animal models. The withdrawal model is "dependent" on alcohol, consumes large quantities of the drug, and subsequently suffering from heightened anxiety. The voluntary drinking model is a moderate alcohol drinker that has never experienced stress while consuming alcohol, and therefore, may not associate alcohol with reduced anxiety. Hence, in this latter model it could be the case that acute CRH administration increases anxiety, but not alcohol intake because the animals have not learned the anxiolytic properties of alcohol. Perhaps chronic stress or chronic CRH administration are

necessary to allow the animal to associate drinking alcohol with a reduction in anxiety. Although this would justify why CRH does not increase alcohol consumption, it does not explain the CRH-induced reduction of alcohol intake. The decrease in alcohol intake may be due to a reduced drive to consume energy. It is widely accepted that CRH reduces energy intake and alcohol is an energy-dense macronutrient.

An alternative explanation is that the complexity of the CRH system is responsible for the discrepant findings. The CRH system consists of two receptor subtypes, a binding protein and four known endogenous ligands: CRH, urocortin I, II, and III. CRH binds with stronger affinity to the CRHR1, urocortin I binds strongly to both receptors, and urocortin II and III bind exclusively to CRHR2 (Lewis et al., 2001; Reyes et al., 2001; Vaughan et al., 1995). Hence, pharmacological studies can be difficult to interpret. For instance, when a nonspecific CRHR antagonist is administered into the cerebral ventricles it will block the endogenous activity of CRH as well as urocortin in numerous brain regions. This is troublesome for the interpretation of the data because there is much evidence that the CRH receptors and ligands do not regulate behavior in the same manner (e.g., CRHR1 is thought to enhance anxiety (Liebsch et al., 1995; Reul and Holsboer, 2002; Smith et al., 1998; Stenzel-Poore et al., 1994), whereas CRHR2 activity has been shown to be anxiolytic (Bale et al., 2000; Isogawa et al., 2003; Valdez et al., 2003a; Venihaki et al., 2004), anxiogenic (Takahashi et al., 2001), or have no effect on anxiety (Coste et al., 2000; Heinrichs et al., 1997)). Further, the urocortins have an independent impact on alcohol drinking. The Edinger-Westphal nucleus, where urocortin I is densely expressed, is activated during voluntary alcohol consumption

(Bachtell et al., 2003; Ryabinin et al., 2001; Ryabinin et al., 2003; Weitemier et al., 2001) and lesions of this region reduce alcohol preference in C57bl/6 mice (Bachtell et al., 2004). Central administration of urocortin III is reported to reduce anxiety associated with alcohol withdrawal and thereby attenuate withdrawal enhanced alcohol self-administration in rats (Valdez et al., 2004). What can be concluded from the current literature is that the CRH system as a whole is involved in the regulation of alcohol intake. The participation of this system is likely linked to the regulation of stress-responsive behaviors including anxiety and ingestive behavior. It is important in future studies to tease apart the specific roles of the two receptors, endogenous ligands and brain regions involved the CRH system's regulation of alcohol consumption.

Neuropeptide Y

Neuropeptide Y (NPY) is a highly conserved peptide found abundantly throughout the CNS (Larhammar et al., 1998; Tatemoto et al., 1982). In the rat brain, NPY is expressed densely in the hypothalamus, nucleus accumbens, periaqueductal gray, locus coeruleus, and amygdala (Adrian et al., 1983; Allen et al., 1983; de Quidt and Emson, 1986a; de Quidt and Emson, 1986b). There are six known receptors (Y1 - Y6) that bind to NPY, as well as to the structurally analogous peptides, peptide YY and pancreatic polypeptide, with varying affinities (Blomqvist and Herzog, 1997). Like NPY, the Y receptors are found throughout the CNS (Dumont et al., 1998; Parker and Herzog, 1999). Consistent with the profuse and widespread distribution of NPY and its receptors in the brain, this peptidergic system has been implicated in many neurobiological functions including energy balance (Gehlert, 1999; Schwartz et al.,

2000), anxiety (Heilig et al., 1989; Kask et al., 2002), memory (Morley and Flood, 1990), reproduction (McDonald, 1990), and circadian rhythms (Moore and Card, 1990; Yannielli and Harrington, 2001).

Not surprisingly, NPY has also been implicated in the regulation of alcohol intake (Heilig and Thorsell, 2002; Pandey et al., 2003a; Thiele and Badia-Elder, 2003; Thiele et al., 2004a; Valdez and Koob, 2004). In 1988 it was reported that a relatively small dose of NPY (0.37 μg) injected into the Lv of the golden hamster increased alcohol consumption (Kulkosky et al., 1988). Since this initial study, NPY has been reported to increase, decrease, or have no effect on alcohol drinking in rodents. These conflicting findings can be explained by many factors including the genetic background of the animal, the amount of experience the animal has with alcohol, and the brain region investigated. When these differences are controlled, tentative conclusions can be drawn. First, there is evidence that hypothalamic NPY activity increases alcohol intake in the same way it augments the consumption of other macronutrients, through its regulation of energy balance. Second, amygdalar NPY activity is hypothesized to decrease alcohol consumption secondarily to its ability to reduce anxiety.

There are a few reports that NPY increases alcohol drinking, but for the most part these are limited to hypothalamic administration of the peptide. For example, administration of NPY into the PVN increased alcohol intake and preference over water in rats, and this effect was attenuated by an NPY receptor antagonist. Others have reported similar findings for the impact of NPY administration in the hypothalamus on

alcohol intake in rats (Gilpin et al., 2004b; Kelley et al., 2001). One study did report an increase in alcohol intake following NPY administration into the Lv. This study used a relatively small dose of NPY in a hamster model (Kulkosky et al., 1988). Because the finding that NPY increases alcohol consumption is generally limited to hypothalamic administration of the peptide, it is believed to be a reflection of NPY's regulation of energy balance which is known to occur in this brain region.

Many studies support the hypothesis that NPY regulates alcohol consumption via its anxiolytic properties. NPY knock-out mice exhibit anxiogenic behavior (Palmiter et al., 1998) and consume more alcohol relative to wildtype mice (Thiele et al., 1998). Similarly, the P line of alcohol-preferring rats are innately more anxious (McKinzie et al., 2000; Stewart et al., 1993) and consume less alcohol following central NPY administration than their low alcohol-preferring counterparts (nP) (Badia-Elder et al., 2001). The amygdala is thought to be central to the anxiolytic effects of NPY (Sajdyk et al., 2004) and thus is also implicated in the effect of NPY on alcohol consumption (Pandey, 2003). For instance, reduced expression of NPY in the amygdala of P rats is though to underlie their heightened anxiety and alcohol preference (Hwang et al., 1999; Suzuki et al., 2004). Reducing NPY levels in the amygdala of rats by infusing protein kinase A increased both anxiety and alcohol intake and this was blocked by co-administration of NPY (Pandey et al., 2003b). Opposite from CRH, alcohol withdrawal is associated with reduced cellular expression of NPY in the amygdala (Roy and Pandey, 2002) and central NPY administration attenuates at least the physical symptoms of alcohol withdrawal (Woldbye et al., 2002) . Finally, augmented alcohol drinking that

follows chronic alcohol vapor exposure is reduced by central NPY administration (Thorsell et al., 2005a; Thorsell et al., 2005b). These data provide support for the involvement of NPY, specifically in the amygdala, in alcohol withdrawal-induced anxiety and alcohol intake. Interestingly, in models that are not innately deficient in NPY and do not display heightened anxiety-like behavior (i.e., unselected rodents and AA rats), central NPY administration has no effect on alcohol consumption (Caberlotto et al., 2001; Katner et al., 2002b; Rodgers and McClearn, 1962; Slawecki et al., 1999) even when it is administered directly into the amygdala (Katner et al., 2002a). Therefore, it seems that a derangement of the endogenous NPY system (via selective breeding or chronic alcohol exposure), and the heightened anxiety accompanying this, is a prerequisite for the ability of NPY to reduce alcohol intake.

All of the data do not fit nicely into this picture. NPY over-expressing mice consume less alcohol than wildtypes, but the genotype has no effect on anxiety (Thiele et al., 1998). Further, although the HAD rats have less amygdalar NPY levels (Hwang et al., 1999) and consume less alcohol following central NPY administration than the LAD rats, no differences have been observed between the two lines in anxiety-like behavior (Badia-Elder et al., 2003). Therefore, NPY can decrease alcohol consumption independent of its effect on anxiety. Because NPY has been demonstrated to enhance alcohol induced sedation (Gilpin et al., 2004a; Thiele et al., 1998), it has been proposed that the reduction of alcohol drinking caused by central NPY signaling may be due in part to an increased level of sedation (Thiele et al., 2004a).

Opioid system

It is well-established that the opioid system is involved in the motivation to consume alcohol. Non-selective blockade of opioid receptors reliably reduces alcohol consumption in rodent and non-human primate models (Altshuler et al., 1980; Hubbell et al., 1986; Kornet et al., 1991; Marfaing-Jallat et al., 1983; Myers et al., 1986; Samson and Doyle, 1985; Sandi et al., 1988b). Further, the non-selective opioid antagonist, Naltrexone, decreases relapse and alcohol intake in recovering alcoholics when administered in combination with psychosocial therapy. Consequently this drug was approved by the Food and Drug Administration as an adjunctive treatment for alcoholism (O'Malley et al., 1992; Volpicelli et al., 1992). Although it is not definitively known how the opioid system regulates alcohol consumption, the most common explanation proposed is that opioid transmission mediates the rewarding properties of alcohol (Herz, 1997; Oswald and Wand, 2004). As with all drugs of abuse, alcohol causes an increase in dopamine release into the nucleus accumbens (Di Chiara and Imperato, 1986) and this is believed to underlie the rewarding aspects of alcohol intake (Wise, 1996). Because opioidergic activity regulates dopamine release in the nucleus accumbens, it has been postulated that endogenous opioids are necessary for alcohol-induced dopamine release in this brain region (Herz, 1997; Oswald and Wand, 2004). Reports on the effects of alcohol on the opioid system have been somewhat inconsistent (Froehlich and Li, 1994), but one finding clearly supports this theory. Alcohol-induced dopamine release can be blocked by an opioid antagonist (Acquas et al., 1993; Widdowson and Holman, 1992). Other possible mechanisms by which the opioid system might regulate alcohol consumption include its role in anxiety and ingestive behavior.

Because of the success of Naltrexone, the non-selective opioid receptor antagonist, in the treatment of alcohol dependence, much effort has been focused on understanding the role of the individual opioid receptors in the regulation of alcohol drinking. There are three major categories of endogenous opioid peptides which are post-translational products of three separate precursor genes: the endorphins as well as other non-opioid peptides (e.g., ACTH and α-melanocyte-stimulating hormone) are derived from POMC (Nakanishi et al., 1979), the enkephalins originate from proenkephalin (Noda et al., 1982), and the dynorphins are processed from prodynorphin (Kakidani et al., 1982). These opioid peptides interact with at least three receptor subtypes: mu-opioid receptor (MOR), delta-opioid receptor (DOR), and kappa-opioid receptor (KOR) (Knapp et al., 1995; Reisine and Bell, 1993). Although none of the opioid peptides bind exclusively to one receptor, β-endorphin binds with greatest affinity to MOR, enkephalins bind more strongly to DOR, and dynorphins bind selectively to KOR. In general, activation of the MOR produces similar outcomes to that of the DOR. On the other hand, KOR activation produces opposite results. This is illustrated by the impact of each opioid receptor on dopamine release into the nucleus accumbens. DA release is augmented by MOR or DOR activity and diminished by KOR activity (Di Chiara and Imperato, 1988; Spanagel et al., 1990; Spanagel et al., 1991; Spanagel et al., 1992). This finding has lead to the hypothesis that the MOR and DOR facilitate the rewarding properties of alcohol, while the KOR diminishes alcohol reward.

Selective antagonists as well as genetic deletion of MOR have demonstrated that the receptor does indeed play a role in alcohol drinking. The MOR knock-out mouse consumes less alcohol than the wildtype control (Becker et al., 2002; Hall et al., 2001; Roberts et al., 2000b). Selective MOR antagonists have been reported to reduce alcohol intake in rats breed for high alcohol preference (i.e., HAD, P, AA) as well as unselected wistar rats (Honkanen et al., 1996; Hyytia and Kiianmaa, 2001; June et al., 2004; Krishnan-Sarin et al., 1998). Blockade of MOR in the alcohol preferring C57BL/6 mouse was first reported to have no effect on alcohol consumption (Le et al., 1993), but a more recent study found that a different MOR antagonist was effective at reducing alcohol intake in these animals in a similar paradigm (Kim et al., 2000). Interestingly, one study found that selective knock-down of β-endorphin, the main endogenous MOR ligand, caused an increase in alcohol intake in mice (Grisel et al., 1999). This is counterintuitive to the proposed positive impact MOR signaling is thought to have on alcohol consumption. However, the findings that mice lacking β-endorphin drink more alcohol might be indicative of the role of the peptide in the general stress response. Altogether, these studies present substantial evidence for the MOR's involvement in the regulation of alcohol intake.

Although it was believed that the DOR would regulate alcohol consumption in a similar manner to the MOR, the studies investigating this receptor have resulted in inconsistent findings. Some studies support the hypothesis that DOR signaling increases the motivation to consume alcohol. Selective antagonists of the DOR have been reported to reduce alcohol consumption in C57BL/6 mice (Kim et al., 2000; Le et al., 1993;

Middaugh et al., 2000) as well as two strains of alcohol-preferring rats (P and AA) (June et al., 1999; Krishnan-Sarin et al., 1995a; Krishnan-Sarin et al., 1995b). In addition, operant responding for alcohol was reduced in both wistar and AA rats by selective DOR antagonism. On the other hand, there are reports that DOR blockade does not effect alcohol intake in Wistar rats (Stromberg et al., 1998) or in AA rats (Honkanen et al., 1996; Ingman et al., 2003). However, one of these reports may be due to sub-threshold dosing with the selective DOR antagonist naltrindole (1mg/kg) (Honkanen et al., 1996). Genetic knock-out models have not supported the prediction that DOR augments alcohol intake. There is no effect of the loss of preproenkephalin (Koenig and Olive, 2002) or the dual loss of enkephalin and β-endorphin (Hayward et al., 2004) on alcohol drinking behavior, while mice lacking the DOR actually self-administer more alcohol than wildtype mice (Roberts et al., 2001). The authors of this last finding suggested that the DOR knock-out mice drank more alcohol due to an increased level of anxiety. DOR knock-out mice display increased anxiety-like behavior in the elevated plus maze and the light-dark box (Filliol et al., 2000), and this is attenuated by alcohol self-administration (Roberts et al., 2001). These findings are intriguing and may explain the inconsistent reports on DOR and alcohol intake. That is, the DOR may increase reward, while reducing anxiety, and these two activities could have opposing effects on the motivation to consume alcohol with one overriding the other in specific situations (i.e., depending on the animal model utilized, the amount of alcohol experience, and the drinking procedure).

The KOR is generally believed to oppose the actions of the MOR and DOR. Therefore, KOR activity was hypothesized to reduce alcohol consumption by decreasing

the rewarding value of the drug. A few studies might be considered consistent with this hypothesis. Administration of dynorphin or a selective KOR agonist increased alcohol drinking behavior in outbred strains of rats (i.e., Wistar and Lewis) (Holter et al., 2000; Sandi et al., 1988a) and KOR knock-out mice consumed less alcohol than the wildtype mice (Kovacs et al., 2005). However, the experimental procedures used in these studies introduce some caveats to this conclusion. First, one study utilized a forced alcohol drinking procedure by severely water-restricting the rats (Sandi et al., 1988a). Therefore, the results of the study do not shed light on the effect of KOR signaling on voluntary alcohol consumption and the rewarding aspects of alcohol. Although the other study used a voluntary drinking procedure, they were interested in the motivation to consume alcohol during a withdrawal period. Further, they found that the KOR agonist increased alcohol intake, but there was no impact of the KOR antagonist on alcohol drinking suggesting that endogenous KOR activity is not involved in the motivation to consume alcohol (Holter et al., 2000). Finally, the reduced alcohol preference reported in the KOR knock-out mouse may be due to an underlying change in taste preference; KOR knock-out mice were found to drink less of a sweet saccharin solution and more of a bitter quinine solution than wildtype mice (Kovacs et al., 2005). Two reports are in direct opposition to the hypothesis that KOR signaling negatively impacts alcohol drinking, finding that KOR activation reduced voluntary alcohol intake in rats (Lindholm et al., 2001; Nestby et al., 1999). It is obvious that more studies are required to understand the role of the KOR in the motivation to consume alcohol.

In addition to the three traditional opioid receptors, a fourth receptor, the opioid receptor-like 1 receptor (NOP), is also believed to play a role in alcohol drinking. Activation of NOP by its endogenous ligand, nociceptin/orphanin FQ (N/ORQ), results in signaling events that are very similar to those which occur following the activation of the traditional opioid receptors. However, the classic opioid peptides do not activate this receptor and naloxone does not block N/ORQ signaling (Darland et al., 1998; Henderson and McKnight, 1997). Functionally, NOP activation is thought to mediate "anti-opioid" activities. For example, N/ORQ attenuates morphine induced analgesia and reduces the development of morphine tolerance (Lutfy et al., 2001; Mogil et al., 1996a; Mogil et al., 1996b). N/ORQ is reported to have a negative impact on the reinforcing value of drugs of abuse, attenuating the conditioned place preference formation to cocaine, morphine, and alcohol (Ciccocioppo et al., 1999; Kotlinska et al., 2002; Sakoori and Murphy, 2004). Studies investigating the role of N/ORQ in alcohol intake have utilized the Marchigian Sardinian alcohol-preferring (msP) strain of rat as a model. The hypothesis proposed is that N/ORQ reduces the rewarding value of alcohol thereby reducing alcohol consumption. Indeed, when given chronically, N/ORQ was found to reduce voluntary alcohol intake (Ciccocioppo et al., 2000; Ciccocioppo et al., 2003; Ciccocioppo et al., 1999) as well operant responding for alcohol (Ciccocioppo et al., 2004). The authors believe this effect is was not due to an impact on ingestive behavior in general, as N/ORQ only increases food intake in higher doses (Olszewski and Levine, 2004). Further, N/ORQ did not impact operant responding for sucrose (Ciccocioppo et al., 2004). Strangely, it has been reported that N/ORQ given acutely increases alcohol consumption (Ciccocioppo et al., 1999). Another possible mechanism by which N/ORQ

might impact the motivation to consume alcohol is via attenuating anxiety. N/ORQ is reported to reduce anxiety-like behavior in general (Jenck et al., 1997) and to inhibit stress-induced alcohol-seeking behavior in rats trained to consume alcohol (Martin-Fardon et al., 2000).

Melanocortin system

The melanocortin peptides (melanocortins) are produced via post-translational modification of the POMC pro-hormone. Central melanocortins activate the melanocortin 3 receptor (MC3R) and the melanocortin 4 receptor (MC4R), while the receptor's activity is blocked by endogenous antagonists, agouti and agouti-related protein (AGRP) (Cone, 2005; Gantz and Fong, 2003). The central melanocortin system is best known for its role in energy homeostasis; the POMC product α-melanocyte-stimulating hormone (α-MSH) activates the MC3R and MC4R to signal a state of positive energy balance leading to a reduction in energy intake. The endogenous antagonists oppose this effect of α-MSH on energy balance (Seeley et al., 2004). There is evidence that the melanocortin system modulates the rewarding value of drugs of abuse, but the direction of the effect is not uniform across different classes of drugs. For example, activation of melanocortin system was reported to attenuate morphine tolerance and dependence (Contreras and Takemori, 1984; Szekely et al., 1979), while augmenting cocaine reward (Cabeza de Vaca et al., 2002).

The first report that the melanocortin system was involved in the regulation of alcohol drinking found that the AA alcohol preferring strain of rats had increased levels

of POMC, reduced levels of AGRP, and differential expression of the central MC receptors, compared to ANA rats (Lindblom et al., 2002). These findings lead to the prediction that the melanocortin system would augment alcohol intake. However, pharmacological studies have actually found the opposite. Non-selective activation of central MC receptors reduces alcohol consumption in AA rats (Ploj et al., 2002), as well as C57BL/6 mice (Navarro et al., 2003). Further, this effect is believed to be mediated via the MC4R because MC3R knock-out mice do not differ from wildtypes in their alcohol preference, and administration of a non-selective MCR agonist reduces alcohol intake similarly in MC3R knock-out mice compared to wildtypes (Navarro et al., 2005). In addition, a selective MC4R agonist reduces alcohol consumption in C57BL/6 mice (Navarro et al., 2005). Pretreatment with the endogenous MCR antagonist, AgRP blocks the effect of MCR activation on alcohol intake (Navarro et al., 2003), and increases alcohol consumption by itself in doses that do not effect food intake (Navarro et al., 2005). This last finding suggests that the effect of the melanocortin system on alcohol intake may be independent from its regulation of energy homeostasis. In contradiction to these findings it has been reported that the synthetic MC4R antagonist, HS014, had no effect on alcohol intake in AA rats, but only one dose was tested (Ploj et al., 2002). Two feasible mechanisms have been proposed for the effect of the melanocortin system on the drive to consume alcohol. First, it is possible that alcohol intake is modulated via an impact on energy regulation (Navarro et al., 2005). Second, the melanocortin system might reduce the rewarding properties of alcohol by modulating endogenous opioid levels in the mesolimbic dopamine system (Ploj et al., 2002).

Galanin

Galanin is a neuropeptide that is widely distributed in the CNS with a dense expression in the hypothalamus (Kordower et al., 1992; Melander et al., 1986; Michener et al., 1990; Skofitsch and Jacobowitz, 1985; Skofitsch and Jacobowitz, 1986). There are three known receptors for this neuropeptide: galanin receptor 1, 2, and 3. The galanin system has been implicated in many regulatory functions. Pertinent to this review, galanin regulates ingestive behavior by increasing food intake (Crawley, 1999; Kyrkouli et al., 1990) and reducing water consumption (in water restricted rats) (Brewer et al., 2005). Further, galanin is believed to be important for regulating anxiety, although the findings are not consistent. There are reports that galanin reduces (Bing et al., 1993), increases (Moller et al., 1999), or has no effect (Karlsson et al., 2005; Kuteeva et al., 2005) on anxiety-like behavior in rodents. The inconsistency seems to be dependent on the brain region, the anxiety model, and the baseline stress level of the animal (Barrera et al., 2005; Holmes et al., 2003). Finally, PVN administration of galanin is reported to increase the levels of extracellular dopamine in the nucleus accumbens (Rada et al., 1998) suggesting a role for hypothalamic galanin signaling in reward processes.

Recently galanin was found to increase alcohol consumption in rats when administered i3vt (Lewis et al., 2004) or PVN (Rada et al., 2004). In addition, both reports found that the non-specific galanin receptor antagonist, M40, blocked the galanin-induced alcohol consumption, and reduced alcohol intake when administered alone (Lewis et al., 2004; Rada et al., 2004). Only one of the studies reported the effect of galanin on food intake alongside alcohol intake, unexpectedly finding that i3vt galanin

administration did not increase in food intake (Lewis et al., 2004) . In other words, galanin selectively augmented alcohol drinking in these animals. Water intake was unchanged by galanin or M40 administration in this study (Lewis et al., 2004). These data demonstrate that endogenous galanin signaling enhances alcohol consumption. Because galanin usually augments caloric intake at the doses used in the study, it is possible that galanin increased the drive to consume alcohol via its effect on energy balance. Of course, the impact of galanin on anxiety or reward could also contribute to the effect of the peptide on alcohol intake. More studies are needed to determine the underlying mechanism by which galanin enhances alcohol intake.

Leptin

Leptin is a hormone, made largely by white adipocytes and the stomach, which crosses the blood brain barrier to relay information to the brain regarding energy balance (Banks et al., 1996). The role of leptin as a central adiposity signal and regulator of energy homeostasis has been well-described (Schwartz et al., 2000; Woods and Seeley, 2000). More recently it has been proposed that leptin is involved in the rewarding aspects of ingestive behavior (Figlewicz, 2003). It has long been known that energy status in general can impact the reinforcing values of drugs of abuse (Carr, 2002; Carroll, 1984) and so it is logical that an adiposity signal would impact reward value. In support of this hypothesis, leptin receptors are found in brain regions involved in reward (Figlewicz et al., 2003). Further, leptin has been reported to diminish lateral hypothalamic stimulation (Fulton et al., 2000) as well as conditioned place preference to palatable food (Figlewicz et al., 2004).

The logical prediction from the above findings is that leptin would cause a reduction in alcohol consumption by reducing the rewarding value of the drug. However, the few studies that have been done reported either no effect of the hormone on alcohol drinking, or a positive relationship between leptin and alcohol intake. For instance, serum leptin levels were elevated during alcohol withdrawal and positively correlated with subjective craving of alcohol and early relapse (Kiefer et al., 2001a; Kiefer et al., 2005). Further, leptin significantly augmented alcohol intake in mice following alcohol withdrawal (Kiefer et al., 2001b) and mice deficient for leptin (ob/ob) or the leptin receptor (db/db) display a lower preference for alcohol (Blednov et al., 2004). On the other hand, leptin treatment in the ob/ob mice did not increase alcohol preference. Further, leptin administration does not effect alcohol intake in wildtype mice (Blednov et al., 2004; Kiefer et al., 2001b) or msP rats (Polidori et al., 2003).

Insulin

Like leptin, insulin is a central adiposity signal (Woods and Seeley, 2000) implicated in the rewarding aspects of ingestive behavior (Figlewicz, 2003; Figlewicz et al., 2004; Figlewicz et al., 2003). As with leptin, this leads to the prediction that insulin would reduce alcohol intake by decreasing the rewarding value of the drug. Only one study has been reported on the effect of insulin signaling on alcohol intoxication. In drosophila melanogaster, mutations in the insulin receptor that caused a 25-50% reduction in insulin receptor kinase activity without altering flies' size or locomotor function, significantly increased alcohol sensitivity (Corl et al., 2005). Because studies in

humans suggest that initial sensitivity to alcohol might predict the likelihood of developing a dependence to alcohol later in life (Schuckit and Smith, 1996) the finding that insulin receptor signaling regulates alcohol sensitivity could be important. To date, there have been no reports of the effects of insulin on alcohol drinking in mammalian models.

Angiotensin II

Angiotensin II (AII) is the major bioactive peptide of the renin-angiotensin system, and is a critical regulator of blood pressure and fluid homeostasis peripherally and centrally. It is formed in a two-step reaction in which the enzyme renin cleaves angiotensinogen to generate angiotensin-I that is in turn hydrolyzed by angiotensin-converting enzyme to form AII. The peptide binds with high affinity to the angiotensin-1 receptor (AT-1) and the angiotensin 2 receptor (AT-2). Both receptors are expressed in the brain. AT-1 mediates the most well-described actions of AII (e.g., increases in blood pressure, renal salt and water retention, thirst, and sodium appetite) (Fitzsimons, 1998; Lavoie and Sigmund, 2003; Stroth and Unger, 1999). The function of the AT-2 receptor is not well-characterized. The receptor is hypothesized to oppose the effect of AT-1 on blood pressure (Carey et al., 2000) and reduce anxiety (Okuyama et al., 1999).

As a stimulator of thirst, it seems logical that AII would increase alcohol intake. Thirst caused by water deprivation has long been used to force alcohol drinking in animals (Deutsch and Eisner, 1977; Veale and Myers, 1969). The first study investigating the effect of AII on alcohol intake found that centrally administered AII

augmented alcohol consumption similar to 24-hr water deprivation (Johnson and Anderson, 1973). If the effect of AII on alcohol consumption was merely secondary to its ability to increase thirst, it might not be very important in the grand scheme of alcohol research, assuming that alcoholism in humans is not a result of a lack in fluid availability. However, it turns out that the role of AII in the motivation to consume alcohol is more complex and may include other factors beyond thirst.

There is considerable controversy as to how AII impacts alcohol consumption. Contrary to the prediction that AII would increase alcohol intake via thirst, it has been repeatedly found that peripheral administration of AII diminishes alcohol intake (Grupp, 1992; Grupp and Harding, 1994; Grupp et al., 1988a; Grupp et al., 1988b; Grupp et al., 1989; Grupp et al., 1991b; Kulkosky et al., 1996). Because the majority of these studies reported an increase in water intake alongside the reduction in alcohol consumption, a major criticism has been that the effect of AII in this situation is an artifact of the animals' drive to consume water (Fitts, 1993). This criticism is supported by the report that the AT-1 antagonist, Losartan, reduced alcohol intake (Kraly and Jones, 1999). Central administration of AII has resulted in much different findings than peripheral administration. In the absence of water, central administration of AII increases alcohol consumption in mice (Weisinger et al., 1999b). Further, centrally administered AII increases alcohol intake when water is available simultaneously in rat models (Fitts, 1993; Weisinger et al., 1999a; Weisinger et al., 1999b)). It is not clear whether location of administration (i.e., central vs. peripheral) or methodological differences are responsible for the disparate findings. Some studies have reported no effect of centrally

administered AII on alcohol drinking in rodents (Blair-West et al., 1996; Grupp and Harding, 1995).

Recently, genetic models have been used to help shed light on the role of AII in the regulation of alcohol consumption. Utilizing rats that have been genetically altered to have less angiotensinogen translation in the brain, Maul et. al. have proposed that AII acts on the AT-1 receptor in the brain to enhance the drive to consume alcohol (Maul et al., 2005; Maul et al., 2001). Interestingly, they do not believe that these findings are the caused by alterations in fluid homeostasis because the angiotensinogen knock-down rats had a significantly lower preference for alcohol despite a slightly elevated total fluid consumption, compared to wildtype rats. The authors propose that AII might be regulating alcohol intake by modulating the dopamine system and found that the angiotensinogen knock-down rat had less dopamine in the ventral tegmental area (VTA) (Maul et al., 2005).

Gut-Brain Peptides

Many peptides produced by the gut in response to nutrient content relay information to the brain, and thereby modulate ingestive behavior (Strader and Woods, 2005). A few of these peptides have been demonstrated to regulate alcohol consumption in a similar manner. Cholecystokinin (CCK), a well-described satiety factor (Moran and Kinzig, 2004), was first reported to reduce alcohol intake in water deprived rats over two decades ago (Kulkosky, 1984). These results have been replicated many times in various models of alcohol intake including a limited access/ non-deprivation procedure

(Kulkosky et al., 1991; Toth et al., 1990) and two different strains of selectively breed alcohol-preferring rats (Geary et al., 2004; Kulkosky et al., 1993). Another established satiety factor that reduces alcohol consumption is bombesin (Dietze and Kulkosky, 1991; Kulkosky et al., 1993). Other satiety factors (e.g., glucagon-like peptide-1, amylin, or apolipoprotein-AIV) may also share this characteristic, but no studies have investigated this possibility. Interestingly, the one gastrointestinal hormone that is known to augment food intake, ghrelin, is reportedly reduced by acute intravenous alcohol administration in healthy subjects (Calissendorff et al., 2005). Further, in abstaining alcoholics, fasting plasma ghrelin levels are increased in comparison to healthy subjects of similar body weight, body mass index and fasting plasma glucose levels (Kim et al., 2005). The authors of the latter report speculated that ghrelin might play a role in the pathogenesis of alcohol dependence, but more studies are needed to substantiate this hypothesis.

Summary and Conclusion

In summary, numerous neuropeptidergic systems have been implicated in the regulation of alcohol drinking. These peptides modulate alcohol consumption because of their involvement in pre-existing regulatory systems that maintain an organism in homeostatic balance in a given environment. The regulation of four main factors was focused on in this review: anxiety, reward, energy and fluid balance. Neuropeptides which are involved in the regulation of anxiety have been implicated in alcohol withdrawal-induced anxiety, a major trigger of relapse in abstaining alcoholics. The data suggest that the CRH and NPY systems in the amygdala are important for the development of alcohol withdrawal-induced anxiety. Neuropeptides, such as opioids,

have been implicated in processing the rewarding aspects of alcohol. Evidence supports a role for endogenous MOR signaling in increasing alcohol reward and thus augmenting alcohol consumption. Neuropeptides that regulate energy balance have been reported to impact alcohol drinking in the same way that they modulate feeding behavior (e.g., NPY, CRH and CCK). Finally, thirst stimulated by peripheral administration of AII preferentially increases water intake over alcohol consumption, but in the absence of water, alcohol will be consumed as a source of fluid. In conclusion, the take-home message is that the regulation of alcohol intake is complex, involving numerous factors including anxiety, reward, fluid and energy balance. Because neuropeptidergic systems can be involved in the regulation of more than one of these factors, the impact of any given neuropeptide on alcohol drinking will be the sum of its effects on all of these factors. It is important to understand how these neuropeptides regulate alcohol intake as each system provides a novel target for the development of pharmacological treatments for alcohol use disorders.

CHAPTER 3

Central administration of melanin-concentrating hormone increases alcohol and sucrose/quinine intake in rats. [†]

[†] Data presented in this chapter appears in the following publication:

Duncan, E. A., Proulx, K., and Woods, S. C. (2005). Central administration of melanin-concentrating hormone increases alcohol and sucrose/quinine intake in rats. *Alcohol: Clin Exp Res*, 26, 958-64.

INTRODUCTION

Alcohol dependence is a major health problem characterized by increased tolerance to the effects of ethanol, impaired control over drinking, and continued drinking despite aversive consequences (Association, 1994). In the United States, alcohol is the 3rd major non-genetic cause of death, behind tobacco and obesity (Mokdad et al., 2004), and the annual cost to society is estimated to be almost 185 billion dollars (Harwood et al., 1998). Further, few pharmacological treatments are available (Anton and Swift, 2003). The opiate antagonist Naltrexone has been the most promising, having been reported to reduce craving and relapse in humans when administered in conjunction to psychotherapy (O'Brien et al., 1996; Swift, 1999). However, because alcoholism is a complex condition with multiple genetic and environmental causes, it is unlikely that one therapy will suffice in treating all patients. It is therefore important to explore novel pharmacological targets in the treatment of alcohol dependence.

The energetic or caloric properties of alcohol (i.e., ethyl alcohol) may provide a novel approach for the treatment of alcohol use disorders (Egli, 2003). Alcohol contains 7 kCal/g, and is more energy dense than carbohydrate or protein. These calories can significantly contribute to total energy intake in rodents and humans (Buemann and Astrup, 2001; Caton et al., 2004; Caton et al., 2005). Therefore, systems that evolved to regulate food intake and energy expenditure also likely contribute to the motivation to consume alcohol. This is supported by research demonstrating that many of the systems involved in energy homeostasis are disrupted during chronic alcohol consumption or abstinence, and in rodent models of high alcohol preference (Kim et al., 2005; Kinoshita et al., 2000; Kraus et al., 2004; Lindblom et al., 2002; Suzuki et al., 2004). In addition,

some peptides involved in the regulation of energy homeostasis also modulate alcohol consumption (Badia-Elder et al., 2001; Bell et al., 1998; Blednov et al., 2004; Kulkosky et al., 1993; Navarro et al., 2003; Thiele et al., 2003). These peptidergic systems represent novel targets in the development of treatments for alcohol dependence.

Melanin-concentrating hormone (MCH) is a hypothalamic neuropeptide best known for its role in energy homeostasis; it increases energy intake and decreases energy expenditure (Della-Zuana et al., 2002; Ludwig et al., 2001; Marsh et al., 2002; Pissios and Maratos-Flier, 2003; Rossi et al., 1997; Shearman et al., 2003; Shi, 2004; Shimada et al., 1998; Suply et al., 2001). In addition, MCH is reported to increase fluid intake (Clegg et al., 2003) and decrease anxiety (Kela et al., 2003; Monzon and De Barioglio, 1999; Monzon et al., 2001). Importantly, the regulation of energy homeostasis, fluid balance, and anxiety have all been implicated in the etiology of alcoholism (Grupp et al., 1991a; Markou et al., 1998; Swendsen and Merikangas, 2000; Swendsen et al., 1998; Thiele et al., 2003; Thomas et al., 2003). Moreover, the anatomical distribution of MCH-producing neurons and MCH receptors is indicative of involvement in reward and motivation. Neurons that synthesize MCH are found only in the lateral hypothalamus and zona incerta, and they project throughout the CNS, contacting areas involved in reward and addiction (e.g., nucleus accumbens, amygdala, hippocampus, and prefrontal cortex (Pissios and Maratos-Flier, 2003)). MCH receptors are densely distributed within the nucleus accumbens shell, amygdala, hippocampus, and cortex (Saito et al., 2001). A recent report found that direct injection of MCH into the shell of the nucleus accumbens, an area linked to the reward pathway, increased food intake for up to 4 hours (Georgescu et al., 2005) . Hence, it is possible that MCH regulates the motivation to consume

alcohol via its involvement in energy and fluid intake, anxiety, and/or reward. Despite the numerous characteristics that suggest a role for MCH in the drive to consume alcohol, the effect of the peptide on alcohol intake has not been explored.

The purpose of these experiments was to investigate the impact of centrally administered MCH on the motivation to consume alcohol in rats. We generated hypotheses as to how MCH might influence alcohol intake from previous experiments on an analogous hypothalamic peptide, neuropeptide Y (NPY). NPY is like MCH in that its administration increases acute food intake (Schwartz et al., 2000; Woods et al., 1998) and reduces anxiety (Heilig et al., 1989), and there are reports that NPY inhibits alcohol consumption in rodent models (Badia-Elder et al., 2001; Thiele et al., 1998). Further, NPY polymorphisms are correlated with the occurrence of alcoholism in humans (Mayfield et al., 2002); (Ilveskoski et al., 2001; Kauhanen et al., 2000). Consistent with the self-medication hypothesis of alcohol dependence, it has been hypothesized that the role of NPY in the motivation to consume alcohol is secondary to its ability to decrease anxiety (Suzuki et al., 2004; Thiele et al., 2004a). That is, alcohol is a potent anxiolytic and in some cases, alcoholics may be motivated to consume alcohol to treat an underlying or co-existent pathology, such as anxiety and/or depression (Markou et al., 1998); (Merikangas et al., 1998; Thomas et al., 2003). We therefore hypothesized that MCH would also decrease the motivation to consume alcohol secondarily to a reduction in anxiety.

METOHDS

Subjects

Male Long-Evans rats (Harlan, Indianapolis, IN) weighing 250-300 g at the start of an experiment were housed individually in plastic tubs and maintained on a 12:12-hr light/dark cycle in a temperature controlled room (21^{o} C). Purina pelleted rat chow and water were provided ad libitum except when otherwise noted. Following arrival to the animal facility rats were handled daily for 1 week before the beginning of an experiment.

Procedure

Experiment 1

Training. Rats were trained to consume 10% alcohol (n = 9) using a modified version of the sucrose-fading technique (Grant and Samson, 1985; Samson, 1986). During this procedure animals were first provided with 20% sucrose for 15 days. Over the next 20 days increasing amounts of alcohol were added to the solution (i.e. 2%, 4%, 6%, 8%, and 10%, every 4th day). Simultaneously, sucrose was subtracted from the solution (i.e., 20% for 2 days and then 15%, 10%, 5%, 2.5%, and 0% every 4th day following). As a caloric control, a second group of rats (n = 10) received an amount of sucrose, equal in caloric density to the alcohol-sucrose solution on any given day of training. A limited access procedure was used throughout the experiment in which animals were given 2-hr access to the alcohol or sucrose beginning 7 hr after light onset. During the access period water was removed. In addition, the alcohol and sucrose solutions contained one of two distinct flavors (grape or cherry Kool-Aid, in counterbalanced order across animals). Final solutions for each group were: alcohol group = 10% alcohol, 0.25% Kool-Aid, 0.1% saccharin in water and sucrose group = 17.75% sucrose and 0.25% Kool-Aid in water.

Surgery. Following training, rats were anesthetized using a mixture of intraperitoneal ketamine (86 mg/kg) and xylazine (12.9 mg/kg). Bilateral stainless steel 23-gauge cannulas were implanted into the 3rd-cerebral ventricle (i3vt) using a stereotaxic procedure (coordinates: anteroposterior: -2.2 mm from bregma and dorsoventral: -7.5 mm from the dura) (Paxinos, 1998). Following surgery animals were given 7 days to recover, remaining on the daily 2-hr schedule with alcohol or sucrose. Cannula placement was then verified by infusing 10 ng angiotensin II (AII) in 2 μl saline into the i3vt. Animals that consumed at least 5 ml water within 1 hr following this infusion were used in the study.

Baseline. Immediately after the AII infusion, animals remained on the same 2-hr daily access schedule for 10 days. During this time a second bottle was given containing the opposite flavor (0.25% grape or cherry Kool-Aid) plus 0.1% saccharin. Therefore during the baseline period animals had 2-hr daily access to a flavor paired with calories (alcohol or sucrose), to a different flavor paired with saccharin, and to chow.

Ingestive Behavior. Saline, 1, 5, or 10 μg of MCH (Phoenix Pharmaceuticals) was infused i3vt, in a 2 μl volume, immediately prior to the 2-hr access period. The intake of alcohol or sucrose, non-caloric solution, and chow were measured after 2 hr and chow was measured again after 24 hr. The experiment was a within-subjects design such that all of the animals received all 4 treatments in a counterbalanced order across 4 infusion days spaced 4 days apart.

Experiment 2

Surgery. A naïve group of animals, never exposed to alcohol or sucrose, was implanted with i3vt cannulas as described in Experiment 1.

Elevated Plus Maze. Following recovery from surgery animals were transported from their housing room to a behavioral testing room 4 hr following the light onset. After allowing the animals to settle for 3 hr, saline (n = 6) or 10 μg of MCH (n = 6) in a 2 μl volume was administered i3vt. Treatment groups were assigned randomly. Thirty-min following this infusion, animals were placed in an elevated plus maze (EPM) (50 cm above ground, arm dimensions: closed = 15 cm high, 6 cm wide, and 30 cm long, open = 0 cm high, 6 cm wide, and 30 cm long) for 5 min. All rats were placed in the center of the maze facing the same closed arm. Between each rat the maze was cleaned thoroughly with 1% acetic acid. A video recorder mounted on the ceiling over the maze recorded the animal's behavior and the tapes were blindly scored for time spent in the open arms, closed arms, and center of the maze. After the EPM assessment, the rats were returned to their home cage and food intake was measured 2 hr following the infusion. This procedure was repeated one week later, except animals that had received MCH now received saline, and animals that had received saline now received 10 μg NPY.

Experiment 3

Training. Animals were trained to consume 10% alcohol (n = 21) as described in Experiment 1. A separate cohort of rats (n = 19) received sucrose in an isocaloric amount

to the alcohol throughout the experiment as described in Experiment 1. In addition, the sucrose solution contained sufficient quinine to reduce the baseline caloric intake to that of the alcohol group, without negatively affecting food intake or body weight (Kratz et al., 1978; Sclafani and Ackroff, 1994; Tolliver et al., 1988). Final solutions for each group were as follows: alcohol group = 10 % alcohol, 0.1% saccharin, and 0.25% Kool-Aid in water, sucrose/quinine group = 17.75% sucrose, 350 μM quinine, and 0.25% Kool-Aid in water. During this experiment animals were never food or water deprived, even during the 2-hr access period. Since in Experiment 1 animals did not drink from the additional bottle containing the opposite flavor and 0.1% saccharin, this bottle was not used in Experiment 2.

Surgery. Animals were implanted with i3vt cannulas as described in Experiment 1.

Ingestive Behavior. Following a 10-day baseline period in which animals were maintained on the 2-hr alcohol or sucrose access schedule, animals with similar baseline intake of sucrose/quinine or alcohol were randomly assigned to receive i3vt saline or 10 μg of MCH immediately prior to the 2-hr access period. This experiment used a between-subjects design such that each animal received one treatment only. Intake of alcohol or sucrose/quinine, water, and chow were measured at 2 hr.

Statistical Analysis

The results were analyzed with parametric tests (ANOVA and t-tests, with $p < 0.05$, 2-tailed, taken as significant).

RESULTS

Experiment 1

Baseline Period. Baseline measurements were defined as the mean intake on the final baseline day. There was a significant difference ($t(17) = 23.0$, $p < 0.001$) in the baseline intake of alcohol (1.2 ± 0.1 g/kg body weight) and sucrose (4.9 ± 0.3 g/kg body weight) between the alcohol and sucrose groups. The saccharin solution provided in the second bottle was negligibly sampled. Body weight did not differ between the groups (alcohol group = 489.6 ± 10.6 g, sucrose group = 506.2 ± 12.7 g, $t(17) = 1.19$, n.s.).

Behavioral Measures. As expected, MCH significantly increased 2-hr chow intake in both the alcohol group, ($F(3, 24) = 3.65$, $p < 0.05$) and the sucrose group, ($F(3, 27) = 5.42$, $p < 0.01$), but had no effect on 24-hr chow intake (Table 1). Contrary to the hypothesis, MCH dose dependently increased 2-hr alcohol intake (Figure 1A., Table 1, $F(3, 24) = 5.56$, $p < 0.01$) but had no effect on 2-hr sucrose intake (Figure 1B., Table 1, $F(3,27) = 2.94$, n.s.). MCH also had no effect on consumption of the saccharin solution in either group.

Table 1			Intake Data				
	Group	Treatment	Alcohol (g/kg)	Sucrose (g/kg)	2-hr Chow (g/kg)	24-hr Chow (g/kg)	Water (ml)
Experiment 1	Alcohol	Saline	0.73 ± 0.1	n/a	1.92 ± 0.4	45.4 ± 7.8	n/a
		1 μg MCH	0.96 ± 0.1	n/a	1.52 ± 0.4	50.0 ± 4.8	n/a
		5 μg MCH	1.24 ± 0.1	n/a	2.97 ± 0.6	58.0 ± 2.1	n/a
		10 μg MCH	1.76 ± 0.3**	n/a	4.56 ± 1.5 *	48.8 ± 5.8	n/a
	Sucrose	Saline	n/a	4.93 ± 0.4	2.66 ± 0.8	38.3 ± 6.0	n/a
		1 μg MCH	n/a	4.55 ± 0.4	2.22± 0.8	45.1 ± 2.8	n/a
		5 μg MCH	n/a	4.86 ± 0.3	5.64 ± 1.6 *	41.4 ± 4.9	n/a
		10 μg MCH	n/a	5.64 ± 0.4	5.48 ± 1.3 *	50.3 ± 5.1	n/a
Experiment 3	Alcohol	Saline	0.95 ± 0.3	n/a	2.24 ± 0.3	n/a	0.90 ± 0.1
		10 μg MCH	1.45 ± 0.4 *	n/a	6.47 ± 1.6 **	n/a	2.53 ± 0.6 **
	Sucrose/ Quinine	Saline	n/a	0.97 ± 0.3	2.22 ± 0.3	n/a	0.88 ± 0.1
		10 μg MCH	n/a	1.67 ± 0.5 *	9.82 ± 1.5 **	n/a	3.84 ± 0.6 **

significantly different from saline, * = $p < 0.05$, ** = $p < 0.01$.

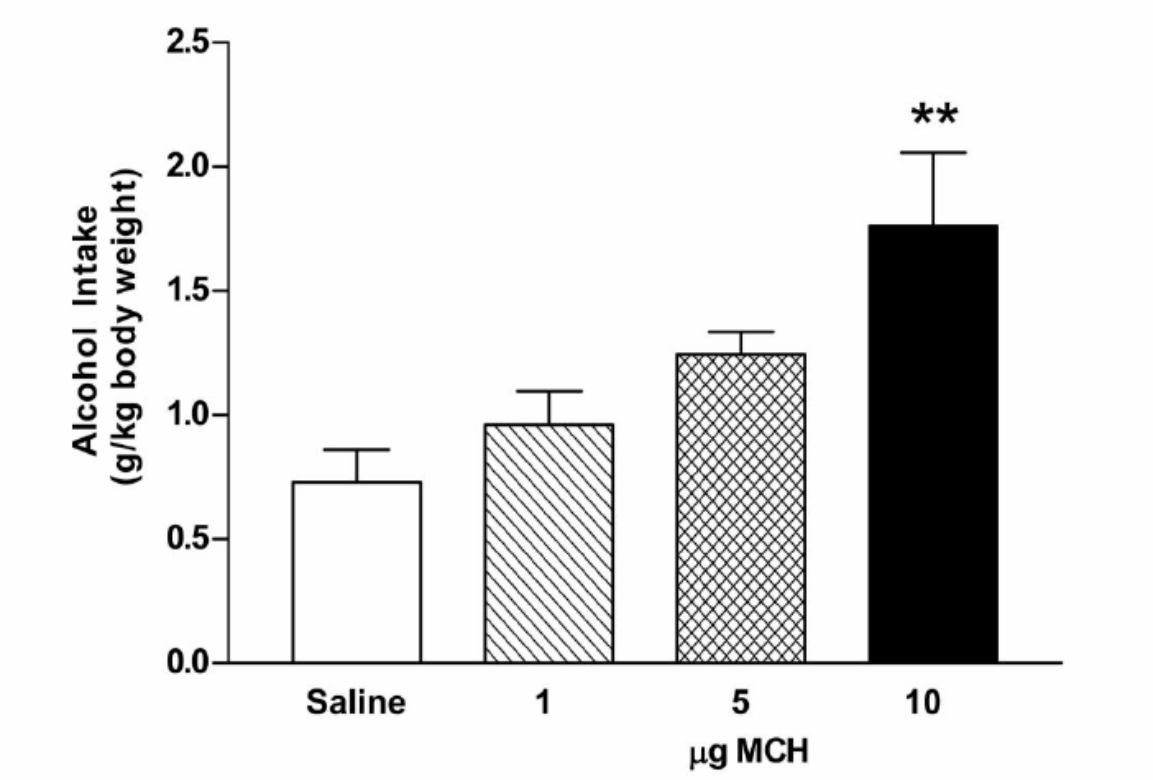

Figure 1a. Mean (± SEM) 2-hr alcohol intake (g alcohol per kg body weight) after the administration of i3vt saline or 1, 5, or 10 μg MCH.

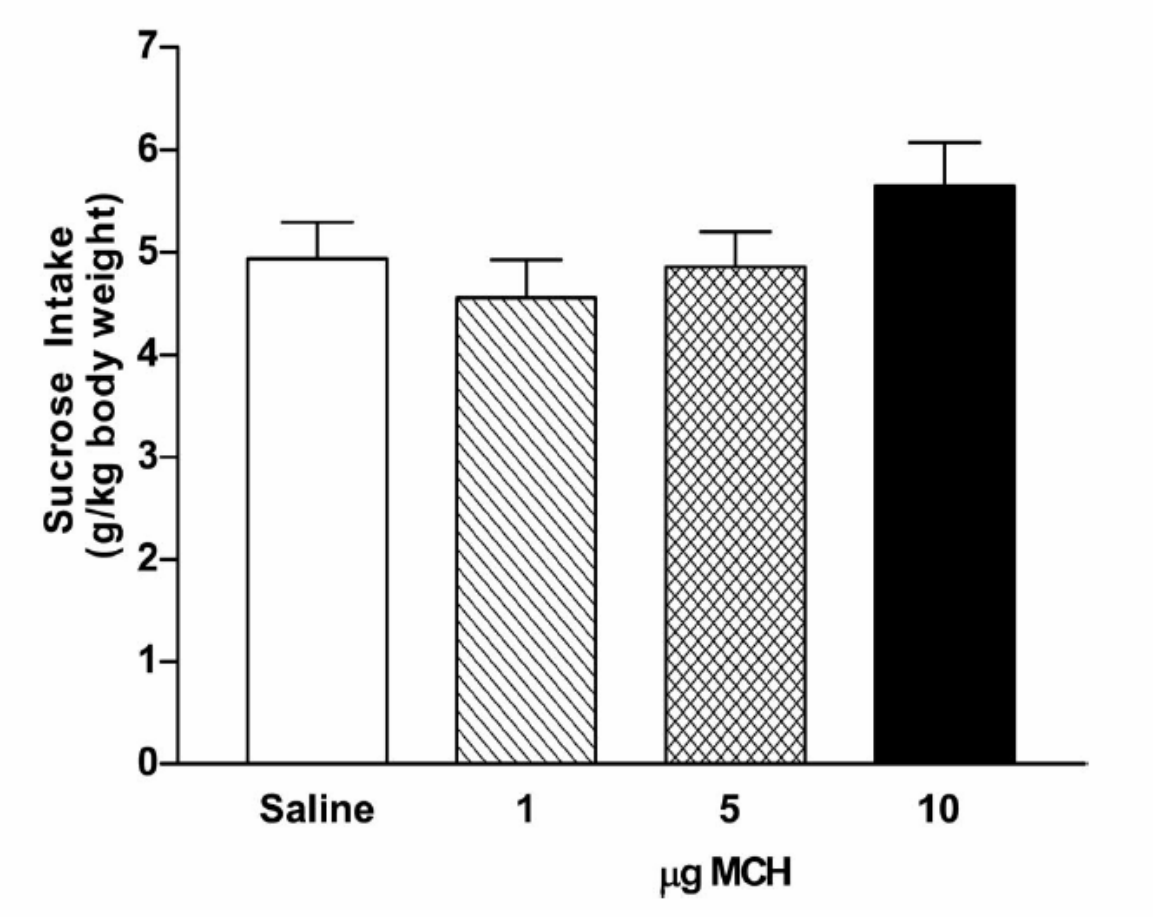

Figure 1b. Mean (±SEM) 2-hr sucrose intake (g sucrose per kg body weight) after the administration of i3vt saline or 1, 5, or 10 μg MCH.

Experiment 2

Elevated Plus Maze. As expected, animals that received NPY, which has documented anxiolytic properties, spent more time in the open arms than animals that received saline (NPY = 109.0 ± 22.0 s, saline = 51.0 ± 14.0 s, Figure 2A., $t(9) = 2.57$, $p < 0.05$). In contrast, MCH, at a dose that increased both food and alcohol consumption in Experiment 1, did not alter time spent in the open arms (MCH = 54.3 ± 11.5, saline = 58.2 ± 23.8, Figure 2B., $t(10) = -0.15$, n.s.). NPY and MCH both increased 2-hr chow intake when the plus maze assessment was finished (NPY = 9.9 ± 0.9 g, saline = 0.8 ± .4 g, $t(9) = 8.96$, $p < 0.001$), (MCH = 2.9 ± 0.2 g, saline = 0.5 ± 0.2 g, $t(10) = 8.39$, $p < 0.001$).

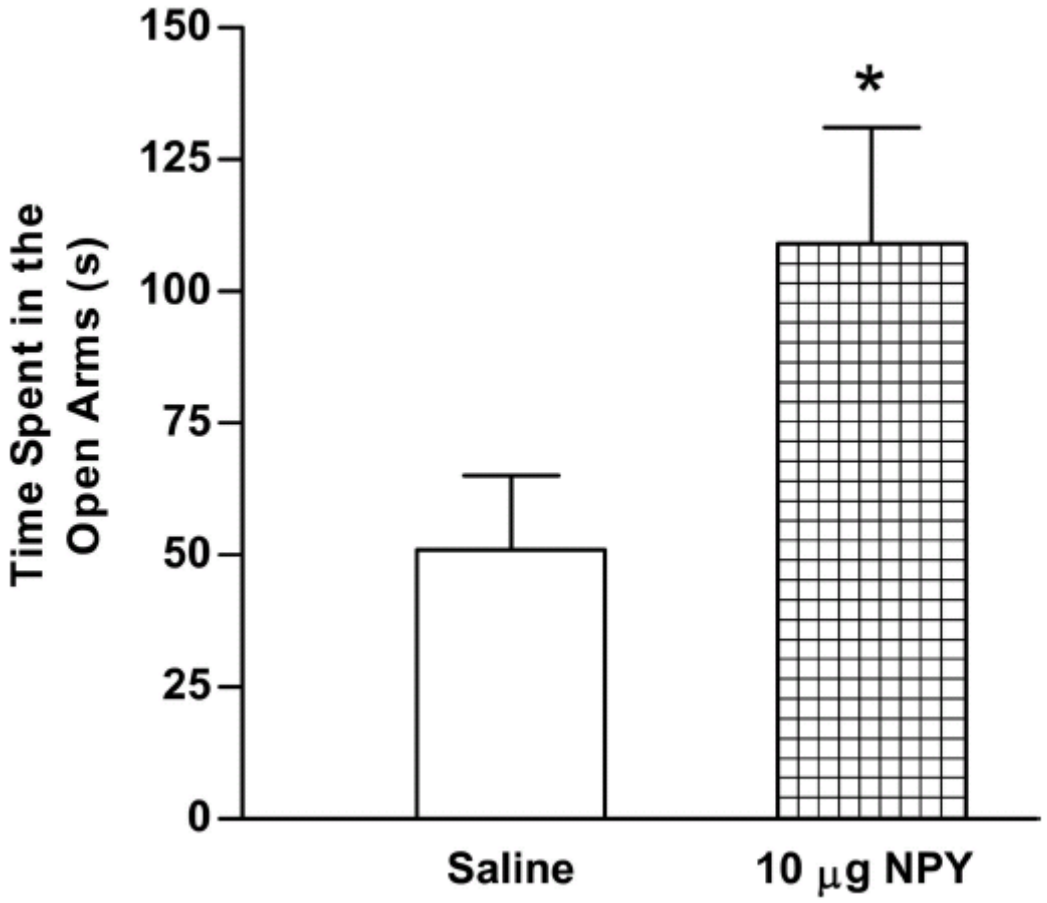

Figure 2a. Mean (± SEM) time (s) spent in the open arms of the EPM after i3vt administration of saline or 10 μg NPY.

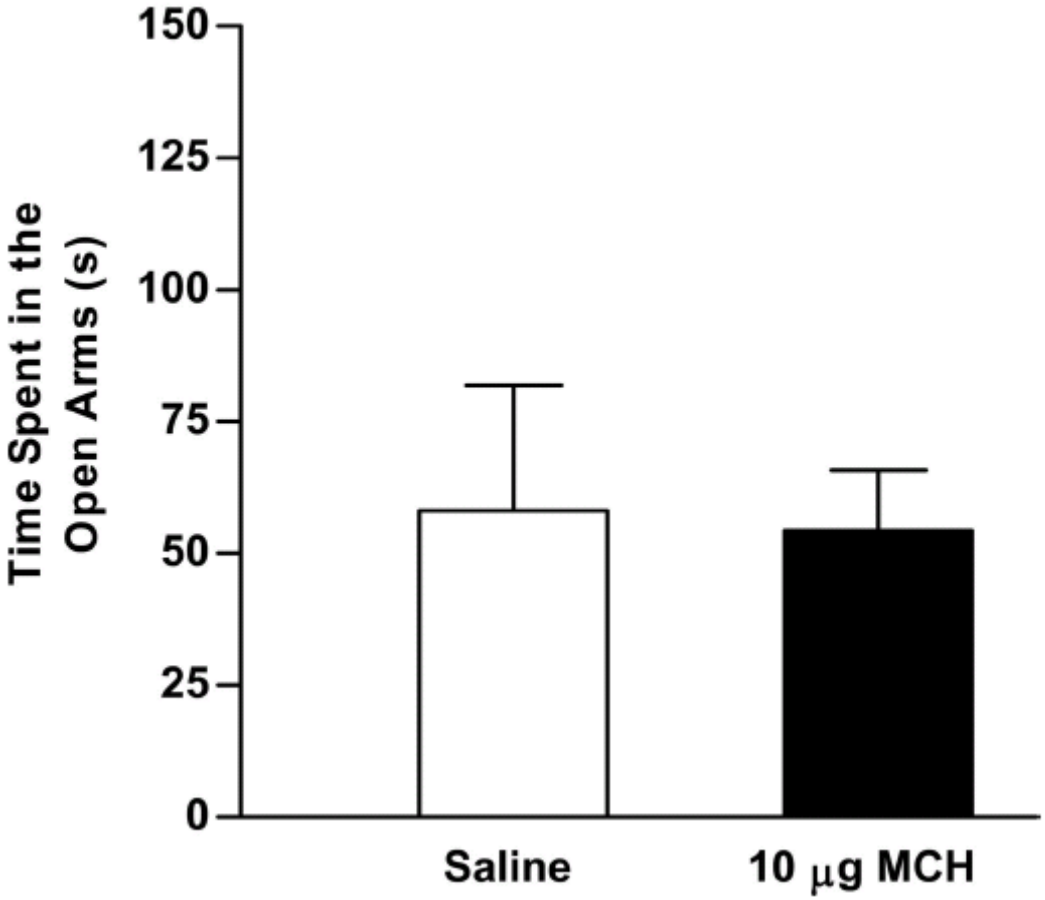

Figure 2b. Mean (± SEM) time (s) spent in the open arms of the EPM after i3vt administration of saline or 10 μg MCH.

Experiment 3

Baseline Period. There was no difference of baseline intake of alcohol (0.9 ± 0.1 g/kg body weight) or sucrose/quinine (1.3 ± 0.2 g/kg body weight), although there was a trend ($t(38) = 1.84$, $p < 0.07$) for the sucrose group to drink more. Body weight did not differ between the two groups on the final day of the baseline period (alcohol group = 393.5 ± 8.4 g, sucrose/quinine group = 398.1 ± 6.1 g, $t(38) = 0.45$, ns).

Ingestive Behavior. MCH increased 2-hr chow intake in both groups when compared to saline (Table 1, $F(36, 1) = 29.2$, $p < 0.01$). In a replication of Experiment 1, MCH significantly increased alcohol intake, while in contrast to what occurred in Experiment 1, MCH also increased sucrose/quinine intake (Figure 3, Table 1, $F(36, 1) = 4.77$, $p < 0.05$). MCH increased water intake in both groups compared to saline (Table 1, $F(36, 1) = 6.45$, $p < 0.01$).

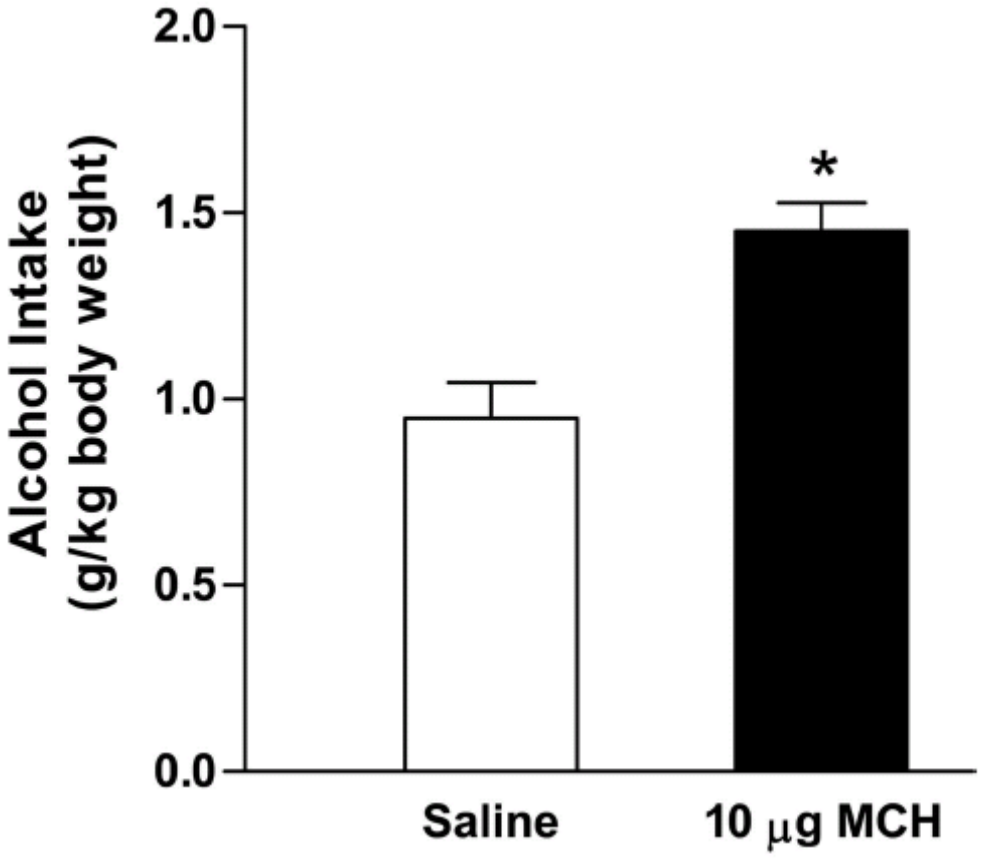

Figure 3a. Mean (± SEM) 2-hr alcohol intake (g alcohol per kg body weight) after the administration of i3vt saline or 10 μg MCH.

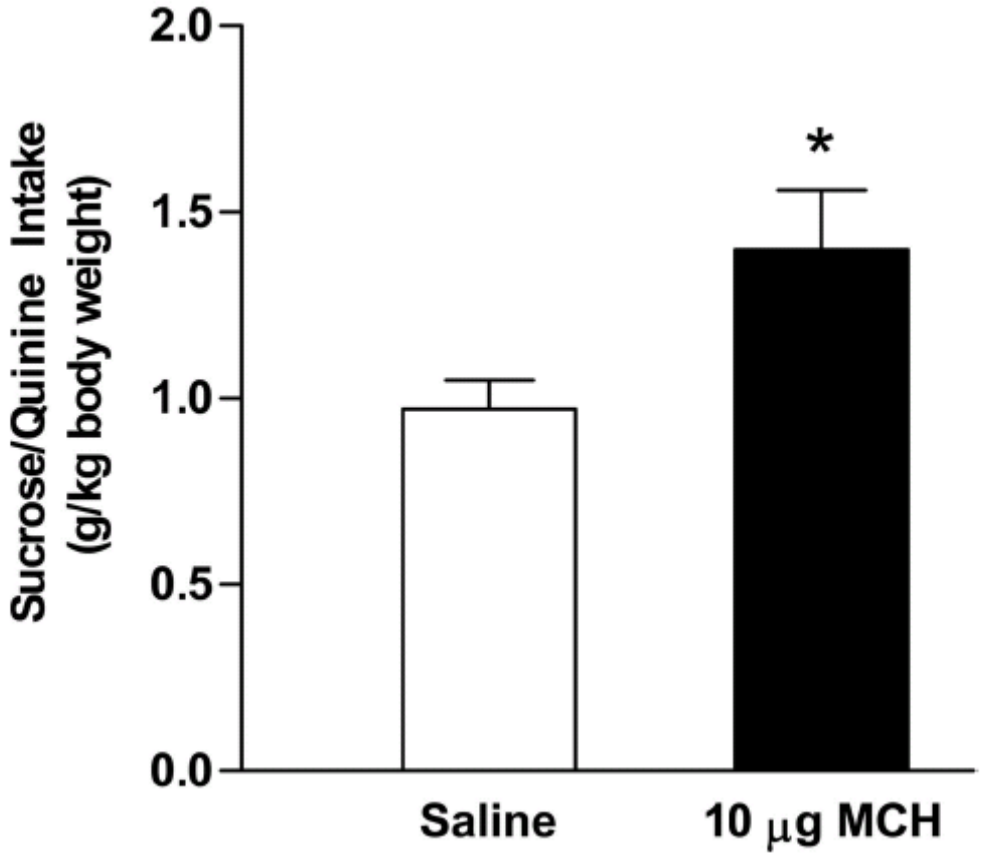

Figure 3b. Mean (± SEM) 2-hr sucrose/quinine intake (g sucrose per kg body weight) after the administration of i3vt saline or 10 μg MCH.

DISCUSSION

Although we hypothesized that MCH would reduce alcohol intake, central administration of MCH actually increased consumption of 10% alcohol in rats with simultaneous access to both food and water. Intake of chow, and water when it was available, was also increased by central MCH, consistent with reports that MCH is both an orexigen and a dipsogen (Clegg et al., 2003; Della-Zuana et al., 2002; Rossi et al., 1997). MCH also augmented sucrose intake, but only when baseline intake was reduced by the addition of quinine. We interpret this to mean that in Experiment 1, MCH was unable to augment sucrose consumption further due to the high baseline intake, and that animals were already drinking as much sucrose as possible in the 2-hr period. Hence, MCH increased intake of whatever ingestible was available.

Our results suggest that MCH augments alcohol consumption independent of any effect it might have on anxiety. Contrary to previous reports (Kela et al., 2003; Monzon and De Barioglio, 1999; Monzon et al., 2001), we found in Experiment 2 that central MCH did not act as an anxiolytic in alcohol-naïve rats, at least in the EPM paradigm. For anxiety to be the underlying factor responsible for the ability of MCH to increase alcohol intake, MCH would have to act as an anxiogenic, and our results do not support this. One caveat is that the rats were allowed to eat food prior to being placed on the elevated maze in Experiment 2, such that differential food intake might have been a factor. It is important to note that the role of MCH in anxiety is not well established in any event. There are a few reports that MCH reduces anxiety (Kela et al., 2003; Monzon and De Barioglio, 1999; Monzon et al., 2001). However, there were notable differences in the

experimental design of these studies from the present experiment. For instance, the previous studies used different strains of rats (Wistar or Sprague-Dawley), and different injection sites (lateral ventricle, amygdala, or hippocampus), and in some cases a smaller dose of MCH (Monzon et al., 2001), or a different behavioral test of anxiety (Kela et al., 2003). These experimental differences may explain the unexpected negative results found with MCH on anxiety in the present study. It is also important to point out that , inconsistent with the reports that MCH reduces anxiety, an MCH receptor antagonist has also been reported to possess anxiolytic properties (Borowsky et al., 2002); (Doggrell, 2003). Therefore, more work will be required to understand the role of MCH in anxiety.

Another possible mediator for the effect of MCH on alcohol consumption is thirst since MCH has been implicated in the regulation of fluid balance. Osmotic stimulation in rats has been reported to decrease MCH mRNA expression (Presse and Nahon, 1993), and central administration of MCH has been demonstrated to increase water intake independent of food intake in rats (Clegg et al., 2003). Therefore, MCH might augment alcohol intake by increasing the drive to consume fluids. We attempted to directly test this possibility in Experiment 1 utilizing a flavor-choice paradigm where the animals could choose between a flavor associated only with saccharin (i.e., fluid only) and a different distinct flavor associated with ethanol or sucrose. However, animals greatly preferred the flavor associated with alcohol or sucrose and never consumed appreciable amounts of the alternate solution. In Experiment 3 water was left on the cage (as opposed to the alternate saccharin solution) during the 2-hr alcohol or sucrose/quinine access period, the reasoning being that if the effect of MCH on alcohol consumption were

merely an artifact of thirst, the animals would likely consume water in the place of alcohol. However, MCH increased alcohol consumption even in the presence of water, implying that fluid balance is not a key factor in the effect of MCH on the alcohol consumption.

There are other plausible explanations for MCH's effect. Because alcohol contains calories, and MCH is orexigenic, it is possible that the effect of MCH to increase alcohol intake is linked to energy homeostasis. Alternatively, because MCH neurons and receptors are found in areas involved in reward and motivation (e.g., the lateral hypothalamus, nucleus accumbens shell, amygdala, and cortex), MCH might act by augmenting the reward value of alcohol. It is difficult to parse the role of caloric need from reward because the two are necessarily intertwined. Calories comprise a potent natural reward and changes of energy balance can alter the rewarding value of drugs of abuse (Cabeza de Vaca and Carr, 1998).

The present results do not rule out or support either of these possibilities. The fact that the caloric control in Experiment 2 (i.e., the sucrose/quinine group) consumed equivalent calories in response to MCH as the alcohol group consumed alcohol may be interpreted to support the position that MCH is merely acting to increase caloric intake independent of whether the calories come from alcohol or carbohydrate. However, sucrose itself is a powerfully rewarding substance having similar effects on the brain as drugs of abuse (Avena and Hoebel, 2003a; Avena and Hoebel, 2003b; Colantuoni et al., 2002; Colantuoni et al., 2001; Spangler et al., 2004). Therefore, all that we can conclude

at this time is that MCH non-specifically increases the consumption of many substances, including alcohol, and that it is not clear whether alcohol and sucrose intake were augmented by changes in their reward status, changes in the animals desire to consume calories in general, or a combination of the two.

It is tempting to speculate that MCH enhances the reward value of all caloric compounds or even other rewarding stimuli. MCH neurons are located in the lateral hypothalamus, a nucleus long believed to be involved in reward and motivation. Animals will self-administer electrical stimulation into the lateral hypothalamus, and when given this stimulation passively have been demonstrated to engage in behaviors historically regarded as rewarding (i.e., sex, eating, aggression) (Hoebel and Teitelbaum, 1962; Margules and Olds, 1962; Valenstein et al., 1968; Woodworth, 1971). It is likely that one of the many neuropeptides found in the lateral hypothalamus will be implicated in reward and MCH is a logical candidate(Valenstein et al., 1968). That said, more work needs to be done to determine the role of MCH in reward and motivated behavior.

CHAPTER 4

Pharmacological studies exploring the impact of MCH on alcohol consumption: endogenous MCH signaling and reward

INTRODUCTION

Melanin-concentrating hormone (MCH) is a cyclic peptide first isolated in the salmon pituitary and originally named for its ability to lighten the skin of teleost fish by inducing aggregation of melanocytes (Kawauchi et al., 1983). Although there is no indication that MCH performs this function in mammals, the peptide is present and its structure is highly conserved; there are only a few amino acids difference between the salmon and human form of MCH. In the mammal, MCH is produced by magnocellular neurons in the lateral hypothalamus and zona incerta (Pissios and Maratos-Flier, 2003). There are two known G-coupled protein receptors that carry out the functions of MCH, MCH receptor 1 and 2 (MCHR1 and MCHR2). MCHR1 is conserved among the mammalian species, while MCHR2 expression is limited to primates and some carnivorous species (e.g., dogs) (Tan et al., 2002). The broad expression pattern of MCHR1 throughout the central nervous system (CNS), as well as the extensive monosynaptic projections of MCH neurons, foreshadowed the many functions for which the neuropeptide is believed to be involved.

The most established action of MCH is in energy homeostasis. MCH acts as a signal of negative energy balance in the brain leading to increased energy consumption (Qu et al., 1996). Another function proposed for the peptide is in the regulation of fluid balance. Changes in osmotic environment causes an activation of MCH-producing neurons in multiple species (i.e., frog, teleost fish and rodents) (Francis and Baker, 1995; Francis et al., 1997; Presse and Nahon, 1993). Further, central infusion of MCH increases water consumption independently of food intake in rats (Clegg et al., 2003). In

addition to energy and fluid balance, MCH has been proposed to regulate anxiety. Administration of MCH into the brain has been reported to reduce (Kela et al., 2003; Monzon and De Barioglio, 1999; Monzon et al., 2001), have no effect (Duncan et al., 2005) or increase (Smith et al., 2005a) anxiety-like behavior in rodents, while genetic deletion or pharmacological blockade of MCHR1 has been reported to reduce measures of rodent anxiety (Borowsky et al., 2002; Chaki et al., 2005; Roy et al., 2006; Smith et al., 2005a). Hence, there is no clear consensus on how MCH regulates anxiety. Perhaps even more speculative, is the proposed role for MCH in reward and motivation. The possibility that MCH might regulate reward was proposed based on the anatomical position of the neurons that make MCH (i.e., the lateral hypothalamus) and the receptors that are activated by the peptide (e.g., the shell of the nucleus accumbens (NAc)) (DiLeone et al., 2003). Some recent reports have bolstered this theory finding that MCH injected directly into the shell of the NAc increases food intake in rodents (Georgescu et al., 2005) and that rodents lacking MCHR1 have increased D1 and D2 binding in the NAc (Smith et al., 2005b). Both of these studies demonstrate a functional relationship between MCH and brain regions known to be involved in the regulation of reward. Most recently we demonstrated a novel function for MCH in the regulation of alcohol intake. MCH administered into the 3rd cerebral ventricle increased consumption intake in rats (Duncan et al., 2005).

In our previous experiments we demonstrated that MCH enhanced the consumption of rewarding solutions; namely 10% alcohol and its caloric equal, 17.75% sucrose(Duncan et al., 2005). It is conceivable that MCH is influencing the consumption

of these solutions through its impact on anxiety, reward, energy and/or fluid balance. Because the dose of MCH that enhanced alcohol intake had no apparent impact on anxiety-like behavior (Duncan et al., 2005), anxiety is probably not an essential factor in MCH's effect on alcohol drinking. Further, MCH increased alcohol intake in the presence of water (Duncan et al., 2005), suggesting that fluid balance is not pertinent in MCH-induced alcohol consumption. Therefore, the two remaining possibilities for how MCH augments alcohol intake are by regulating energy balance and/or reward. It is difficult to parse the impact of MCH on caloric need from reward because the two are not mutually exclusive. Calories can serve as a potent natural reward, while changes of energy balance can enhance the rewarding value of drugs of abuse (Cabeza de Vaca and Carr, 1998). Because energetic need and reward are necessarily intertwined, it is possible that MCH is working through both systems to regulate alcohol drinking.

The purpose of the present study was two-fold. First, we were interested in testing the hypothesis that one mechanism by which MCH acts to augments alcohol drinking is by increasing the rewarding properties of alcohol. In an attempt to isolate the role of reward from energy need we utilized free-feeding animals that were trained to lever press for alcohol under a progressive ratio schedule, a classic measure of reward value (Hodos, 1961). Second, we wanted to determine if the impact of MCH on alcohol intake was physiologically relevant, or merely a pharmacological result of exogenous MCH administration. We predicted that endogenous MCH signaling would increase alcohol consumption and tested this by administering an MCHR1 antagonist to rats that were voluntarily consuming alcohol.

METHODS

Subjects

Male Long-Evans rats (Harlan, Indianapolis, IN) weighing 250-300 g at the start of each experiment were housed individually in plastic tubs, and maintained on a 12:12-hr light/dark cycle in a room controlled for temperature (21^{o} C) and humidity (40-60%). Pelleted rat chow (Harlan Teklad, Indianapolis, IN) and tap water were provided *ad libitum*, except when otherwise noted. Experiments commenced 1 week following the arrival of the rats to the animal housing facility. Research was conducted in AAALAC-approved facilities conforming to NIH and USDA regulations, with the approval of the University of Cincinnati Institutional Animal Care and Use Committee.

Experimental Procedures

Experiment 1

Voluntary alcohol intake training procedure. Rats (n = 42) were trained to voluntarily consume alcohol or sucrose using a sucrose-fading procedure adapted from Grant and Samson (Grant and Samson, 1985). A limited access procedure was used such that rats had daily 2-hr access to solutions in their home cage in the middle of the light cycle for the duration of the study. For the first 10 days all rats were given 10% sucrose (w/v). Every 4 days thereafter for 32 days the rats were presented with a novel solution. Half of the rats (ALC group) were transitioned from 10% sucrose to 10% alcohol by incrementally adding alcohol to 10% sucrose (alcohol (w/v) = 0 to 2.5% to 5% to 7.5% to 10% alcohol), and then decrementally reducing the sucrose concentration (i.e., 10% to

7.5% to 5% to 2.5% to 0 sucrose). After the 32-day fading process the rats were given 10 additional days of 2-hour access to 10% alcohol to establish stable drinking. As a caloric control, the remaining 24 rats (SUC group) received an amount of sucrose equal in caloric density to the solution presented to the ALC group on any given day during the 2-hour drinking session. In addition, the sucrose solution contained a small amount of quinine (25 μM – 750 μM) to limit sucrose consumption to a caloric level similar to the amount of alcohol calories consumed by the ALC rats. This amount of quinine does not affect food intake or body weight (Kratz et al., 1978; Sclafani and Ackroff, 1994; Tolliver et al., 1988). The final solution presented to the SUC group was 17.75% sucrose (w/v) containing 750 μM quinine.

Intracerebroventricular cannulation and injection procedure. Following training, rats were anesthetized using a mixture of intraperitoneal ketamine (86 mg/kg) and xylazine (12.9 mg/kg). Stainless steel 23-gauge cannulas were implanted into the 3rd-cerebral ventricle using a stereotaxic procedure (coordinates: anteroposterior: -2.2 mm from bregma and dorsoventral: -7.5 mm from the dura) (Paxinos, 1998). Following surgery animals were given 7 days to recover with continuing daily 2-hr access to solutions. Intracerebroventrical (i3vt) infusions were administered manually in a 1 μl volume using a 25 μl Hamilton syringe (Hamilton, Reno, NV). Cannula placement was verified by an i3vt infusion of angiotensin II (7.5 ng). Animals that consumed at least 5 ml water within 1 hr following this infusion were used in the study.

Impact of MCHR1 antagonist on ingestive behavior. To reestablish stable drinking levels, rats were allowed to recover from the surgery for two weeks. On the final 3 days of the recovery period rats were handled immediately before their routine alcohol drinking period. On day 15 after the surgery rats were randomly assigned to receive one of three doses of the MCHR1 antagonist, Compound B (Merck Research Laboratories, Rahway, NJ; 10, 20, or 30 μg) or saline immediately before the presentation of solutions on the cage. Ingestive behavior (i.e., alcohol or sucrose/quinine and chow intake) was measured after 2 hr and the alcohol and sucrose/quinine bottles were taken off the cages. Chow intake was then monitored at 4, 6, 12, and 24 hr post-infusion.

Experiment 2

Ability of MCHR1 antagonist to block MCH-induced ingestive behavior. A separate cohort of animals (n = 21) was trained to drink alcohol and implanted with i3vt cannulas as described in Experiment 1. The SUC group was not included in this experiment. Following surgery animals were given 2 weeks to recover. Animals were handled daily during the latter 7 days of the recovery period, immediately prior to receiving alcohol. On the 15th day after surgery, 10 μg of the MCHR1 antagonist, Compound B, or its vehicle, saline were administered 15 min before an i3vt infusion of 10 μg MCH or saline. This dose of compound B is reported to have no effect on food intake when administered into the lateral cerebral ventricle (Shearman et al., 2003). Rats were randomly assigned to receive one of the following infusion combinations: saline-saline (n = 5), saline-MCH (n = 6), Compound B- saline (n = 5), Compound B – MCH (n = 5). Immediately following the second infusion alcohol bottles were presented to the animals. Alcohol,

chow and water intake were measured 2 hr post-infusion, while simultaneously the alcohol bottles were removed from the cages. Chow and water intake were measured again at 6 and 24 hr post-injection.

Experiment 3

Surgery. A naïve group of animals, never exposed to alcohol or sucrose, was implanted with i3vt cannulas as described in Experiment 1.

Effect of MCHR1 antagonist on anxiety-like behavior. Following recovery from surgery animals were transported from their housing room to a behavioral testing room 4 hr following the light onset. After allowing the animals to settle for 3 hr, saline (n = 6), 10 μg of Compound B (n = 7), or 10 μg of NPY (n = 4) were administered i3vt. Treatment groups were assigned randomly. Following the infusion, the animals were placed back in their home cage without food or water for 30 min prior to being placed on an elevated plus maze (EPM) (50 cm above ground, arm dimensions: closed = 15 cm high, 6 cm wide, and 30 cm long, open = 0 cm high, 6 cm wide, and 30 cm long) for 5 min. All rats were placed in the center of the maze facing the same closed arm. Between each rat the maze was cleaned thoroughly with 0.1% acetic acid. A video recorder mounted on the ceiling over the maze recorded the animal's behavior and the tapes were blindly scored for time spent in the open arms of the maze. After the EPM assessment, the rats were returned to their home cage and food intake was measured 2 hr following the infusion.

Experiment 4

Animals and apparatus. A naïve cohort of animals was trained to self-administer 10% alcohol or a 45-mg sucrose pellet (Formula F, PJ Noyes Co, Inc, Lancaster, NH,). The experiment utilized 4 operant chambers composed of polycarbonate walls and stainless steel rod floors (Lafayette Instrument, Lafayette, IN). Two stainless steel levers, a pellet dispenser and a liquid dispenser were incorporated into one wall of the chamber. The chambers were interfaced with an IBM-compatible computer that recorded data (i.e., lever presses and rewards) using the Animal Behavior Environment Test (ABET) software (Lafayette Instrument, Lafayette, IN).

Operant training for sucrose on a progressive ratio (PR) schedule. Rats (n = 10) were restricted to 85% of their normal daily food intake (assessed by a 3-day mean of 24-hr chow intake) to initiate lever pressing for 2 days prior to the beginning of training. Training sessions lasted for 1 hr and occurred once daily. For the first 2-4 days of training the animals were rewarded with 1 sucrose pellet for every lever press (i.e., fixed ratio 1:1, FR1). In the event that the animal did not press the lever for a 5-min period, the pellet dispenser would automatically drop 1 pellet into the reservoir (fixed interval (FI) 5 min). After an animal had established reliable lever-pressing for sucrose on this schedule it was returned to *ad libitum* food intake and put through 2 FR1 training sessions. Subsequently, animals were trained to lever-press for sucrose on the following schedules for 2 sessions each: FR2, FR3 and FR4. Once all rats were lever-pressing for sucrose on the FR4 schedule they were placed on a progressive ratio (PR) schedule in which the response requirement for 1 sucrose pellet increased arithmetically by 2 lever presses

following each reward (i.e., 1, 3, 5, 7, 9, 11, 13, etc…). The break point was defined as the number of reinforcements received prior to an interval of 20 min without responding. Animals remained in the operant chamber and were able to respond for a total of 4 hr per session. Every rat had completed the PR schedule (i.e., stopped responding) within the 4-hr period. Once all animals had established a stable baseline (< 20% mean variation per subject) of responding on the PR schedule, surgery was performed to implant i3vt cannulas (as described in experiment 1).

Operant training for alcohol on a PR schedule. To initiate lever pressing rats (n = 15) were water restricted overnight (16 hr) prior to the first training session. All training sessions lasted for 1 hr and each animal was subjected to 1 session per day. To start, rats were trained to lever press for 100-µl of sucrose (10% w/v) in a combined FR1, FI 5 min schedule as describe above. Water access was restricted to 3 hr per day (following training sessions) until reliable lever pressing was established (no more than 3 days). Subsequently the FR schedule for 10% sucrose was increased every two days as follows: FR1, FR2, FR3 and FR4. Once on the FR4 schedule rats were trained to self-administer alcohol (10% w/v) by using a sucrose fading procedure (Samson, 1986). The fading process was similar to the procedure described above for home cage alcohol training except that solutions were made available for 1 hr in the operant chamber on an FR4 schedule. After sucrose fading, the rats were allowed to self-administer 10% alcohol on an FR4 schedule for 10 days. On the 7th day blood samples were collected from the tip of the tail 15 min after the beginning of the session to validate the measurement of alcohol consumption (see below section on blood alcohol measurement). Finally, animals were

trained to respond for 10% alcohol on the PR schedule described above. After approximately 2 weeks animals had established a stable baseline of responding on the PR schedule and were implanted with i3vt cannulas as described above.

Blood alcohol measurement. Headspace gas chromatography was used to analyze blood alcohol levels using a Carbopack B/5% gas chromatography column, Shimadzu gas chromatography machine, and Hewlett Packard Integrator. Immediately following blood collection, 20 µl of whole blood was mixed with 80 µl of an internal standard, N-propanol, and placed on ice until analysis later that day.

Impact of MCH on progressive ratio responding for alcohol or sucrose. Rats were given 1 week to recover from surgery and were then allowed 4-hr daily access to sucrose or 10% alcohol in the operant chambers for 2 weeks to reestablish responding on the PR schedule. Rats were randomly assigned to receive MCH (5 or 10 µg) or saline. The treatment groups had equivalent average baseline responding rates on the PR schedule. Infusions were administered immediately prior to placing the animal in the operant chamber. The number of lever presses an animal made prior to reaching the break point and total presses over the 4-hr session were recorded as the dependent variables.

Statistical Analysis

The data were analyzed with ANOVAs using the appropriate between- and within-subjects factors followed by post hoc (Tukey HSD) tests. Significance was set at $p < 0.05$.

RESULTS

Experiment 1

Impact of MCHR1 antagonist on ingestive behavior. The selective MCHR1 antagonist, Compound B, significantly reduced 2-hr chow intake in both the ALC and SUC rats at the 30 μg dose (0.24 ± 0.62 g/kg body weight) compared to saline (2.41 ± 0.56 g/kg body weight), $F(3, 38) = 6.6$, $p = 0.001$, Fig 4c. Neither the 10, nor the 20 μg dose was significantly different from saline at this time point, Fig 4c. Acute administration of 30 μg of Compound B, unlike the reported impact of a chronic infusion at this dose (Shearman et al., 2003), is short acting because at all the later measurements (i.e., 4, 6, 12 and 24 hr following the infusion) there was no significant effect of Compound B on chow intake compared to saline, Table 2.

Contrary to the ability of an MCH R1 antagonist to reduce caloric intake, there was no change in the consumption of calorie-rich solutions following the administration of Compound B. There was no significant effect on alcohol (Fig 4a) or sucrose/quinine (Fig 4b) intake at any of the doses administered compared to saline, Table 2.

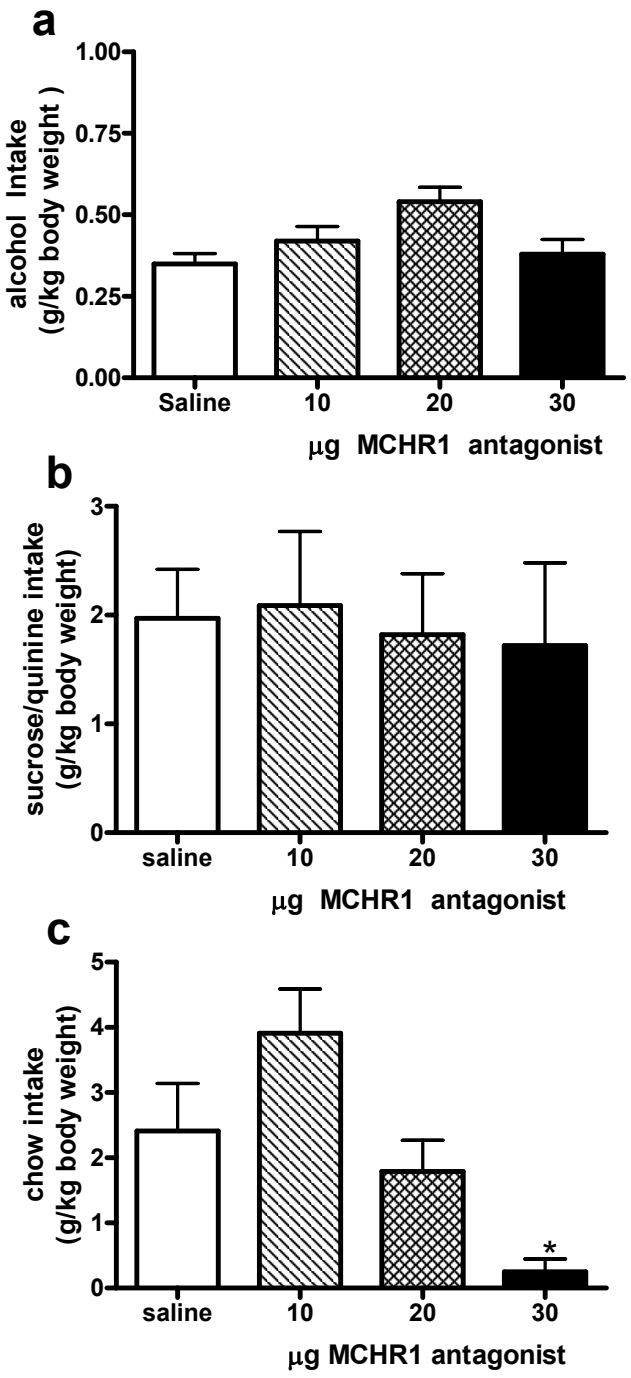

Figure 4. Mean (± SEM) alcohol intake (a), sucrose/quinine intake (b), and chow intake (c) in g/kg body weight 2 hr following i3vt administration of saline, 10, 20, or 30 µg of the MCHR1 antagonist, Compound B. Significantly different from saline, * $p < 0.05$.

Table 2
Impact of MCHR1 antagonism on ingestive behavior.

treatment	alcohol g/kg 2-hr	sucrose g/kg 2-hr	chow g/kg 2-hr	chow g/kg 4-hr	chow g/kg 6-hr	chow g/kg 12-hr	chow g/kg 24-hr
saline	0.4 ± 0.1	2.0 ± 0.6	2.4 ± 0.6	5.2 ± 0.9	10.2 ± 1.3	25.0 ± 2.1	45.4 ± 3.7
10 µg Comp B	0.4 ± 0.1	2.1 ± 0.6	3.9 ± 0.6	5.2 ± 0.9	9.3 ± 1.2	29.0 ± 2.2	49.8 ± 3.7
20 µg Comp B	0.5 ± 0.1	1.8 ± 0.6	1.8 ± 0.6	4.0 ± 0.9	7.2 ± 1.3	20.8 ± 2.2	38.3 ± 3.7
30 µg Comp B	0.4 ± 0.1	1.4 ± 0.6	0.2 ± 0.6*	2.2 ± 1.0	6.5 ± 1.4	19.6 ± 2.4	37.1 ± 4.0

Significantly different from saline, * $p < 0.05$.

Experiment 2

Ability of an MCHR1 antagonist to block MCH-induced ingestive behavior. A dose of Compound B (10 μg) that has no impact on chow intake alone, significantly attenuated MCH-induced chow intake 2-hr following administration, $F(3, 17) = 4.6$, $p = 0.01$, Fig 3b, Table 3. Chow intake remained significantly elevated by MCH 6 hr after administration, but the effect was gone after 24 hr, Table 3. In addition, this dose of Compound B significantly attenuated MCH-induced water intake 2 hr after administration $F(3, 17) = 3.3$, $p < 0.05$, Fig 3c, Table 3. Interestingly, the effect of MCH on water intake was still present 6 hr after treatment ($F(1, 17) = 3.8$, $p < 0.05$, post hoc = $p < 0.05$), but the ability of Compound B to attenuate this effect had disappeared; water intake of the antagonist-MCH group was significantly elevated compared to the antagonist-saline control group, $p < 0.05$, Table 3. There was no effect of treatment on 24-hr water intake, Table 3.

There was a significant overall effect of treatment on alcohol drinking, $F(3, 17) = 3.3$, $p < 0.5$. Consistent with previous reports (Duncan et al., 2005), the post-hoc analysis revealed that animals which received MCH preceded by saline drank significantly more alcohol compared to the saline-saline control animals, $p < 0.001$, Fig 3a. However, rats treated with the antagonist followed by MCH were not different compared to their respective control (antagonist-saline). These data are not easy to interpret because the lack of effect of MCH in the group receiving the antagonist seems to be due to an increase in alcohol intake in the control (antagonist-saline) group as opposed to an attenuated effect of MCH. That is, the post-hoc analysis found that the antagonist-MCH group drank significantly more alcohol compared to the saline-saline control, $p < 0.05$, Fig 3a, Table 3.

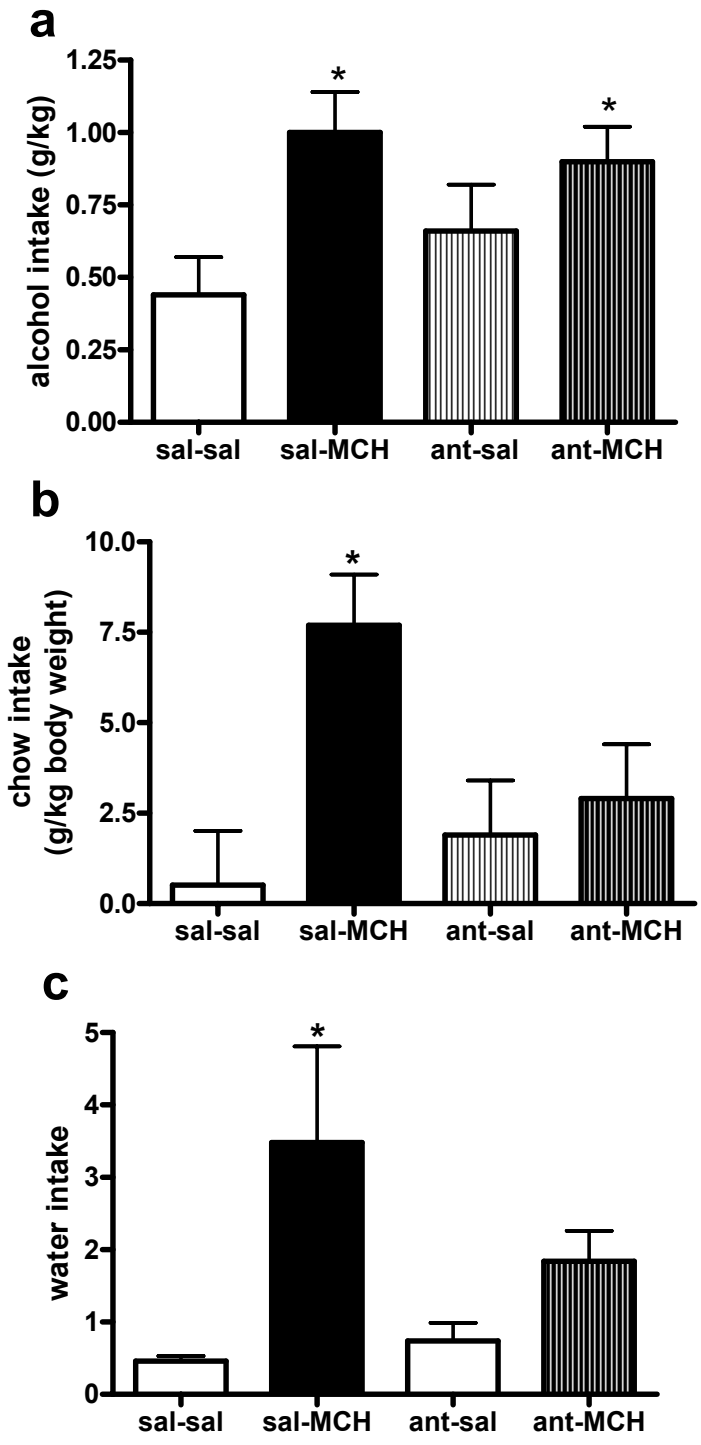

Figure 5. Mean (± SEM) alcohol intake (a) chow intake (b) and water intake (c) intake 2 hr following a double infusion of saline-saline (sal-sal), saline-MCH (sal-MCH), the MCHR1 antagonist-saline (ant-sal), or the MCHR1 antagonist-MCH (ant-MCH). Significantly different from sal-sal, * $p < 0.05$.

Table 3.
Impact of MCHR1 antagonism on MCH-induced ingestive behavior.

treatment	alcohol (g/kg) 2-hr	chow (g/kg) 2-hr	water (ml) 2-hr	chow (g/kg) 6-hr	water (ml) 6-hr	chow (g/kg) 24-hr	water (ml) 24-hr
saline – saline	0.4 ± 0.1	0.5 ± 1.5	0.5 ± 0.8	7.4 ± 3.1	2.8 ± 1.1	43.9 ± 8.9	15.5 ± 3.8
saline – MCH	1.0 ± 0.1*	7.7 ± 1.4*	3.5 ± 0.7*	21.2 ± 2.9*#	6.8 ± 1.0*#	63.0 ± 8.1	20.8 ± 3.5
10 µg Comp B - saline	0.7 ± 0.1	2.0 ± 1.5	0.74 ± 0.8	12.1 ± 3.1	2.7 ± 1.1	51.0 ± 8.9	16.9 ± 3.8
10 µg Comp B - MCH	0.9 ± 0.1*	2.9 ± 1.3	1.8 ± 0.8* #	15.6 ± 3.1*	6.0 ± 1.1#	63.2 ± 8.9	23.4 ± 3.8

Significantly different from saline-saline, * $p < 0.05$, ** $p < 0.01$ or from10 µg Comp B – saline # $p < 0.05$.

Experiment 3

Effect of MCHR1 antagonist on anxiety-like behavior. There was an overall effect of treatment on time spent in the open arms of the EPM, $F(2, 14) = 3.9$, $p < 0.05$. However, post-hoc analysis revealed that there was no effect of the MCH R1 antagonist, Compound B, on anxiety-like behavior. The animals given an i3vt infusion of 10 µg of Compound B spent slightly more time in the open arms of the EPM (54.8 ± 18.7s) compared to the saline control (37.1 ± 19.5 s), but the difference was not statistically significant. On the other hand, rats administered 10µg of NPY, a known anxiolytic, spent more time in the open arms than the saline control group as well as the antagonist group, $p < 0.05$, Fig 6.

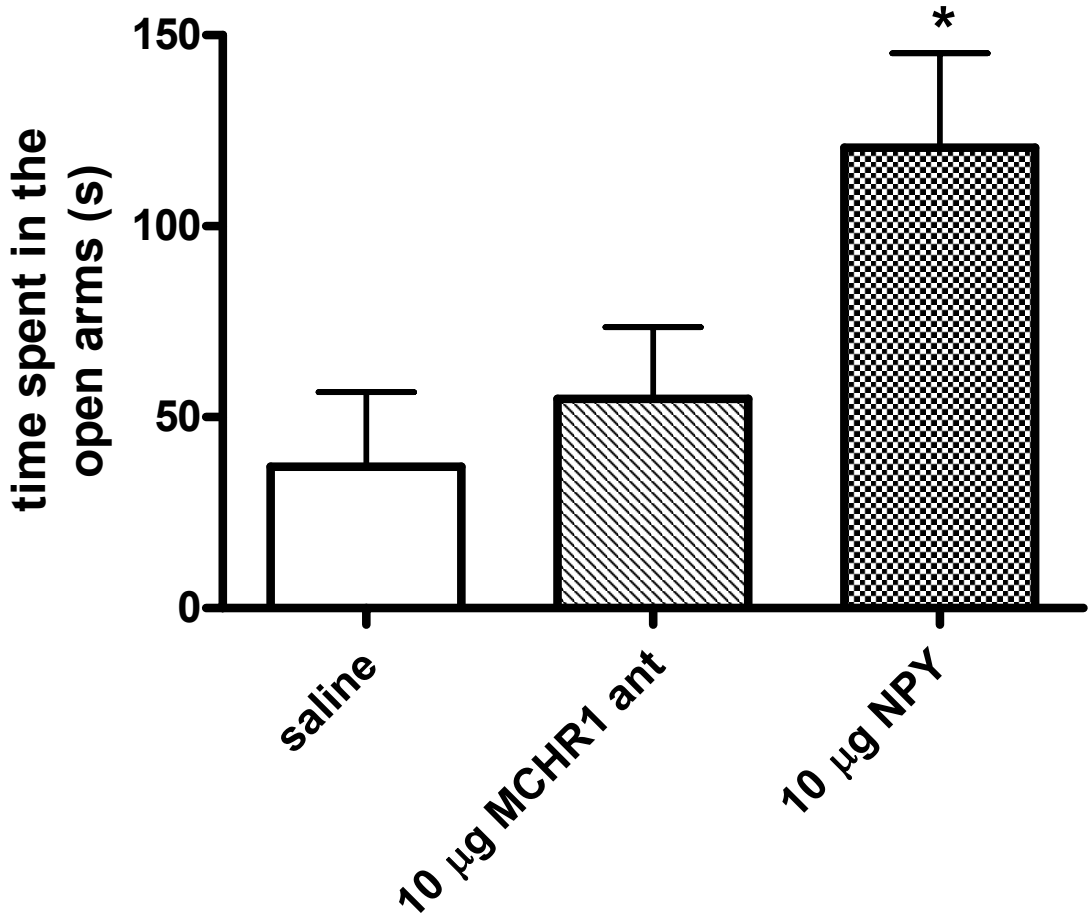

Figure 6. Mean (± SEM) time spent in the open arms (s) of the EPM following the administration of saline, 10 μg MCHR1 antagonist or 10 μg NPY. Significantly different from saline, * $p < 0.05$.

Experiment 4

Blood alcohol levels (BAL). The BALs of animals responding on an FR4 schedule for 10% alcohol were significantly correlated with both the number of rewards collected during the 15 min period ($r^2 = 0.70$) and the amount of alcohol consumed ($r^2 = 0.84$). BALs ranged from 0 – 48.4 mg/dl (mean = 12.5, median = 14.2 mg/dl), while alcohol intake ranged from 0 – 0.77 g/kg body weight (mean = 0.32, median = 0.33 g/kg body weight) during the first 15 min of responding.

Effect of MCH on progressive ratio responding for alcohol or sucrose. There was a significant effect of MCH on lever pressing for sucrose pellets prior to reaching the break point, $F(2, 7) = 11.0$, $p > 0.01$. Lever pressing following the highest dose of MCH (10 µg MCH = 855.3 ± 130.0 lever presses) was significantly elevated compared to saline (88.5 ± 112.6 lever presses), $p = 0.01$, and the lower dose of the peptide (5 µg MCH = 187.7 ± 130.0), $p < 0.05$. Responding increased for all groups during the time following the break point, $F(1, 7) = 7.9$, $p < .05$, Fig 7a. In contrast to the effect of MCH on responding for sucrose, MCH did not increase responding for alcohol prior to the break point. However, there was a significant effect of the peptide on responding for 10 % alcohol over the entire 4-hr period, $F(2, 12) = 4.2$, $p < 0.05$, with the highest dose of MCH (10 µg MCH = 315.4 ± 61) significantly increasing responses compared to saline (87.2 ± 32), $p < 0.05$. There was a significant interaction between treatment and time in regards to lever pressing for alcohol, $F(2, 12) = 5.9$, $p < 0.05$. Animals that received the highest dose of MCH responded significantly more after reaching the break point when compared to the other two groups, $p < 0.01$, Fig 7b.

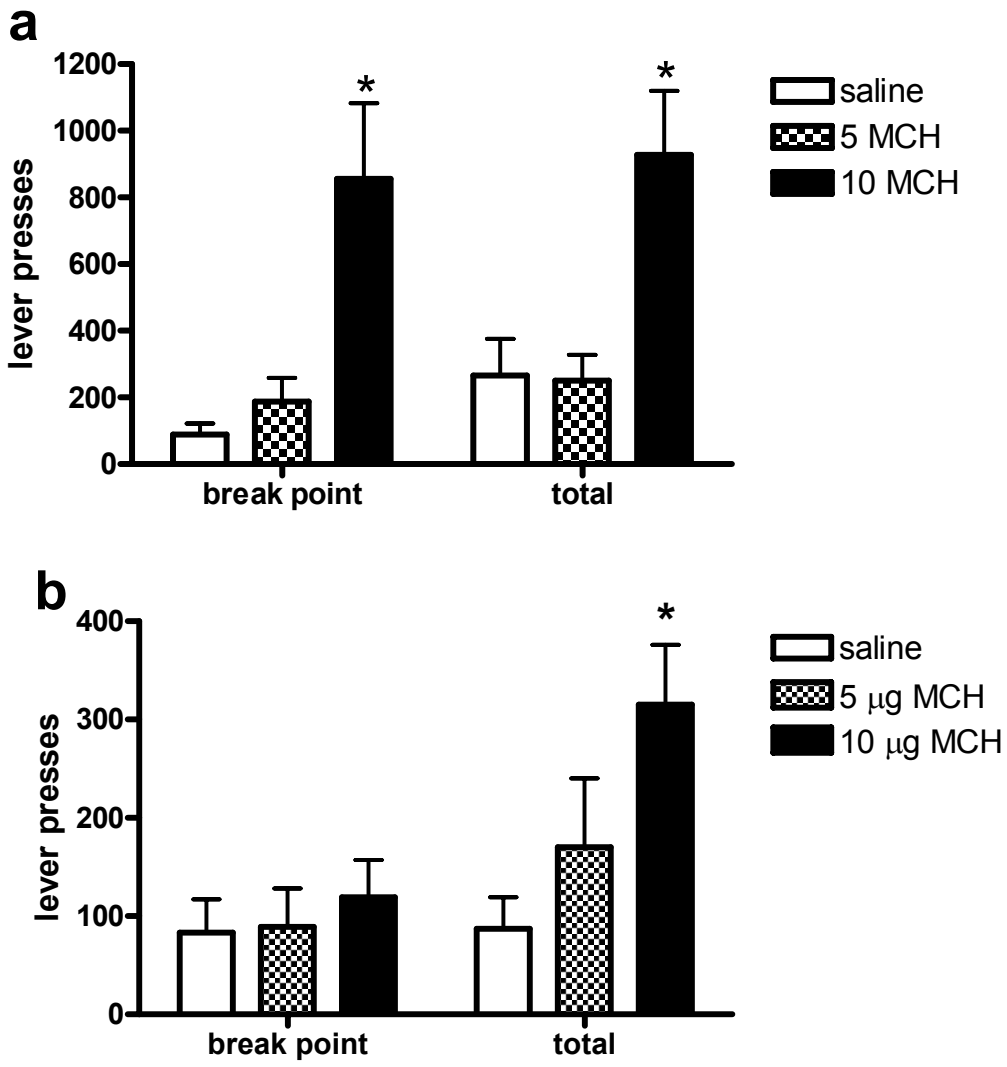

Figure 7. Mean (± SEM) lever press responses for a 45 mg sucrose pellet (a) or 100 μl of 10% alcohol (b) on a PR schedule following the administration of saline, 5 μg MCH, or 10 μg MCH, either prior to the break point (break point), or over the entire 4-hr session (total). Significantly different from saline, * $p < 0.05$.

DISCUSSION

One goal of these experiments was to test the hypothesis that endogenous MCH signaling regulates alcohol drinking. The results are not consistent with this hypothesis. While administration of a selective MCHR1 antagonist effectively reduced 2-hr chow intake, it had no impact on 10% alcohol intake, or on the consumption of a sucrose solution, equal in calories to 10% alcohol. Further, it is not clear that the MCHR1 antagonist was able to attenuate MCH-induced alcohol intake. Consistent with previous reports (Morens et al., 2005; Shearman et al., 2003), a dose of the MCHR1 antagonist that had no effect on ingestive behavior alone, significantly blocked MCH-induced chow and water intake. However, administration of the MCHR1 antagonist prior to administration of MCH resulted in a level of alcohol intake that did not significantly differ from the appropriate control condition (i.e., animals that received the antagonist followed by saline). These data are difficult to interpret because the apparent lack of difference between these two groups was associated with a slight elevation of alcohol intake in the control group (antagonist-saline) as opposed to an attenuation of the effect of MCH in the animals receiving the antagonist followed by the peptide. In other words, the antagonist-MCH group drank significantly more alcohol compared to the saline-saline group.

Because of the numerous functions proposed for MCH, a feasible explanation for these findings is that antagonizing central endogenous MCHR1 signaling throughout the brain does not have a simple unidirectional impact on alcohol intake. Because there are a few reports that MCH acts as an anxiolytic (Kela et al., 2003; Monzon and De Barioglio,

1999; Monzon et al., 2001), it is possible that the MCHR1 antagonist was increasing anxiety, thereby causing an unexpected augmentation in the drive to drink alcohol. In other words, the antagonist might increase alcohol intake via an impact on anxiety, while simultaneously reducing alcohol consumption through a reward or energy balance mechanism. However, the data were not consistent with this hypothesis. At the lowest dose used in both our pharmacological experiments, the MCHR1 antagonist did not affect anxiety-like behavior. Thus, changes in anxiety caused by the MCHR1 antagonist cannot explain the present findings.

Another possible reason for the failure of blockade of MCHR1 signaling to affect alcohol and sucrose intake, at least when the antagonist was given alone, is activation of a counteracting learned anticipatory response. The experience of receiving access to alcohol and sucrose in a routine manner, daily for months, is analogous to the phenomenon of meal-feeding. Meal-fed rats, or rats given their entire daily food ration in a restricted, but predictable time each day, are believed to acquire specific anticipatory responses (e.g., insulin secretion (Woods et al., 1970; Woods et al., 1977), changes in blood glucose levels (Woods and Kuskosky, 1976) and ghrelin secretion (Drazen et al., 2006)) through learning, allowing them to consume a larger amount of calories during a single meal (Woods, 1991; Woods and Ramsay, 2000). In addition, rats fed ad libitum, but given limited access to a palatable source of calories, also display entrained changes in brain activation and behavior (Mendoza et al., 2005a; Mendoza et al., 2005b; Mistlberger and Rusak, 1987). As a specific example of how this is relevant to the present findings, one study found that meal-feeding reduced the ability of an anorexic

agent (melanocortin agonist) to decrease food intake (Benoit et al., 2003). In the same way, the animals trained to consume alcohol and sucrose at the same time each day may have specific learned anticipatory responses which facilitate the consumption of these solutions and these may stifle the effect of an MCHR1 antagonist. If this is the case, it might require a larger dose to reduce consumption of these anticipated fluids.

The above speculation on why the MCHR1 antagonist did not reduce alcohol and sucrose intake requires further experimentation. At present, our data seem to suggest that endogenous MCH signaling does not regulate the consumption of rewarding solutions (i.e., alcohol and sucrose). However, the reports that MCHR1 antagonists reduce the consumption of a sweetened condensed milk, which is highly palatable (Borowsky et al., 2002; Morens et al., 2005), are not consistent with this interpretation. Further, as described in the introduction, there is evidence for a role of MCH in the rewarding aspects of food intake (Georgescu et al., 2005). Because of the fact that the antagonist did not clearly attenuate MCH-induced alcohol consumption, we are not confident in concluding that endogenous MCH signaling is not involved in alcohol and sucrose drinking. Future experiments are aimed at utilizing a genetic mouse model to further address this hypothesis.

The second aim of these studies was to test the hypothesis that one way by which MCH augments alcohol intake is via increasing alcohol's rewarding properties. The initial analysis of the data revealed that MCH augmented responding for sucrose, but not alcohol, on the progressive ratio schedule prior to reaching the defined break point (i.e.,

20 min without responding). At first glance, these data suggest that reward and motivation are not involved in MCH-mediated alcohol intake. However, analysis of the entire 4-hr operant session revealed that MCH in fact did increase total responding for alcohol. That is, unlike control animals, animals given 10 μg MCH began responding again after they had reached the defined break point. This phenomenon was not observed in the sucrose experiment; the highest dose of MCH increased lever-pressing for sucrose prior to the break point, but no further elevation in responding during the 4-hr session occurred.

These finding suggest that there is a fundamental difference between how MCH motivates the intake of alcohol vs. sucrose. The differences between the two macronutrients are substantial. Obviously both are rewarding substances, but alcohol has many properties that are not present in sucrose, such as the ability to induce locomotor sedation. This is an important distinction to make in light of the present data because the sedating properties of alcohol might provide a logical explanation for the delayed effect of MCH on lever-pressing for alcohol under the progressive ratio schedule. However, the average dose of alcohol self-administered prior to the break point in this experiment was very low (0.2 g/kg alcohol, range 0.02 – 0.3 g/kg alcohol). If anything, this low dose of alcohol would be expected to increase locomotor activity (Colombo et al., 1998; Moore et al., 1993; Paivarinta and Korpi, 1993; Read et al., 1960). Therefore, it is not likely that alcohol-induced sedation is responsible for MCH's relatively sluggish impact to increase lever pressing for alcohol, compared to sucrose.

Another important difference between these two rewards is the amount of calories they offer. The standard 45 mg sucrose pellet contains 3.6 Kcal/g (i.e., 0.16 Kcal/pellet), while the alcohol contains 7.1 kCal/g (i.e., 0.07 Kcal/ 0.1 ml 10% alcohol, w/v). Thus if it were the case that the effect of MCH on responding for these rewards was purely energy-motivated, one would expect equivalent amounts of calories to be received. The lever-pressing for 10% alcohol was slightly lower than for the sucrose pellet following saline administration, but there was no significant difference. However, following MCH administration, lever pressing for the sucrose pellet increased by around 800% compared to saline, while responding for the lesser calorie source, alcohol, only increased by 300%. Further, augmentation in lever-pressing for alcohol occurred only after responding had stopped for more than 20 minutes. Hence, caloric density did not determine the effect of MCH on responding for these two macronutrients.

Finally, the most obvious difference between the alcohol and sucrose rewards was palatability. Perhaps MCH stimulated the animals to work harder for sucrose than alcohol because of its greater palatability. However, in light of the findings of another study investigating the role of MCH in diet preference this does not seem likely. MCH administration stimulated the consumption of a high fat diet equally to that of low fat diet. Further, the effect of MCH on food intake was found to be independent of the opioid system, which is thought to be involved in signaling the relative palatability of food (Clegg et al., 2002).

In summary, this study found that pharmacological antagonism of the MCHR1 was unable to reduce alcohol or sucrose/quinine consumption at a dose that effectively reduced chow intake. Although these findings suggest that endogenous MCH signaling is not involved in the consumption of rewarding solutions, previous reports with sweetened milk do not support this interpretation (Borowsky et al., 2002; Morens et al., 2005). Further, because the MCHR1 antagonist also did not block MCH-induced alcohol intake, there may be a complex bi-directional effect of endogenous MCH on alcohol drinking, which make the data difficult to interpret. The broad expression of the MCHR1 throughout the CNS as well as the peptide's involvement in numerous regulatory systems that can modulate alcohol drinking in different ways (e.g., reward, anxiety and energy balance) provide reason to believe that endogenous MCH signaling in different brain regions might have opposite effects on alcohol drinking.

We attempted to isolate the role of reward in the effect of MCH on alcohol drinking by utilizing a progressive ratio operant self-administration model. MCH increased responding for both sucrose and alcohol during the 4-hr session. However, the motivational effect of MCH on alcohol intake seemed to be delayed in comparison to sucrose. The effect of MCH on lever pressing did not seem to be related to the caloric properties of the reward. More work needs to be done to elucidate the brain regions and motivational factors involved in MCH-induced alcohol drinking.

CHAPTER 5

Alcohol consumption of MCH receptor 1 deficient mice

INTRODUCTION

Melanin-concentrating hormone (MCH) is a peptide made by neurons in the lateral hypothalamus and zona incerta (Pissios and Maratos-Flier, 2003). In rodents MCH acts solely through the MCH receptor 1 (MCHR1 – also known as GPR24 or SLC-1) (Lembo et al., 1999; Saito et al., 1999) which is expressed throughout the central nervous system (Saito et al., 2001). This extensive receptor distribution is consistent with the numerous functions proposed for the neuropeptide. MCH is best known as a signal of negative energy balance. When administered into the brain of rodents, MCH increases food intake (Qu et al., 1996). Further, genetic deletion of MCH in mice leads to a hypophagic and lean phenotype (Shimada et al., 1998), while overexpression of the peptide results in hyperphagia and increased body weight. Following genetic deletion of MCHR1 mice are lean, but surprisingly hyperphagic compared to wildtype mice (Marsh et al., 2002). It is counterintuitive that the loss of MCHR1 would result in increased food intake, but the hyperphagia could be due to compensation for an elevated level of energy expenditure (Marsh et al., 2002). That is, MCHR1 knock-out mice have to eat all of the time to maintain a lean phenotype. A recent report found that MCHR1 knock-out mice have increased sensitivity to dopamine receptor binding in the mesolimbic dopamine system. Thus, the hyperphagia may also be caused by an increase in motivated behavior in general (Smith et al., 2005b).

Beyond its role in energy balance, there are many other proposed functions for MCH. The peptide is reported to increase water intake independently from food intake,

suggesting that it may regulate fluid balance (Clegg et al., 2003). MCH is also thought to be involved in anxiety although the findings are equivocal. The peptide has been reported to reduce (Kela et al., 2003; Monzon and De Barioglio, 1999; Monzon et al., 2001), increase (Gonzalez et al., 1996) or have no effect (Duncan et al., 2005) on anxiety-like behavior in rats. On the other hand, MCHR1 signaling is reported to increase anxiety in mice (Roy et al., 2006; Smith et al., 2005a) and MCHR1 antagonists have anxiolytic properties in rats and guinea pigs (Borowsky et al., 2002; Chaki et al., 2005). Anatomically, there is evidence to suggest that MCH is involved in reward. The lateral hypothalamus where MCH is synthesized has long been thought to be important for motivated behavior (Margules and Olds, 1962). Further, MCHR1 is densely expressed within the shell of the nucleus accumbens (NAc), a brain region known to be important for processing reward (Saito et al., 2001). A recent study found that MCH signaling within this brain region increased food intake indicating a role for MCH in the rewarding aspects of feeding behavior (Georgescu et al., 2005).

All of the abovementioned functions of MCH are also implicated in the etiology of alcoholism. Alcohol dependence is considered to be a major health problem which consists of increased tolerance to the effects of alcohol, impaired control over drinking, and continued drinking despite adverse consequences (Association, 1994). In order to develop better pharmacological therapies for alcohol dependence it is important to understand the underlying neural mechanisms that regulate alcohol consumption. It is not likely that the brain evolved a specific means to regulate alcohol intake per se. Instead, preexisting circuits within the brain probably modulate alcohol drinking after

having experience with the drug. For example, alcohol consumption is positively reinforced through its rewarding effects, and negatively reinforced by the anxiolytic or tension reducing properties of the drug. In addition, alcohol contains energy (i.e., 7 Kcal/g) and thus it is possible that the systems in the brain used to regulate energy balance are secondarily involved in the drive to drink alcohol. The same thing can be said of systems that regulate fluid balance, as alcohol is an orally consumed fluid.

Because MCH is implicated in the regulation of reward and anxiety, and is known to modulate energy and fluid balance, we were interested in how MCH would impact alcohol consumption. We have demonstrated that administration of exogenous MCH into the brain increases alcohol intake, at a dose that has no effect on anxiety-like behavior in rats (Duncan et al., 2005). These results suggest that MCH might enhance alcohol drinking secondarily to its effect on energy balance and/or through reward circuitry within the brain. However, we do not know if this effect of MCH is merely pharmacological or if endogenous MCH signaling plays a role in regulating alcohol intake. In order to test the hypothesis that endogenous MCH signaling enhances alcohol drinking, we utilized mice deficient in MCHR1, predicting that the absence of MCH signaling would result in depressed alcohol intake in this model.

METHODS

Subjects

Mice lacking a functional MCHR1 on a mixed genetic background (C57bl/6 x 129/SvJ) were obtained from Dr. Bernard Lakaye at the University of Liege in Belgium. The generation of these mice has been described previously (Adamantidis et al., 2005). For the purposes of the present experiments, mice were back-crossed for 5 generations onto a C57bl/6 background, an alcohol-preferring strain (Rodgers and McClearn, 1962). Heterozygote breeding pairs were then used to generate 3 experimental groups: wildtype (WT), heterozygous (HET), and homozygous (KO) MCHR1 deficient mice. Mice were group-housed in plastic tubs with corn-cob bedding in humidity (40-60%) and temperature (21° C) controlled rooms on a 12:12 hr light/dark cycle. For experiments measuring ingestive behavior, mice were individually housed in the same environment. Pelleted rat chow (Harlan Teklad, Indianapolis, IN) and tap water were provided *ad libitum*. Research was conducted in AAALAC-approved facilities conforming to NIH and USDA regulations, with the approval of the University of Cincinnati Institutional Animal Care and Use Committee.

Drugs

Alcohol solutions were made from 95% ethanol (w/v), diluted in tap water for drinking, or diluted to a 20% solution in saline for intraperitoneal (i.p.) injections. Sucrose/quinine solutions were made by dissolving quinine sulfate (Sigma-Aldrich, St Louis, MO) and sucrose (w/v) in tap water. MCH (Phoenix Pharmaceuticals, Belmont,

CA) and Neuropeptide Y (NPY: American Peptide Company, Sunnyvale, CA) were dissolved in a saline.

Ad libitum alcohol access procedure

Adult male MCHR1 WT (n = 8), HET (n= 8), and KO (n = 7) mice were placed in individual plastic tub cages with corn-cob bedding and two water bottles. Mice were given 7 days to become accustomed to the new housing arrangement. After this period, a bottle of 2 % alcohol (w/v) was presented in place of one of the water bottles. Every 5th day thereafter the concentration of alcohol was increased as follows: 4, 6, 8, 10, 15, 20%, w/v alcohol. To avoid the formation of a "side preference" the bottle containing ethanol and the bottle containing water were rotated to different sides of the cage every day. Fresh tap water was provided in a clean bottle each time a new alcohol solution was presented. Chow, water, and alcohol intake were measured daily.

Limited alcohol access procedure

Immediately following the last day of access to 20% alcohol animals were placed on a restricted alcohol access schedule. For 7 days at the onset of dark animals received a bottle of 10% alcohol (w/v) for 1 h. Animals had free access to food and a water bottle for 24 hours a day. Alcohol intake was recorded for the 1-hr alcohol access period, while chow and water intake were measured during that hour as well as the remaining 23 hours of the day.

Ad libitum sucrose/quinine access procedure

Animals that had been given limited access to alcohol were again given 2 bottles containing water for 7 days. Following this wash-out period, a solution of 17.75% sucrose and 0.7 mM quinine was given in one of the bottles. The quinine concentration was augmented by 0.2 mM every 3rd day until the solution preference (i.e., ratio of sucrose/quinine to water consumption) was similar to that of 10% alcohol for the WT mice. The final solution (17.75% sucrose + 1.3 mM quinine) was then given for 5 days. The relative position of the two bottles was rotated and ingestive behavior (i.e., chow, water, and sucrose/quinine) was measured each day.

Measurement of body weight and composition

The mice used in the above experiments were weighed every 2-3 days throughout the study, and at the end (i.e., following the last day of *ad libitum* sucrose/quinine access) body composition was measured using an EchoMRI machine (Waco, TX). The machine utilizes nuclear magnetic resonance to assess the density of hydrogen nuclei thereby estimating the physical state of the tissue (i.e., total body fat, lean mass, free fluids and water). Mice were immobilized in plastic restrainer tubes and then put into the EchoMRI machine. This procedure takes less than 1 minute and does not require anesthesia or sedation.

Measurement of alcohol metabolism

A naïve cohort of adult male MCHR1 WT (n = 10) and KO mice (n = 8) were injected (i.p.) with 3.0 g/kg body weight alcohol (20% in a sterile saline solution). Blood samples were collected from the tip of the tail 30, 60, 180 and 480 min following alcohol administration. Blood alcohol level (BAL) was measured using headspace gas

chromatography in a Shimadzu gas chromatography machine equipped with a Carbopack B/5% gas chromatography column and Hewlett Packard Integrator. Immediately following blood collection, 10 µl of whole blood was mixed with 40 µl of an internal standard, N-propanol, and stored at -20° C until analysis. All samples were analyzed within 48 hr of collection.

Measurement of anxiety-like behavior

Naïve adult male and female MCHR1 WT (n = 12), HET (n = 12), and KO (n = 12) mice were tested for anxiety-like behavior in an elevated plus maze (EPM) in the middle of the light period. Each mouse was placed in the center of EPM facing the same closed arm and allowed to explore the maze for 5 min. A video camera was used to record behavior and time spent in each arm was scored by a blind observer.

Intracerebroventricular cannulation surgery

Mice were anesthetized with tribromomethanol (250 mg/kg) and surgically implanted with a 26-gauge stainless steel cannula (Plastics One, Roanoke, VA) aimed at the third cerebral ventricle using a stereotaxic procedure (coordinates measured from bregma: 4.8 mm anterior, 0.8 mm dorsal. Following a 1-week recovery period cannula placement was verified by intracerebroventricular (i3vt) infusion of 5 µg of NPY. Mice that consumed more than 0.5 g of chow within 1 hr following NPY administration were included in the study. All infusions were administered manually in a 1 µl volume using a 10 µl Hamilton syringe.

Measurement of alcohol intake following an ICV infusion of MCH

A naïve cohort of adult female WT (n = 4) and MCHR1 HET (n = 4) were individually housed with two water bottles for 1 week and then given continuous access to an increasing concentration of alcohol as described above. After the mice had been drinking 10% alcohol solution for 5 days they were implanted with i3vt cannulas and allowed to recover for 1 week. NPY was then used to assess cannula placement (as described above). Alcohol was available during the recovery period and for 1 additional week prior to the experiment. Mice were randomly assigned to receive an i3vt infusion of 5 μg MCH or sterile saline, 5 hr after the onset of light. The intake of 10% alcohol, chow, and water were measured 1 hr and 24 hr following the infusion. Four days later the group assignments were reversed so animals that had been given saline were now given 5 μg MCH and vice versa. Ingestive behavior was again monitored at 1 and 24 hr following the injection.

Measurement of sucrose/quinine intake following an ICV infusion of MCH

Mice from the above experiment were given 2 water bottles for 2 weeks. Sucrose/quinine was then added to one bottle as described in the *ad libitum* sucrose/quinine experiment described above. The final solution (i.e.,17.75% sucrose and 1.3 mM quinine) was given for 7 days prior to drug infusions. Mice were again randomly assigned to two groups receiving 5 μg MCH or saline 5 hr after the onset of the light period. Intakes of sucrose/quinine, chow and water were measured 1 and 24 hr following the injection. The mice were injected again 4 days later with the opposite treatment and ingestive behavior was again measured after 1 and 24 hr.

RESULTS

Ingestive behavior during *ad libitum* alcohol access

Alcohol intake. There was a significant effect of genotype ($F(2, 20) = 3.87$, $p < 0.05$) on alcohol intake over the duration of the experiment. MCHR1 KO mice drank significantly more alcohol than WT or HET mice at the 4% (WT = 5.19 ± 0.50, HET = 5.06 ± 0.5, KO = 6.87 ± 0.53 g/kg body weight mean 24-hr alcohol intake), $F(2,20) = 3.77$, $p < 0.05$, and 10% concentrations (WT = 6.26 ± 1.23, HET = 6.49 ± 1.23, KO = 11.50 ± 1.31 g/kg body weight mean 24-hr alcohol intake), $F(2,20) = 5.3$, $p < 0.05$. There was a trend for the KO mice to drink more 8% alcohol (WT = 8.51 ± 1.08, HET = 10.13 ± 1.08, KO = 12.49 ± 1.16 g/kg body weight mean 24-hr alcohol intake), $F(2, 20) = 3.13$, $p = 0.07$, Figs 8 and 9a.

Water intake. There was no effect of genotype on water intake over the course of the experiment (WT = 2.16 ± 0.19, HET = 2.08 ± 0.19, KO = 2.02 ± 0.21 ml mean 24-hr water intake), Fig 10b. However, there was an interaction between genotype and days ($F(72, 702) = 2.1$, $p < 0.001$), Fig 2d. Total fluid intake was increased in the KO (4.86 ± 0.14 ml mean 24-hr fluid intake) compared to the WT (4.22 ± 0.14 ml mean 24-hr fluid intake) and HET (4.15 ± 0.14 ml mean 24-hr fluid intake), $F(2, 20) = 7.2$, $p < 0.01$, Fig 9b.

Chow intake. KO mice were hyperphagic compared to WT and HET mice throughout the experiment (WT = 146.94 ± 3.67, HET = 149.13 ± 3.67, KO = 168.83 ± 3.93 g/kg body weight mean 24-hr chow intake), $F(2, 20) = 9.5$, $p < 0.01$, Fig 9c.

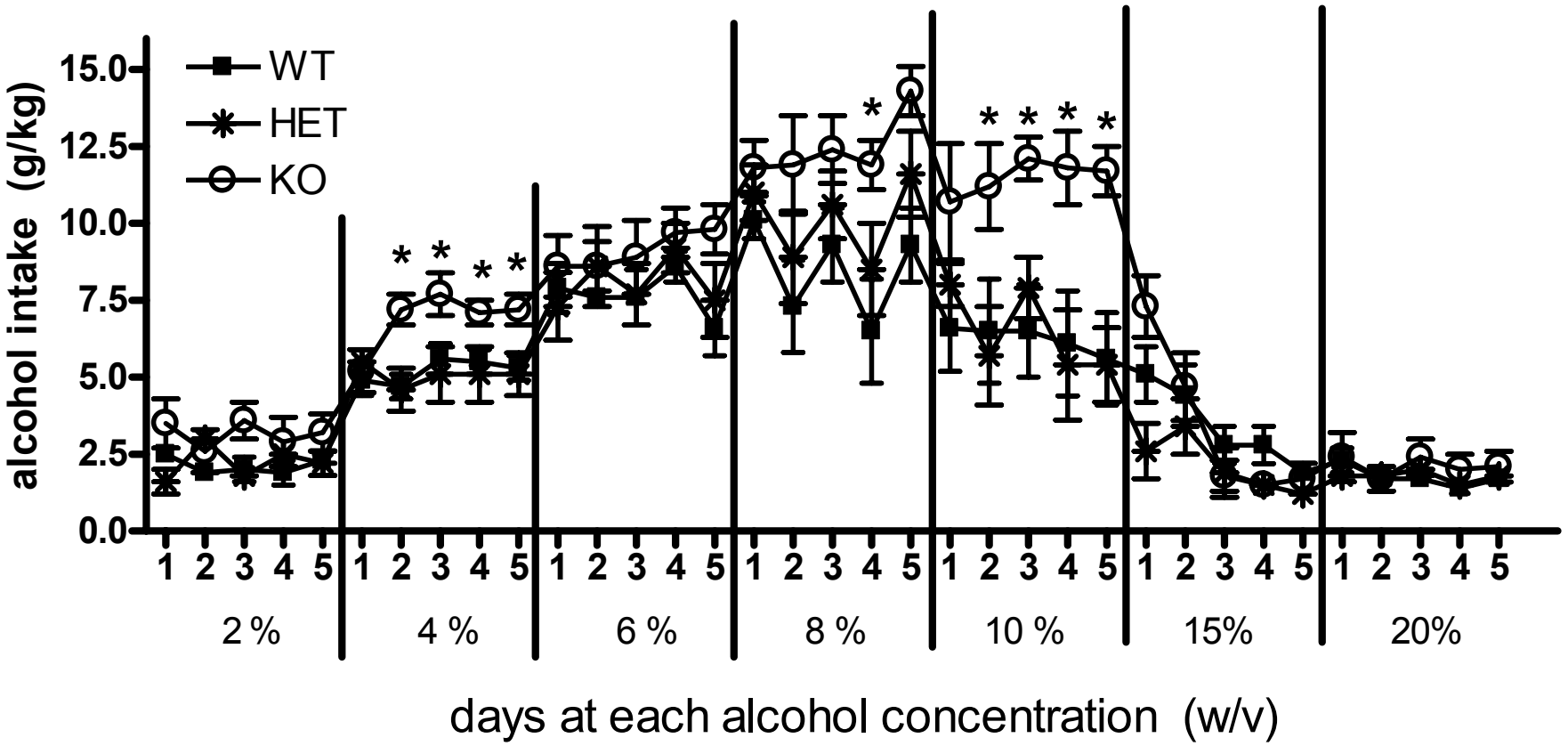

Figure 8. Mean (± SEM) daily alcohol intake across the *ad libitum* alcohol procedure for WT, HET, and KO MCHR1 mice. * Significantly different from WT and HET mice, $p < 0.05$.

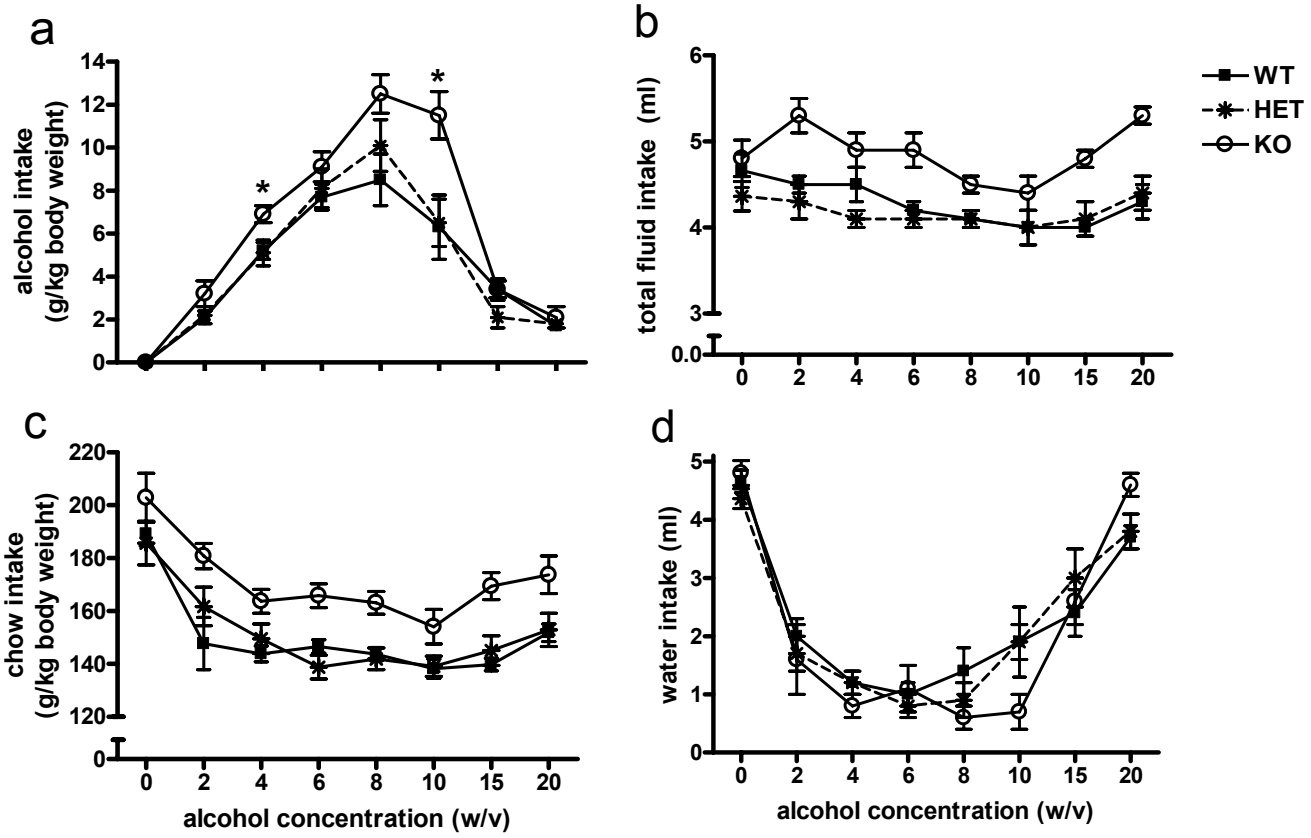

Figure 9. Mean (± SEM) consumption of alcohol (a) total fluid (b), chow (c) and water (d) by MCHR1 WT, HET and KO mice. Each data point represents a 5-day average. * Significantly different from WT and HET, $p < 0.05$.

Ingestive behavior during limited alcohol access

Alcohol intake. There was no effect of genotype on alcohol intake during the limited access procedure (WT = 2.0 ± 0.3, HET = 2.5 ± 0.3, KO = 2.6 ± 0.3 mean daily 1-hr g/kg body weight alcohol intake), Fig 11a. Over the week, daily 1-hr alcohol intake increased over days for all groups (Day 1 = 0.5 ± 0.1, Day 6 = 3.1 ± 0.2 mean 1-hr g/kg body weight alcohol intake of all genotypes combined, $F(5, 100) = 3.6$, $p < 0.001$), Fig 10a.

Water intake. There was no effect of genotype on water intake during the 1-hr alcohol access period (WT = 0.30 ± 0.05, HET = 0.26 ± 0.05, KO = 0.41 ± 0.06 ml), Fig 10c.

Chow intake. During the 1-hr period in which mice received alcohol there was no difference between the genotypes in chow intake (WT = 13.2 ± 1.3, HET = 12.2 ± 1.3, KO = 15.0 ± 1.4 mean 1-hr g/kg body weight chow intake), Fig 11b. There was a significant effect of days in that the 1-hr chow intake decreased for all groups as they became accustomed to receiving alcohol during that hour (Day 1 = 15.2 ± 1.4, Day 6 = 10.6 ± 1.3 mean 1-hr g/kg chow intake of all genotypes combined, $F(5, 100) = 3.6$, $p < 0.01$), Fig 10b. Throughout the limited alcohol access experiment the KO mice consumed more chow compared to WT and HET mice (WT = 151.0 ± 4.6, HET = 152.6 ± 4.6, KO = 174.1 ± 5.0 mean 24-hr g/kg body weight chow intake, $F(2, 20) = 7.1$, $p < 0.01$), Fig 10d.

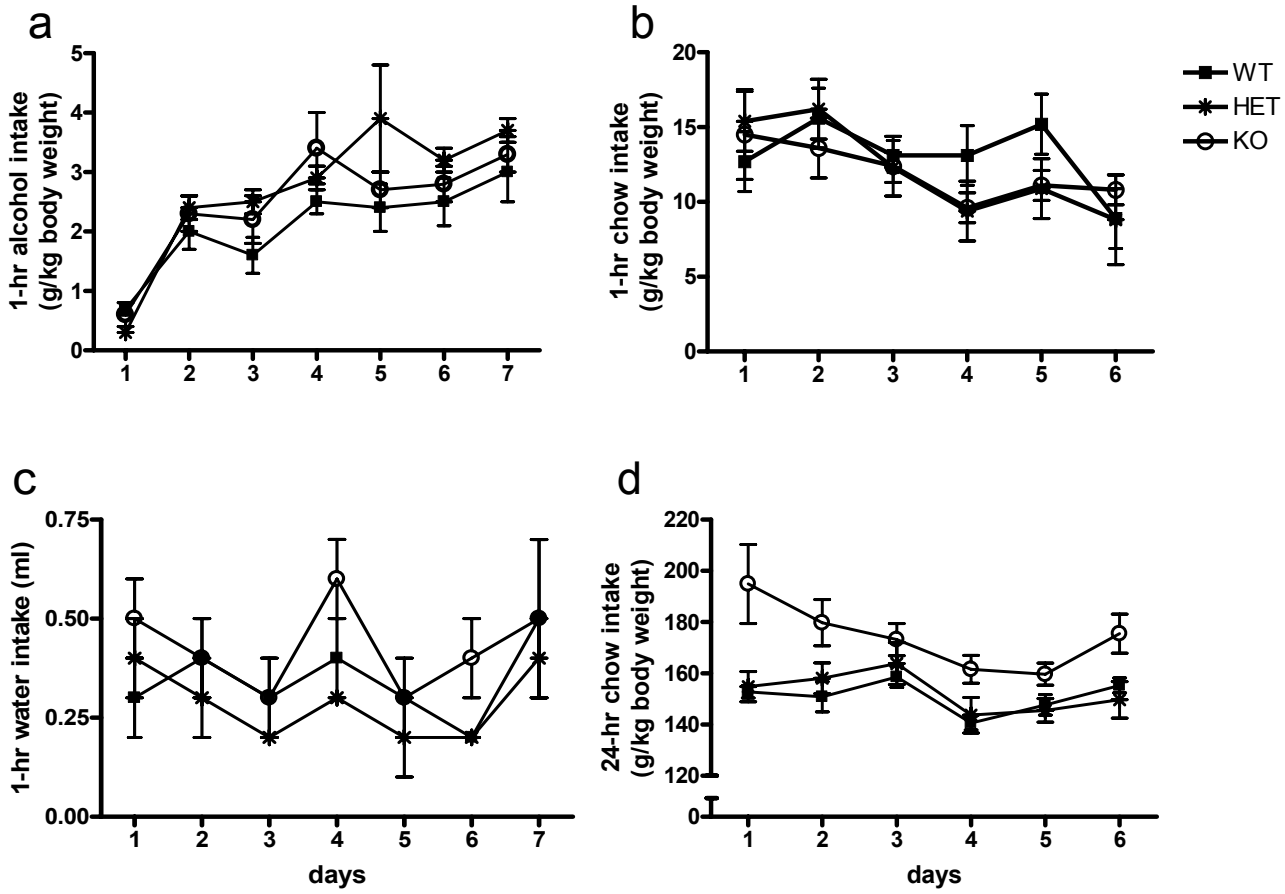

Figure 10. Mean (± SEM) 1-hr consumption of alcohol (a) chow (b) and water (c) and mean (± SEM) 24-hr consumption of chow (d) by MCHR1 WT, HET and KO mice. * Significantly different from WT and HET, $p < 0.05$.

Ingestive behavior during *ad libitum* sucrose/quinine access

Sucrose/quinine intake. Although there was no effect of genotype on sucrose/quinine intake during the 14 day period, there was a significant interaction between the genotype and day variables, $F (26, 260) < 0.001$. The KO mice tended to consume more sucrose/quinine than the WT and HET mice until the amount of quinine reached the 1.3 mM concentration. At this point the KO mice drank less than that of WT and HET, Fig 12a. The 1.3 mM quinine + 17.75 % sucrose solution was the last offered because at this level of quinine the WT mice consumed a similar amount (10.68 ± 3.58 g/kg body weight mean daily sucrose/quinine intake) compared to their intake of 10% alcohol (6.26 ± 1.2 g/kg body weight mean daily alcohol intake). On the other hand the KO mice drank less of the sucrose/quinine solution containing 1.3 mM quinine (KO = 4.1 ± 1.0 g/kg body weight mean daily sucrose/quinine intake) compared to 10% alcohol (11.50 ± 1.31 g/kg body weight mean daily alcohol intake), $p < 0.05$. Finally, there was a significant overall effect of days, $F (12, 260) = 28.6$, $p < 0.001$; sucrose/quinine intake decreased over the 14 day period, Fig 11a.

Water intake. There was no effect of genotype on water intake over the 14 day period. However, there was an effect of days ($F (13, 260) = 31.0$, $p < 0.001$ and an interaction between days and genotypes ($F (26, 260) = 4.5$, $p < 0.001$). Changes in water intake over the 14 period were a reflection of the changes in sucrose/quinine consumption. That is, as the mice drank less sucrose/quinine they consumed more water, Fig 11d. Total fluid intake during the experiment did not differ between genotypes, but did decrease across days, $F (13, 260) = 12.7$, $p < 0.001$, Fig 12b.

Chow intake. Over the 14-day period there was no effect of genotype on chow intake. However, there was a significant increase of chow intake for all groups as the quinine concentration was elevated, $F(13, 260) = 59.8$, $p < 0.01$, Fig 12c. In addition, there was a significant interaction between the genotype and day variables over the 14-day period, $F(26, 260) = 2.1$, $p < 0.01$, Fig 11c. During the last 5 days of the experiment, when the sucrose/quinine solution had become relatively aversive (i.e., 1.3 mM quinine concentration), the KO mice ate significantly more chow (182.1 ± 6.3 g/kg body weight mean daily intake) compared to WT (151.7 ± 5.9 g/kg body weight mean daily intake) and HET (154.3 ± 5.9 g/kg mean daily intake) mice, $F(2, 20) = 7.5$, $p < 0.01$, Fig 11c.

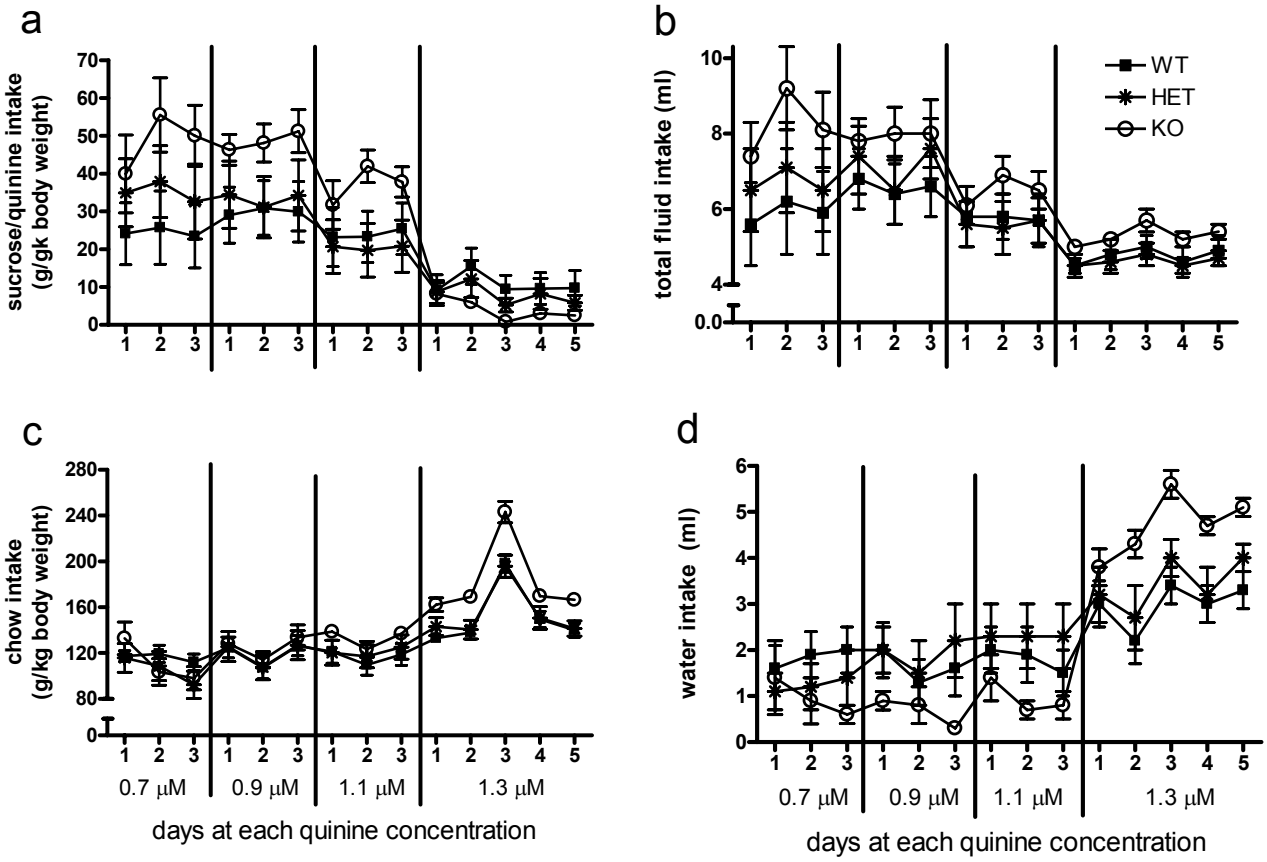

Figure 11. Mean (± SEM) daily consumption of sucrose/quinine (a) total fluid (b), chow (c) and water (d) by MCHR1 WT, HET and KO mice. The quinine concentration was increased every 3 days as indicated on the x axis. * Significantly different from WT and HET, $p < 0.05$.

Body weight and composition

Although KO mice tended to be leaner than WT and HET, there was no significant difference in body weight throughout the study (WT = 24.6 ± 0.5, HET = 24.6 ± 0.5, KO = 24.0 ± 0.6 g mean body weight between 8-14 weeks of age), Fig 12a. Body composition was similar to previous reports (Marsh et al., 2002). KO mice had around 2/3 less body fat (2.0 ± 0.4% fat) than WT (6.3 ± 0.6% fat) and HET (5.9 ± 0.8% fat), $F(2, 20) = 13.1$, $p < 0.001$, Fig 12b, and more lean tissue (WT = 88.1 ± 0.6, HET = 87.9 ± 0.7, KO = 92.0 ± 0.8), F (2, 20) = , $p < 0.001$, Fig 12c.

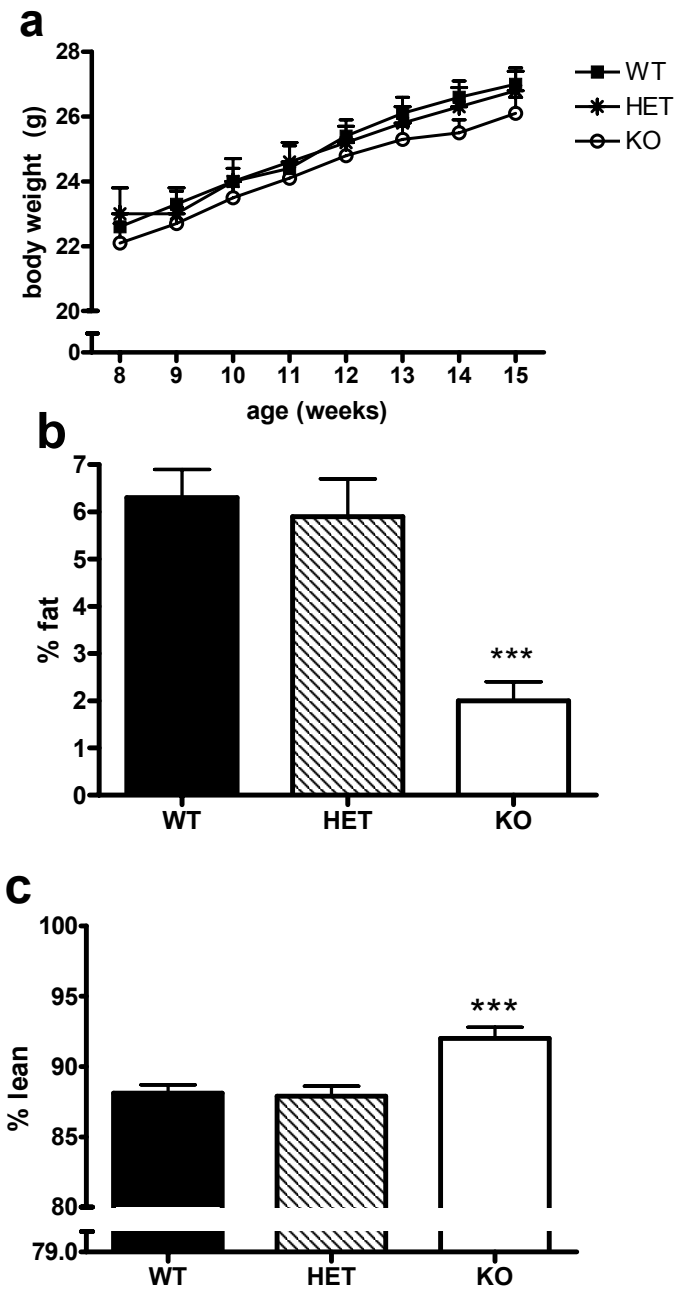

Figure 12. Mean (± SEM) age-matched body weight during the *ad libitum* alcohol access, limited alcohol access and *ad libitum* sucrose/quinine access experiments (a). Mean (± SEM) percent body fat (b) and percent body lean (c) following *ad libitum* sucrose/quinine access. *** Significantly different from WT and HET mice, $p < 0.001$.

Alcohol metabolism

There was no difference between MCHR1 KO and WT mice in blood alcohol levels at any of the time points measured following the administration of 3g/kg body weight alcohol (30 min: WT = 281.9 ± 18.3, KO = 274.3 ± 20.5, 1 h: WT = 284.0 ± 13.2, KO = 286.8 ± 14.7, 3 h: WT = 164.1 ± 14.1, KO = 154.2 ± 15.7, 8 h: WT = 0.7 ± 0.2, KO = 0.8 ± 0.2), data not illustrated.

Anxiety-like behavior

There was a trend for females to spend more time in the open arms than male mice, $F(1, 30) = 4.0$, $p = 0.054$ (female = 67.5 ± 8.6 s, male = 43.3 ± 8.6 s), but no interaction between sex and genotype. There was a significant effect of genotype on time spent on the open arms of the elevated plus maze, $F(2, 30) = 5.39$, $p = 0.01$. KO mice spent more time in the open arms of the EPM (80.5 ± 14.5 s) than HET (53.8 ± 8.9 s) or WT (31.9 ± 8.9 s) mice, $F(2, 30) = 5.4$, $p < 0.05$, Fig 13. These findings are consistent with previous reports of anxiety-like behavior of these genotypes (Roy et al., 2006).

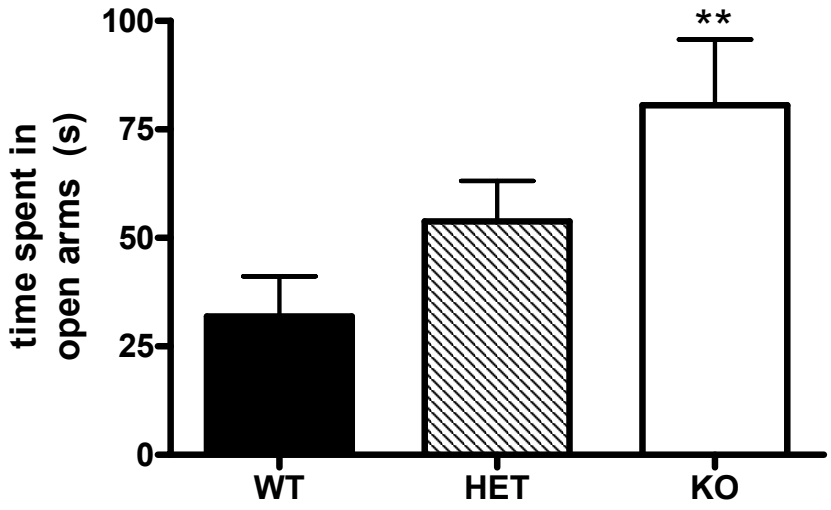

Figure 13. Mean (± SEM) time spent on the open arms of the EPM for MCHR1 WT, HET and KO mice. ** Significantly different than WT mice, $p < 0.01$.

Ingestive behavior following ICV MCH during alcohol access

There was no effect of genotype (WT vs. HET) on any measure of ingestive behavior following MCH administration and thus the two groups were combined for analysis.

Alcohol intake. Central infusion of 5 μg MCH significantly increased 10% alcohol intake (1.01 ± 0.20 g/kg body weight) compared to saline (0.48 ±0.08 g/kg body weight) 1-hr after the infusions, $F(1, 7) = 7.68$, $p < 0.05$, Fig 14a. The effect of MCH on alcohol intake was no longer present 24-hr following the infusion (5 μg MCH = 8.62 ± 2.22, saline = 6.78 ± 1.96 g/kg body weight).

Water intake. There was no effect of MCH on water intake either 1-hr following (5 mg MCH = 0.2 ± 0.1, saline = 0.3 ± 0.1 ml, $F(1, 7) = 0.9$, NS) (Fig 14c) or 24 h following the infusions (5 mg MCH = 1.9 ± 0.6, saline = 2.7 ± 0.6 ml).

Chow intake. Central MCH (5 μg) significantly increased 1-hr chow intake (13.9 ± 3.4 g/kg body weight) compared to saline (3.4 ± 1.2 g/kg body weight), $F(1, 7) = 14.7$, $p < 0.01$, Fig 14b. The effect of MCH was no longer significant 24 hr following the infusion (5 μg MCH = 141.5 ± 20.9, saline = 144.1 ± 13.5 g/kg body weight).

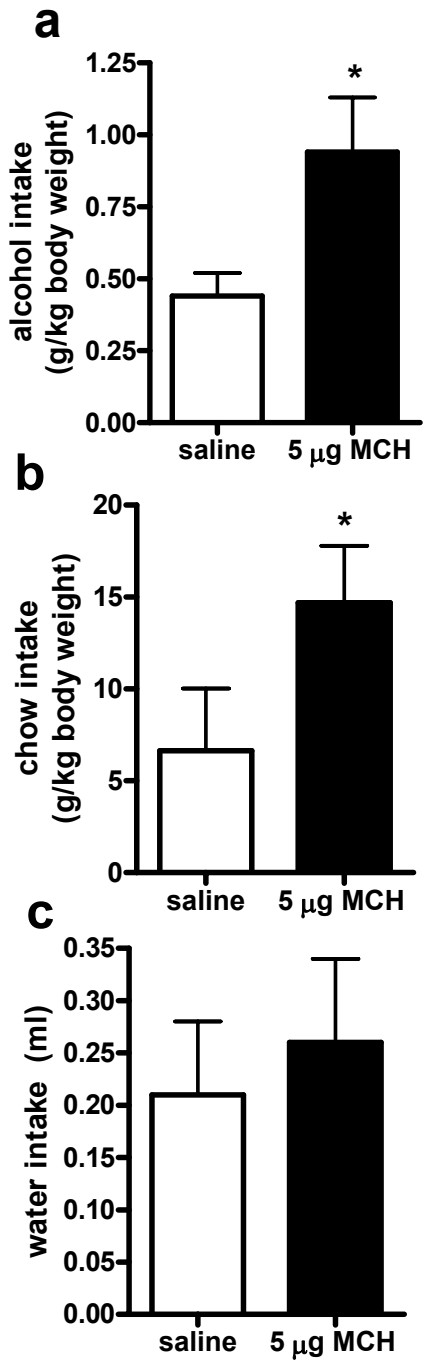

Figure 14. Mean (± SEM) 1-hr consumption of alcohol (a), chow (b) and water (c) following i3vt administration of saline or 5 μg MCH in MCHR1 WT and HET mice.

* Significantly different from saline, $p < 0.05$.

Ingestive behavior following ICV MCH during sucrose/quinine access

Sucrose/quinine intake. Central MCH (5 mg) increased sucrose/quinine intake 1 hr following the infusion (1.9 ± 0.6 g/kg body weight) compared to saline (0.3 ± 0.2 g/kg body weight), $F(1, 7) = 8.4$, $p < 0.05$, Fig 15 a. The effect of MCH on sucrose/quinine intake was no longer present after 24 hr (5 µg MCH = 21.8 ± 7.3, saline = 30.8 ± 6.5 g/kg body weight).

Water intake. Although the animals drank very little water in the first hour following drug infusion there was a significant effect of MCH (0.1 ± 0.03 ml) compared to saline (0.04 ± 0.01), $F(1, 7) = 5.6$, $p < 0.05$, Fig 15 c. There was no effect of MCH on water intake 24 h following the infusion (5 mg MCH = 1.8 ± 0.6 ml) compared to saline (2.2 ± 0.5 ml).

Chow intake. There was only a trend for MCH to increase food intake during the first hour of measurement (5 µg MCH = 7.1 ± 3.1, saline = 2.0 ± 0.7 g/kg body weight), Fig 15 b. There was also no significant effect of MCH on 24-hr chow intake (5 µg MCH = 126.9 ± 13.6, saline = 121.1 ± 15.9 g/kg body weight).

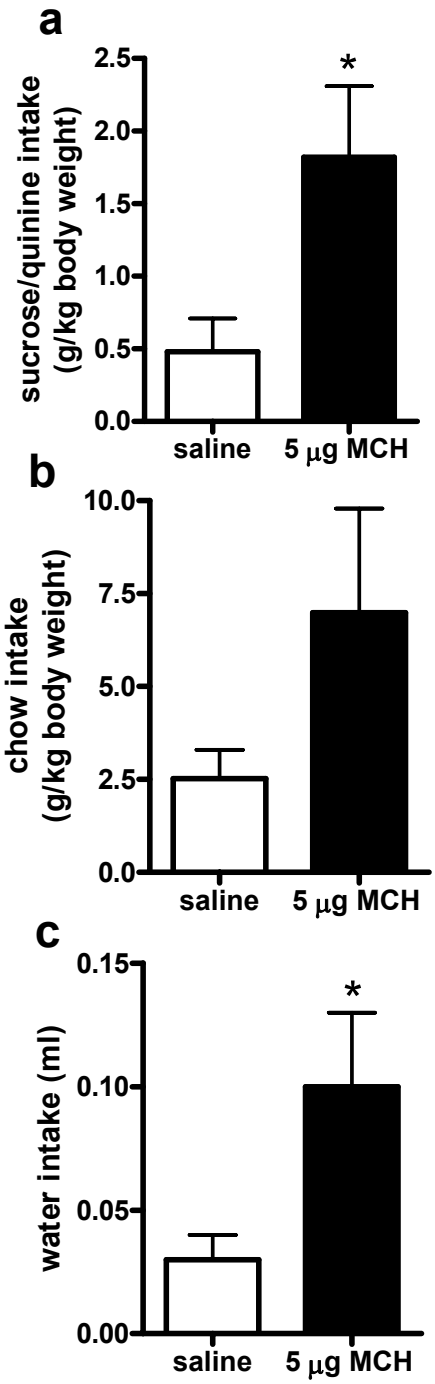

Figure 15. Mean (± SEM) 1-hr consumption of sucrose/quinine (a), chow (b) and water (c) following i3vt administration of saline or 5 μg MCH in MCHR1 WT and HET mice.

* Significantly different from saline, $p < 0.05$.

DISCUSSION

The goal of these studies was to test the hypothesis that endogenous MCH signaling enhances alcohol drinking. Consistent with previous reports, the MCHR1 KO mice utilized in the study were lean and hyperphagic (Chen et al., 2002; Marsh et al., 2002), and displayed less anxiety-like behavior relative to the WT and HET mice (Roy et al., 2006). Although there was an effect of a loss of endogenous MCH signaling on alcohol consumption, it was not in the anticipated direction. MCHR1 KO mice consumed more alcohol than WT and HET mice. The difference in alcohol consumption among genotypes was not due to an effect of MCH signaling on alcohol metabolism, as all mice cleared a bolus of alcohol at the same rate. Further, because the KO mice exhibited less anxiety-like behavior than WT or HET mice, anxiety is not likely involved in the alcohol drinking phenotype. Finally, based on the sucrose/quinine consumption data, it is not likely that differences in taste preference account for the alcohol drinking phenotype of the KO mice. The KO mice tended to drink more of the sucrose/quinine solution compared to WT and HET mice when it was relatively sweet (i.e., low quinine concentration). However, when the quinine concentration was elevated to the point where the WT mice consumed similar amounts to 10% alcohol, the KO mice drank less than both WT and HET mice. Thus, the KO mice had a lower preference a bitter tasting solutions than the WT mice.

Because our previous finding, that MCH enhanced alcohol intake, was in a rat model (Duncan et al., 2005), it became important to control for a possible species effect of MCH on alcohol intake in the present study. The results were clear and demonstrate

that mice and rats respond in an analogous way to central MCH administration. Rats consume more alcohol, sucrose/quinine, water and chow, 2-hr following i3vt administration of 10 μg MCH (Duncan et al., 2005), while in the present study mice consumed more alcohol, sucrose/quinine, water, and chow, 1-hr following i3vt administration of 5 μg MCH. Thus, either genetically reducing or pharmacologically increasing MCH signaling in mice augments alcohol drinking.

The findings of the this study are reminiscent of the seemingly paradoxical observations that the MCHR1 KO mice are hyperphagic compared to the WT mice (Chen et al., 2002; Marsh et al., 2002). It is counterintuitive that an absence of MCH signaling would result in either increased chow intake or augmented alcohol consumption. In many ways, alcohol is similar to food; it is a macronutrient with more calories per gram than chow, and it is consumed orally. Therefore, it is possible that the mechanism responsible for the hyperphagic phenotype of the MCHR1 KO mice is also leading the mice to drink more alcohol. However, the actual underlying cause of the hyperphagia is not clear. It has been proposed that the KO mice eat more merely to compensate for their hyperactivity and increased lean mass (Marsh et al., 2002). That is, they must consume more calories just to maintain a healthy body weight. The hyperactivity of the MCHR1 KO mice is thought to be a result of an increased sensitivity to mesolimbic dopamine. The MCHR1 KO mice are more sensitive to the locomotor-enhancing effects of amphetamine and have increased D1 dopamine receptor binding in the mesolimbic dopamine system compared to WT mice (Smith et al., 2005b). The involvement of the mesolimbic dopamine system implicates that the hyperactivity may be the result of

altered reward or motivation processing (Smith et al., 2005b). Thus, the elevation in mesolimbic dopaminergic signaling in the KO might increase goal-directed behavior in general resulting in hyperactivity, hyperphagia, and increased alcohol drinking.

The hypothesis that altered reward processing is responsible for the increased alcohol intake in the MCHR1 KO mouse may be correct. The rewarding properties of alcohol are believed to be a result of increased extracellular dopamine levels in the NAc. Oral alcohol self-administration has been demonstrated to increase mesolimbic dopamine release in both rodents and humans (Boileau et al., 2003; Weiss et al., 1993), and pharmacological blockade of dopamine receptors in the NAc is reported to reduce alcohol self-administration (Samson et al., 1992). As the MCHR1 KO mice are more sensitive to D1 receptor binding in the NAc, it is possible that they experience the rewarding aspects of alcohol more intensely than WT mice and are therefore more motivated to ingest the drug. Indeed, it has been reported that MCHR1 KO mice are more sensitive to the locomotor activating effects of alcohol (Ferraro, 2004).

Some of the data in the present study also suggest that elevated alcohol intake in the MCHR1 KO mice is due to altered reward processing. First, the consumption of chow does not seem to be offset by the extra calories provided by alcohol, at least at the 10% alcohol concentration. The same cannot be said of the sucrose/quinine data. During the first 9 days of sucrose/quinine access, when the quinine content was relatively low, the KO mice choose more of the sucrose/quinine solution, but equal amount of chow, compared to WT and HET mice. These data suggest that the MCHR1 KO mice might be

drinking more alcohol for its pharmacological properties instead of the calories provided by the alcohol. On the other hand, the finding that there was no effect of genotype on alcohol intake in the limited access procedure goes against a reward-mediated mechanism. If the KO mice were consuming more alcohol solely due to a change in the drugs rewarding properties the increase in alcohol intake found in the KO mice would not be limited to *ad libitum* alcohol access procedure.

The major finding of the current study is that MCHR1 KO mice consume more alcohol than WT and HET mice. This finding does not support the hypothesis that endogenous MCH enhances alcohol drinking. However, the peculiar hyperphagic phenotype of these mice is also inconsistent with the established role of MCH as a signal of negative energy balance. Therefore, it is premature to label the hypothesis null based exclusively on the findings in this study. Regardless of whether endogenous MCH signaling is involved in alcohol consumption, the present findings lend some support to the idea that the MCH is involved in the regulation of reward processing in general.

CHAPTER 6

General Discussion and Future Directions

The overall objective of this dissertation was to explore the effect of MCH on alcohol consumption. Compared to the classic neurotransmitter systems (e.g., dopamine, glutamate, serotonin), little is known about how neuropeptidergic systems regulate alcohol intake. A better understanding of the role of neuropeptides, including MCH, in the motivation to drink alcohol could provide novel pharmacological targets for the treatment of alcohol use disorders. In the case of MCH, there were many factors indicating that the peptide would be involved in the regulation of alcohol consumption. MCH is known to be critically involved in the regulation of energy balance (Pissios and Maratos-Flier, 2003). In addition, the peptide is believed to modulate anxiety, reward and fluid balance (Carlini et al., 2006; Clegg et al., 2003; Georgescu et al., 2005). As all of these are factors that are implicated in the etiology of alcoholism (Spinosa et al., 1988; Swendsen et al., 1998; Thiele et al., 2003; Wise, 1987), it seemed likely that MCH would be involved in the regulation of alcohol intake..

Initially it was hypothesized that the ability of MCH to modulate alcohol intake would be related to the peptide's anxiolytic properties. Hence, unlike its action to increase food and water intake, MCH was predicted to decrease alcohol intake. Experiments in Chapter 3 tested this hypothesis by centrally administering MCH into a rodent model of voluntary alcohol intake. The findings did not support the hypothesis. MCH potently enhanced alcohol, as well as sucrose/quinine intake in rats. Further, the dose of MCH that significantly increased alcohol drinking had no effect on anxiety-like behavior assessed in the elevated plus maze (Duncan et al., 2005). Although the data did not support the initial hypothesis, they demonstrated a novel role for MCH in the

regulation of alcohol drinking. Following these initial experiments, the remainder of the dissertation research focused on investigating two specific aims. First, experiments were devised to explore the involvement of endogenous MCH signaling in alcohol consumption. Second, an attempt was made to understand the underlying factors responsible for MCH's impact on alcohol drinking.

Endogenous MCH signaling and alcohol intake

Experiments in Chapters 4 and 5 tested the hypothesis that endogenous MCH signaling enhances alcohol intake. Chapter 4 tested this hypothesis pharmacologically, utilizing an MCHR1 antagonist. The antagonist effectively blocked chow intake, but had no impact on alcohol or sucrose/quinine consumption in rats. At first glance, these data suggested that endogenous MCH signaling did not regulate the ingestion of rewarding solutions. However, the antagonist was also ineffective in blocking MCH-induced alcohol drinking. These data suggest that blocking MCHR1 signaling does not have a simple unidirectional impact on alcohol drinking. For instance, the antagonist could have acted in the hypothalamus to reduce the drive for energy intake (Abbott et al., 2003), while acting in the preoptic nucleus to increase anxiety (Gonzalez et al., 1996), resulting in both a decrease and an increase in the drive to consume alcohol. No clear conclusion regarding the role of endogenous MCH signaling in alcohol drinking could be drawn from these data. Thus an additional experiment utilizing a different model was incorporated to explore the hypothesis.

Chapter 5 utilized mice lacking MCHR1 to test the hypothesis that endogenous MCH signaling increased the drive to drink alcohol. The prediction was that the loss of MCH signaling would lead to a reduction in alcohol drinking in this model. However, the MCHR1 KO mice actually drank more alcohol than wildtype mice. Although these data do not support the hypothesis, there is an important caveat that must be addressed with this model. Genetic mouse models in general can have phenotypic characteristics (e.g., change in baseline anxiety, change in sensitivity to alcohol, change in alcohol metabolism or change in caloric intake) that can determine the amount of alcohol they will consume. These phenotypes might be the direct result of a loss of function of the particular gene, but they also could be the result of developmental compensation. In Chapter 5, the MCHR1 KO mice had some phenotypic characteristics that seem to be the result of developmental compensation as opposed to a loss of MCHR1 signaling. These specific phenotypes might explain the increased alcohol drinking. First, although a loss of MCH would be expected to result in a reduction in feeding behavior, the MCHR1 mice are hyperphagic (Chen et al., 2002; Marsh et al., 2002). The increased drive to obtain calories might be responsible for the increase in alcohol consumption. Second, the MCHR1 KO mice are more sensitive to dopamine signaling within the mesolimbic dopamine pathway (Smith et al., 2005b). This disarrangement of signaling within the so-called reward pathway may be responsible for the elevation of alcohol drinking and/or an increase in goal-directed behaviors in general.

Studies investigating delta-opioid receptor (DOR) signaling, and its role in alcohol drinking, have resulted in strikingly similar findings to those reported in this

dissertation for MCHR1. Analogous to the hypothesis guiding this dissertation, activation of the DOR by endogenous opioid peptides was predicted to increase alcohol consumption. However, pharmacological experiments that selectively blocked endogenous DOR signaling resulted in inconsistent findings. While some studies did report a reduction of alcohol intake (Kim et al., 2000; Krishnan-Sarin et al., 1995a; Le et al., 1993; Middaugh et al., 2000), others reported no effect of the DOR antagonist on alcohol drinking (Honkanen et al., 1996; Ingman et al., 2003; Stromberg et al., 1998). Further, in direct opposition to the hypothesis, DOR knock-out mice were found to drink more alcohol than wildtype mice (Roberts et al., 2001). The elevated alcohol consumption observed in the DOR knock-out mice is thought to be a result of an anxiogenic phenotype (Filliol et al., 2000). The similarities between the DOR and MCHR1 stories illustrate the difficulty of these types of experiments. It is likely that much work will be necessary to fully understand the role of these endogenous peptidergic signals in alcohol drinking.

Future studies investigating this problem should focus on the location, duration and/or extent of receptor blockade in the animal models utilized. The importance of the specificity of receptor blockade cannot be underestimated. The neuropeptide Y (NPY) system is a good example of how the brain region investigated is crucial to the outcome of the study; amygdalar NPY signaling reduces the drive to drink alcohol by reducing anxiety (during withdrawal),(Pandey, 2003) while hypothalamic NPY increases alcohol intake by signaling a need for energy consumption (Kelley et al., 2001). The localization of action could be an important factor in understanding the role of endogenous MCH

signaling in alcohol drinking. MCHR1 is expressed ubiquitously, but does not serve the same purpose throughout the CNS. For example, microinjections of MCH into discrete nuclei within the hypothalamus can either increase food intake, or have no effect (Abbott et al., 2003).

As it is likely that role of MCH in alcohol consumption will be region-specific it is important to consider the location or extent of blockade of the MCHR1 in the interpretation of the data collected in Chapters 4 and 5. Both experiments utilized models that were intended to block endogenous MCHR1 signaling throughout the CNS. The genetic mouse model provided a complete CNS deletion of the MCHR1. Conversely, the extent of diffusion of the MCH and the MCHR1 antagonist following intracerebroventricular (i3vt) administration is not known, and thus it is not clear which MCHR1s were actually reached in the pharmacological studies. Although the cerebrospinal fluid circulates throughout the brain, it is likely that only the brain regions surrounding the 3rd ventricle were contacted by the administered compounds (Fenstermacher and Kaye, 1988; Nagaraja et al., 2005). This could provide a simple explanation for the finding that both pharmacologically increasing and genetically decreasing MCH signaling in the mouse results in an increase in alcohol consumption. Developmental compensation aside, the MCHR1 knock-out mice had a complete lack in MCH signaling throughout the CNS, while the mice that received MCH into the 3rd cerebral ventricle likely only had enhanced signaling in a subset of MCH receptors. The receptors that are not contacted by i3vt administration, but are deleted in MCHR1 knock-out mice might be responsible for the different outcomes.

Additionally, it cannot be assumed that administration of MCH reached the same receptors as the MCHR1 antagonist, as the two have slightly different structures, and thus different rates of diffusion and break-down. Hence, there is a small possibility that the MCHR1 antagonist did not attenuate MCH-induced alcohol intake because it did not reach the receptors involved, or it was broken down more quickly than MCH itself. Indeed, the ability of the antagonist to block MCH-induced water intake was short-lived (i.e., gone after 6-hr) suggesting that Compound B (the MCHR1 antagonist) was broken down more quickly than MCH during the study.

Because of the issues described above, it is important in future studies to investigate discrete brain regions in order to better understand the impact of endogenous MCH in alcohol drinking. Little has been done to investigate the functions of discrete populations of MCH receptors outside of the hypothalamus. Brain regions that have the most intense expression of MCHR1 are the hippocampus, amygdala and the shell of the nucleus accumbens (Saito et al., 2001). There are only a handful of studies that deal with the function of MCH signaling in these brain regions. Administration of MCH into the shell of the nucleus accumbens is reported to augment feeding behavior and increase depressive-like behavior in the forced swim test (Georgescu et al., 2005). Hence, in this region MCH could stimulate alcohol drinking either through activating the circuitry involved in feeding or depression. In the amygdala MCH is reported to reduce anxiety (Monzon et al., 2001) and increase food intake (Carlini et al., 2006). Therefore, amygdalar MCH might reduce the need for alcohol due to the reduced level of anxiety, or

increase alcohol intake via its impact on ingestive behavior. Finally, hippocampal MCH signaling is reported to reduce anxiety (Monzon et al., 2001) increase memory retention (Monzon et al., 1999), but have no impact on feeding behavior (Carlini et al., 2006). Hence, if anything, hippocampal MCH signaling would reduce alcohol consumption through an anxiety-specific mechanism. The thrust of these predictions is that modulation of alcohol consumption by endogenous MCH signaling will be dependent on the brain region tested.

Factors underlying the impact of MCH on alcohol drinking

Anxiety

None of the evidence in this dissertation has supported a role for anxiety in the impact of MCH on alcohol drinking. The findings that both MCH and the MCHR1 antagonist did not impact anxiety-like behavior in rodents at doses used to modulate alcohol intake indicate that anxiety is not an important factor in MCH-induced alcohol consumption. Further, the finding that MCHR1 KO mice drink more alcohol, but are less anxious than wildtype mice provides more evidence that anxiety is not involved in the regulation of alcohol drinking by MCH. However, it is important to point out that in this dissertation, only models of voluntary alcohol intake were studied. The data do not speak to the possible role of MCH in anxiety-induced alcohol intake during alcohol withdrawal. Other neuropeptides, such as corticotropin-releasing hormone and NPY, which have been demonstrated to be important regulators of anxiety, have been implicated in motivation to consume alcohol following alcohol withdrawal (Hwang et al., 2004; Pandey, 2003;

Valdez et al., 2002). The role of MCH in the regulation of anxiety is still a matter of debate, but it is likely to be significant player (Hervieu, 2003). Therefore, it is possible that MCH will be implicated in the motivation to consume alcohol during alcohol-withdrawal through an anxiety-specific mechanism. A better understanding of the effect of MCH on anxiety is advisable before exploring this possibility.

Fluid balance

There are data to support a role for MCH in the regulation of fluid homeostasis. MCH administration in rats increases water intake independently of food intake (Clegg et al., 2003; Sakamaki et al., 2005). Therefore, it might be suggested that MCH-induced alcohol consumption is motivated by thirst. However, angiotensin II administration, which increases volumetric thirst, has actually been reported to reduce alcohol intake. When given a choice between water and alcohol following angiotensin II administration, thirsty animals chose water exclusively (Blair-West et al., 1996; Grupp et al., 1988a). In this dissertation, it was demonstration that MCH increased alcohol consumption along with water intake when the two were simultaneously present. Therefore, it is not likely that the augmentation of alcohol intake was a reflection of thirst.

Energy balance and reward

Recently there has been newfound interest for the involvement of systems that regulate energy balance in the etiology of alcoholism (Thiele et al., 2003; Thiele et al., 2004b). However, the possibility that the energetic properties of alcohol are important for the regulation of alcohol intake is longstanding (Richter, 1953). As MCH is an

established signal of negative energy balance in the CNS, it is feasible that the impact of MCH on alcohol intake reported in this dissertation is merely a result of an increased drive to consume energy. There is some evidence that would support this hypothesis. A study investigating the impact of MCH on the consumption of various types of solutions found that MCH augmented the ingestion of sweet solutions that contained calories (i.e., sucrose and glucose), while not affecting consumption of a saccharin solution (Sakamaki et al., 2005). This study suggests that effect of MCH on ingestive behavior is driven solely by its influence on energy balance. Further, another study found that MCH administration increased the ingestion of both a high fat and low fat diet, suggesting that the peptide does not discriminate based on dietary preference or the relative reward value the caloric source (Clegg et al., 2002). Both of these studies utilized i3vt administration of MCH and thus it could be that this mode of administration results in a preferential activation of MCHR1s important for energy balance. If this is the case, then the ability of MCH to increase alcohol intake following i3vt administration is probably due to an increased drive to consume calories.

Nevertheless, it is difficult to parse the role of energy balance from reward and motivation. Calories comprise a natural reward for the body, while energy status can alter the rewarding properties of other non-caloric rewards (Cabeza de Vaca and Carr, 1998). Data collected in Chapter 4 seem to support the idea that reward is a factor involved in the effect of MCH on alcohol drinking. I3vt administration of MCH increased the amount an animal would work to obtain either sucrose or alcohol over a 4-hr period suggesting that MCH enhances the rewarding properties of both sucrose and

alcohol. Perhaps i3vt administration of MCH enhances the rewarding properties of calories in general, regardless of macronutrient type, or relative palatability.

MCH and other drugs of abuse

In future studies, a possible way to separate the role of energy balance from reward in the effect of MCH on alcohol drinking is to study the impact of MCH on the self-administration of another drug of abuse. Alcohol and other drugs of abuse are thought to elicit reward by a universal mechanism (Wise and Bozarth, 1987). Thus, it is likely that if MCH is increasing alcohol drinking through a reward-specific mechanism, it will also increase the self-administration of other drugs of abuse, which are not consumed orally and do not provide the body with energy. On the other hand if MCH is merely increasing the drive to take in calories, independently from reward, the peptide would not impact the self-administration of such a drug. Moreover, if MCH is lowering reward thresholds in general in the brain one might expect that the peptide itself would be self-administered. However, self-administration experiments with endogenous substrates as opposed to drugs of abuse (i.e., cocaine, heroin, morphine) are difficult to carry out because the peptides are broken down too quickly to have a substantial effect (personal communication, Ikemoto, S., Behavioral Neuroscience Branch, National Institute of Drug Abuse). Still, it might be feasible to investigate this possibility using a long-lasting MCH receptor agonist.

The importance of elucidating the role of MCH in reward processes in general gains support from the findings that have been reported recently for the orexin system.

Orexin is a neuropeptide that has a very similar anatomical profile to MCH. Like MCH, orexin is produced by neurons in the lateral hypothalamus, but the two neuropeptides are not co-expressed. That is, MCH and orexin are made by separate populations of neurons (Broberger et al., 1998). Further, the two orexin receptors (orexin receptor 1 and 2) are expressed in a strikingly similar pattern to that of the MCHR1 in the rodent brain (Kilduff and de Lecea, 2001). These anatomical similarities suggest that the peptides may serve as redundant signals in the CNS. Indeed, orexin was named for its ability to increase food intake (Rodgers et al., 2002), but it is now more widely noted for its role in sleep and arousal (Siegel, 2004).

Recently, many studies have reported that orexin is able to regulate reward by activating ventral tegmental (VTA) dopamine neurons. Orexin-containing neurons project directly to the VTA (Fadel and Deutch, 2002), and increase the firing rate of dopamine neurons (Korotkova et al., 2003). Further, orexin is important for the rewarding properties of morphine. Prepro-orexin knock-out mice are less prone to become dependent to morphine or suffer withdrawal symptoms from the drug (Georgescu et al., 2003). In addition, blocking orexin signaling in the VTA of rats abolishes the formation of a conditioned place preference to morphine (Narita et al., 2006). Unlike orexin, MCH was found to have no direct impact on VTA dopaminergic neuron firing (Korotkova et al., 2003). However, the fact that mice lacking MCHR1 have increased dopamine receptor sensitivity (Smith et al., 2005b) supports the possibility that MCH is a modulator of the mesolimbic dopamine system. Because of the dense expression of MCHR1 in the shell of the nucleus accumbens it might be the case that MCH modulates

dopaminergic activity indirectly by acting on neurons in the dopaminergic terminal field, as opposed to the dopamine neurons themselves (like orexin). More work is necessary to understand the relationship between the MCH and dopamine neuronal networks and the implications for this interaction on the reward system.

Conclusion

In conclusion, the research described in this dissertation demonstrates a novel role for MCH in the regulation of alcohol consumption. The findings suggest that the effect of MCH on alcohol intake is due to the peptide's role in reward and/or energy balance, but not fluid balance or anxiety. The hypothesis, that endogenous MCH signaling increases the motivation to consume alcohol is likely too simple to sufficiently describe the role of MCH in alcohol drinking. Although it is apparent that MCH is able to increase alcohol intake, the role of endogenous MCH in the regulation of alcohol intake remains vague. Future studies should focus on discrete brain regions that receive projections from MCH neurons and express the MCHR1, in order to elucidate the role of endogenous MCH signaling in alcohol drinking.

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