Effects of CRF-R1 and Opioid Antagonism on Alcohol Intake in C57BL/6J Mice

Tala Kayyali
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Tufts University, Medford, MA

Tala M. Kayyali, Lara. S. Hwa, Joseph F. DeBold, Klaus A. Miczek

Abstract

Modulation of corticotropin-releasing factor (CRF) neural pathways and the opioid system has been promising for reducing ethanol intake in alcohol-dependent rodents and humans. Specifically, CRF receptor 1 (CRF-R1) antagonism in brain sites such as the dorsal raphé nucleus (DRN) has been suggested to alter output involved in alcohol drinking. Naltrexone, an opioid antagonist, is a drug that is currently being used in the treatment of

alcoholism. This research explores the role of antagonism of CRF-R1 and the opiate system in the DRN as they affect ethanol consumption in C57BL/6J mice. In a series of experiments, animals were given intermittent access to alcohol. After establishing stable drinking levels, mice received systemic injections of naltrexone (0.1, 1.0, 10.0 mg/kg) and then were implanted with guide cannulae into the DRN. Different doses of a CRF-R1 antagonist and naltrexone were infused intra-DRN to test for a reduction of alcohol consumption. Measures of withdrawal and blood ethanol concentration were taken to verify the alcoholism model in mice. Another batch underwent surgery after achieving stable drinking levels, then received intra-DRN infusions using different doses of CP-154,526, naltrexone, or a combination. Current findings indicate that both CP 154,526 and naltrexone are effective in significantly reducing ethanol intake, but do not affect water intake. The combination of both did not show an additive effect, but it did result in a greater reduction in ethanol intake than each drug had individually. This could mean that CRF and opioid receptors work independently on serotonin in the DRN. Future studies could investigate other areas where CRF and opioid receptors are co-localized such as the central amygdala or the locus coeruleus.

Effects of CRF-R1 and Opioid Antagonism on Alcohol Intake in Mice

Alcohol, Alcoholism and Society

Alcohol is a ubiquitous substance that can be found all over the world; however patterns of its use and abuse vary by region and culture. Alcohol dependence extends beyond mere drug use. Alcoholism involves the excessive consumption of alcohol and a shift from controlled to

uncontrolled heavy drinking with chronic cycles of abstinence and relapse (Funk et al. 2007). The study of how alcohol affects the body is extremely important because of its tangible effects on everyday life (Kamdar et al., 2007). The detrimental consequences of alcohol abuse range from domestic violence, to crime, and tragic accidents. They affect the user, their family, friends, and society (Müller et al., 2010). It continues to be a tenacious issue in many different countries.

Epidemiological data

According to the World Health Organization (WHO), global alcohol consumption has increased in recent decades (WHO, 2010). Worldwide, alcohol causes 2.5 million deaths per year and it ranks as the fifth highest risk factor for premature death and disease (WHO, 2010). The WHO also estimates that about 55% of adults have consumed alcohol (WHO, 2010).

There are several factors that contribute to problems related to alcohol use and abuse. Social, cultural, political, environmental, and genetic factors in addition to employment status and socioeconomic standing all influence drinking behavior (Bloomfield et al., 2003). According to the WHO, the worldwide total consumption of alcohol was equal to 6.13 liters of pure alcohol per person (15 years and older) in 2005. They estimate that unrecorded consumption accounts for nearly 30% of the worldwide total adult consumption. In terms of heavy drinking, about 1.5% of drinkers across the world have weekly heavy *episodic* drinking occasions. On average, males tend to consume more alcohol than females, and they drink more frequently (Chen et al., 2009). Consumption levels vary such that middle and upper-middle classes typically consume more that their working class counterparts. One of the main groups of concern is teenagers. The rates and patterns of alcohol consumption for under-aged individuals are of critical importance. According to a report by Eaton et al. (2008), 75% of high school students have tried alcohol. Previous

studies have shown that the age when one initially tries alcohol affects future use (Faden, 2006). Since alcohol affects so many groups in society, determining the neurobiological basis of alcoholism could provide an effective means to tackle alcohol-related problems.

Animal models of alcohol intake

Animal models attempt to understand alcoholism at the physiological, biochemical and molecular levels (Tabakoff & Hoffman, 2000). Developing translational models of excessive alcohol drinking could help better understand human alcohol drinking (Hwa, 2011). Animal models allow for a systematic evaluation of potential pharmacotherapies to reduce ethanol intake and reward (Middaugh et al., 1999). They allow us to screen new compounds for potential efficacy and to explore the etiology of excessive alcohol drinking (Kamdar et al., 2007).

C57BL/6J is an inbred strain of mice that has a high avidity for ethanol solutions (McClearn & Rodgers, 1959; Elmer et al., 1988; Lê et al., 1994; Phillips et al., 1994Phillips, Wenger & Dorow, 1997; Middaugh et al., 1999). This strain has also been shown to respond to ethanol reward (Elmer, Meisch, & George 1986; Elmer et al., 1988). It has been used in alcohol research for more than five decades due to its high alcohol preference (Belknap et al., 1993; Rodgers, 1966). In addition, several studies have demonstrated that C57BL/6J mice drink to pharmacologically relevant levels (Becker & Lopez, 2004). For example, studies found that C57BL/6J mice achieved blood ethanol levels of greater than 80 mg/dL following the drinking in the dark procedure (Sparta et al., 2008). Other studies have found that these mice consume more alcohol after repeated cycles of chronic ethanol exposure and withdrawal experiences (Becker & Lopez, 2004). They exhibit clear signs of behavioral intoxication (Rhodes et al., 2005, 2007;

Lowery & Thiele, 2010), dependence (Becker & Lopez, 2004) and withdrawal (Becker & Lopez, 2004).

Method	Description	Origin	Main Advantages	Main Disadvantages
One bottle forced(Ethanol liquid diet)	Animal only has an alcoholic drinking fluid	Lieber et at., 1963	Creates elevated alcohol drinking	Forced: only source of nutrition + fluid
Vapor Chamber	Forced vapor inhalation	Schulteis et al., 1995	Achieves high levels of drinking dependence, withdrawal	Lack of choice Human drinking does not involve inhalation
Sucrose fading	A sweet solution (e.g., saccharin) is used to introduce animals to alcohol and the concentration of sweetness is gradually reduced	Samson, 1986	Animals prefer sweet solutions and readily consume alcohol if it is combined with sugar	Does not assess aversion to taste Not alcohol is naturally sweet
Two bottle choice Intermittent Access	Episodic presentations of alcohol that represent repeating cycling of ethanol access and deprivation	Le Magnen, 1960	Mimic repeated cyclical alcohol access and deprivation: Cardinal feature of human drinking High voluntary consumption	Difficult to demonstrate motivation
Drinking in the Dark	The water bottle is replaced with a bottle containing 20% ethanol for 2 or 4 hours in the home cage starting 3 hours into the dark cycle.	Kamdar et al., 2006	Demonstrates voluntary alcohol drinking. Finding: animals drink the highest levels 3 hours into the dark cycle	Limited access to alcohol

In order to gain a better understanding of alcoholism, different models of animal drinking have evolved since 1940 (Spanagel, 2000). The following table presents a summary of the main methods used to mimic human alcohol drinking in animals:

Intermittent Access Protocol

Over the past decades, different models have been developed to capture the cardinal features of alcoholism in animals yet no procedure fully represents all aspects of human alcohol

disorders. Cultural and social aspects are especially difficult to attain, yet high levels of drinking have been observed using the different methods described in the table above. It is therefore important to use a model that mimics deliberate, free-choice, preferential alcohol drinking compared to another fluid.

Escalated alcohol drinking tends to be highly episodic in both humans (WHO, 2010) and animals (Breese et al., 2005). Dependence may be driven by repeated cycles of high ethanol intake followed by deprivation, similar to electrophysiological kindling (Ballenger and Post, 1978). 'Kindling' was modeled after the increasingly heightened responses (sensitization) to repeated electrical brain stimulation (Goddard et al., 1969). In the context of alcohol dependence, it is hypothesized that each withdrawal-induced episode of central nervous system hyperexcitability may serve as a stimulus, in a kindling-like process (Ballenger and Post, 1978). A vicious cycle of escalated alcohol drinking and alcohol abstinence/withdrawal may develop over time, where individuals drink increasing amounts to manage increasing numbers and severity of stressors. According to this model, an increase in alcohol drinking is not due to repeated alcohol exposures, but rather, repeated experiences of alcohol withdrawal and its symptoms underlie the progression in drinking. The kindling-driven schedule of access to alcohol may generate dependence over repeated cycles (Ballenger and Post, 1978).

In addition, the alcohol deprivation effect (ADE) could be an important factor in the success of the intermittent access method. ADE comprises periods of ethanol access which alternate with periods of deprivation in weekly cycles (Spanagel ,1996; Melendez, 2006). One of the results of the alcohol deprivation effect includes a pronounced increase in ethanol consumption and preference (after imposed abstinence). In line with the kindling hypothesis and the alcohol deprivation effect; intermittent presentations of alcohol have been shown to

substantially elevate 2-bottle choice drinking in rats (Pinel and Huang, 1976; Wayner and Greenberg, 1972; Wise, 1973). Recently, Simms and colleagues (2008, 2010) revived the method that was used in the 1970s that pertains to intermittent access. The procedure demonstrated that intermittent access to 20% ethanol in outbred rats induced high ethanol intake without the need of sucrose fading (Samson, 1986) or water deprivation (Meisch & Thompson, 1972). In a study by Li et al. (2009), intermittent access to 10% ethanol increased motivation for operant ethanol self-administration in rats. Li et al. (2009) speculated that intermittent ethanol exposure is involved in increasing motivation for ethanol responding by affecting neurotransmitter activity.

Neural mechanisms involved in excessive drinking

Alcohol has important pharmacological effects on different neurotransmitter systems including the opioidergic, dopaminergic, serotonergic and GABAergic systems (Volpicelli et al., 1995). This study will focus on the opioid system and corticotrophin-releasing factor as they relate to alcohol drinking.

The Opioid System

a. Anatomy

The discovery of the opioid system is rooted in ancient history as a consequence of opium use (Le Merrer et al., 2009). Opium, which is extracted from poppy seeds (*Papaver somniferum*) has strong pain relieving properties and produces euphoria (Le Merrer et al., 2009). Opioid binding sites in the brain were identified in 1973 by Pert and Snyder; Simon et al., and Terenius almost simultaneously. Since then, endogenous ligands: enkephalin (Hughes et al., 1975), dynorphin (Goldstein et al., 1979), and β -endorphine (Bradbury et al., 1976), and opioid peptides have been discovered. Research reveals that the opioid receptor gene family

includes four members encoding mu, delta, kappa and the non-opioid orphaninFQ/nociceptin receptors (Le Merrer et al., 2009).

b. Receptors

Opioid receptor protein and mRNA distribution have been mapped extensively with regard to brain region and opioid receptor subtype (Mansour et al., 1995). Opioid receptors are membrane receptors with a 7-transmembrane topology (Le Merrer et al., 2009). They are G-protein coupled receptors (Le Merrer et al., 2009). In terms of the distribution of the different opioid receptors, previous studies have found that mu, delta, and kappa opioid receptors are found in the nucleus accumbens (Wamsley et al., 1980; George et al., 1994; Tuchan et al., 1999; Meshul and McGinty, 2000). The different receptors correspond to converging and diverging roles in different brain areas.

c. Functions

The opioid system has a central role in nociception and analgesia as well as pain regulation (Le Merrer et al., 2009). It is also involved in the regulation of responses to stress, respiration, gastrointestinal transit, endocrine and immune functions, mood and well-being, and addictive behaviors (Dickenson & Kiefer, 2005; Le Merrer et al., 2009; Zollner & Stein, 2007).

Opioids and Alcohol

Opioids play a key role in modulating addictive behaviors (Le Merrer et al., 2009). The opioid system is a major component of brain reward pathways (Koob, 1992; Self and Nestler, 1995). One of the important pharmacological actions of ethanol includes increases in opioid signaling (Froehlich and Li, 1994) possibly by stimulating the release of endogenous opioids,

which may produce the euphoric feelings associated with alcohol consumption (Volpicelli et al., 2004). Volpicelli et al. (1994) explain that endogenous opioids (such as β-endorphin) are released into the synapse and stimulate activity at opiate receptors, which produces a signal in the target neuron. It has been hypothesized that this activates the dopamine reward pathway by inhibiting GABAergic cells that normally disinhibit the dopamine system (Froehlich, 1996). The neurochemical and behavioral effects of alcohol may be mediated by the opioid systems (Middaugh et al., 1999). The blockade of central opioid receptors can inhibit ethanol-induced increases in extracellular dopamine concentrations in brain areas associated with reinforcement and reward such as the nucleus accumbens (Hodge et al., 1992, 1997; Weiss et al., 1993). This mechanism could explain the suppressive effects of naltrexone, an opioid antagonist, on ethanol intake (Gonzales and Weiss, 1998).

Opioid Antagonists

Opioid antagonists could be important therapeutic agents with regards to alcohol abuse (Middaugh et al., 1999). Opioid receptor antagonists have been found to reduce ethanol consumption or preference in mice, rats, monkeys and humans (Altshuler et al., 1980; De Witte, 1984; Samson & Doyle, 1985; Volpicelli et al., 1988, 1992; Weiss et al., 1990; Lê et al., 1993; O'Malley et al., 1992). Different receptors may have different roles with regard to alcohol consumption. However, research on the role of the mu receptor in alcohol intake is inconclusive. One study found that blocking the receptor using the irreversible mu antagonist β-funaltrexamine (β-FNA) had no effect on alcohol consumption (Lê et al., 1993). However, Hyytia (1993) reported reductions in ethanol intake in AA rats (an alcohol preferring strain) (Gianoulakis, de Waele & Kiianmaa, 1992) after the blockade of mu opiate receptors.

Froehlich et al. (1991) saw a substantial reduction in ethanol intake of rats after

peripheral administration of the delta opiate receptor antagonist ICI 174864. Pretreatment with the delta opiate receptor antagonist, naltrindole, also resulted in a significant reduction in alcohol intake in C57BL/6J mice (Lê et al., 1993). Both Froehlich et al. (1991) and Lê et al. (1993) suggest that enkephalin systems may be the most important endogenous opioid ligands in the regulation of ethanol reinforcement in rodents. It has been established that this system and its different receptors play an important role in affecting alcohol intake. As such, one of the current Food and Drug Association (FDA) treatments is an opioid antagonist, naltrexone.

Naltrexone

Naltrexone (*N*-cyclopropylmethyloroxymorphone, EN 1639A) was first synthesized by Blumberg et al. (1965). It is a nonselective competitive opioid antagonist (Volpicelli et al., 1992; Kiefer et al., 2003). It has a preferential affinity for mu opiate receptors but it can act on other opiate receptors depending on the administered dose (Kosterlitz, Corbett & Paterson, 1989). According to Verebey and Mule (1975), naltrexone does not have any addictive properties. A review by Volipicelli at al. (1995) confirmed that naltrexone is safe and well tolerated by humans. It has been FDA approved since 1995 (National Institutes of Health, 2010). It is used to reduce the rate of relapse to uncontrolled alcohol intake in alcohol-depende nt individuals (Volpicelli et al., 1992; Phillips, Wenger & Dorrow, 1997). Moreover, naltrexone has been found to be effective in reducing alcohol consumption in C57BL/6J mice even up to 50% relative to baseline control groups (Lê et al., 1993). This strain has heightened opioid function (De Waele & Gianoulakis, 1992, 1994), and they possess higher levels of β-endorphin (Gianoulakis & Gupta, 1986).

Naltrexone and Alcohol

In a study on the effects of naltrexone on ethanol drinking acquisition and established ethanol consumption in C57BL/6J mice, Phillips, Wenger and Dorrow (1997) found that naltrexone reduced ethanol preference in established drinkers. Higher doses showed diminished effects on reducing alcohol intake (Phillips, Wenger & Dorrow, 1997). The reduced efficacy of higher doses could be due to naltrexone-induced opiate receptor changes (Phillips, Wenger & Dorrow, 1997) since naltrexone is thought to block opioids from activating opiate receptors (Volpicelli at al., 1994). Lê et al. (1993) hypothesize that most of the effects of naltrexone on ethanol drinking can be attributed to its ability to block delta opiate receptors. Naltrexone was also found to slow the acquisition of ethanol drinking (Phillips, Wenger & Dorrow, 1997). This effect however could not be replicated when naltrexone was re-administered after a phase when ethanol was offered without any naltrexone treatment (Phillips, Wenger & Dorrow, 1997). Chronic administration of naltrexone through naltrexone pellets resulted in greater amounts of ethanol consumption possibly due to naltrexone-induced opioid receptor changes (Phillips, Wenger & Dorrow, 1997). Importantly, the effects of naltrexone are specific to ethanol intake as it does not affect water intake (Middaugh et al., 1999). It has also been suggested that opioid signaling plays a specific role in ethanol drinking behaviors, not in 10% sugar-water drinking or plain-water drinking (Kamdar et al, 2007). This may be evidence that supports a different pathway for reward for ethanol than natural rewards such as sugar-water or plain-water (Kelley and Berridge, 2002; Kamdar et al., 2007).

Naltrexone has been an effective treatment for alcoholism due to its ability to reduce craving in abstinent, dependent individuals and because it reduces the pleasure associated with subsequent alcohol intake (Phillips, Wenger & Dorrow, 1997). When ethanol is consumed, dopamine levels transiently increase in extracellular spaces (Melendez et al., 2002). This increase

possibly contributes to ethanol's reinforcement properties and to addiction (Melendez et al., 2002). Since increases in opioid signaling are a consequence of alcohol drinking (Froehlich and Li, 1994), these increases activate the dopamine reward pathway (Froehlich, 1996). Consequently, it has been suggested that naltrexone may reduce ethanol drinking by blocking ethanol-induced increases in dopamine signaling, thereby diminishing any rewards from ethanol (Froehlich, 1996). Naltrexone patients reported reductions in the "high" experience when they drank alcohol (Volpicelli et al., 1995; Phillips, Wenger & Dorrow, 1997). Other studies have also reported that it reduces cravings for alcohol (O'Malley et al., 1992, 1996; Volpicelli et al., 1992).

Although reward properties of alcohol are very important, discriminative stimulus or cue properties are also considered an important component of alcohol abuse (Middaugh et al., 1999). Studies suggest that opioid antagonists have little influence on the interoceptive effects of ethanol in operant ethanol discrimination experiments (Altshuler et al., 1981; Spanagel, 1996).

Summary adapted from substance abuse treatment advisory, 2005:

Name	Naltrexone (Revia)
Mechanism of Action	• Blocks brain opioid receptors • Eliminates euphoria associated with alcohol use • Makes alcohol use less rewarding • Does not cause sickness of alcohol is ingested • Reduces craving
Possible Drug Interactions	Opioid containing medications, thioridazine, yohimbine and oral hypoglycemic.
Side Effects	Most common: opioid withdrawal symptoms (insomnia, nausea, vomiting, anxiety, headache, abdominal pain, muscle aches, rash, dizziness, fatigue, constipation, and chills). Less common but more serious: potential liver toxicity (especially at high doses) and suicidal ideation.
Contraindications and Cautions	Hypersensitivity to the drug, any use of narcotic analgesics, liver disease, and acute opioid withdrawal, FDA pregnancy category C (potential fetal risk or no conclusive results).

As noted above, alcohol is influenced by a variety of peptides and systems in the brain. In

addition to the opioid system, another important peptide that affects alcohol intake is corticotrophin-releasing factor (CRF).

Corticptropin Releasing Factor (CRF)

a. Anatomy and Distribution

Corticotropin-releasing factor (CRF) is a peptide consisting of 41 amino acids (Vale et al., 1981). It is a neuromodulator that is widely expressed throughout the central nervous system (Sparta et al., 2008) and was first isolated from ovine hypothalamus (Vale et al., 1981). According to Cooper, Bloom and Roth (2003) the cellular origin of mammalian CRF was traced to the portion of the paraventricular nucleus that projects to the median eminence but not into the posterior pituitary. CRF-containing circuits were identified as innervating an extensive group of neurons in the pons, medulla, cortex and amygdala, possibly representing a stress-related circuitry (Cooper, Bloom & Roth, 2003). It is a very potent adrenocorticotropic hormone (ACTH) secretogogue (Cooper, Bloom & Roth, 2003). Its effects are significantly augmented by vasopressin as well as norepinephrine and angiotensin (Cooper, Bloom & Roth, 2003). Initial efforts to identify CRF receptors focused on ligand binding autoradiography which replicated the tissue patterns of CRF immunoreactivity with highest binding in the cortex, amygdala, hippocampus, and pons (Cooper, Bloom & Roth, 2003 as well as systems that regulate the output of the pituitary and the autonomic nervous system (Koob, 2008). According to Koob (2008), the longer central fibers course either ventrally through the medial forebrain bundle and its caudal extension in the reticular formation, or dorsally through a periventricular system in the thalamus and brainstem. It is also expressed in the projection from the inferior olive to the cerebellar cortex containing CRF and glutamate (Cooper, Bloom & Roth, 2003).

b. Functions

CRF is involved in food intake, stress and anxiety-like behaviors as well as neurobiological responses to ethanol (Sparta et al., 2008). One major role for CRF is to activate the hypothalamic-pituitary-adrenal stress response (Vale et al., 1981). A second major role for CRF is to act extra-hypothalamically. High concentrations of CRF can be found in the hypothalamus, the bed nucleus of the stria terminalis (BNST), the hippocampus, and amygdala; brain regions involved in mediating stress-related cognitive functions (Swanson et al., 1983; Contarino & Papaleo, 2010). The functions of CRF are mediated by two receptor subtypes and the binding protein.

c. Receptors

The CRF system has a complex role in affect regulation due to the presence of two G-protein-coupled receptor subtypes: CRF1 receptor (CRF-R1) and CRF2 receptor (CRF-R2) as well as the CRF binding protein (Risbrough et al., 2004; Contarino & Papaleo, 2010). In terms of the expression of receptor subtypes, CRF-R1 is ubiquitous throughout the brain with high density found in hypothalamic, cortical and limbic regions while CRF-R2 expression is limited to the raphé nuclei, lateral septum and sub regions of the amygdala and hypothalamus (Hauger et al., 2006; Lowery & Thiele, 2010).The two receptor subtypes may have opposite functions in their role in affective-like behaviors (Contarino & Papaleo, 2010) or could be acting sequentially or in concert (Risbrough et al., 2004). While CRF-R1 may be involved in increased anxiety-like responses, CRF-R2 may be responsible for anxiolytic–like and anti-depressant like effects. (Risbrough et al., 2004; Contarino & Papaleo, 2010).

d. Binding properties

CRF binds to both the CRF1 receptor (CRF-R1) and CRF2 receptor (CRF-R2) but has a greater affinity to CRF-R1 (Ryabinin et al., 2002; Hauger et al., 2006; Pioszak et al., 2008; Lowery & Thiele, 2010). Urocortin is a 40-amino acid peptide that is highly related to CRF as it has 45% sequence identity (Vaughan et al., 1995; Cooper, Bloom & Roth, 2003; Lowery and Thiele, 2010).The urocotrin family includes Urocortin 1 (Ucn1), Urocortin 2 (Ucn2), and Urocortin 3 (Ucn3) (Lowery & Thiele, 2010). Urocortin is the endogenous ligand for CRF-R2 (Cooper, Bloom & Roth, 2003). Ucn1 displays equal affinity for both CRF receptor types while Ucn2 and Ucn3 display affinity primarily for CRF-R2 (Ryabinin et al., 2002; Venihaki et al., 2004; Hauger et al., 2006; Lowery & Thiele, 2010).

e. CRF and Alcohol

According to Contarino and Papaleo (2010), CRF and its pathways may mediate the motivational effects of drug dependence. Previous studies have found that both acute and chronic ethanol exposure activate central CRF pathways (Koob et al. 1993; Ramussen et al., 2000; Rivier et al., 1984). Studies have also reported increased levels of CRF during ethanol withdrawal (Merlo Pich et al., 1995).

Current pharmacological and genetic research provides evidence to support the hypothesis that CRF exerts its effects on ethanol consumption through the activation of the CRF-R1 rather than CRF-R2 or the CRF binding protein. In terms of pharmacological manipulations, CRF receptor antagonists have been found to reverse the anxiogenic effects of ethanol withdrawal in ethanol dependent rats (Breese et al., 2004; Knapp et al., 2004; Overstreet et al., 2004; Rassnick et al., 1993). They have also been found to reduce voluntary alcohol drinking after forced alcohol exposure (Rimondini et al., 2002; Valdez et al., 2002; Gehlert et al., 2007) and reduce stress-related symptoms and affective states associated with acute ethanol withdrawal

(Baldwin et al., 1991; Valdez et al., 2003; Contarino & Papaleo, 2010). In terms of genetic manipulations, studies have shown that CRF-R1 deficient mice did not show increased levels of ethanol consumption following the acquisition of ethanol dependence and a period of abstinence as was observed in wild-type mice (Chu et al., 2007). In addition, a genetic polymorphism at the Crhr1 locus was found to be significantly linked to alcoholism (Treutlein et al., 2006). Treutlein et al. (2006) analyzed 14 genetic variations of the CRH-receptor 1 gene . Specific Crhf1 genotypes and both htSNPs were associated with higher amounts of binge drinking. Altered availability of CRH-receptor1 could predispose juveniles to alcohol drinking when under stress. This could be important if different CRF-related treatments for alcohol affect people differently.

Acute alcohol withdrawal induces anxiety-like responses that are correlated with increased CRF levels in sites that mediate behavioral stress responses such as the central nucleus of the amygdala and the BNST (Olive et al., 2002; Gehlert et al., 2007). Accordingly, anxiogenic effects of alcohol withdrawal are reversed by CRF antagonism (Baldwin et al., 1991). Several CRF-R1 antagonists have been used to alter ethanol consumption in rodents (Koob et al. 1993). One of the prominent features of CRF-R1 antagonists is their selective action on alcohol consumption in operant-conditioning for alcohol reinforcement or two-bottle choice alcohol consumption in post-dependent animals (Funk et al., 2007; Gehlert et al., 2007). In their study, Gehlert et al. (2007) used Wistar and Marchegian Sardinian (mSP) rats that were trained to drink alcohol. Operant self-administration of ethanol was used to establish stable drinking levels. Rats then received 0, 3.0 and 10.0 mg/kg MTIP (a selective CRF-R1 antagonist), 30 minutes before self-administration sessions. MTIP decreased lever presses for ethanol reinforcement in ethanol-dependent rats to non-dependent levels of consumption (Gehlert et al., 2007).

Importantly, CRF manipulations have been found to affect ethanol-dependent but not

independent rodents (Funk et al., 2007; Gehlert et al., 2007). Funk et al. (2007) trained Wistar rats to orally self-administer ethanol. The rats were also exposed intermittently to ethanol vapors to induce dependence (control rats were exposed to air). After a 2-hour withdrawal period, all animals were systemically administered anatalarmin, MJL-1-109-2 or R121919 (CRF-R1 antagonists), and ethanol self-administration was measured. They found that the CRF-R1 antagonists selectively reduced excessive self-administration of ethanol in dependent animals during acute withdrawal but had no effects on the nondependent rats. Gehlert et al. (2007) reported that rats without a history of dependence were not affected by any drug dose.

Another CRF-R1 antagonist that is currently being used in alcohol studies is CP-154,526, the first non-steroidal CRF antagonist to be used (Schultz et al., 1996). Using systemic injections of CP-154,526, Sparta et al. (2009) observed dose-dependent reductions in operant responding for ethanol with C57BL/6J mice that had undergone 4 days of alcohol deprivation. C57BL/6J mice received intraperitoneal injections of 4 doses of CP-154,526 (0, 1.0, 3.0, 10.0 mg/kg), 30 minutes before receiving their ethanol bottle. Their results revealed that CRF-R1 signaling modulates high but not moderate levels of ethanol drinking.

CRF and the Opioid System

CRF-opioid interactions have been demonstrated in different areas of the brain including the locus coeruleus and hypothalamus (Contarino & Papaleo, 2010). Studies by Land and colleagues (2008; 2009) have suggested that the dysphoric properties of stress are encoded by CRF-induced activation of the endogenous dynorphin opioid system. Stein and Lang (2009) have suggested that CRF could elicit opioid increases by binding to CRF receptors. The combination of medications and therapies may offer the best approach to the treatment of alcohol-dependant

patients (Volpicelli et al., 1995). One brain site at which both CRF and opioid receptors have been documented is the dorsal raphé nucleus. The study of the interaction between CRF and opioid receptors could be important as an alternative treatment. The following section will explain the importance of this brain area and its main neurotransmitter, serotonin, in respect to alcohol.

Previous studies have targeted specific neural receptors that are more prone to stimulation by alcohol in order to understand the neural foundations of episodic, escalated alcohol drinking. An important monoamine that relates to alcohol consumption is serotonin (5-HT).

Serotonin

a. Anatomy

Serotonin (5-HT) was first isolated in the brain by Twarog and Page (1953). The serotonergic system is a collection of neurons and nuclei that send ascending and descending projections: the rostral mainly ascending in the forebrain and the caudal mainly into the spinal cord (Hornung, 2010). Serotonin-containing neurons originate in the brainstem, mainly in the raphé nuclei (Dahlström & Fuxe, 1964). The dorsal raphé is a crucial area in terms of brain serotonin as it contains the largest collection of forebrain-projecting 5-HT neurons (Molliver, 1987; Jacobs & Azmitia, 1992). It is the primary source of serotonin in the mammalian brain containing a third of all serotonergic neurons in the brain, and its projections reach structures involved in the regulation of stress, affective state and analgesic responses (Land et al., 2009, Amat et al., 2004; Zhao et al., 2007, Hornung, 2010). The anterior DRN projects primarily to the cerebral cortex, neostriatum, amygdala and substania nigra (Steinbusch et al., 1980; van der Kooy & Hattori, 1980; Waterhouse et al., 1986; Vertes, 1991; Corvaja et al., 1993). The caudal division of the dorsal raphé, extending to the pons, projects to the hippocampus, the entorhinal

cortex and the locus coeruleus (Kohler and Steinbusch, 1982; Imai et al., 1986; Datiche et al., 1995, Vertes & Crane, 1997). Other targets of the dorsal raphé include the prefrontal cortex and the nucleus accumbens (Van Bockstaele et al., 1993), superior colliculus (Villar et al., 1988), thalamus (Westlund et al., 1990), lateral septum or striatum (Waselus et al., 2006), somatosensory, auditory, or vestibular nuclei (Thompson et al., 1995; Simpson et al., 2003; Halberstadt & Balaban, 2006; Lee et al., 2008), the motor nuclei of the brainstem (Li et al., 1993), the cerebellum and the spinal cord (Kazakov et al., 1993; Li et al., 1993; Petit et al., 1995).The largest population of serotonergic cell bodies is located within the ventral portion of the periaqueductal gray area (PAG) in the DRN (Tao & Auerbach, 2002; Basbaum & Fields, 1984). Overall, the serotonergic sub nuclei are integrated in many different systems including the cardio-respiratory system, the pain system, the motor system and the limbic system.

b. Functions

Serotonin has many different functions including roles in appetite and ingestion control, mood, brain reward, development, basal sensory-motor control, sexual behavior, sleep, pain, aggression and others (Müller et al., 2010). It also plays a role in disorders related to these adaptive functions including drug addiction, emotion and anxiety disorders, eating disorders and others (Müller et al., 2010). Previous studies have established that the serotonin system has a major role in stress-related psychiatric disorders (Cowen, 1993; Heils et al., 1997; Lesch, 1991; Mann, 1999; Nordstrom and Asberg, 1992, van Praag, 1984). It has also been connected to the reinforcing effects of alcohol (McBride, 2010). The PAG is an area innervated with opioids and it is involved in integrating responses to stress (Tao & Auerbach, 2002; Basbaum & Fields, 1984). Drug addiction also produces profound changes in extracellular 5-HT and 5-HT receptor function (Müller et al., 2010). Furthermore, serotonin influences other neurotransmitter systems

(Adell et al., 2010). The DRN is a very important player in the 5-HT system as its afferent connections provide the neuroanatomical substrate for the interaction between different brain areas and serotonergic neurons (Adell et al., 2010). Therefore, the DRN is a likely site of 5-HT dysfunction (Heils et al., 1997; Arrango et al., 2002; Zalsman et al., 2006) and its study offers further insight into ingestion behavior and drug abuse.

c. Serotonin and alcohol

Alcohol connects several different functions of serotonin including its role in ingestion behavior, drug abuse, and the stress response. Previous studies have found that 5-HT is involved in the regulation of alcohol preference in animals (Naranjo et al., 1986; Sellers et al., 1992; McBride & Li, 1998) and humans (Gorelick & Paredes, 1992; Underwood et al., 2004; Wong et al., 2003). Koob and colleagues (Koob et al., 1997; Koob & Le Moal, 1997) suggested that cessation of drug use would cause serotonin neurotransmission in the nucleus accumbens to decrease, resulting in a dysphoric negative state of withdrawal. Alcohol preferring (P) rats appear to have fewer raphé neurons compared to non-preferring rats due to reduced innervations and raphé neurons (Zhou et al., 1991; Müller et al., 2010). The general overall reduction in 5-HT modulation may promote higher levels of alcohol intake in P rats (Müller et al., 2010). The inverse relationship between 5-HT function and alcohol intake implies that increasing brain serotonin should result in decreased ethanol intake (Müller et al., 2010). `Murphy et al. (1985, 1988) and McBride et al. (1990, 1992) reported a reduction in ethanol drinking in alcohol preferring (P) rats following the administration of fluoxetine, a 5-HT reuptake inhibitor. Additionally, the administration of low doses of 8-OH-DPAT, a 5-HT_{1A} receptor agonist acting on somato-dendritic autoreceptors, increased ethanol intake due to inhibition of 5-HT impulse flows (Tomkins et al., 1994; McKenzie-Quirk & Miczek, 2003). Overall, serotonin seems to

have a negative modulatory effect on brain reward systems such that activity in the DRN reduces rewarding effects of alcohol (McBride, 2010).

d. Serotonin interactions with neurochemical systems

Given the established role of serotonergic DRN neurons in escalated alcohol consumption, neuropeptides that affect this system are prime targets for stress-related modulations of alcohol drinking. Corticotropin-releasing factor (CRF)-immuno-reactive fibers densely innervate the DRN in a topographically intricate manner (Kirby et al., 2000, Sakana et al., 1987; Swanson et al., 1983). The organization of CRF fibers within the DRN is such that they are poised to regulate specific forebrain regions in accordance with their topographical distribution (Waselus and Bockstaele, 2007). Both CRF-R1 and CRF-R2 receptor subtypes are found in the DRN, but there is a relatively high expression of CRF2 receptors in that area (Chalmers et al., 1995). According to Waselus and Bockstaele (2007), the interaction of CRF immunolabeled axon terminals with postsynaptic targets varies in accordance with the specific DRN sub regions. They explain that CRF labeled axon terminals were most frequently found with somatodendritic processes in the dorsolateral DRN, whereas axon terminals containing CRF in the ventromedial DRN were most often associated with other axon terminals including GABAergic or glutamatergic ones (Waselus and Bockstaele, 2007).

Different mechanisms underlie the effects of stress of the DRN-5-HT system and subsequent substance abuse. One of these mechanisms is CRF-R1 mediated inhibition of the DRN 5-HT that occurs during acute stress (Price et al., 1998; Price & Lucki, 2001). CRF-R1 mediated inhibition results in a hyposertonergic state that could contribute to the initiation of substance abuse by possible promoting impulsive behavior (Price et al., 1998; Price & Lucki, 2001). Price and Lucki (2001) and Lukkes et al. (2008) have shown that low doses of CRF (that

are more selective for CRF-R1) led to decreased extracellular serotonin in the forebrain while high doses of CRF (that are more selective for CRF-R2) led to higher concentrations of serotonin (Amat et al., 2004).

Another peptidergic system that influences serotonin is the opioid peptide system. According to Le Merrer et al., (2009) mu opioid receptors are present in moderate density in the DRN. Immunocytochemical (Kalyuzhny et al., 1996) and neurochemical (Tao & Auerbach, 1995) studies have demonstrated a role for opioids in modulating serotonergic neuronal activity (Tao & Auerbach, 2002). Stressful stimuli activate opioidergic neurons in the PAG, which may modulate the activity of serotonergic neurons (Tao & Auerbach, 2002; Grahn et al., 1999). Also, Land et al. (2009) hypothesize that the effects of opioid receptors in the nucleus accumbens on brain dopamine could be mediated via DRN projections to the nucleus accumbens. Adverse effects of stress may converge on the serotonergic system. Mansour et al. (1988) report that a moderate density of the mu receptors could be found in the DRN. According to Tao and Auerbach (2002), opioid receptor subtypes may play different roles in modulating serotonin neurotransmission. Their study used the selective mu receptor antagonist, β-funaltrexamine to block extracellular 5-HT increases in the DRN using in vivo microdialysis. They demonstrated that mu ligands have a selective influence on serotonergic neurons in the DRN as they did not find an effect in the median raphé nucleus or the nucleus accumbens. According to Wang et al. (1998), mu opioid receptors are localized on the postsynaptic membrane of dendrites, extra-synaptic plasma membrane, and the surface of the small, clear vesicles in axon terminals in the DRN.

Given the connections of CRF and the opioid system, specifically the mu opioid receptors, to 5-HT in the DRN, and their influence on alcohol intake, the current study aims to

explore the interaction between opioid antagonist, naltrexone, and CRF-R1 antagonist, CP 154,526, as treatments for alcoholism. The study focuses on the role of CRF1 and mu opioid receptors in the DRN in escalated alcohol consumption using intermittent access. Since both CP 154,526 and naltrexone reduce alcohol intake, the hypothesis is that a combination of both may result in a further reduction: an additive effect or that the two systems work independently of one another. Another aim is to evaluate intermittent access as a model that captures escalated ethanol drinking by measures of withdrawal and blood ethanol concentrations (BECs). The hypothesis is that as the animals establish stable drinking levels, they may become dependent and exhibit signs of withdrawal. Their ethanol intake should also be reflected in their BECs. A series of experiments was conducted to test the aforementioned hypotheses. The following section describes these experiments in detail.

Methods

Animals and Housing

Adult male C57BL/6J mice (n=36) (Jackson Laboratories, Bar Harbor, ME) were eight weeks old upon arrival and were initially housed with a female in polycarbonate cages (28 × 17 × 12 cm) with stainless steel wire mesh lids and pine shavings covering the floor. Animals were given one week to habituate to the vivarium conditions on a 12-hour reversed light/dark cycle (lights off at 7 AM) and constant temperature (21 ± 2°C) and humidity (25%). Standard rodent chow (LabDiet 5001 Rodent Diet; PMI Nutrition International, Brentwood, MO) and tap water were available ad libitum. All procedures were approved by the Tufts University Institutional Animal Care and Use Committee and were in accordance with the NIH Guide for Care and use

of Laboratory Animals (http://www.nap.edu/openbook.php?record_id=5140&page=R1).

Ethanol Intake Procedures

Ethanol solutions (w/v) were prepared in tap water from 95% ethyl alcohol (Pharmaco-AAPER, Brookfield, CT). Males were changed to individual housing at least 24h before the presentation of two drinking tubes of water on the cage lid for one week for habituation to drinking from sipper tubes. Fluids were presented in 50-ml plastic centrifuge tubes (Nalgene) with no. 5 rubber stoppers (Fisher Scientific, Agawam, MA 01001) containing stainless steel ball-bearing sipper nozzles (Ancare Corp., Bellmore, NY). Centrifuge tubes were securely held through the wire mesh cage lid and presented to mice 3 hours into the dark cycle, the optimal time at which mice consume ethanol (Kamdar et al., 2006). Bottles were weighed to the nearest hundredth of a gram 24 hours after the fluids were given (post weights). To determine final mL intake/24 hours, post weights were subtracted from initial bottle weights (taken before mice were give alcohol access). An additional cage without animals was used to control for spillage due to experimenter handling or evaporation. Weekly "drip" averages (loss of fluid in the empty cage) were subtracted from individual fluid intakes. Mice were weighed to the nearest tenth of a gram before every ethanol drinking session to calculate the grams of ethanol intake per kilogram of body weight (g/kg).

Drugs

CP-154,526 [butyl-(2,5-dimethyl-7-[2,4,6-trimethypheynul]-7H-pyrrolo[2,3-d]pyramidin-4-yl)-ethylamine] (Pfizer, Groton, CT) was suspended in a vehicle of artificial cerebral spinal fluid (aCSF). CP-154,526 displays high affinity for the CRF-1 receptor (Ki < 10

nM) and blocks CRF-stimulated adenylate cyclase activity in rodent pituitary and cortical membranes (Lundkvist et al., 1996; Schulz et al., 1996). Systemic administration of CP-154,526 reaches the brain as the drug crosses the blood brain barrier and reaches peak concentration 20 minutes after administration (Keller et al., 2002). Significant levels of the drug were observed in the cortex, striatum, cerebellum, and hippocampus (Keller et al., 2002).

Naltrexone (*N*-cyclopropylmethyloroxymorphone, EN 1639A) (NIDA) was also suspended in a vehicle of aCSF. Naltrexone is a nonselective competitive opioid antagonist (Volpicelli et al., 1992; Kiefer et al., 2003) that has a preferential affinity for mu opiate receptors (Kosterlitz, Corbett & Paterson, 1989). Naltrexone can also act on other opiate receptors depending on the administered dose (Kosterlitz, Corbett & Paterson, 1989). The mean elimination half-life (T-1/2) value for naltrexone is 4 hours (Mason et al., 2002).

Experiment 1

Male mice (n=12) were given intermittent access to 20% ethanol in a recently revived drinking protocol (Simms et al., 2008), originally established by Wise (1973) in rats. Mice received increasing concentrations of alcohol until the 20% concentration was reached. Specifically, on the first Monday, Wednesday and Friday they received 3%, 6%, and 10% (w/v) ethanol solutions in one bottle and water in the second bottle. After the first week of fading in ethanol (Wise, 1973; Simms et al., 2008), animals were presented with one bottle of 20% ethanol and one bottle of water every Monday, Wednesday, and Friday for 24 hours. The next day, bottles were removed at the same hour that they were given, weighed and the ethanol solution in one bottle was thoroughly washed out and replaced by water. The animals received two water bottles on Tuesday, Thursday, Saturday and Sunday until the next drinking session. Bottles were

alternated each ethanol drinking session to avoid the development of side preferences.

After establishing stable drinking level, animals were assessed for alcohol withdrawal using handling induced convulsions described in the upcoming ethanol withdrawal section.

In preparation for systemic μ-opioid manipulations of intermittent access, animals were handled and habituated to injections using intraperitoneal (i.p.) injections of 0.9% saline (1 ml/100g), first in the afternoons (to avoid changes in fluid consumption) and then before drinking sessions. After establishing a stable baseline of drinking post injections, animals received 0.1, 1.0, or 10.0 mg/kg naltrexone i.p. in a counterbalanced order to avoid order effects. Injections were given on alternate sides to prevent any associations with stress.

After the completion of systemic injections, mice underwent stereotaxic surgery where a cannula was implanted in the dorsal raphé nucleus of the brain as described below.

Stereotaxic Surgery

Mice were anesthetized with a mixture of ketamine (100 mg/kg of ketamine) and xylazine (6 mg/kg) both being injected together (1 ml/100g, i.p.) and placed in a stereotaxic frame (David Kopf Instruments) with the incisor bar set at –3.3 mm. A 6 mm, 26 ga. guide cannula; MD-2250 (BaSi) was sterotaxically implanted unilaterally 1 mm above the dorsal raphé nucleus (anterior-posterior: –4.6 mm from bregma; medial-lateral: + 1.5 from midline) at a 26º angle (Paxinos & Watson, 1986). Coordinates for the dorsalventral (DV) measurement (Paxinos & Watson, 1986) were taken from the dura matter, and the cannula was placed at DV: –1.9 mm. All cannulae were fixed with three layers of dental cement composed of Durelon. Mice were administered a 1 ml saline subcutaneous injection (s.c.) to prevent dehydration at the conclusion of each surgery. They also received a subcutaneous (s.c) injection of carporfen (Rimadyl™) (dose: 5 mg/kg, concentration: 0.5 mg/ml) after 5 minutes of anesthesia (1ml/100g)). The end concentration that the mice receive is. All mice were allowed at least 3 days of recovery after the

surgery before they were given access to alcohol solutions. Once they reestablished baseline drinking levels, mice were habituated to 'mock' microinjections where their obdurators would be replaced by an injector to prepare them for drug microinjections, 10 minutes before ethanol access.

Intracerebral mu opioid and CRF-R1 manipulation of intermittent ethanol drinking

On test days, naltrexone (NIDA), or CRF-R1 antagonist CP-154,526 (Pfizer, Groton, CT) or combinations of CP-154,526 + naltrexone (combo1 and combo2) were freshly suspended in 0.5 ml artificial cerebral spinal fluid (aCSF). The following table demonstrates the 5 different treatments with their respective doses:

Treatment	Dose
Vehicle	(aCSF)
Naltrexone	6 µg
CP 154,526 (CP)	0.24 µg
Combo1	0.12 µg CP + 3 naltrexone
Combo2	0.24 µg CP + 6 naltrexone

A 26 ga. injector (Plastics One, Roanoke, VA) was connected via flared polyethylene tubing (size P50) to a glass syringe controlled by an automatic CMA/100 microinjection pump (CMA Microdialysis, Sweden). 0.2 µl volume of aCSF, 6 µg naltrexone, 0.12 µg or 0.24 µg CP-154,526, combo1: 0.12 µg CP-154,526 + 3 µg naltrexone or combo2: 0.24 µg CP-154,526 + 6 µg naltrexone was microinfused at a flow rate of 0.1 µl/min into the DRN. Mice were able to move freely during the infusion process. The 6 mm injector (Plastics One, Roanoke, VA) extended 0.5 mm beyond the cannula tip. After completed infusions, the injector remained in the injections site for 1 minute to allow for diffusion and minimize vertical capillary action. 10 minutes post-infusion, alcohol and water bottles were provided to mice. Both bottles were

weighed 2 hours, 4 hours and 24 hours after the presentation of the bottles post microinjections to assess CRF-R1 and mu opioid receptor manipulation of alcohol drinking and fluid consumption.

Histology

After microinjection testing, the animals were sacrificed. Histology was performed to confirm proper cannula placement into the dorsal raphé nucleus. Mice were deeply anesthetized with a lethal injection of (0.5 ml/kg, i.p.) of the ketamine/xylazine combo (1 ml/100g, i.p.). Mice were intracardiacally perfused with 0.9% saline and 4% paraformaldehyde so their brains could be removed. Brain tissue was left to affix in 4% paraformaldehyde then 10% sucrose for at least 2 days. Brains were later sliced in 60 µm coronal sections using a Cryostat (Leica CM1900, Bannockburn, IL). Slices were mounted on gelatin-coated slides or VWR charged micro slides (Radnor, PA), dried, stained using the cresyl violet acetate (Nissl) stain and examined under a microscope to check for proper placement.

Ethanol Withdrawal Assessments:

Handling-Induced Convulsion (HIC)

After 6 weeks of intermittent access to ethanol, reactions to ethanol withdrawal were used to assess alcohol dependence (Goldstein and Pal, 1971). Handling-induced convulsions (HIC) were elicited by lifting a mouse by the tail; they are scored for severity on a scale from 0 – 4 (Goldstein in 1972). According to Goldstein (1972), the characteristic tonic convulsion consists of tightening of facial muscles (grimace), head thrown back, forelegs flexed and hind legs extended laterally, with generalized body tremor. A slight twist is used to evoke minimal signs. The table below describes the scoring system that was used.

Scoring system for convulsions elicited by lifting a mouse by the tail (Adapted from Goldstein, 1972)

Score	
0	No convulsions when the mouse is lifted.
1	Tonic convulsions when the mouse is lifted and given a gentle 180° turn.
2	Tonic-clonic convulsions elicited by the gentle spin, *or* tonic convulsions when the mouse was lifted without turning.
3	Tonic-clonic convulsions not requiring any spin.
4	Violent tonic-clonic convulsions, often continuing after release of the mouse.

According to this scale, a score of 0 signifies no withdrawal signs and a score of 4 signifies the most severe withdrawal reactions. Observations were made 8 hours after the ethanol bottle had been withdrawn, a time point demonstrated to show the strongest signs of withdrawal in this strain after intermittent ethanol (Hwa et al., 2010). A two-day average was used for final HIC scores.

Blood Ethanol Concentration Analysis

Another batch of males (n=12) was subjected to the same protocol of intermittent access to drinking. When they maintained a stable baseline of drinking, blood samples were collected from the submandibular vein 2 hours after they were given ethanol. Blood samples were collected and centrifuged at 4°C for 10 min at 3000 r.p.m. Blood ethanol concentrations (mg/dl) were determined using a colorimetric assay kit that uses an alcohol oxidase coupled reaction (BioVision, Mountain View, CA). Absorbance at 570 nm was determined with an ELx808 microplate reader and analyzed with Gen5 analysis software (BioTek Instruments, Winooski, VT).

Intra-DRN modulation of ethanol drinking without naltrexone history

Another group of C57BL/6J mice (n=12) was put on the intermittent access protocol described above. After establishing stable levels of drinking under intermittent access, mice underwent stereotaxic surgery where a cannula was implanted in the DRN. After recovery, intra-

DRN microinjections of 0.6 µg CP 154, 526, 6 µg naltrexone and a combination of both (combo: 0.6 µg of CP 154,526 and 6 µg of naltrexone) were used to modify alcohol drinking behavior (doses based on studies by Varaschin et al., 2005). Animals were sacrificed after receiving all treatments and histological analysis was conducted to check placement. The following table demonstrates the 4 different treatments with their respective doses:

Treatment	**Dose**
Vehicle	(aCSF)
Naltrexone	6 µg
CP 154,526 (CP)	0.6 µg
Combo	0.6 µg CP + 6 naltrexone

Results

Acquisition of 20% ethanol drinking under intermittent access

C57BL/6J mice (n=36) were given a 2-bottle choice of 20% ethanol or water on Mondays, Wednesdays, and Fridays for 24 hours. Ethanol intakes increased over time, and over approximately 20 drinking sessions, mean ethanol consumption increased to levels observed by Simms et al. (2008). The mean group intake (g/kg/24hr) was 24.49 ± 1.21 [Figure 1]. After recovery from stereotaxic surgery, animals continued to maintain stable drinking levels.

Experiment 1

Ethanol Withdrawal Assessment

Handling-induced Convulsion (HIC)

After establishing stable levels of drinking under intermittent access, severity of ethanol withdrawal was judged at the peak of withdrawal at 8 hours after withdrawal (Hwa et al., 2011) using the HIC rating scale described by Goldstein (1972). C57BL/6J mice (n=12), were assessed for HIC ratings after 6 weeks of intermittent access to ethanol. All mice that underwent HIC assessments showed stable ethanol intake over 4 weeks before they were deprived of ethanol.

Higher ethanol intake (g/kg) was associated with a higher HIC score, indicating more severe withdrawal [Figure 2].

Systemic injections of naltrexone

The same group of mice (n=12) received systemic injections of saline (vehicle) and naltrexone: 0.1, 1.0 and 10.0 mg/kg (based on doses used in previous studies such as Phillips, Wenger and Dorrow, 1997; Lê et al., 1993,). There was a dose-dependent decrease in ethanol intake at 2 hours, 4 hours, and 24 hours post injection [Figure 3a]. One-way ANOVA with repeated measures indicated that there was a dose-dependent attenuation of ethanol drinking in the first 2 hours after the i.p. injection of naltrexone that was statistically significant ($F(3, 33) = 3.63$, $p = 0.02$). There was also a dose-dependent attenuation of ethanol drinking in the first 4 hours after the i.p. injection of naltrexone that was statistically significant ($F(3, 33) = 3.00$, $p = 0.04$). Post-hoc Bonferroni *t*-tests showed that only the highest dose (10.0 mg/kg) resulted in a significant decrease in ethanol intake 2 hours post injection ($t = 3.12$, $p = 0.01$) and 4 hours post injection ($t = 2.56$, $p = 0.05$). The decrease in ethanol intake (g/kg) after 24 hours was not statistically significant for any of the doses, p=0.12.

When comparing the consumption of water and ethanol intake, one-way ANOVA with repeated measures also demonstrated a dose-dependent decrease in ethanol drinking (ml) at 2 hours post injection [($F(3, 33) = 3.67$, $p = 0.02$], and 4 hours post injection [(F (3, 33) =3.00, $p = 0.04$] but not after 24 hours ($p = 0.30$). There was no significant change in water drinking (ml) across all time points: 2 hours ($p = 0.87$), 4 hours ($p = 0.07$), 24 hours ($p = 0.45$) [Figure 3b].

Intra-DRN modulation of ethanol drinking

After undergoing systemic injections, the same group (n=12) underwent stereotaxic surgery where a cannula was implanted in the dorsal raphé nucleus (DRN) of the brain. After

recovery, intra-DRN microinjections of CP-154,526 and naltrexone were used to modify alcohol drinking behavior. Histological analysis revealed that only 3 of the original 12 had correct placement. Keeping in mind that this group has a history of naltrexone, the results of these microinjections remain inconclusive.

Experiment 2: Blood ethanol concentration differences between saline and naltrexone-injected mice

Blood ethanol concentrations

After establishing stable levels of drinking under intermittent access, blood ethanol concentrations (BEC) were determined. C57BL/6J mice (n=12) received systemic injections of saline (vehicle) and 10.0 mg/kg naltrexone, and blood ethanol concentrations were assessed 2 hours post injection. Ethanol intake (g/kg) was positively correlated with BEC (mg/dL) such that higher ethanol intake was associated with higher BEC (mg/dL). Naltrexone was associated with lower BEC compared to saline [Figure 4]. The mean BEC (mg/dL) after saline injections (M = 6.26, SD = 1.43) was higher than the mean BEC after a 10.0, i.p. naltrexone injection (M = 2.53, SD = 0.77).

Experiment 3: Intra-DRN modulation of ethanol drinking

Another group of C57BL/6J mice (n=8) was put on the intermittent access protocol described above. After establishing stable levels of drinking under intermittent access, mice underwent stereotaxic surgery where a cannula was implanted in the DRN [Figure 5]. After recovery, intra-DRN microinjections of 0.6 μg CP-154, 526, 6 μg naltrexone and a combination of both (combo: 0.6 μg of CP-154,526 and 6 μg of naltrexone) were used to modify alcohol drinking behavior. All treatments were associated with a decrease in ethanol drinking compared

to the aCSF vehicle. One-way ANOVA with repeated measures indicated that there was an attenuation of g/kg ethanol drinking in the first 2 hours after intra-DRN infusions of CP-154,526 that was statistically significant ($F(1, 7) = 30.20$, $p = <0.01$), and 4 hours after infusions ($F(1, 7) = 3.63$, $p = 0.02$) but not at 24 hours after infusions $p = 0.61$. Naltrexone also led to a significant decrease in g/kg intake after 2 hours ($F (1, 7) = 20.93$, $p < 0.01$), 4 hours ($F (1, 7) = 36.39$, $p < 0.01$), but not 24 hours, $p = 0.16$. The combination of both drugs led to the biggest decrease in consumption, and it was significantly different than the aCSF vehicle after 2 hours ($F (1, 7) = 22.59$, $p < 0.01$), 4 hours ($F(1, 7) = 12.32$, $p = 0.01$), but not 24 hours, $p = 0.98$ [figure 6].

At 2 hours post-infusion, CP 154,526 led to a 47.79% reduction from baseline levels of g/kg ethanol intake; naltrexone led to a 45.77% reduction in g/kg ethanol intake while the combo led to a 59.12% reduction from baseline levels of g/kg ethanol intake [figure 7].

Looking at the consumption of water and ethanol intake, one-way ANOVA with repeated measures also demonstrated a significant decrease in ethanol drinking (ml) at 2 hours post infusions of CP-154,526 [$F(1, 7) = 30.02$, $p < 0.01$], and 4 hours post injection [$F (1, 7) = 59.976$, $p < 0.01$] but not after 24 hours ($p = 0.30$). One-way ANOVA with repeated measures also demonstrated a significant decrease in ethanol drinking (ml) at 2 hours post infusions of naltrexone [$F(1, 7) = 21.01$, $p < 0.01$], and 4 hours post injection [$F (1, 7) = 36.08$, $p < 0.01$] but not after 24 hours ($p = 0.21$). Lastly, there was a significant decrease in ethanol drinking (ml) at 2 hours post infusions of the combination at 2 hours [$F(1, 7) = 23.24$, $p < 0.01$], and 4 hours post injection [$F (1, 7) = 12.32$, $p = 0.01$] but not after 24 hours ($p = 0.36$). There was no significant change in water drinking (ml) across all time points: 2 hours ($p = 0.87$), 4 hours ($p = 0.07$), 24 hours ($p = 0.45$), except for the combo at 4 hours ($t = 3.66$, $p < 0.01$) as confirmed by a post-hoc Bonferroni *t*-tests.

Blood ethanol concentrations

Blood ethanol concentrations (BEC) were determined at 2 hours post combo intra-DRN infusions. There was a strong positive correlation between g/kg intake and BEC (mg/dL) $r(6) = 0.78$, $p < 0.01$. Higher ethanol intake (g/kg) was associated with higher BEC (mg/dL) [Figure 8].

Discussion

The present investigation confirms that male C57BL/6J mice given intermittent access to 20% ethanol and water will voluntarily and preferentially consume high quantities of alcohol. After 4 weeks, the intermittent access procedure, alternating daily limited access and deprivation can induce a reliable and steady escalation of *per os* alcohol intake where C57BL.6J mice drink an average of 25 g/kg/24h. Under these conditions, significant signs of dependence begin to emerge; the relevance of alcohol intake is reflected in blood ethanol concentrations (BEC), and handling induced convulsions (HIC) scores. This replicates findings by Hwa et al. (2011).

Naltrexone dose-dependently reduces ethanol intake following the intermittent access protocol when administered i.p. This reduced intake is also associated with lower BECs compared to values after saline injections. Intra-DRN microinjections of CP 154,526 and naltrexone both reduce ethanol without affecting water intake. Microinjections of the combined drug also resulted in an attenuation in ethanol intake, but no additive effect was observed.

The results of the present experiment complement previous studies that generated high ethanol consumption with the same intermittent access 20% ethanol protocol in rats (Simms et al., 2008; Wise, 1973). The current study, like Simms and colleagues (Simms et al., 2008; Simms et al., 2010), revives a protocol that demonstrated that intermittent access to 20% ethanol induced high ethanol intake without the need of sucrose fading (Samson, 1986) or water deprivation (Meisch and Thompson, 1972).

Intermittency, a cardinal feature of access to drinking, has been implemented in various protocols that ultimately lead to elevated ethanol consumption (Tomie et al., 2006). In addition, other protocols that share some of the features of intermittent access include the alcohol deprivation effect (ADE), where periods of ethanol access alternate with periods of deprivation in weekly cycles (Melendez et al., 2006; Rodd-Henricks et al., 2000; Sinclair & Senter, 1968). Relapse-like drinking has been studied in the expression of ADE (Breese et al., 2005; Heyser et al., 1997; Sanchis-Segura and Spanagel, 2006), which is measured by a pronounced increase in ethanol preference and consumption after imposed abstinence. Though the ethanol drinking after the ADE (O'Dell et al., 2004) is substantially escalated, the current procedure has the potential to lead to ethanol dependence without forced ethanol vapor exposure (Becker and Lopez, 2004; Schulteis et al., 1995) that incorporates free-choice drinking.

In the present experiments, some animals given intermittent access to alcohol exhibited high withdrawal scores, indicative of dependence. Withdrawal scores were positively correlated with ethanol intake such that higher intakes were associated with higher HIC scores, suggesting more severe withdrawal. Similar to alcoholic patients who show withdrawal symptoms as severe as insomnia, nausea, autonomic hyperactivity, and even tonic-clonic seizures (Cornish et al., 2001; Hillbom et al., 2003), ethanol-dependent animals display enhanced anxiety-like behaviors, handling-induced convulsions (Goldstein and Pal, 1971) and heightened startle responses (Macey et al., 1996) during acute and protracted withdrawal (Baldwin et al., 1991; Kliethermes et al., 2004; Overstreet et al., 2002; Valdez et al., 2002). For now, whether excessive drinking observed in the current intermittent access procedure induces dependence has yet to be characterized more comprehensively.

In the current series of experiments, repeated cycling of high ethanol consumption followed by deprivation is hypothesized to induce persistent neuroadapdations. Previous studies have showed that naltrexone reduced ethanol preference in established C57BL/6J drinkers (Phillips, Wenger & Dorrow, 1997). Higher doses showed diminished effects on reducing alcohol intake (Phillips, Wenger & Dorrow, 1997). The literature also depicts the effects of naltrexone as specific to ethanol intake as it does not affect water intake (Middaugh et al., 1999; Kamdar et al, 2007). Previous research also implicates neuropeptides corticotropin-releasing factor (CRF) and neuropeptide Y as prominent candidates for the modulation of dependent, intermittent drinking behavior (Heilig & Koob, 2007; Koob et al., 1993; Rasmussen et al., 2000; Rivier et al., 1984). Increased levels of CRF have been observed in the amygdala during ethanol withdrawal (Merlo et al., 1995), and some anxiogenic-like effects of withdrawal in dependent rats can be potently reversed by CRF receptor antagonists (Breese et al., 2004; Knapp et al., 2004; Overstreet et al., 2004; Rassnick et al., 1993). In the current investigation of central CRF and naltrexone during intermittent access drinking in C57BL/6J mice, results show that antagonism of the CRF1 receptor (using CP-154,526), and the mu opioid receptor (using naltrexone) leads to selective suppression of alcohol drinking behavior.

Additionally, the results show that both naltrexone and CP 154,526 were effective at 2 hours, and 4 hours, but not at 24 hours after administration. These findings were expected since both drugs antagonize mu-opioid receptors and CRF-R1 receptors respectively moments after infusion. In addition, it can be assumed that both drugs become degraded after 24 hours and are therefore no longer effective. The present results are consistent with previous findings that have revealed a role for CRF receptor signaling in neurobiological responses to ethanol. In 2008, Sparta et al. found that pretreatment with CP 154,526 prevents binge-like drinking in C57BL/6J

mice. The findings reported in the current investigation complement previous studies that have explored the relationship between CRF-R1 signaling and escalated ethanol drinking due to relieving negative symptoms of withdrawal.

Previous studies have reported that CRF and the opioid system may significantly contribute to neural mechanisms for excessive alcohol consumption seen after intermittent access and may interact with serotonin in the dorsal raphé nucleus (DRN) (Kirby et al., 2000; Valentino et al., 2010). With regards to the interaction between serotonin and CRF, low doses of CRF (that are more selective for CRF-R1) are associated with lower extracellular serotonin levels in the forebrain (Price & Lucki, 2001; Lukkes et al., 2008) while high doses of CRF (that are more selective for CRF-R2) are associated with higher concentrations of serotonin (Amat et al., 2004).

However, the results of the present investigation show that there was no additive effect when CP 154,526 and naltrexone were combined suggesting that the two work independently. Because of the neurochemical heterogeneity of the DRN and the presence of multiple CRF and opioid receptor subtypes on both 5-HT and non-5-HT neurons, the regulation of the DRN-5-HT system as it relates to CRF and the opioid systems has been difficult to understand fully. Although these two drugs influence serotonergic activity, they may play different roles in ethanol reinforcement. As discussed by Sabino et al. (2006), high ethanol intake may be positively reinforced by an opioid-dependent mechanism whereas CRF may be involved in negative affective state associated with ethanol withdrawal. Also, Gilpin et al. (2008) found that brain mu-opioid-receptor systems are involved in baseline alcohol-drinking behavior, but their role is not altered following the transition to dependence. As such, we may not be able to see an additive effect when combining both.

The current study presents further insight into alcohol and its connections with CRF, the

opioid system, and serotonin in the DRN. The intermittent access protocol provides and externally valid method to assess drinking in rodents. It is expected that chronic intermittent access should produce higher levels of binge-like drinking, due to potential mechanisms driven by dependence. In line with the kindling hypothesis of escalated alcohol intake (Ballenger and Post, 1978; Becker, 1998), the chronic intermittent access protocol allows for longer periods of deprivation paired with longer episodes of high drinking, repeated for several weeks, which may contribute to the higher intake values during the initial 2-hour access period. These features constitute a strong binge-like drinking model making this model useful because these features are pertinent to alcoholism. This model may therefore be a step closer to approximating human behavior.

The intermittent access model captures important features of alcoholism such as drinking to the point of intoxication (Rhodes et al., 2007) and rapid initiation to high levels (Rhodes et al., 2005) and dependence as measured by signs of withdrawal (HIC). However, it does not capture important features of drinking behavior. For example, this protocol does not measure motivation for drinking. Volume of fluid consumed is not an appropriate indicator of motivation because the animals may have adjusted the volume to reach a desired level of intoxication, which could vary across subjects. Other features such as tolerance have not yet been demonstrated in using this specific protocol. Another limitation is blood-ethanol levels. Kamdar et al. (2007) explain that single bottle animals reach higher blood-ethanol levels than two bottles (Rhodes, 2007). This could explain the low BEC obtained in this study.

In terms of pharmacological manipulations, the lack of an additive effect could be explained by the fact that mu opioid receptors and CRF1 receptors are present on different parts of serotonin neurons in the DRN. Wang et al. (1998) report that mu opioid receptors are localized

on the postsynaptic membrane of dendrites, extra-synaptic plasma membrane, and the surface of the small, clear vesicles in axon terminals in the DRN. The localization of CRF-R1 receptors has been difficult to ascertain because of the neurochemical heterogeneity of the DRN and the presence of multiple CRF receptor subtypes on both 5-HT and non-5-HT neurons. Studies show that there is a relatively high expression of CRF2 receptors in that area (Chalmers et al., 1995) which may play a role in altering CRF-R1 antagonism. Previous studies have suggested that CRF1 and CRF2 may regulate the DRN-5-HT system in opposing manners (Valentino et al., 2009). This is evident in the level of neuronal activity as well as 5-HT release in forebrain regions where low doses of ovine CRF that are more selective for CRF1 decrease, and higher doses that begin to activate CRF2 receptors or selective CRF2 agonists increase 5-HT extracellular levels in forebrain (Amat et al., 2004; Lukkes et al., 2008; Price et al., 1998; Price & Lucki, 2001). Further studies need to be conducted to better understand the interaction between CRF-R1 and CRF-R2 in the DRN.

Further work to expand on the current project could include systemic injections of CP 154,526. It is important to determine if this drug has the same effects as naltrexone systemically (as it appears so in the current microinjection experiments). Since CRF and the opioid system densely innervate different areas in the brain, future studies could look into other areas in the brain where these systems are co-localized. One such area includes the nucleus accumbens which involves the dopamine system. Several studies have suggested that the blockade of central opioid receptors can reverse ethanol-induced increases in extracellular dopamine concentration in the nucleus accumbens (Hodge et al., 1992, 1997; Weiss et al., 1993).

Other target areas may include the locus coeruleus as one hypothesis for the mechanism of CRF and opioid antagonists could involve GABA. Reyes et al. (2007) used

immunofluorescence and dual immunoelectron microscopy to show that the locus coeruleus was a site where both CRF and opioid mu opioid receptors were co-localized. Other studies have also focused on the central amygdala as an important brain site that involves both systems. Zhou et al. (2010) suggest that the availability of mu opioid receptors in GABAergic dendrites of the bed nucleus of the stria terminalis is dictated in part by the expression of CRF-R1 receptors. Studies have also found a connection between corticotropin- releasing hormone and the opioid system via β-enkephalin which primarily affects mu opioid receptors in the central amygdala (Ciado & Morales, 2000; Funk et al., 2006; Lam et al., 2008). Taking all these findings together, a complex picture emerges where the interaction between CRF-R1 and mu opoid receptors spans different brain areas, and acts on different neurotransmitter systems.

One of the limitations of the study is a lack of neurotransmitter measurement for each treatment. Microdialysis experiments to determine the effects of these drugs on serotonin levels in the brain, targeting specific areas (such as the DRN) measuring changes in extracellular concentrations following specific treatments could be important to determining the specific effects of these drugs on serotonin levels in the brain.

In conclusion, intermittent access is a procedure that incorporates ethanol-preferring C57BL/6J mice within a schedule that allows frequent limited access to alcohol during the week, thereby generating excessive, preferential, voluntary alcohol drinking. This procedure complements the intermittent access 20% ethanol drinking protocol using outbred rats, though the observed levels of alcohol drinking can be up to three times higher in the inbred mice. This protocol could be used to mimic the escalation to alcoholic-like drinking. CRF-R1 and mu opioid antagonism decreased alcohol intake in these mice. However, it remains difficult for one pharmacological agent to be effective for all patients and all aspects of alcoholism. Individuals

drink for a variety of reasons which most likely involve a variety of neurotransmitters. Some people may drink to alleviate symptoms of anxiety or depression which may be mediated by the GABA-ergic or serotonergic systems, as well as the dopaminergic system. More studies should be conducted to create a better picture for the interactions of these systems with current treatments, and treatments on the horizon.

Figures

1

Figure 1: Ethanol consumption and gradual acquisition of alcohol drinking behavior to 20% of ethanol (g/kg/ 24) over time. Male C57BL/6J mice (n=36) were given intermittent access to 20% ethanol in a 2-bottle choice drinking paradigm.

2

Figure 2: Alcohol intake (g/kg) and withdrawal assessment as measured by handling induced convulsions (HIC). Male C57BL/6J (n=12) were assessed for severity of withdrawal 8 hours after withdrawal using the HIC rating scale after 6 weeks of intermittent access to ethanol. A 2-day average of 2 HIC assessment sessions is shown.

3 (a)

Figure 3: (a) Alcohol intake (g/kg) following systemic injections. Male C57BL/6J (n=12) received systemic injections of saline (vehicle) and naltrexone: 0.1, 1.0 and 10.0 mg/kg. *$p < .05$ compared to vehicle. (b) Fluid intake (ml) 2 hours, 4 hours, and 24 hours after injections. *$p < .05$ compared to vehicle.

4

Figure 4: Blood ethanol concentration (mg/dL) after saline or naltrexone systemic injections. Male C57BL/6J mice (n=12) received systemic injections of saline (vehicle) and 10.0 mg/kg naltrexone, and blood ethanol concentrations were assessed 2 hours post injection.

5 (a)

Figure 5: Intra-dorsal raphé nucleus (DRN) placements. C57BL/6J (n=8) underwent stereotaxic surgery and were implanted with a cannula into the DRN. Dots represent correct cannula placements revealed after histological analysis.

(b)

Figure 5: (b) Photomicrograph of an intra-dorsal raphé nucleus (DRN) placement. C57BL/6J (n=8) underwent stereotaxic surgery and were implanted with a cannula into the DRN. The photomicrograph above shows correct cannula placement revealed after histological analysis.

6

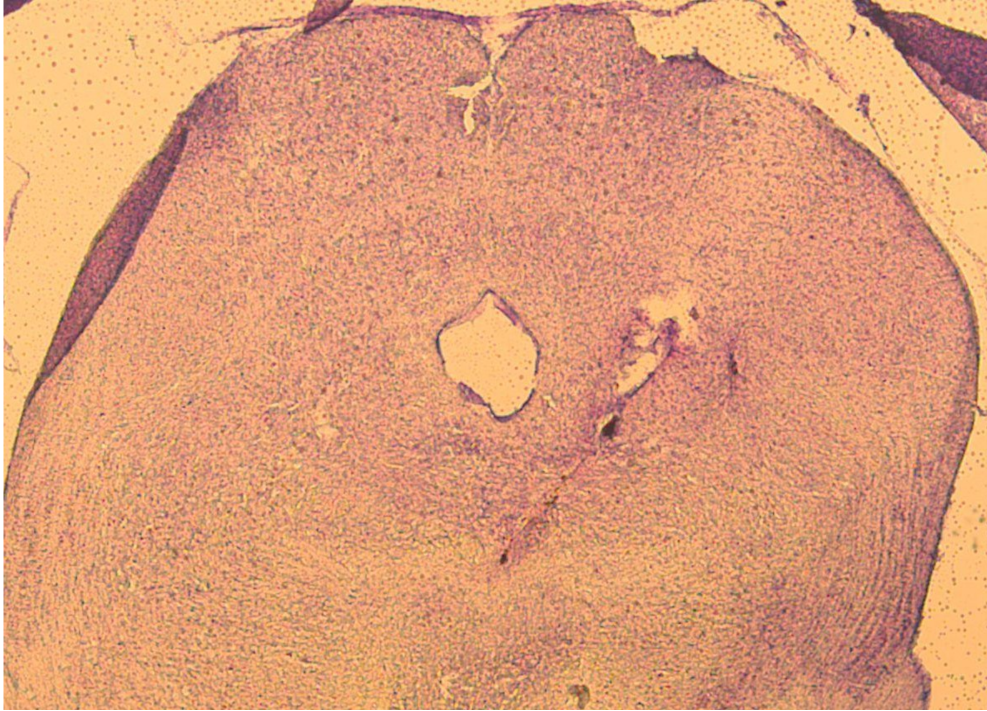

Figure 5: Alcohol intake (g/kg) following intra-dorsal raphé nucleus (DRN) microinjections. Male C57BL/6J (n=8) received intra-DRN injections of artificial cerebrospinal fluid (aCSF; vehicle) and 0.6 µg CP 154,526 6 µg naltrexone, and a combo: and 0.6 µg CP 154,526 + 6 µg naltrexone. *p < .05 compared to vehicle

Figure 6: Change in ethanol intake in with intra-DRN micoinjections using intermittent access. Male C57BL/6J (n=8) received intra-DRN injections of artificial cerebrospinal fluid (aCSF; vehicle) and 0.6 µg CP-154,526, 6 µg naltrexone, and a combo: and 0.6 µg CP 154,526 + 6 µg naltrexone.

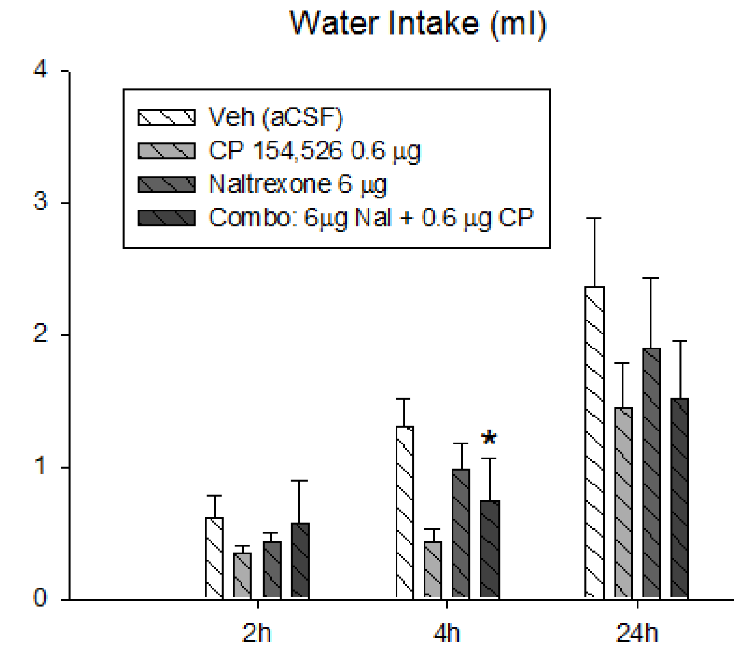

Figure 7: Fluid intake (ml) 2 hours, 4 hours, and 24 hours after intra-DRN microinjections. Male C57BL/6J (n=8) received intra-DRN injections of artificial cerebrospinal fluid (aCSF; vehicle) and 0.6 µg CP 154,526 6 µg naltrexone, and a combo: and 0.6 µg CP 154,526 + 6 µg naltrexone. $*p < .05$ compared to vehicle.

9

Figure 8: Blood ethanol concentration (mg/dL) after a combination: 0.6 µg CP 154,526 + 6 µg naltrexone intra-dorsal raphé nucleus (DRN) microinjection. Male C57BL/6J mice (n=8) received intra-DRN injections of 0.6 µg CP 154,526 + 6 µg naltrexone, and blood ethanol concentrations were assessed 2 hours post microinjection.

References

Adell, A., Bortolozzi, A., Diaz-Mataix, L., Santana, N., Celada, P., Artigas, F. (2010). Serotonin interaction
with other transmitter systems. In: Handbook of the behavioral neurology of serotonin, pp.
259-276.

Altshuler, H. L., Phillips, P. E., Feinhandler, D. A. (1980). Alteration of ethanol self-administration by
naltrexone. *Life Sciences* 26679-26688.

Altshuler, H. L., Applebaum, E., Shippenberg, T.S. (1981). The effects of opiate antagonists on the
discriminative stimulus properties of ethanol. *Pharmacology Biochemistry and Behavior* 14: 97-
100.

Amat, J., Tamblyn, J. P., Paul, E. D., Bland, S. T., Amat, P., Foster, A. C., Watkins, L. R., and Maier, S. F.
(2004). Microinjection of urocortin 2 into the dorsal raphé nucleaus activates serotonergic
neurons and increases extracellular serotonin in the basolateral amygdala. *Neuroscience* 129:
509-519.

Amat, J., Baratta, M. V., Paul, E., Bland, S. T., Watkins, L. R., and Maier, S. F. (2005). Medial prefrontal

cortex determines how stressor controllability affects behavior and dorsal raphé nucleus. *Nature Neuroscience* 8: 365-371.

Arrango, V., Underwood, M. D., and Mann, J. J. (2002). Chapter 35 Serotonin brain circuits involved in
major depression and suicide. *Progress in Brain Research*, 136: 446-453.

Baldwin, H. A., Rassnick, S., Rivier, J., Koob, G. F., and Britton, K. T. (1991). CRF antagonist reverses the "anxiogenic" response to ethanol withdrawal in the rat. *Psychopharmacology*103: 22 -232

Ballenger J. C., Post, R. M. (1978) Kindling as a model for alcohol withdrawal syndromes. *British Journal*
of Psychiatry 133: 1-14.

Beaman, C. M., Hunter, G. A., Dunn, L. L., and Reid, L. D. (1984). Opioids, benzodiazepines and intake of
ethanol. *Alcohol* 1: 39-42.

Becker, H.C., and Lopez, M. F. (2004). Increased ethanol drinking after repeated chronic ethanol exposure and withdrawal experience in C57BL/6J mice. *Alcoholism: Clinical and Experimental*
Research 28: 1829-1838.

Belknap, J. K., Crabbe, J. C., Young, E. R. (1993). Voluntary consumption of ethanol in 15 inbred
mouse strains. *Psychopharmacology* (Berlin) 112: 503-510.

Bradbury, A. F., Smyth, D. G., Snell, C. R. (1976). C fragment of lipoprotein has a high affinity for brain
opiate receptors. *Nature* 260: 793-795.

Becker, H. C. (1998). Kindling in alcohol withdrawal. *Alcohol Health & Research World* 22:25-33.

Breese, G. R., Knapp, D. J., and Overstreet, D. H. (2004). Stress sensitization of ethanol withdrawal-
induced reduction in social interaction: inhibition by CRF-1 and benzodiazepine receptor
antagonists and a 5-HT1A-receptor agonist. *Neuropsychopharmacology* 29: 470-482.

Breese, G. R., Chu, K., Dayas, C. V., Dunk, D. Knapp, D. J., Koob, G. F., Le, A. D., O'Dell, L., Overstreet,
D. H. Roberts, A. J., Sinha, R., Valdez, G. R., Weiss, F. (2005). Stress enhancement of craving
during sobriety: A risk for relapse. *Alcoholism: Clinical and Experimental Research* 29: 185-195.

Chalmers, D. T., Lovenberg, T. W. and De Souza, E. B (1995). Localization of novel corticotrophin
-releasing factor receptor (CRF2) mRNA expression to specific subcortical nuclei in rat brain:
comparison with CRF1 receptor mRNA expression. *Journal of Neuroscience* 15: 6340-6350.

Chu, K., Koob, G. F., Cole, M., Zorilla, E. P., and Roberts, A. J. (2007). Dependence-induced increases in

ethanol self-administration in mice are blocked by the DRF1 receptor antagonist antalarmin and by CRF1 receptor knockout. *Pharmacology Biochemistry and Behavior* 86: 813-821.

Ciado, J. R., Morales, M. (2000). Acute ethanol induction of c-Fos immunoreactivity in pre-pro-enkephalin expressing neuronsof the central nucleus of the amygdala. *Brain Research* 861: 173 -177.

Contarino, A., and Papaleo, L. L. (2010). The corticotropin-releasing factor receptor-1 pathway mediates the negative affective states of opiate withdrawal. *Proceedings of the National Academy of Sciences of the United States of America* 102 (51): 18649-18654.

Cooper, J. R., Bloom, F. E., Roth, R. H. (2003). The biochemical basis of neuropharmacology. New York USA: Oxford University press.

Cornish, J. W., McNicholas, L. F., O'Brien, C. P. (2001). Treatment of substance-related disorders, in *Essentials of clinical psychopharmacology.* (Schatzberg AF, Nemeroff CB eds), pp. 519-538. American Psychiatric Press, Washington (DC).

Corvaja, N., Doucet, G., Bolam, J. P. (1993). Ultrasonic and synaptic targets of the raphé –nigral projection in the rat. *Neuroscience*, 55: 417-427.

Cowen, P. J. (1993). Serotonin receptor subtypes in depression: evidence from studies in neuroendocrine regulation. *Clinical Neurophamacology* 16 Supplement 3: S6-18.

Dahlström, A. and Fuxe, K. (1964). Localization of monoamines in the lower brain stem.

Expereintia

20:398-399.

Datiche, F., Luppi, P. H. and Cattarelli, M. (1995). Serotonergic and non-serotonergic projections from

the raphé nuclei to the piriform cortex in the rat: a cholera toxin B subunit (CTb) and 5-HT

immunohistochemical study. *Brain research*, 671: 27-37.

De Witte, P. (1984). Naloxone reduces alcohol intake in a free-choice procedure even when both

drinking bottles contain saccharin sodium or quinine substances. *Neuropsychobiology* 12:73-77.

Dickenson, A. H., and Kieffer, B. L. (2005). Opiates: basic mechanisms. In: text book of pain, edited by

McMahon, S. B, Koltzenburg, M. New York: Elsevier, pp. 427-442.

Elmer, G. I., Meisch, R. A., and George, F. R. (1986). Oral ethanol reinforced behavior in inbred mice.

Pharmacology Biochemistry and Behavior 24: 1417-1421.

Elmer, G. I., Meisch, R.A., Goldberg, S.R., George, F.R. (1988). Fixed-ratio schedules of oral ethanol self-

administration in inbred mouse strains. *Psychopharmacology* 96:431-436.

Froehlich, J.C., Zqeifel, M., Harts, J., Lumeng, L. and Li, T.K. (1991). Importance of delta opioid receptors

in maintaining high alcohol drinking, *Psychopharmacology,* 103: 467-472.

Froehlich, J.C., and Li, T. K. (1994). Opioid involvement in alcohol drinking. *Annuals of the*

New York

Academy of Sciences. 739: 156–167.

Froehlich, J. C. (1996). The neurobiology of ethanol-opioid interactions in ethanol reinforcement.

Alcoholism: Clinical and Experimental Research 20: 181A–186A.

Funk, C. K., O'Dell, L. E., Crawford, E. F., Koob, G. F. (2006). Corticotropin-releasing factor within the

central nucleus of the amygdala mediates enhanced ethanol self-administration in withdrawn,

ethanol-dependent rats. *Journal of Neuroscience* 26: 11324-11332.

Funk, C K., Zorrilla, E. P., Lee, M. J., Rice, K. C., and Koob, G. F. (2007). Corticotropin-releasing factor 1

antagonists selectively reduce ethanol self-administration in ethanol-dependent rats. *Biological*

Psychiatry 61: 78-86.

Garcia, J., Kimeldorf, D. J. and Hunt, E. L. (1957). Spatial avoidance in the rat as a result of exposure to

ionizing radiation. *British Journal of Radiology* 30: 318-321.

George, S. R., Zastawny, R. L., Briones-Urbina, R., Cheng, R., Nguyen, T., Heiber, M., Kouvelas, A., Chan,

A. S., and O'Dowd, B. F. (1994). Distinct distributions of mu, delta and kappa opioid receptor

mRNA in rat brain. *Biochemical and Biophysical Research Communications* 205: 1438–1444.

Gehlert, D. R., Cippitelli, A., Thorsell, A., Le, A. D., Hipskind, P. A., Hamdouchi, C., Lu, J., Hembre, E. J., Cramer, J., Song, M., McKinzie, D., Morin, M., Ciccocioppo, R., and Heilig, M. (2007). 3-(4-Chloro-2-morpholin-4-yl-thiazol-5-yl)-8-(1-ethylpropyl)-2,6-dimethyl- imidazo[1,2-b]pyridazine: a novel brain-penetrant, orally available corticotropin-releasing factor receptor 1 antagonist with efficacy in animal models of alcoholism. *Jounral of Neuroscience* 27:2718-2726.

Gianoulakis, C. and Gupta, A. (1986). Inbred strains of mice with variable sensitivity to ethanol exhibit

differences in content and processing of/3-endorphin, *Life Sciences.,* 39: 2315-2325.

Gianoulakis, C., de Waele, J.P, and Kiianmaa, K. (1992). Differences in the brain and pituitary/3-endorphin system between the alcohol preferring AA and alcohol-avoiding ANA rats, *Alcoholism:*

Clinical and Experimental Research, 16: 453-459.

Gilpin, N. W., Richardson, H. N, and Koob, G. F. (2008). *Alcoholism: Clinical and Experimental Research*,

32: 1535-1542.

Goddard, G. V., McIntyre, D. C., Leech, C. K. (1969). A permanent change in brain function resulting from

daily electrical stimulation. *Experimental Neurology* 25 :295-330.

Goldstein, D. B., Pal, N. (1971). Alcohol dependence produced in mice by inhalation of ethanol: grading
the withdrawal reaction. *Science* 172: 288-290.

Goldstein, A., Tachibana, S. Lowney, L. I., Hunapiller, M.Hood, L. (1979). Dynorphin-(1-13), an extraordinary potent opioid peptide. *Proceedings of the National Academy of Science*. 76: 6666
-6670.

Gonzales, R. A., Weiss, F. (1998). Suppression of ethanol-reinforced behavior by naltrexone is associated
with attenuation of the ethanol-induced increase in dialysate dopamine levels in the nucleus
accumbens. *Journal of Neuroscience* 18: 10663–10671.

Gorelick, D. A., and Paredes, A. (1992). Effect of fluoxetine on alcohol consumption in male alcoholics.
Alcoholism: Clinical and Experimental Research 16, 261-265.

Grahn, E. R., Maswood, S., McQueen, M. B., Watkins, L. R. and Maier, S. F. (1999). Opioid-dependent
effects of inescapable shock on escape behavior and conditioned fear responding are mediated
by the dorsal raphé nucleus. *Behavioral Brain Research* 99: 153-167.

Halberstadt, A. L., and Balaban, C. D. (2006). Serotonergic and nonserotonergic neurons in the dorsal
raphé nucleus send collateralized projections to both the vestibular nuclei and the central

amygdaloid nucleus. *Neuroscience*, 140: 1067-1077.

Hauger, R. L., Risbrough, V., Brauns, O., and Dautzenberg, F. M., (2006). Corticotropin releasing factor

(CRF) receptor signaling in the central nervous system: new molecular targets. *CNS & Neurological Disorders Drug Targets* 5: 453-79.

Heilig M., and Koob, G. F. (2007). A key role for corticotropin-releasing factor in alcohol dependence.

Trends in Neurosciences 30: 399-406.

Heils, A., Teufel, A., Petri, S., Stober, G., Riederer, P., Bengel, D. and Lesch, K. P. (1996). Allelic variation

of human serotonin transporter gene expression. *Journal of Neurochemistry*, 66: 2621-2624.

Heyser, C. J., Schulteis, G., Koob, G. F. (1997). Increased ethanol self administration after a period of

imposed ethanol deprivation in rats trained in a limited access paradigm. *Alcoholism: Clinical*

and Experimental Research 21: 784-791.

Hillbomm M., Pieninkeroinen, I., and Leone, M. (2003). Seizures in alcohol-dependent patients:

epidemiology, pathophysiology and management. *CNS Drugs* 17:1013-1030.

Hodge, C. W., Samson, H. H., Haraguchi, M. (1992). Microinjections of dopamine agonists in the nucleus

accumbens increase ethanol reinforced responding. *Pharmacology Biochemistry and*

Behavior

43:249–254.

Hodge C. W., Samson, H. H., Chappelle, A. M. (1997). Alcohol self-administration: further examination

of the role of dopamine receptors in the nucleus accumbens. *Alcoholism: Clinical Experimental*

Research, 21: 1083–1091.

Hornung, J.P (2010) The neuroanatomy of the serotonergic system. Handbook of Behavioral

Neurobiology of Serotonin. USA. pp. 51-64.

Hughes, J., Smith, T., Morgan, B., Fothergill, L. (1975). Purification and properties of enkephalin – The

possible endogenous ligand for the morphine receptor. *Life Sciences* 16: 1753-1758.

Hwa, L. S. Chu, A. C., Levinson, S. A., Kayyali, T. M. DeBold, J. F. and Miczek, K. A. (2010). Persistent

escalation of alcohol drinking in C57BL/6J mice with intermittent access to 20% ethanol. *In*

press.

Hyytia, P. (1993). Involvement of [mu]-opioid receptors in alcohol drinking by alcohol-preferring AA rats,

Pharmacology Biochemistry and Behavior, 45: 697-701.

Imai, H. Steindler, D. A. and Kitai, S. T. (1986). The organization of divergent axonal projections from the

midbrain raphé nuclei in the rat. *Journal of Comparative Neurology*, 243: 363-380.

Jacobs, B. L. and Azmitia, E. C. (1992). Structure and function of the brain serotonin system. *Physiological reviews*, 72: 165-229.

Kamdar, N. K., Miller S. A., Syed, Y. M., Bhayana, R., Gupta, T., and Rhodes, J. S. (2007). Acute effects of Naltrexone and GBR 12909 on ethanol drinking-in-the-dark in C57BL/6J mice. *Psychopharmacology* 192: 207–217.

Kazakov, V. N., Kravtsov, P. Y. Krakhotkina, E. D., and Maisky, V. A, (1993). Sources of cortical, hypothalamic, and spinal serotonergic projections: topical organization, within the nucleus raphé dorsalis. *Neuroscience*, 56: 157-164.

Kelley, A. E., and Berridge, K. C. (2002). The neuroscience of natural rewards: relevance to addictive drugs. *Journal of Neuroscience* 22: 3306–3311.

Kiefer, F., Jahn, H., Tarnaske, T., Helwig, H., Briken, P., Holzbach, R., Kampf, P., Stracke, R., Baehr, M., Naber, D., and Wiedemann, K. (2003). Comparing and combining naltrexone and acamprosate in relapse prevention of alcoholism: a double-blind, placebo-controlled study. *Archives of General Psychiatry*, 60:92– 99.

Kirby, L. G., Rice, K. C., Valentino, R. J. (2000). Effects of corticotropin-releasing factor on

neuronal

activity in the serotonergic dorsal raphé nucleus. *Neuropsychopharmacology* 22: 148 – 162.

Kliethermes, C. L., Cronise, K., and Crabbe, J. C. (2004). Anxiety-like behavior in mice in two apparatuses

during withdrawal from chronic ethanol vapor inhalation. *Alcoholism: Clinical and Experimental*

Research 28:1012-1019.

Knapp, D. J., Overstreet, D. H., Moy, S. S., and Breese, G. R. (2004). SB242084, flumazenil, and CRA1000

block ethanol withdrawal-induced anxiety in rats. *Alcohol* 32: 101-111.

Kohler, C. and Steinbusch, H. (1982). Identification of serotonin and non-serotonin-containing neurons

of the mid-brain raphé projecting to the entorhinal area and the hippocampal formation. A

combined immunohistochemical and fluorescent retrograde tracing study in the rat brain.

Neuroscience, 7: 951-975.

Koob, G. F. (1992). Drugs of abuse: Anatomy, pharmacology and function of reward pathways. *Trends in*

Pharmacological Sciences 13: 177-184.

Koob, G. F. (1992). Dopamine, addiction and reward. Seminars in the Neurosciences 4: 139-148.

Koob, G. F., Caine, S. B, Parsons, L., Markou, A., and Weiss, F. (1997). Opponent process model and

psychostimulant addiction. *Pharmacology Biochemistry and Behavior*, 57: 513-521.

Koob, G. F., and Le Moal, M. L. (1997). Drug abuse, dysregulation of reward, and allostasis. *Neurpsychopharmacology*, 24: 97-129.

Koob, G. F. (2003). Alcoholism: allostasis and beyond. *Alcoholism: Clinical and Experimental Research* 27: 232-243.

Koob, G. F. (2008). A role for brain stress systems in addiction. *Neuron* 59: 11-34.

Koob, G. F., Heinrichs, S. C., Pich, E. M., Menzaghi, F., Baldwin, H., Miczek, K., Britton, K. T. (1993). The role of corticotropin-releasing factor in behavioural responses to stress. *Ciba Foundation Sympoisum* 172:277-289

Kosterlitz, H.W., Corbett, A.D. and Paterson, S.J. (1989). Opioid receptors and ligands, *NIDA Research Monograph Index,* 95: 159-166.

Lam, M. P., Marinelli, P. W., Bai, L., Gianoulakis, C. (2008). Effects of acute ethanol on opioid peptide release in the central amygdala: an in vivo microdialysis study. *Psychopharmacology* (Berlin) 208.

Land, B. B., Bruchas, M. R., Lemos, J. C., Xu, M., Melief, E. J., and Chavkin, C. (2008). The dysphoric component of stress is encoded by activation of the dynorphin kappa-opioid system. *Journal of*

Neuroscience 28: 407-414.

Land, B. B., Bruchas, M. R., Schattauer, S., Giardino, W. K., Aita, M., Messinger, D., Hnasko, T. S.,

Palmiter, R. D., and Chavkin, C. (2009). Activation of the kappa Opioid receptor in the dorsal

raphé nucleus mediates the aversive effects of stress and reinstates drug seeking. *Proceedings*

of the National Academy of Sciences of the United States of America, 106(45): 19168-19173.

Le Magnen, J. (1960). Study of some factors associated with modifications of spontaneous ingestion of

ethyl alcohol by the rat. *Journal of Physiology* (Paris) 52: 873-884.

Lê, A. D., Poulos, C. X., Quan, B., and Chow, S. (1993). Alcohol consumption by C57BW6, BALBk, and

DBN2 mice in a limited access paradigm. *Pharmacology Biochemistry and Behavior* 47: 375-378

Lê, A. D., Ko, J., Chow, S., Quan, B. (1994). Alcohol consumption by C57BW6, BALBk, and DBN2 mice in a

limited access paradigm. *Pharmacology Biochemistry and Behavior* 47: 375-378

Lee, S. B., Lee, H. S. and Waterhouse, B. D. (2008).The collateral projection from the dorsal raphé

nucleus to whisker-related, trigeminal sensory and facial motor systems in the rat. *Brain Research*, 1214: 11-22.

Leiber, C.S., Jones, D.O., Menderlson, J., and DeCarli, L.M. (1963). Fatty liver, hyperlipemia and

hyperuricemia produced by prolonged alcohol consumption, despite adequate dietary intake.

Transactions f the Association of American Physicians 76: 289-300.

Lesch, K. P. (1991). 5-HT1A receptor responsivity in anxiety disorders and depression. Progress in

Neuropsychophamacology & Biological Psychiatry 15: 723-733.

Li, Y. Q., Takada, M. and Mizuno, N. (1993). The sites of the origin of serotonirgic afferent fibers in the

trigeminal motor facial, and hypoglossal nuclei in the rat. *Neuroscience Research*, 17: 307-313.

Lowery, E. G., and Thiele, T. E., (2010). Pre-clinical evidence that corticotrophin-releasing factor (CRF)

receptor antagonists are promising targets for pharmacological treatment of alcoholism. *CNS*

& Neurological Disorders Drug Targets 9: 77-86.

Lukkes, J. L., Forster, G. L., Renner, K. J., Summers, C. H. (2008). Corticotropin-releasing factor 1 and 2

receptors in the dorsal raphé differentially affect serotonin release in the nucleus accumbens.

European Journal of Pharmacology 578: 185-193.

Macey, D. J., Schulteis, G., Heinrichs, S. C., and Koob, G. F. (1996). Time-dependent

quantifiable withdrawal from ethanol in the rat: Effect of method of dependence induction. Alcohol 13:163-170.

Mann, J. J. (1999). Role of the serotonergic system in the pathogenesis of major depression and suicidal behavior. *Neuropsychopharmacology* 21: 99S-105S.

Mansour, A., Fox, C. A., Akil, H., and Watson, S. J. (1995). Opioid-receptor mRNA expression in the rat CNS: Anatomical and functional implications. *Trends in Neuroscience* 18:22-29.

Mayson, B. J., Goodman, A. M., Dixon, R. M., Abdel Hameer, M. H., Hulot, T., Wesness, K., Hunter, J. A., and Boyeson, M. G. (2002) A pharmacokinetic and pharmacodynamic drug interaction study of acamprosate and naltrexone, *Neuropsychopharamcology,* 27: 596-606.

McBride, W. J. (2010). Role of serotonin in brain reward and regulation of alcohol drinking behavior. In: Handbook of Behavioral Neurobiology of Serotonin. USA. pp. 399-415.

McBride, W. J. and Li, T. K. (1998). Animals models of alcoholism: neurobiology of high alcohol drinking behavior. *Critical Reviews in Neurobiology*, 12(4): 339-369.

McBride, W. J., Murphy, J. M., Lumeng, L., and Li, T. K. (1990). Serotonin, dopamine and GABA involvement in alcohol drinking of selectively bred rats. *Alcohol*, 7: 199-205.

McBride, W. J., Murphy, J. M., Lumeng, L., and Li, T. K. (1992). Serotonin and alcohol

consumption. In

Naranjo: C. A. and Sellers, E. M. (Eds.), Novel Pharamcological Interventions for Alcoholism.

Springer-Verleng, New York, NY pp.59-67.

McClearn, G. E., Rodgers, D. A. (1959). Differences in alcohol preference among inbred strains of mice.

Journal of Studies on Alcohol 20: 691-695.

McKenzie-Quirk, S. D. and Miczek, K. A (2003). 5-HT1A agonists: alcohol drinking in rats and squirrel

monkeys. *Psychopharmacology* (Berlin) 167: 145-152.

Meisch, R.A., and Lemaire, G.A. (1993). Drug self-administration. In: F. van Haaren, Editor. *Methods in*

Behavioral Pharmacology. Elsevier, Amsterdam, 257-300.

Meisch R. A., and Thompson, T. (1972). Ethanol intake during schedule-induced polydipsia. *Physiology &*

Behavior 8:471-475

Melendez, R. I., Middaugh, L. D., Kalivas, P. W. (2006). Development of an alcohol deprivation and

escalation effect in C57Bl/6J mice. *Alcohol: Clinical and Experimental Research* 30: 2017-2035.

Merlo Pich, E., Lorang, M., Yeganeh, M., Rodriguez, D. F., Raber, J., Koob, G. F., and Weiss, F. (1995).

Increase of extracellular corticotropin-releasing factor-like immunoreactivity levels in

the

amygdala of awake rats during restraint stress and ethanol withdrawal as measured by microdialysis. *Journal of Neuroscience* 15: 5439-5447.

Meshul, C. K., and McGinty, J. F. (2000). Kappa opioid receptor immunoreactivity in the nucleus

accumbens and caudate-putamen is primarily associated with synaptic vesicles in axons. *Neuroscience*, 96 :91–99.

Middaugh, L.D., Kelley, B. M., Eduardo, R. C. Jr., and Groseclose, C. H. (1999). Naltrexone effects on ethanol reward and discrimination in C57BL/6 mice. *Alcoholism: Clinical and Experimental*

Research 23: 456-464.

Molliver, M. E. (1987). Serotonergic neuronal systems: what their anatomic organization tells us about

function. *Journal of Clinical Psychopharmacology*, 7 (6 Suppl): 3S-23S.

Mϋller, C. P., Pum, M. E., Schumannm G., Huston, J. P. (2010). The role of serotonin in drug addiction.

Handbook of Behavioral Neurobiology of Serotonin 507-545.

Murphy, J. M., Waller, M. B., Gatto, G. J., McBride, W. J., Lumeng, L., and Li, T. K. (1985). Monoamine

uptake inhibitors attenuate ethanol intake in alcohol-preferring (P) rats. *Alcohol*, 2: 349-352.

Murphy, J. M., Waller, M. B., Gatto, G. J., McBride, W. J., Lumeng, L., and Li, T. K. (1988). Effects of

fluoxetine on the intragastric self-administration of ethanol in the alcohol preferring P line of

rats. *Alcohol*, 5: 283-286.

Myers, R. D., and Lankford, M. F. (1996). Suppression of alcohol preference in high alcohol drinking rats:

efficacy of amperozide versus naltrexone. *Neuropsychopharmacology* 14(2): 139-149.

Naranjo, C. A., Sellers, E. M., and Lawrin, M. O. (1986). Modulation of ethanol intake by serotonin uptake

inhibitors. *Journal of Clinical Psychiatry*, 47: 16-22.

National Institute of Health (NIH) Retrieved from:

http://www.niaaa.nih.gov/NewsEvents/NewsReleases/naltre.htm

Nordstrom, P. and Asbery, M. (1992). Suicide risk and serotonin. International Clinical

Psychopharmacology 6 Suppl 6: 12-21.

O'Dell, L. E., Roberts, A. J., Smith, R. T., Koob, G. F. (2004). Enhanced alcohol self

-administration after intermittent versus continuous alcohol vapor exposure. *Alcoholism Clinical and Experimental Research* 28: 1676-1682.

O'Malley ,S. S., Jaffe, A..J., Chang, G., Schottenfeld, R. S., Meyer, R. E., and Rounsaville, B. (1992).

Naltrexone and coping skills therapy for alcohol dependence. A controlled study. *Archives of*

General Psychiatry 49: 881-887.

O'Malley, S. S., Jaffe, A. J., Rode, *S.,* and Rounsaville, B. J. (1996). Experience of a "slip"

among alcoholics

treated with naltrexone or placebo. *American Journal of Psychiatry* 153: 281-283.

Olive, M. F., Koenig, H. N., Nannini, M. A., and Hodge, C. W. (2002). Elevated extracellular CRF levels in

the bed nucleus of the stria terminalis during ethanol withdrawal and reduction by subsequent

ethanol intake. *Pharmacology Biochemistry and Behavior* 72: 213–20.

Overstreet, D. H., Knapp, D. J., and Breese, G. R. (2002). Accentuated decrease in social

interaction in rats subjected to repeated ethanol withdrawals. Alcoholism: Clinical and

Experimental Research 26: 1259-1268.

Overstreet, D. H., Knapp, D. F., and Breese, G. R. (2004). Modulation of multiple ethanol withdrawal-

induced anxiety-like behavior by CRF and CRF1 receptors. *Pharmacology Biochemistry and*

Behavior 77: 405-413.

Paxinos, G., and Watson, C. (1986). The rat brain in stereotaxic coordinates. Sydney: Academic

Press.

Pert, C. B. and Snyder, S. H. (1973). Opiate receptor: Demonstration in nervous tissue. *Science* 179,

1011-1014.

Petit, J. M., Luppi, P. H. Peyron C., Rampon, C. and Jouvet, M. (1995). VIP-like immunoreactive

projections from the dorsal raphé and caudal linear raphé nuclei to the bed nucleus of

the stria

terminalis demonstrated by a double nucleus of the stria terminalis demonstrated by a double

immunohistochemical method in the rat. *Neuroscience Letters*, 193: 77-80.

Phillips, T.J., Crabbe, J.C., Metten, P., and Belknap, J. (1994). Localization of genes affecting alcohol

drinking in mice. *Alcoholism: Clinical and Experimental Research* 18:931-941.

Phillips, T. J., Wenger, C. D., and Dorow, J. D. (1997). Naltrexone Effects on Ethanol Drinking Acquisition

and on Established Ethanol Consumption in C57BL/6J Mice. *Alcoholism: Clinical and Experimental Research* 21: 691-702.

Pioszak, A. A., Parker, N. R., Suino-Powel, K., and Xu, H. E. (2008). Molecular recognition of corticotropin

-releasing factor by its G-protein-coupled receptor DRFR1. *The Journal of Biological Chemistry*

283(47): 32900-39212.

Price, M. L. and Lucki, I. (2001). Regulation of serotonin release in the lateral septum and striatum by

corticotrophin-releasing factor. *Journal of Neuroscience* 21: 2833-2841.

Price, M. L., Curtis, A. L., Kirby, L. G. Valentino, R. J., and Lucki, I. (1998). Effects of corticotopin-releasing

factor on brain serotonergic activity. *Neuropsychopharmacolloogy* 18: 492-502.

Rasmussen, D. D. , Boldt, B. M., Bryant, C. A., Mitton, D. R., Larsen, S. A., and Wilkinson,

C.W. (2000).

Chronic daily ethanol and withdrawal: 1. Long-term changes in the hypothalamo-pituitary

-adrenal axis. *Alcoholism: Clinical and Experimental Research* 24: 1836–1849.

Rassnick, S., Heinrichs, S. C., Britton, K. T., and Koob, G. F. (1993). Microinjection of a corticotropin-

releasing factor antagonist into the central nucleus of the amygdala reverses anxiogeneic-like

effects of ethanol withdrawal. *Brain Research* 605: 25-32.

Reyes, B. A., Glaser, J. D., and Van Bockstaele, J. (2007). Ultrastructural evidence for co-localization of

corticotrophin-releasing factor receptor and mu opioid receptor in the rat nucleus locus

coeruleus. *Neuroscience Letters,* 413: 216-221.

Rhodes, J. S., Best, K., Belknap, J. K., Finn, D. A., and Crabbe, J. C. (2005). Evaluation of a simple model of

ethanol drinking to intoxication in C57BL/6J mice. *Physiology and Behavior* 84: 53-63.

Rhodes, J. S., Ford, M. M., Yu, C. H., Brown, L. L., Finn, D. A., Garland, T. Jr., and Crabbe, J. C. (2007).

Mouse inbred strain differences in ethanol drinking to intoxication. *Genes Brain and Behavior* 6:

1-18.

Rimondini, R,, Arlinde, C., Sommer, W., and Heilig, M. (2002) Long-lasting increase in voluntary ethanol

consumption and transcriptional regulation in the rat brain after intermittent exposure to alcohol. *The Journal of the Federation of American Socities for Experimental Biology* 16:27-35

Risbrough, V. B., Hauger, R. L., Roberts, A. L., Wylie, Vale, W., and Geyer, M. A. (2004). Corticotropin-
releasing factor receptors CRF1 and CRF2 exert both additive and opposing influences on
defensive startle behavior. *The Journal of Neuroscience*, 24(29): 6545– 6552.

Rivier, J., Rivier, C., and Vale, W. (1984). Synthetic competitive antagonists of corticotropin-releasing
factor: effects on ACTH secretion in the rat, *Science*, 224: 889–891.

Rodd-Henricks, Z. A., McKinzie, D. L., Murphy, J. M., McBride, W. J., Lumeng, L., Li, T. K. (2000). The
expression of an alcohol deprivation effect in the high-alcohol-drinking replicate rat lines is
dependent on repeated deprivations. Alcoholism: Clinincal and Experimental Research 24:747-
753.

Ryabinin, A. E., Bachtell, R. K., Heinrichs, S. C., Lee, S., Rivier, C. Olive, M. F., Mehmert, K. K., Camarini, R.,
Kim, J. A., Koenig, H. N, annini, M. A., Hodge, C. W., Roberts, A. J., and Koob, G. F. (2002). The
corticotropin-releasing factor/urocortin system and alcohol. *Alcoholism: Clinical and*

Experimental Research 26: 714-722.

Sabino, V., Cottone, P., Koob, G. F., Steardo, L., Lee, M. J., Rice, K. C., and Zorilla, E. P. (2006).
Dissociation between opioid and CRF1 antagonist sensitive drinking in Sardinian
alcohol
-preferring rats. *Psychopharmacology*, 189: 175-186.

Sakana, M., Shibasaki, T., Lederis, K. (1987). Corticotropin-releasing factor-containing afferents
to the
inferior colliculus of the rat brain. *Brain Research* 41: 68-76.

Samson, H.H., (1986). Initiation of ethanol reinforcement using a sucrose-substitution procedure
in
food-and water-sated rats. *Alcoholism: Clinical and Experimental Research* 10:436-442.

Samson, H. H., and Doyle T. F. (1985). Oral ethanol self-administration in the rat: Effect of
naloxone.
Pharmacology Biochemistry and Behavior 22: 91-99.

Sanchis-Segura C., and Spanagel, R .(2006). Behavioural assessment of drug reinforcement and
addictive
features in rodents: an overview. *Addiction Biology* 11:2-38.

Self , D. W., and Nestler, E. J. (1995). Molecular mechanisms of drug reinforcement and
addiction. *The*
Annual Review of Neuroscience 18:463-495.

Sellers, E. M., Higgins, G. A. and Sobell, M. B. (1992). 5-HT and alcohol abuse. *Trends in*
Pharmacological
Sciences. 13: 69-75.

Schulteis, G., Markou, A., Cole, M., and Koob, G. F. (1995). Decreased brain reward produced by ethanol
withdrawal. *Proceedings of the National Academy of Sciences of the United States of America*
92: 5880-5884.

Schulz, D. W., Mansbach, R. S., Sprouse, J., Braselton, J. P., Collins, J., Corman, M., Dunaiskis, A., Faraci,
S., Schmidt, A. W., Seeger, T., Seymour, P., Tingley, F. D. III, Winston, E. N., Chen, Y. L., and
Heym, J. (1996). CP-154,526: a potent and selective nonpeptide antagonist of corticotropin
releasing factor receptors. *Proceedings of the National Academdy of Sciences of the United*
States of America 93: 10477-10482.

Simon, E. J., Hiller, J. M. & Edelman, I.(1973). Stereospecifc binding of the potent narcotic
analgesic [3H]
–etorphine to rat-brain homogenate. *Proceedings of the National Academy of Sciences of the*
United States of America 70 1947-1949.

Sinclair J. D., Senter, R. J. (1968). Development of an alcohol-deprivation effect in rats.
*Quarterly Journal of Studies on Alcoho*l 29: 863-867.

Spanagel, R. (1996). The influence of opioid antagonists on the discriminative stimulus effects of
ethanol. *Pharmacology Biochemistry and Behavior* 54: 645-649.

Spanagel, R. (2000). Recent Animal Models of Alcoholism. Alcohol Research and Health 24: 124-131.

Sparta, D. R., Sparrow, A. M., Lowery, E. G., Fee, J. R., Knapp, D. J., and Thiele, T. E. (2008). Blockade of
the corticotropin releasing factor type 1 receptor attenuates elevated ethanol drinking
associated with drinking in the dark procedures. *Alcoholism: Clinical and Experimental Research* 32: 259-265.

Steinbusch, H. W., can der Kooy, D., Verofstad, A. A., and Pellegrino, A. (1980). Serotonergic and non-
serotonergic projections from the nucleus raphé dorsalis to the caudate-putamen complex in
the rat, studied by a combined immunofluorescence and flurescent retrograde axonal labeling
technique. *Neuroscience*, 19: 137-142.

Swanson, L. W., Sawchenko, P. E., Rivier, J., Vale, W. W. (1983). Organization of ovine corticotropin-
releasing factor immunoreactive cells and fibers in the rat brain: an immunohistorchemical
study. *Neuroendocrinology* 36: 165-186.

Tao, R., and Auerbach, S. B. (1995). Involvement of the dorsal raphé but not median raphé nucleus in
morphine-induced increases in serotonin release in the rat forebrain. *Neuroscience* 68: 553-561.

Tao, R., and Auerbach, S. B. (2002). Opioid receptor subtypes differentially modulate serotonin efflux in

the rat central nervous system. *The Journal of Pharmacology and Experimental Therapeutics*

303: 549-556.

Terenius, L. (1973). Characteristics of the 'receptor' for narcotic analgesics in synaptic plasma membrane

from rat brain. *Acta Pharmacologica et Toxicologica*. 33, 377-384.

Thompson, A. M., Moore, K. R. and Thompson, G. C. (1995). Distribution and origin of serotonergic

afferents to guinea pig cochlear nucleus. Journal of Comparative Neurology, 351: 104-116.

Tomkins, D. M., Higgins, G. A., and Sellers, E. M. (1994). Low doses of the 5-HT1A agonist 8-hydroxy-2

-(di-n-propylamino)-teteralin (8-OH DPAT) increase ethanol intake. *Psychopharmacology* (Berlin)

115: 176-179.

Tomie A., Miller, W. C., Dranoff, E., and Pohorecky, L. A. (2006). Intermittent presentations of ethanol

sipper tube induce ethanol drinking in rats. *Alcohol* 41:225-230.

Treutlein, J., Kissling, C., Frank, J., Wiemann, S., Dong, L., Depner, M., Saam, C., Lascorz, J., Soyka, M.,

Preuss, U. W., Rujescu, D., Skowronek, M. H., Rietschel, M., Spanagel, R., Heinz, A.,

Laucht, M.,

Mann, K., and Schumann, G. (2006). Genetic association of the human corticotropin releasing

hormone receptor 1 (CRHR1) with binge drinking and alcohol intake patterns in two independent samples. *Molecular Psychiatry* 11: 594–602.

Turchan, J., Przewlocka, B., Toth, G., Lason, W., Borsodi, A., and Przewlocki, R. (1999). The effect of

repeated administration of morphine, cocaine and ethanol on mu and delta opioid receptor

density in the nucleus accumbens and striatum of the rat. Neuroscience 91: 971–977.

Twarog B. M., Page, I. H. (1953). Serotonin content of some mammalian tissues and urine and a method

for its determination. *American Journal of Physiology* 175: 157–161.

Underwood, M. D., Mann, J. J., and Arango, V. (2004). Serotonergic and noradrenergic neurobiology of

alcohol suicide. *Alcoholism: Clinical and Experimental Research*, 28: 57S-69S.

Valdez, G. R., Roberts, A. J., Chan, K., Davis, H., Brennan, M., Zorrilla, E. P., and Koob, G. F. (2002).

Increased ethanol self-administration and anxiety-like behavior during acute ethanol withdrawal and protracted abstinence: Regulation by corticotropin-releasing factor. *Alcoholism-*

Clinical and Experimental Research 26:1494-1501.

Valdez, G. R., Zorrilla, E. P., Roberts, A. J., and Koob, G. F. (2003). Antagonism of

corticotropin-releasing

factor attenuates the enhanced responsiveness to stress observed during protracted ethanol

abstinence. *Alcohol* 29:55-60.

Vale, W., Spiess, J., Rivier, C., Rivier, J. (1981). Characterization of a 41-residue ovine hypothalamic

peptide that stimulates secretion of corticotropin and beta-endorphin. *Science* 213: 1394–1397.

Van Bockstaele, E. J., Biswas A., and Pickel. V. M. (1993). Topography of serotonin neurons in the dorsal

raphé nucleus that send axon collaterals to the rat prefrontal cortex and nucleus accumbens.

Brain Research, 624: 188-198.

van der Kooy, D. and Hattori, T. (1980). Dorsal raphé cells with collateral projections to the caudate

-putamen and substania nigra: a fluorescent retrograde double labeling study in the rat. *Brain*

Research, 186: 1-7.

van Praag, H. M. (1982). Depression, suicide and themetabolism of serotonin in the brain. *Journal of*

Affective Disorders 4: 275-290.

Varaschin, R. K., Wazlawik, E., and Morato, G. S. (2005). Systemic and intra-accumbens microinjections

of naltrexone interfere with tolerance to ethanol in rats. *Psychopharmacology*, 182: 336-374.

Vaughan, J., Donaldson, C., Bittencourt, J., Perrin, M. H., Lewis, K., Sutton, S., Chan, R., Turnbull, A. V.,
Lovejoy, D., Rivier, C., Rivier, J., Sawchenko, P.E., Vale, W. (1995). Urocortin, a mammalian
neuropeptide related to fish urotensin I and to corticotropin-releasing factor. *Nature* 378:287–
292.

Venihaki, M., Sakihara, S., Subramanian, S., Dikkes, P., Weniger, S. C., Liapakis, Graf, T., and Majzoub, J.
A. (2004). Urocortin III, a brain neuropeptide of the corticotropin-releasing hormone family:
modulation by stress and attenuation of some anxiety-like behaviors. *Journal of Neuroendocrinology* 16: 411-422.

Vertes R. P. (1991). A PHA-L analysis of ascending projections of the dorsal raphé nucleus in the rat.
Journal of Comparative Neurology, 313: 643-668.

Vertes, R. P., and Crane, A. M. (1997). Distrubution, quantification, and morphological characterstics of
serotonin-immunoreactive cells of the supralemniscal nucleus (B9) and pontine and
mesencephalic reticular formation in the rat. *Journal of Comparative Neurology*, 378: 411-424.

Villar, M. J., Vitale, M. L., Hokfelt, T. and Verhofstad, A. A. J. (1998). Dorsal raphé serotonergic branching

neurons projecting both to the lateral genticulate body and superior colliculus: a combined

retrograde tracing-immunohistochemical study in the rat. *Journal of Comparative Neurology*,

227: 126-140.

Volpicelli, J. R., O'Brien, C. P., Alterman, A. I., Hayashida, M. (1988). Naltrexone and the treatment of

alcohol-dependence: Initial observations, in Reid LD (ed): Opioids, Bulimia, and Alcohol Abuse

and Alcoholism. New York. Springer-Verlag, 195-214.

Volpicelli, J. R., Alterman, A.I., Hayashida, M., and O'Brien, C. P. (1992). Naltrexone in the treatment of

alcohol dependence. *Archives of General Psychiatry* 49: 876- 880.

Volpicelli, J. R., Clay, K. L., Watson, N. T., and Volpicelli, L. A. (1994). Naltrexone and the treatment of

alcohol dependence. *Alcohol Health & Research World* 18: 272-278.

Volpicelli, J. R., Volpicelli, L. A., and O'brien, C. P. (1995). Medical management of alcohol dependence:

Clinical use and limitations of naltrexone treatment. *Alcohol & Alcoholism* 30(6): 789-798.

Volpicelli, J. R., Watson, N. T., King, A.C., Sherman, C.E., and O'Brien, C.P. (1996). Effect of

naltrexone on

alcohol "high" in alcoholics. American Journal of Psychiatry 152: 613-615.

Wamsley, J. K., Young, W. S III., Kuhar, M. J. (1980). Immunohistochemical localization of enkephalin in

rat forebrain. *Brain Research* 190: 153–174.

Walterhouse, B. D., Milhailoff, G. A., Baack, J. C., and Woodward, D. J. (1986). Topographical distribution

of dorsal and median raphé neurons projecting to motor, sensorimotor and visual cortical areas

in the rat. *Journal of Comparative Neurology*, 249: 460-476.

Wang, Q. P., Ochiai H., Guan J. L., and Nakai Y. (1998). Ultrastructural localization of mu-1 opioid

receptor in the dorsal raphé nucleus of the rat. *Synapse*, 29(3): 240-247.

Waselus, M. and Van Bockstaele, E. J. (2007). Co-localization of corticotropin-releasing factor and

vesicular glutamate transporters within axon terminals of the rat dorsal raphé nucleus. *Brain*

Research 1174:53-65.

Waselus, M. Galvez, J. P., Valentino, R. J., and Van Bockstaele, E. K. (2006). Differential projections of

dorsal raphé nucleus neurons to the lateral septum and striatum. *Journal of Chemical Neuroanatomy,* 31: 233-242.

Weiss, F., Mitchiner, M., Bloom, F. E., and Koob, G. F. (1990). Free-choice responding for

ethanol versus

water in alcohol preferring (P) and unselected Wistar rats is differentially modified by naloxone,

bromocriptine, and methysergide. *Psychopharmacology* 101:178-186.

Weiss, F., Lorang, M. T., Bloom, F. E., and Koob, G. F. (1993). Oral alcohol self-administration stimulates

dopamine release in the rat nucleus accumbens: genetic and motivational determinants.

Journal of *Pharmacology* and Experimental Therapeutics 267: 250–258.

Westlund, K. N., Lu, Y., Coggeshall, R. E. and Wilis, W. D. (1992). Serotonergic and noradrenergic

projections to the ventral posterolateral nucleus of the monkey thalamus. *Journal of Comparative Neurology*, 295: 197-207.

Wong, D. F., Maini, A., Rousset, O. G. and Brasic, J. R. (2003). Positron Emission Tomography – A tool for

identifying the effects of alcohol dependence on the brain. *Alcohol Research and Health*, 27(2):

161-173.

World Health Organization (2010): Retrieved from: http://www.who.int/research/en/

Zalsman, G., Huang, Y., Oquendo, M. A., Burke, A. K., Hu, X., Brent, D. A., Ellis, Sp. P, Goldman, D. and

Mann, J. J. (2006). Association of a triallelic serotonin transporter gene promoter region (5

-HTTLPR) polymorphism with stressful life events and severity of depression. *American*

Journal

of Psychiatry 163: 1588-1593.

Zhao, Z. Q., Gao, Y. J., Sun, Y. G., Zhao, C. S., GereauIV, R. W., and Chen, Z. F. (2007). Central serotonergic

neurons are differentially required for opioid analgesia but not for morphine tolerance or morphine rewards. *Proceedings of the National Academy of Sciences of the United States of*

America 104: 14519-14524.

Zhou, F. C., Bledsoe, Lumeng, L., and Li, T. K. (1991). Serotonergic immunostained terminal fibers are

lower in selected forebrain regions of alcohol-preferring rats. *Alcohol*, 8: 1-7.

Zollner, C. and Stein, C. (2007). Opioids. *Handbook of Experimental Pharmacology,* 31-63.