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Orexin Neural Activity Associated with Morphine Spontaneous Withdrawal-Induced Anxiety in Rats

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ORX and EPM

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Rats

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May 5, 2011

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Abstract

Hypocretin, also known as orexin, plays a critical role in maintaining and regulating arousal, sleep, and homeostatic functions. Recently, orexin has shown to influence drug addiction neural circuitry, specifically in mediating the hypothalamic response to stress and reinstatement of drug seeking behaviors during withdrawal. Thirty-two male Sprague-Dawley rats were observed on the elevated plus-maze to examine withdrawal-induced anxiety levels. Rats with prior exposure to 6 daily injections of 3mg/kg morphine were compared to saline-treated animals. C-Fos and orexin immunohistochemistry was used to examine lateral hypothalamic (LH) activity as well. There was a statistically significant difference in c-Fos/orexin double-labeled cells between groups, which suggests that orexinergic neurons were activated during spontaneous withdrawal from morphine after being subjected to the elevated plus-maze (EPM). There were no other significant differences found, however general trends were observed in open-arm duration between groups and c-Fos activation. Future studies should incorporate the manipulation of the orexin neural system during induced-spontaneous withdrawal with the orexin antagonist SB334867.

Orexin Neural Activity Associated with Morphine Spontaneous Withdrawal-Induced Anxiety in Rats

There are times in all of our lives when the desire for natural rewards, such as food and sex, strongly motivates our behavior. Although the diagnostic criteria of addiction do not typically apply to seeking food and sex, the neuronal circuitry for natural rewards to motivate behavior is hypothesized to be the same circuitry activated by addictive drugs to reinforce and produce addiction. For example, neuroimaging studies have shown that cues associated with both drugs of abuse and natural rewards activate overlapping cortical and limbic circuitry (Sharf, Sharhan & DiLeone, 2010).

Drug addiction is a chronically relapsing disorder that is defined by two major characteristics: a compulsion to take the drug with a narrowing of the behavioral repertoire toward excessive drug intake, and a loss of control in limiting intake (American Psychiatric Association 1994; World Health Organization 1992). The most problematic drugs are alcohol, nicotine from tobacco, and opioids. About 18 million Americans abuse or are dependent on alcohol with 2.2 million people, approximately 10% currently seeking treatment. The cost to society for alcohol abuse is estimated at \$185 billion per year. In the US, approximately 440,000 persons die per year from cigarette smoking. Cigarette smoking causes about \$75 billion in direct medical costs. Opioid dependency has also been growing rapidly, especially from 1990 to 2001. The number of people who used prescription painkillers recreationally for the first time grew 335%, which includes almost 2.5 million people (Koob & Le Moal 2000).

Illicit drug use in the United States has risen to its highest level in 8 years, according to the 2009 National Survey on Drug Use and Health (NSDUH). Last year, 8.7 percent of Americans that were 12 years and older said they used illegal drugs in the month prior to the

survey. This represents a 9 percent increase or 21.8 million people over the 2008 rate. Addicts continually crave more and more drug, as its effects captivate them. The structure and function of the brain are changed with repeated use. Consequently, over time the act of using drugs is no longer voluntary and no longer for pleasure. Addicts often self-medicate to feel “good” or feel “normal” (NIDA 2009).

Drug addiction is not a physiological dysregulation that can be explained by a single component, but rather by different components that interplay to create a cycle (Baumeister, Heatherton & Tice 1994). The addiction cycle has been described as having three factors: preoccupation-anticipation, binge-intoxication and withdrawal-negative affect (Koob & Le Moal 1997). Behaviorally, spiraling distress occurs and builds a negative affect when there is a failure to self regulate (Baumeister, Heatherton & Tice 1994). The focus on the pleasurable effects of taking the drug leads to greater and greater use, with increasing intoxication and negative consequences of drug use. At this point the effort to control intake is made difficult by withdrawal symptoms, leading to continued use of the drug and more and more negative feelings about being addicted.

Spiraling distress can also be described as a progressive dysregulation of the brain reward system within the context of repeated addiction cycles. While this dysregulation is difficult to study in humans, there are animal models that have been validated for different symptoms or constructs associated with elements of the addiction cycle, addiction criteria and sources of reinforcement associated with addiction (Koob & Le Moal 1997).

The addiction cycle is also composed of multiple sources of positive and negative reinforcements that can contribute to compulsivity for drug use. Positive reinforcement, in the context of reward is often associated with pleasurable effects of drugs in the absence of a

deprived or distress state. For example, pleasurable components of drug addiction include the presence of the drug and the ability to use the drug and achieve a high. Positive reinforcement also occurs when the sight of the drug at its receptor increases the addict's craving, increasing the probability of taking the drug again. Secondary positive reinforcement is when there is a conditioned pairing of previously neutral stimuli with acute positive reinforcing effects. In contrast, negative reinforcement occurs with alleviation of an existing aversive state or of withdrawal (Koob & Le Moal 2001). Secondary negative reinforcing effects occurs with the removal of the conditioned negative reinforcing effects of conditioned abstinence. Conditioned positive and negative reinforcement generally contributes to the preoccupation and anticipation stage of the drug addiction cycle (Baumeister, Heatherton & Tice 1994). The neurobiological basis for acute positive reinforcing effects of drugs of abuse, negative reinforcing effects imparted by the dependent state and the conditioned reinforcing effects associated with protracted abstinence and relapse have provided insight into the basis of addiction. Protracted abstinence is defined as a state of residual reward dysregulation after acute withdrawal (Koob & Le Moal 2000).

Drug addiction, or substance dependence has been hypothesized to occur when there is a detachment from homeostatic brain regulatory mechanisms that control normal everyday emotional states (Sharf, Sharhan & DiLeone 2010). Specifically, two factors have been hypothesized to comprise the break from homeostasis, the under-activation of brain reward neurotransmitters and the recruitment of brain stress systems. The reward and stress systems all interact at different stages of the addiction cycle to potentiate the dysregulation (Edwards & Koob 2010). Similar to chronic physiological disorders, the effects worsen over time and are subject to significant environmental influences and leave a lasting neuroadaptive trace that

allows rapid reinstatement of addictive behaviors even years after detoxification and abstinence. However, the characteristics of drug addiction are more than just a homeostatic dysregulation of hedonic function and executive function, rather a dynamic break with homeostasis of these systems toward a new set point, often noted as allostasis (Volkow, Fowler, Wang & Swanson 2004).

Allostasis was originally conceptualized to explain the persistent illness of arousal and autonomic function associated with chronic stress. Allostasis is defined as ‘stability through change’ in which continuous readjustment of physiological mechanisms occurs toward a new state (McEwen 2000). The state of chronic regulatory system abnormality from its normal or homeostatic operating level can be defined as an ‘allostatic state.’ Two components of the brain are hypothesized to adjust to the challenges imposed by drugs of abuse to produce an allostatic-like state: under-activation of reward circuitry and activation of the stress response (McEwen 2000). Repeated challenges, such as drugs of abuse, lead to attempts of the brain via biochemical, cellular and neurocircuitry changes to maintain stability. For drug addiction, the residual deviation from normal brain reward threshold regulation is termed an ‘allostatic state’, and the cost leading to sustained pathology is the ‘allostatic load.’ The allostatic state represents motivational perception by decreased function of reward circuits and recruitment of brain stress systems, which lead to the compulsivity of drug seeking and drug taking to prevent withdrawal-like symptoms.

Drug addiction is a chronically relapsing disorder characterized by: compulsion to seek and take drugs, loss of control in limiting intake, and emergence of a negative emotional state reflecting motivational withdrawal syndrome when the drug is not available (Koob 2009). Addiction has been signified as an evolving disorder with three stages; anticipation or

impulsivity, binge/intoxication, and withdrawal/negative affect. When a drug addict moves through these stages, from positive reinforcement driving the motivated behavior to negative reinforcement driving the motivated behavior, removal of the aversive stimulus will increase the probability of a response (Koob & Le Moal 2004). As a commonality, all drugs produce this psychological cycle but different drugs produce different patterns of addiction with different components of the addiction cycle (Koob 2009).

Neuropharmacological studies have shown the importance of the mesolimbic system and its role in reward, specifically the release of dopamine from the ventral tegmental area or the “pleasure center.” The midbrain dopamine system is composed of two major projections: the nigrostriatal system which projects from the substantia nigra to the corpus striatum and the mesocorticolimbic dopamine system which projects from the ventral tegmental area (VTA) to the nucleus accumbens (Koob, Sanna & Bloom 1998). The mesocorticolimbic system has been primarily implicated as mediating the reinforcing actions of drugs of abuse. For example, psychostimulants such as cocaine increase the extracellular concentrations of dopamine by inhibiting the reuptake by the dopamine transporter. The dopamine system can be modulated by endogenous opioids like endorphins, enkephalins and dynorphin, which the body naturally produces. Endogenous opioids have a wide range of functions like regulating reactions to pain and vital functions such as hunger and thirst, mood control, immune response and other processes. One of the main reasons why opiates such as heroin and morphine affect the brain so powerfully is the ability of the exogenous substances to bind to the same receptors as the endogenous opioids. There are three kinds of opioid receptors that are found distributed throughout the brain; mu, delta, and kappa receptors. The receptors are G-protein coupled, functioning via second messengers and can influence the opening of ion channels.

In many cases the opening of the ion channels reduces the excitability of neurons and the reduction is thought to be the reason for the euphoric effect of opiates that bind to mu and delta receptors. The GABA inhibitory interneurons of the VTA are also involved in the euphoric effect. When exogenous opioids, like morphine bind to the mu receptors, the effect decreases the amount of GABA released into the synapse. When there is no morphine, GABA usually reduces the amount of dopamine release in the nucleus accumbens. However, with morphine or any other opiate present, the result is inhibiting the inhibitor, which ultimately increases dopamine levels and the amount of pleasure felt.

The development of addiction and vulnerability to relapse following withdrawal is proposed to be the result of neuroadaptive processes within the central nervous system that oppose the acute reinforcing actions of drugs of abuse (Weiss et al., 2006). These changes lead to impairment in the mechanisms that mediate positive reinforcement and the emergence of affective changes such as anxiety, dysphoria, and depression during withdrawal. Considerable evidence exists implicating perturbations in DA and 5-HT transmission in the nucleus accumbens—neurochemical systems that are activated by cocaine and ethanol self-administration and are deficient during withdrawal—as potential substrates for these affective changes (Logrip, Koob & Zorrilla 2011, Weiss 2006).

One component of the cycle of drug addiction is the transitional development of aversive or negative emotional states during the allostatic load. Negative emotional states consist of certain motivation elements such as irritability, emotional pain, dysphoria and loss of motivation for natural rewards (Edwards & Koob 2010, Weiss et al., 2006). Two clear processes that have been hypothesized to play an active role in the neurological basis of this state: loss of function in the reward systems and hyperactivity of brain stress systems (Volkow, Fowler, Wang &

Swanson 2004). Growing evidence suggests that enhanced CRF release in the central nucleus of the amygdala represents a mechanism underlying the anxiogenic and stress-like consequences of withdrawal that are common to all drugs of abuse. A growing body of evidence implicates dysregulation of this non-neuroendocrine CRF stress system as a common factor in the anxiogenic and aversive consequences of withdrawal from drugs of abuse (Weiss et al., 2006).

Stress can be defined generally as responses to demands upon the body or, specifically on the central nervous system, as alterations in psychological homeostatic processes (Hennessey & Levine 1979). A state of stress is associated with various external and internal challenges to the body and brain, usually termed stressors, and the construct of stress may represent the extreme pathological continuum of over-activation of the normal activation (arousal) or emotional systems of the body. The state of stress is reflected biologically by various physiological changes that include an activation of the pituitary-adrenal axis and release of glucocorticoids into the bloodstream, activation of the sympathetic nervous system, and activation of brain emotional systems (Koob & Le Moal 2000). The hypothalamic-pituitary adrenal axis is the center and the stress regulatory system of an organism that connects the central nervous system with the hormonal system. Cortisol, which is released at the end of the chain, has a wide range of physiological effects in the body. Cortisol plays a critical role in metabolism by mobilizing resources to provide energy. The release of cortisol increases metabolic demands presented by the stressor and allows the organism to adapt and also maintain homeostasis and support normal physiological functioning (Kudielka & Kirschbaum 2005).

When an organism is presented with a stressor, the hypothalamus secretes corticotrophin-releasing hormone (CRH), which produces the release of adrenocorticotrophic hormone (ACTH) from the pituitary. ACTH then triggers the secretion of glucocorticoids from the adrenal cortex.

In humans, the main glucocorticoid is cortisol, which is predominately bound to proteins in blood and about 5-10% of total plasma cortisol circulating as biologically active or “free” cortisol (Kudielka & Kirschbaum 2005). Normally, high levels of glucocorticoids, via negative feedback decrease CRF synthesis at the level of the paraventricular nucleus but activate CRF activity at the level of the central nucleus of the amygdala. Stressful stimuli also activate CRF systems in the basal forebrain, particularly the bed nucleus of the stria terminalis and the central nucleus of the amygdala to help mediate behavioral responses to stressors and to mediate sympathetic activation associated with stressors systems (Koob & Le Moal 2000).

Glucocorticoids, instead of exerting a feedback suppression of CRF synthesis in the central nucleus of the amygdala, actually increase synthesis of CRF providing a means for extending the contribution of the brain stress systems to allostasis (Kudielka & Kirschbaum 2005).

Stress has been hypothesized as a state that contributes tremendously to different stage of the addiction cycle. Specifically, the focus of stress on addiction has been associated with withdrawal or the negative affect stage (Koob & Le Moal 1997). The role of CRF as a component of addiction cycle is based on the opponent process theory, which has been expanded into the different domains of the neurobiology of drug addiction from a neurocircuitry perspective. An allostatic model of the motivational systems has been proposed to explain the changes of motivation that are associated with the dependence in addiction (Koob & Le Moal 2001). This view conceptualizes addiction as a cycle of increasing dysregulation of brain reward/anti-reward mechanisms that results in a negative emotional state, which strengthens compulsive drug use. Counteradaptive processes that are normally part of homeostatic limitation of reward function fail to revert back to normal homeostatic range. The counteradaptive processes are hypothesized to be mediated by two mechanisms: within-system, which is changes

in the reward pathways and between-systems neuroadaptations, which is the recruitment of the brain stress systems (Koob & Bloom 1988). The recruitment of the brain stress systems where CRF most likely a significant component of the negative reinforcement processes that drive the compulsivity of drug addiction (Koob 2009). The opponent process theory assumes that many affective states, whether pleasant or aversive are automatically opposed by centrally mediated mechanisms that reduce the intensity of the affective state (Koob, Markou, Weiss & Shulteis 1993). The opponent process theory hypothesizes that positive reinforcers, like drugs have positive hedonic processes that are counteracted by negative hedonic processes (Koob & Le Moal 2008).

Positive hedonic processes are hypothesized to be stable and simple with a short latency and duration and follow right after drug administration. Negative hedonic processes have longer latencies and build up on strength and diminish slowly and resistant to the development of tolerance (Koob, Markou, Weiss & Shulteis 1993). The example of an opiate “rush” is considered a positive hedonic process and the withdrawal symptoms are a reflection of opponent negative hedonic processes. Self-administration of drugs of abuse have tolerance to euphoric effects while at the same time the withdrawal or abstinence of the drug becomes more intense and of longer duration. The positive reinforcing properties slowly diminish while the negative reinforcing properties strengthen. Specific brain areas that are involved in reward and stress within the basal forebrain like the ventral striatum and amygdala are thought to be dysregulated in addiction. These structures convey the opponent motivational processes that drive dependence in addiction. Neurotransmission in these structures are not only experiencing a decrease in reward neurotransmission from dopamine and opioid peptides in the ventral striatum but there is also a recruitment of brain stress systems such as corticotropin-releasing factor, noradrenaline

and dynorphin in the amygdala (Koob 2009). From a social psychological standpoint, stress contributes to the addiction process known as under regulation such as decreases in strength and sources of misuse such as self-medication, leading to spiraling distress (Baumeister, Heatherton, Tice 1994). From a psychiatric perspective stress is the major state or stimulus associated with vulnerability to relapse (Erb, Shaham & Stewart 1996).

As addiction progresses, the brain's stress system releases corticotropin-releasing factor (CRF), norepinephrine and dynorphin at higher rates which produce aversive or stress-like behavior. At the same time, the mesolimbic system, which drives positive motivation and reward that was associated with the initial use of the drug, declines (Edward & Koob 2010). Consequently, the negative emotional state develops with chronic use, and drugs are subsequently taken to alleviate negative motivational symptoms associated with withdrawal.

The release of corticotropin-releasing factor or CRF during stress is an unavoidable consequence of daily experiences that occur from external environmental stimuli. Therefore the release of CRF or cortisol affects a wide range of critical physiological processes. The body must strictly regulate the activity of the stress response because of the wide range of external stressors. The role of stress during addiction is often recognized during withdrawal or drug-seeking behaviors. Acute withdrawal from addictive drugs has been associated with physical signs that vary depending on the drug of abuse. Recent conceptualizations of addiction have considered that the physical signs of withdrawal serve as only one of the symptoms associated with addiction (Erb, Shaham & Stewart 1996). However, other variables contribute to acute withdrawal that have motivational significance into the cycle of drug addiction; these actions fall under the conceptual framework of stress.

Acute withdrawal is associated with a negative affective state, which includes dysphoria, depression, irritability and anxiety. For example, cocaine withdrawal in humans is characterized by severe depressive symptoms as well as irritability, anxiety and anhedonia, which can last several hours to days. These symptoms are associated with the motivating factors in the maintenance of cocaine dependence (Gawin & Kieber 1986). Opiate and ethanol withdrawal produce dysphoria. It has been suggested that the motivational effects of drug withdrawal may have the same neural substrates and neuropharmacological mechanisms of the neural systems for the positive reinforcing effects of drug abuse. For example, when intracranial self-stimulation was used to measure reward thresholds throughout the course of drug dependence, the results showed that reward thresholds increased following chronic administration of major drugs of abuse such as opiates, psychostimulants, ethanol, and nicotine. The effects appeared to reflect changes in the activity of the same mesocorticolimbic system known for its neural positive reinforcing effects of drugs and can last up to 72 hours depending on the drug and dosage (Koob 2006).

Neuroadaptive mechanisms that are associated with reward function have been hypothesized to be neurochemical changes associated with the same neurotransmitters implicated in the acute reinforcing effects of drugs (Koob & Bloom 1988). When measured by *in vivo* microdialysis, an example of homeostatic within-system adaptive neurochemical events would include decreases in dopaminergic and serotonergic neurotransmission in the nucleus accumbens during drug withdrawal (Parsons, Koob & Weiss 1995 and Weiss, Markou, Lorang & Koob 1992). Increased sensitivity of opioid receptor transduction mechanisms in the nucleus accumbens has been shown during opiate withdrawal and decreased GABAergic and increased

NMDA glutamatergic transmission has been shown during ethanol withdrawal (Stinus, Le Moal & Koob 1990).

Additional evidence supporting a role for brain stress systems being activated during acute withdrawal are studies showing an increase in extracellular levels of CRF in the region of the central nucleus of the amygdala during acute withdrawal from drugs of abuse. Animals exposed to a chronic ethanol liquid diet to induce ethanol dependence showed a time-related increase in CRF in the amygdala as measured by *in vivo* microdialysis (Merio-Pich et al., 1995). Similar results have been observed during withdrawal from cocaine (Richter & Weiss 1999) and precipitated cannabinoid withdrawal (Rodrigues De Fonseca, Carrera, Navarro, Koob & Weiss 1997).

Animal models of symptoms of addiction on specific drugs such as stimulants, opioids, alcohol and nicotine have been used to define the different stages of the addiction cycle (Shippenberg & Koob 2002). For example, animal models focused on the binge/intoxication stage of addiction can be conceptualized as measuring acute drug reward where the reward is a positive reinforcer with additional emotional value. Animal models associated with withdrawal/negative affect stage have used conditioned place aversion to precipitated withdrawal or spontaneous withdrawal thresholds and dependence-induced increases drug seeking (Koob 2008). Furthermore, chronic administration of drugs has been known to cause a dysregulation of the stress responses mediated by CRF not only by the HPA axis but also the brain extra hypothalamic stress system (Koob 2009). In general, a stress response to all drugs of abuse and alcohol and acute withdrawal cause an activated HPA response. However, with chronic usage of drugs of abuse, there is a blunted HPA response but also a sensitized extra hypothalamic CRF stress system response (Koob & Kreek 2007).

In vivo microdialysis during acute withdrawal following chronic administration or self-administration of drugs of abuse have been shown to produce an increase in extracellular CRF in the extended amygdala (Merio-Pich et al. 1995). During alcohol withdrawal, hyperactivity of the extra hypothalamic CRF systems are combined with an increase in extracellular CRF within the central nucleus of the amygdala and bed nucleus of the stria terminalis of dependent rats (Merio-Pich et al. 1995; Olive et al. 2002). Extracellular CRF was also increased in the central amygdala during precipitated withdrawal from chronic nicotine (George et al. 2007), binge cocaine self-administration (Richter & Weiss 1999), and precipitated withdrawal from opioids (Weiss et al., 2001) and cannabinoids (Rodriguez de Fonseca et al. 1997).

Another common response to acute withdrawal from major drugs of abuse is the manifestation of negative emotional state, including anxiety-like responses. There have been animal models in which the dependent variable is commonly a passive response to a novel or aversive stimulus; such as the open field test, elevated plus-maze, defensive withdrawal test or social interaction test; or an active response to an aversive stimulus, such as defensive burying which have shown anxiety like responses to acute withdrawal from all major drugs of abuse. When subjected to repeated exposure and then withdrawal of cocaine, alcohol, nicotine, cannabinoids, and benzodiazepines, they produce anxiogenic-like responses in the elevated plus-maze, defensive withdrawal or defensive burying test. Furthermore, the effects can be reversed by using CRF antagonists (Rodriguez de Fonseca et al. 1997; Basso et al, 1999; Knapp et al. 2004; George et al. 2007).

Elevated-plus maze and Drug Withdrawal

The elevated plus-maze (EPM) has been used to study anxiety-related behavior in rodents (Pellow, Chopin, File & Brilley 1985) and assessing the anti-anxiety effects of pharmacological

agents that are known to produce anxiolytic effects such as chlordiazepoxide and diazepam and ones that cause anxiety such as yohimbine, caffeine and amphetamines (Pellow, Chopin, File & Brilley 1985) and steroid hormones (Walf & Frye, 2007), defining brain regions and mechanisms underlying anxiety-related behavior (Pellow, Chopin, File & Brilley 1985; Hogg 1996; Walf & Frye 2007). The behaviors that are commonly recorded when using the elevated plus maze are the time spent and entries made on open and closed arms (Pellow, Chopin, File & Brilley 1985; Walf & Frye 2007). The EPM consists of two open and two closed arms with a central exposed area where the arms bisect each other (Pellow, Chopin, File & Brilley 1985; Paterson, Iwunze, Davis, Malekiani Hanania 2010). Activity in the open arms is considered to be a conflict between the rodent's preference for protected areas or closed arms that would be considered their innate motivation to explore novel environments. Anti-anxiety behavior which would produce an increase time on the open arm or open arm entries can be determined with a measure of spontaneous motor activity, total and/or closed arm entries. Other ethological measures can also be observed like the number of rears, freezing stretched-attend postures, protected and unprotected head dips, grooming or sniff duration (Rodgers & Johnson 1995).

Normal exploratory behavior in the elevated plus-maze is usually in favor of the closed arms and this tendency to stay in the closed aspects of the maze can be enhanced by drugs like anxiogenics that increase the aversion towards the anxiety-provoking open arms. In contrast, when subjects are administered anxiolytic compounds, the behaviors produced will reduce natural aversion to the open arms and promote the exploration. The critical aspect of the EPM is the number of entries made onto the open arm and time spent on the arms. These variables have been correlated with anxiety (Hogg 1996). Locomotor activity is assessed by monitoring the total or closed number of arm entries (File 1992; Lister 1987).

The elevated plus-maze has frequently been used in research as a tool to measure anxiety-like behaviors during withdrawal. Subjects who have been repeatedly exposed to drugs of abuse are hypothesized to exhibit withdrawal-like symptoms like drug-seeking behavior. Drug-seeking behavior has been generally characterized as the subject more likely spending time in the closed arms compared to the open arms in the elevated plus-maze.

In recent studies, in evaluating withdrawal from chronic exposure to ethanol, the elevated plus-maze has been used to study anxiety-like behaviors and the underlying neurobiology of withdrawal (Taksande et al. 2010) with a decrease in open arm time and percent open arm entries (Jung, Wallis, Gatch & Lal 2000). In general, ethanol withdrawal decreases most of a subject's behavior in the EPM, indicating the occurrence of an anxiogenic-like effect of withdrawal in rodents, although the effects of withdrawal have not always been consistent (Klithernes 2004). However, others argue that the elevated plus-maze experiments using ethanol dependent rats have generally been more consistent in finding presumed anxiogenic-like effects of ethanol withdrawal with most studies observing increased anxiety like behaviors after the cessation of ethanol administration (Baldwin, Rassnick, Rivier, Koob & Britton 1991; Cagetti, Liang, Spigelman & Olsen 2003; File, Andrews, al-Farhan 1993).

Taksande et al (2010) demonstrated the effects of agmatine on acute ethanol anxiolytics and withdrawal anxiety in rats using the EPM. Consistent with previous research, acute administration of ethanol produced a dose-dependent anxiolytic effect where there was an increased percent time spent and entries into the open arms without any changes in the closed-arm entries. However, animals treated with higher dose (2.5 g/kg, i.p.) of ethanol showed decrease in open as well as closed-arm activity in the EPM test, indicating mild sedation or motor incoordination as previously reported. When rats were assessed in the EPM 18h following

ethanol injections, adult rats showed higher anxiogenic effects of acute withdrawal in the elevated plus-maze. The anxiogenic effects were measured as a reduction in percent of open arm entries and open arm time, coupled with increases in percent of protected head dips and stretch attend postures (Doremus, Brunell, Varlinskaya, Patia Spear 2003).

The withdrawal symptoms of hyperactivity to external stimuli and increased anxiety-related behavior in the elevated plus-maze were observed in long-term, voluntary alcohol-drinking rats with frequent deprivation sessions, (Holter, Linthorst, Reul & Spanagel 2000; Jung, Wallis, Gatch & Lal 2000). Furthermore, Holter, Linthorst, Reul & Spanagel (2000) found that rats with repeated alcohol deprivation compared to rats without prior alcohol deprivation experience exhibited higher anxiety-related behaviors in the EPM. Researchers suggested that the blood alcohol levels attained, length of chronic alcohol intake and withdrawal experience may have played a role for the intensity of the withdrawal symptoms.

Addiction to nicotine has also been paired with subsequent addiction to ethanol, especially during adolescence. Smokers consume twice as much alcohol as do nonsmokers and alcoholics who smoke consume more cigarettes than do nonalcoholic smokers (Larsson & Engel 2004). Exploratory use of alcohol occurs typically during adolescence and there has been a strong correlation between the onset of tobacco consumption at an early age and alcohol addiction (Grant 1998). In a recent study, the elevated plus-maze was used to assess short-term and long-term anxiety effects of nicotine and/or ethanol exposure in mice. Researchers found that ethanol was anxiolytic in adolescent mice and that nicotine reversed the effect. Short-term drug withdrawal elicited sex-dependent effects and exposure to nicotine and/or ethanol was only significant in females. Co-administration of ethanol and nicotine also elicited an anxiogenic response, which suggested that the deficient response to the anxiolytic effects of ethanol in

adolescents co-exposed to nicotine might drive higher ethanol consumption. Furthermore, increased anxiety during long-term nicotine and/or ethanol withdrawal may facilitate relapse to drug use (Abreu-Villaca, Nunes, Queiro-Gomes, Manahaes & Filgueiras 2008).

Another study examining the co-consumption of nicotine and ethanol in mice looked at withdrawal symptoms during abstinence using the EPM. Researchers observed aversion to the open arms of the elevated plus-maze and the modification of spontaneous locomotor activity during and following repeated ethanol and/or nicotine administration in mice. Onaivi, Todd & Martin (1989) found that ethanol plus nicotine treated animals increased time spent in the open arms of the maze during treatment relative to controls. When mice were subjected to spontaneous withdrawal, there was a rapid onset of intense aversion to the open arms of the maze and a concomitant reduction in locomotor activity that was greater compared to mice subjected to spontaneous nicotine and/or ethanol withdrawal alone. The study suggested that the combined effects of ethanol and nicotine reduced aversion to the open arms of the elevated plus-maze and may imply an anti-aversive action; however mice also demonstrate an increased aversiveness to the open arms following spontaneous withdrawal of the both nicotine and ethanol together.

Studies concerning the acute and chronic effects of nicotine on the elevated plus-maze in mice have also been a focus in examining anxiety-related symptoms. Biala and Budzynska (2006) examined the effects of nicotine when administered at certain time-points before the mice were subjected to the EPM, 5 minutes after an injection, nicotine had an anxiogenic effect by causing decreases in the percentage of time spent on the open arms and in the percentage of time in open arm entries. Furthermore, after six days of injections, tolerance was observed and on the seventh day of injection, an anxiolytic effect was seen with increases in time spent on the open arms and percentage of open arm entries.

In a separate study, Manhaes, Guthierrez, Filqueiras & Abreu-Villaca (2008) examined the anxiety effects of nicotine withdrawal using the EPM in rats. Researchers also tested if nicotine consumption could be predicted after being subjected to the elevated plus-maze as a model of relapse. Animals were classified as having low anxiety or high anxiety after nicotine withdrawal and immediately after finishing exposure to the EPM, rats were given the free choice between bottles of water or a nicotine solution. Researchers found that nicotine consumption was dependent on the anxiety level where the high anxiety group had lower nicotine consumption than other groups. This study suggested that anxiety-like behavior during nicotine withdrawal is associated with subsequent nicotine self-administration.

Other studies examining cocaine withdrawal, demonstrated that rats that are exposed to chronic administration of cocaine and then one-day withdrawal found an increase in anxiety-like behavior from decreases in delta opioid receptor signaling using the elevated plus-maze. Researchers also found depression-like behaviors using the forced swim test. The results of the study found that with early withdrawal from cocaine, there is an apparent increase in anxiety and depression, which is related to the desensitization of delta opioid receptor function (Perrine, Sheikh, Nwaneshiudu, Schroeder and Unterwald 2008). A similar study using the elevated plus-maze and forced swim test found that after chronic cocaine administration, withdrawal from cocaine resulted in a decrease in time spent in the open arms and entries onto the open arms of the plus maze. They also observed a reduction in escape behaviors and time to first immobility in the forced swim test. However, when researchers administered the nicotinic acetylcholine receptor antagonist bis- (2,2,6,6-tetramethyl-4-piperidiny)l sebacate (BTMPS), cocaine-induced anxiety-like behaviors were reversed in the elevated plus-maze as were depressive-like behaviors in the forced swim test (Hall, Pearson, Buccafusco 2010).

Sorge and Stewart (2005) presented the importance of studying responses during late withdrawal where stressor-induced reinstatement is lower during early withdrawal when “anxiety” responses are most pronounced and is heightened later during withdrawal when “anxiety” responses are likely diminished. In order to investigate anxiety-like behaviors in repeated cocaine administration and withdrawal, Mantsch, Baker, Francis, Katz, Hoks & Serge (2007) examined stress-related responses further into withdrawal. Researchers found that rats that had a shorter exposure to cocaine had an increased activity in the elevated plus-maze. Several weeks after extinction/withdrawal, rats that had a shorter exposure to cocaine still had more activity during times of stress associated with heightened susceptibility to stressor-induced drug-seeking behavior, which may be the result of increases in CRF regulation.

In a separate study Huang, Liu-Chen and Kirby (2010) attempted to characterize the emotional aspects of SR141716-precipitated THC withdrawal in a mouse model of anxiety using the EPM and found a reduction in the percent of open arm exploration compared to vehicle. Marijuana discontinuation has been characterized as anxiogenic; therefore researchers were the first to explore the potential anxiogenic effects of precipitated THC withdrawal. Researchers concluded that the decrease in open arm entries was a result of anxiety-like behavior whereas Pellow, Chopin, File & Brilley 1985 described decreases in open arm entries were indicative of anxiety-like behavior. The experimental design was novel, insofar that the conclusions made by the researchers were dependent on the levels of CRF in mice that were subjected to precipitated THC withdrawal and EPM. Mice treated with SR141716 precipitated a reduction in exploration of the open arms of EPM in mice repeatedly treated with THC vs. vehicle. SR141716 significantly reduced the percent of open arm entries per of the total arm entries and open arm time of total time in arms. This was the first evidence that cannabinoid withdrawal produces

anxiety-like effects in mice. This animal model may help to identify the mechanisms that contribute to adaptations in the neuronal circuitry of the brain that are expressed as emotional symptoms of cannabinoid withdrawal.

Similar to the previous studies, Zhang and Schulteis (2008) used morphine and found that withdrawal from acute morphine at low doses can produce anxiolytic effects with increased exploration in open arm entries. Researchers also found that repeated administration of morphine resulted in a further increase in the magnitude and duration of this anxiolytic-like effect. Three injection regimens were employed: Morphine Naïve four vehicle injections, Acute Morphine with three vehicle injections and the fourth injection of morphine, or Repeat Morphine with four injections of morphine. Acute pretreatment with morphine resulted in time-dependent increases in exploration of the open arms of the plus maze in naloxone-naïve rats when tested at 2, 4 or 8 hr. after the final pretreatment injection, with the effects at the higher dose appearing later four hours later than after the lower dose 2 hr. This pattern of results confirms a significant anxiolytic-like effect of a low dose of morphine 0.56 mg/kg administered 15 min prior to test, suggested that low residual morphine levels remaining in plasma at 2-4 hr. after 5.6 and 10 mg/kg morphine may be sufficient to elicit anxiolytic-like effects. Repeat treatment with either dose of morphine resulted in a further increase in the magnitude and duration of this anxiolytic-like effect. These effects had dissipated by 8 hr. post-morphine, and therefore precipitation of withdrawal by one of several doses of naloxone (0.10-3.3 mg/kg) was assessed in separate cohorts of rats 8 hr. after the final pretreatment under Morphine Naïve, Acute Morphine, or Repeat Morphine conditions. Naloxone resulted in a significant dose-dependent expression of anxiety-like behavior with no effects on general activity after Acute Morphine pretreatment at either 5.6 or 10-mg/kg morphine. A further significant shift in naloxone potency was observed

after Repeat Morphine pretreatment at the 10 mg/kg but not the 5.6 mg/kg dose. Thus, anxiety-like behavior is a prominent feature of the negative emotional consequences of naloxone-precipitated withdrawal from acute opioid dependence.

Another study examining mice undergoing naloxone-precipitated morphine withdrawal, showed behaviors in the elevated plus-maze that were very similar as described in the previous study (Buckman, Hodgson, Hofford and Eitan 2009). Mice exhibited an increase in time spent in the open arms compared to controls, therefore researchers wanted to further inspect the effect of spontaneous morphine withdrawal on the EPM behavior of mice in order to see if increase in open-arm time is classically understood to represent an anxiolytic response.

Researchers found that mice undergoing opioid withdrawal exhibited an increase in EPM open-arm time. Buckman, Hodgson, Hofford and Eitan (2009) reported in mice undergoing both naloxone-precipitated and spontaneous opioid withdrawal exhibited an unexpected outcome where there was an increase time and entries on the elevated plus-maze on the open arm compared to controls. Classically, conditions that decrease the amount of time spent in the open arms of the EPM are associated with anxiety, while increases in open-arm time are associated with an anxiolytic profile. Although an unexpected behavior, since opioid withdrawal is associated with increase in anxiety and known to cause negative symptoms including aversion and increased plasma CRF levels, the results of the study raise the possibility that perhaps the behavioral response in the EPM might be susceptible to modulation by multiple variables and not exclusively anxiety. Buckman, Hodgson, Hofford and Eitan (2009) also demonstrated that mice exhibited an increase in both open-arm time and percent of open-arm entries 8-hr following the last dose of morphine. The increase in open-arm time during withdrawal was not

simply due to an overall increase in total activity, since the amount of exploratory activity did not change significantly during withdrawal.

Opioid withdrawal has been recognized as anxiogenic in humans and the elevated plus-maze has also been used to demonstrate anxiogenic effects in mice. Hodgson, Hofford, Norris & Eitan (2008), characterized EPM behaviors of mice during naloxone-precipitated morphine withdrawal where naloxone did not show an effect in EPM behaviors in drug-naïve mice. Morphine-dependent mice where withdrawal was not precipitated spent less time in the open arms compared to controls. Increased open-arm time was observed in morphine-dependent mice undergoing naloxone-precipitated withdrawal. The increase however was not due to an increase in locomotor activity because significant differences were not seen but morphine dependency was apparent given that there was not a significant increase in open-arm time for mice undergoing acute morphine withdrawal. Observed increase in open-arm duration was unexpected since opioid withdrawal is associated with anxiety. Furthermore, mice that underwent naloxone-precipitated morphine withdrawal are known to be aversive and increase levels of corticosterone. The conclusions of this study were unexpected because of the unusual EPM behavior for mice undergoing naloxone-precipitated morphine withdrawal. As a result, researchers suggest that EPM behaviors observed raise the possibility that anxiety-behavior may be accompanied by withdrawal-induced behaviors as well in the EPM.

In an attempt to characterize EPM behaviors during spontaneous and naloxone-precipitated opiate withdrawal Shulteis, Yackey, Risbrough & Koob (1998) examined whether spontaneous and naloxone precipitated opiate withdrawal could be observed reliably in rats that were dependent on morphine. Previous attempts with rats to model the anxiogenic-like effects of opiate withdrawal using the EPM has been found with mixed success. Rats were implanted with

morphine pellets and 72-hrs after implantation rats were tested in the EPM compared to control. One aspect of the study examined the effects when pellets were removed 8 or 12-hr prior exposure to the EPM. Anxiogenic effects, a reduction in time spent in the open arms was observed in opiate withdrawal in animals that had the pellets removed 8-hr prior but not in the 12-hr prior the EPM. Researchers also examined naloxone-precipitated withdrawal and did not remove the pellets from the cages. Results showed that naloxone dose dependently precipitated a reduction in exploration of the open arms of the EPM. Overall these results suggest that both spontaneous and precipitated withdrawal from continuous morphine administration results in an anxiogenic-like effect in the plus-maze.

Peregud, Vorontsova, Yakovlev, Panchenko, & Gulyaeva (2008) demonstrated the involvement of nitric oxide (NO) in its mediating effects of opiates in opiate withdrawal. The study measured concentrations of the nitergic system and used the elevated plus-maze to measure anxiety during morphine withdrawal. During day six, after morphine withdrawal, rats showed more entries in the open arms of the EPM and also spent a longer time in the open arms. Correlations further showed that measure of the NO system in the hippocampus specifically and behavior of animals in the EPM, may represent one of the molecular mechanisms impairing the behavior of animals in abstinence.

Hypocretins/Orexin Pathways

Drug addiction is a chronic brain disease that has been found to have profound effects on the mesolimbic dopamine system and the release of corticotropin-releasing factor, however growing research has shown the importance of a different circuitry that innervates both systems during withdrawal. The orexins or hypocretins are a pair of neuropeptides that encode for a single precursor, as the endogenous ligands for two G protein coupled receptors that are

expressed in the brain and were previously considered to be orphan receptors (Chemelli et al. 1999). An mRNA encoding the same neuropeptide precursor was independently identified by differential cloning approach and the putative encoded peptides were named as hypocretins. Orexin A and B are neuropeptides of 28-33 amino acids that are produced exclusively by neurons in lateral hypothalamus.

Orexin peptides are unique among hypothalamic peptide neuromodulators that act directly at axon terminals and can increase the release of GABA and glutamate which both are responsible for almost all fast synaptic activity in the hypothalamus. The densest orexin projections are found in locus coeruleus, raphe nuclei, medullary reticular formation, paraventricular thalamic nucleus, septal nucleus and lamina of dorsal horn suggesting a possible role in modulating sensory input. Physiologic roles of orexin have been primarily focused on energy homeostasis because of past evidence of the lateral hypothalamus but based on neuroanatomical data, researchers have suggested a possible role of orexin as sleep wake modulators (Sakurai et al 1998).

Although hypocretin/orexin (HRT/ORX) have been shown to have an essential role in sleep/wakefulness, other fundamental roles have recently been discovered that are not related to sleep. Orexin has been found to innervate important pathways such as neuroendocrine and autonomic function, appetite regulation, cardiovascular function and regulation of reproductive and stress hormone secretion (Samson, Taylor & Ferguson 2005). Orexin innervates several major ascending arousal pathways. A major input to the relay and reticular nuclei of the thalamus originates from cholinergic cell groups in the upper pons, pedunculo pontine (PPT) and laterodorsal tegmental nuclei (LDT), which facilitate thalamocortical transmission. A second pathway activates the cerebral cortex to facilitate the processing inputs from the thalamus, which

comes from neurons in the monoaminergic cell groups, which include the tuberomammillary nucleus (TMN), the A10 cell group containing dopamine, dorsal and median raphe nuclei containing serotonin, and the locus coeruleus containing noradrenaline. The second pathway also receives input from the lateral hypothalamus containing orexin or melanin-concentrating hormone, and from the basal forebrain neurons containing GABA or Ach. Orexin also projects to the ventrolateral preoptic nucleus (VLPO), which includes the TMN, A10 cell group, raphe cell groups and the LC (Saper, Scammell & Lu 2005).

Orexin plays a unique role in autonomic function, researchers showed that hypothalamic and brain stem structures known to play a role in central cardiovascular regulation were innervated by ORX containing axon terminals and that the two receptors of ORX were localized at those sites (Sakurai et al. 1998; Ferguson & Samson 2003). Studies reported that when administration of exogenous orexin resulted in increased blood pressure and heart rate in conscious and anesthetized animals, with the effects mediated by activation of the sympathetic arm of the autonomic nervous system (Samson, Taylor & Ferguson 2005).

Orexin is known to play a role alongside the endocrine system. There are several studies showing the presence of ORX-positive axon terminals and receptors in hypothalamic and extra-hypothalamic sites, which is recognized to be important in the control of anterior pituitary function of neuroendocrine effects of peptides (Samson, & Taylor & Ferguson 2005). Orexin has also been shown to have roles in appetite regulation where administration of ORX into the cerebroventricular system of conscious rats, regardless of fed state, did stimulate significant increases in food intake, however its effects are less than known neuropeptides to stimulate appetite like ghrelin or NPY (Samson, & Taylor & Ferguson 2005). Projections of ORX are

shown to end at the arcuate nucleus and administration at the arcuate nucleus increases NPY mRNA levels in the hypothalamus (Lopez, Seoane, Mdel, Dieguez & Senaris 2002).

However, orexin does not appear to be a critical player in food intake behaviors but rather adapt the arousal and motivation levels for such behaviors to be carried out (Adamantidis & de Lecea 2008). Orexin has more significantly been shown to elicit a certain levels of arousal to engage exploratory and goal-oriented behaviors, which can ultimately strengthen over time and motivation for liquids and food, or more so lead to the reinstatement of a previously extinguished food seeking behavior in operant conditioning paradigms (Boutrel, Cannella & De Lecea 2010; Boutrel et al., 2005; Yamanaka et al. 2003).

The basal forebrain cholinergic system plays a role in several functions of attention. Activation of the system from different afferent inputs is hypothesized to influence how attentional resources are distributed. Projections from the hypothalamus has been recognized as a key player and source of projections to the basal forebrain however, phenotypes of such inputs and conditions under the basal forebrain cholinergic system is unclear. Orexin has been shown to project to the basal forebrain with receptors on cholinergic parts of the basal forebrain affecting cell activity and cortical acetylcholine release. A study examining orexin inputs and the basal forebrain cholinergic system interactions concluded that dysfunction in orexin-acetylcholine may play a role in arousal and attentional deficits that accompany neurodegenerative conditions from drug addiction and age-related cognitive decline (Fadel & Burk 2009).

Hypocretin/Orexin and Stress

Not only has orexin been shown to activate key areas known for reward, recent evidence also suggests that orexins stimulate corticosterone release when injected in the brain ventricles, suggesting a role in the activation of the HPA axis (Stricker-Krongrad & Beck 2002). This

hypothesis has been supported with evidence that neuropeptide Y and corticotropin releasing-hormone, are both involved in the feeding and release of corticosterone are closely interacting with orexin effects on food regulation (Ida et al. 2000). Data has also shown that corticotrophin-releasing factor (CRF) peptidergic system directly innervates the hypocretin-expressing neurons. Several studies further show that the hypocretin cells also terminate to the CRF-system in order for feedback and the CRF activates the hypocretin system which relays the signal to brain stem nuclei involved in maintaining and activating arousal (Winsky-Sommerer, Boutrel & de Lecea 2005).

As described earlier, the hypothalamus-pituitary- adrenal axis is activated and the synthesis of the corticotrophin releasing factor (CRF) is induced in the paraventricular nucleus of the hypothalamus. The CRF stimulates the production of adrenocorticotrophic hormone (ACTH). The release of ACTH then stimulates the release of glucocorticoids, which allows a feedback loop back to the hypothalamus (Koob & Le Moal 2000). Orexin is known to play an important role in the “hyperarousal” state that characterizes stress and when hypocretin1 is centrally administered, the effects show an increase in plasma ACTH and corticosterone levels, similar to the central administration of CRF (Winsky-Sommerer, Boutrel & de Lecea 2005). The role of orexin as a mediator in the HPA axis is further supported when orexin receptors were found at each of the levels of the HPA axis (Blanco et al 2002; Lopez et al. 1999). In the behavioral “resident-intruder test” orexin knockout mice show a decrease in emotional responses to stress as well (Winsky-Sommerer, Boutrel & de Lecea 2005).

The extent to which orexin projects to the HPA axis suggests that orexin neurons constitute a feedback circuit regulating arousal in response to stressful stimuli. Anatomical data between CRF and orexin showed that synapses between the two systems have the CRF

immunoreactive boutons and hypocretin immunopositive perikarya and dendrites in the lateral hypothalamus (Winsky-Sommerer, Boutrel & de Lecea 2005). Furthermore, hypocretin immunoreactive neurons express CRF 1 and 2 receptors, suggesting strong afferent innervation from the paraventricular nucleus, central nucleus of the amygdala, and bed nucleus of the stria terminalis (Winsky-Sommerer et al. 2004).

Hypocretins are also considered regulators of allostasis with their greatest influences in basic homeostatic pressures like hunger, anxiety and drive for sex (Carter, Borg & de Lecea 2009). In contrast to homeostasis, allostasis maintains stability levels outside the normal range and is achieved by varying the internal milieu to match the perceived and anticipated environmental demands (McEwen & Wingfield 2003; Robers, Heyser, Cole, Griffin & Koob 2000). Under allostatic pressure, hypocretins have been shown to inhibit stress-induced depressive symptoms, reinstating homeostatic control of brain arousal (Carter, Borg & de Lecea 2009). Furthermore, orexin has been implicated to mediate an allostatic generalized stress response to calorie restriction that allows an animal to overcome maladaptive depressive symptoms induced by chronic stress (Akiyama, Yuasa, Hayasaka, Horikawa, Sakurai & Shibata 2004). Generally, orexin may not necessarily stimulate food intake, but activation of the orexin neurons mediate allostatic changes in behavior, for example in animals will be awake and motivated to obtain food during limited times when it is available (Carter, Borg & de Lecea 2009).

Hypocretin neurons are also reciprocally connected with neuropeptide Y (NPY) containing neurons, which are another peptidergic system, involved in acute stress (Heilig 2004). NPY and orexin have similar projections including extended amygdala, which is known to have an important role in responses to acute stress (Adrian et al. 1983; Baldo, Daniel, Berridge &

Kelley 2003). Also, when intracerebroventricular administration of NPY increases sedation, and has anxiolytic activity in response to some stimuli (Bannon et al. 2000), NPY was found to hyperpolarize hypocretin neurons *in vitro* (Fu, Acuna-Goycolea & van den Pol 2004). Thus, researchers have hypothesized that the behavioral effects of NPY are mediated by inhibition of the orexin system. The interacting circuits of CRF, orexin and NPY have sparked significant interest in multiple physiological and pathological situations, specifically in hyperaroused states associated with motivation and addiction.

Hypocretin/Orexin and Addiction or Goal Oriented Behaviors

One related aspect that the orexin system has had recent growing interest is the actions on arousal state and reward behaviors, specifically the mesolimbic dopamine neurons of the ventral tegmental area (Samson, Taylor & Ferguson 2005; Winsky-Sommerer, Boutrel & de Lecea 2005) and its role in addiction and goal-oriented behaviors (Boutrel, Cannella & De Lecea 2010; Yamanaka et al 2003). Orexin is considered to play a critical role in regulation of drug-seeking and drug-taking behaviors as much as CRF, opioids, cannabinoids and nociception and has an excitatory effect on the VTA and inhibitory effect on the NAcc (Martin, Gabre, Siggins & De Lecea 2002). Microdialysis studies have shown that drugs of abuse increase extracellular levels of dopamine levels at the level of the NAc and neuroadaptations in the system is thought to underlie elements of addiction. The hypothalamic orexin system has been shown to interact with the mesocorticolimbic pathway, specifically the VTA (Carboni, Imperato, Perezzi & Di Chiara 1989). Furthermore, the orexin projections that are found in the VTA have been localized with their terminals on tyrosine-hydroxylase positive cells with the VTA containing numerous orexin-containing dense core vesicles and a high density of orexin receptors in the VTA and DA-

containing and GABA-containing neurons (Korotkova, Sergeeva, Eriksson, Haas & Brown 2003; Narita et al. 2006).

One of the first studies demonstrating the link between orexin to drug addiction was a report on a decreased sign of precipitated opiate withdrawal displayed by mutant mice deficient in orexin (Gerogescu et al. 2003). Another important discovery was the c-Fos activation of lateral hypothalamic orexin neurons correlated with preference for an environment paired with food and drug rewards. Reinstatement of an extinguished preference for an environment paired with natural and non-natural rewards was also observed when morphine was injected with orexin neurons being activated (Harris, Wimmer & Jones 2005).

Fos activation in orexin neurons and the expression of conditioned place preference (CPP) for drug or natural rewards has shown strong correlations. Rats conditioned with morphine, cocaine or food in a CPP paradigm exhibit substantially increased Fos staining in LH orexin neurons on the drug- and food-free conditioned place preference test (Aston-Jones et al. 2010). Self-administration studies have also shown Fos-activation in orexin neurons after exposure to ethanol-associated stimuli (Dayas, McGranahan, Martin-Fardon & Weiss 2008). Early studies showed that orexin projections include the midbrain dopamine (DA) neurons of the ventral tegmental area (VTA) (Baldo et al. 2003; Fadel & Deutch 2002; Peyron et al. 1998). Studies of drug reward and drug induced neuroadaptations have focused on orexin's effects on the VTA and the mesocorticolimbic target regions such as the nucleus accumbens (NAc) and amygdala (Sharf, Sarhan & DiLeone 2010).

Furthermore, the activation of orexin playing a role in reward-seeking and addiction neural circuitry has given new insights and investigation in determining whether the function or dysfunction of the orexin system may contribute to the overeating related to obesity (Sartor &

Aston-Jones 2010). In several studies, selective intake of high-fat food was induced by the injection of the mu opioid receptor agonist (DAMGO) into the nucleus accumbens core. Researchers concluded that orexin signaling in VTA is involved in DAMGO-induced intake of high-fat diet through a projection from NAc to hypothalamic orexin neurons (Stratford & Kelley 1999; Baldo, Gual-Bonilla, Sijapati, Daniel, Landry & Kelley 2004). Also, previous findings showed that high-fat diet intake induced by inactivation of NAc shell caused an increase in Fos expression in LH and the intake decreased with NMDA antagonism in LH. Altogether, these studies suggest that a neural pathway linking the NAc and LH is involved in reward-based eating in sated rats (Cason et al. 2010).

Hypocretin/Orexin and Stress & Drug Addiction

The relationship between stress and addiction has been well established with the extended amygdala having a key role in mediating both positive and negative reinforcement associated with drug addiction (Kalivas & McFarland 2003; Koob 1999). The extended amygdala is composed of the medial subregion of the nucleus accumbens, bed nucleus of the stria terminalis and the central nucleus of the amygdala. These structures receive several afferent information from the limbic structures like the hippocampus, and basolateral amygdala, which then sends efferents not only to the medial part of the ventral pallidum but also the lateral hypothalamus. These connections, specifically the extended amygdala, provide a connection for the basal forebrain to the reward systems of the lateral hypothalamus through the medial forebrain bundle reward system (Koob 2000; Koob 1999).

The orexin system has been shown to project to all of the major components of the extended amygdala, particularly the shell of the nucleus accumbens and the bed nucleus of the stria terminalis. Hypocretins have also been shown to be involved in GABAergic modulation of

the mesolimbic dopamine system, which provides anatomical and functional criteria for modulating critical connections regulation both positive and negative, reinforcing properties of drugs of abuse (Winsky-Sommerer, Boutrel & de Lecea 2005). Evidence shows that there is a close interaction between the CRF and hypocretin peptidergic systems (Ida et al. 2000; Winsky-Sommerer et al. 2004) which places hypocretin neurons as a key system in the integration of emotional stimuli as well as in the integration of sensory inputs and suggests a role for this system in the stabilization of motivated behaviors. Hypocretin system has therefore been hypothesized to be a component that underlies vulnerability to relapse during a prolonged abstinence from drugs of abuse (Aston-Jones & Harris 2004). Furthermore, the orexin system may act as a cautionary alarm that prepares the organism for withdrawal and its consequences on energy homeostasis and allostasis (Winsky-Sommerer, Boutrel & de Lecea 2005).

Studies have also shown that the orexin system also plays a role in the ability of conditioned stimuli to elicit cocaine-seeking behavior. In one study, rats were self-administered intravenous cocaine during a daily 2-hour session followed by extinction training in an operant chamber or abstinence in a home cage. After extinction, cocaine seeking was reinstated when distinct cues previously paired with cocaine infusions or re-exposure to the original self-administration context. After abstinence, cocaine seeking was evaluated with an exposure again to the self-administration environment. Researchers found that cue-induced reinstatement of extinguished cocaine-seeking behavior was significantly attenuated by systemic administration of orexin antagonist, SB334867 (Smith, See & Aston-Jones 2009).

Other studies examining ethanol intake and the orexin system have shown LH expressing orexin mRNA is increased after ethanol drinking in rats with an increase in Fos expression in orexin neurons following context-induced reinstatement of ethanol seeking (Dayas,

McGranahan, Martin-Fardon & Weiss 2008). A similar study investigated orexin system and its regulation of behavioral and biochemical processes underlying nicotine-induced anxiety like responses and reinstatement of nicotine-seeking behavior (Plaza-Zabala, Martin-Garcia, de Lecea, Maldonado & Berrendero 2010). Researchers found that with acute administration of nicotine induced anxiogenic-like effects were observed in the elevated plus-maze. Furthermore, pretreatment with OXR1-antagonist and in orexin knockout mice, anxiogenic-like effects of nicotine were blocked. Orexins were found to have an anxiogenic effect in acute administration of nicotine and behavioral responses were associated with activation of CRF and AVP neurons in the PVN that was dependent on orexin transmission. The study also supported the idea that activation of orexin can also reinstate nicotine-seeking behavior after induced withdrawal.

To test whether orexin was involved in the acquisition of drug consumption Boutrel et al. 2005 infused 0.2-1.5 nmol of ORX-1 into rat brains that were trained to self-administer cocaine. Researchers found no differences compared to the saline treated group or repeated injections of the peptide and different periods of the circadian cycle and at different exposure times to cocaine. It was concluded that hypocretins did not play a significant role in the consumption of cocaine, however when cocaine was given intracerebroventricularly, ORX-1 led to a dose related reinstatement of an extinguished cocaine seeking behavior. Supporting previously described studies, ORX-1 plays a significant role in reinstatement of cocaine seeking but mechanisms may be different from increased dopamine released (Harris, Wimmer & Jones 2005).

Through recent evidence, it was hypothesized that the hypocretin and stress systems interact closely specifically in regulating drug-seeking behavior (Martin-Fardon, Zorrilla, Ciccocioppo & Weiss 2010; Borgland, Ungless & Bonci 2010). This was shown by blocking the orexin-induced reinstatement of cocaine seeking by CRF/NA antagonism and to a lesser degree,

food-seeking behaviors also increased. In addition, antagonism of ORX-1 receptors blocked footshock-induced reinstatement of previously extinguished cocaine seeking. More importantly, ORX-1 significantly elevated intracranial self-stimulation (ICSS) thresholds in rats, reflecting a decrease in the activity of brain reward systems (Boutrel et al. 2005). The action of ORX-1 on ICSS thresholds is opposite to the well known threshold-lowering effects of cocaine, an index of cocaine-induced excitation of brain reward systems (Kenny, Koob & Markou 2003).

Generally, research has shown a consistent trend that the orexin system is a new mechanism by which stress could influence relapse to drug seeking and drug taking from increased dopamine release (Martin-Fardon, Zorrilla, Ciccocioppo & Weiss 2010; Borgland, Ungless & Bonci 2010; Boutrel & de Lecea 2008; Bonci & Borgland 2009). ORX-1 is shown to reinstate cocaine seeking by mechanisms different from an increase of dopamine release and the blockade of ORX-1 induced reinstatement by CRF/noradrenergic antagonism suggests that orexin and stress systems may closely interact to regulate opiate seeking behaviors (Boutrel et al. 2005). CRF has also been shown to induce glutamate release in the VTA of rats who have been exposed to cocaine but not in cocaine naïve rats (Wang, Shaham, Zitzman, Azari, Wise & You 2005). Hypocretins have been shown to act synergistically with glutamatergic afferents to depolarize neurons in the VTA (Korotkova, Sergeeva, Eriksson, Haas & Brown 2003). Additionally, hypocretin has been shown to critically contribute to cocaine sensitization through the recruitment of NMDA receptors in the VTA.

Altogether, these results suggest that hypocretins in concomitance with CRF could contribute to glutamate release facilitation that ultimately leads to the arousal/motivational systems activation including both adrenaline and noradrenaline. Chronic activation of these brain areas could lead to an allostatic state of brain reward systems that consequently underlies the

vulnerability to relapse for drug seeking after a period of protracted abstinence (Carter, Borg & de Lecea 2009). Furthermore, when the cessation of drugs of abuse does occur, the orexin system may act as an alarm signal that would prepare the organism for withdrawal and its consequences on energy and fluid homeostasis. Evidence has already shown that leptin, which hyperpolarizes orexin neurons also attenuates fasting-induced heroin-seeking behavior (Shalev, Morales, Hope, Yap, Shaham 2001) and mice deficient of orexin has diminished signs of precipitated opiate withdrawal (Georgescu et al. 2004).

As discussed above, many studies have performed experiments in determining the specific effects of orexin in concomitance with CRF in drug addiction. In many studies, the activation of orexin was examined during the reinstatement of drug-seeking behavior or the overall role orexin may play in arousal during the drug addiction process. However, an insufficient number of studies have yet to observe drug-seeking behavior using the elevated plus-maze during withdrawal, precipitated or spontaneous. The elevated plus-maze has been used extensively to study drug withdrawal, specifically in anxiety and drug-seeking behavior. In the present study, it is of interest to see the activity of orexin in rats that have been chronically exposed to morphine and then subjected to the elevated plus-maze. Furthermore, the purpose of the study was to assess the effects of chronically exposed rats to morphine and through spontaneous withdrawal, and exposure to the elevated plus-maze, to examine orexin and Fos activity of the lateral hypothalamus.

Since morphine is known to exhibit both anxiolytic and anxiogenic effects depending on dosage, it was hypothesized that in rats that receive chronic morphine for seven days and who are then subjected to the elevated plus-maze should experience the anxiolytic effects of morphine and histologically have less orexin neurons active. Chronically exposed rats should also

behaviorally be less stressed compared to control. In rats that receive chronic morphine for six days and then do not receive morphine on the seventh day through induced-spontaneous withdrawal, should be more stressed and histologically have more orexin neurons activated and behaviorally may show, anxiogenic effects or drug-seeking behavior compared to the control.

Methods

Subjects

Thirty-two male Sprague-Dawley rats (200-500g; 2-3 months old) were received from Charles River Laboratories (Wilmington, MA). The subjects were housed in groups of two in a humidity-temperature-controlled environment, with 12 hr light/dark cycles with free access to food and water. The rats were handled once three days prior to testing and once again on the day before testing. All procedures were approved by the Connecticut College Institutional Animal Care and Use of Laboratory Animals (National Research Council, 1996).

Materials

EPM, morphine (Sigma-Aldrich), orexin and Fos antibodies (Santa Cruz biotechnology)

Procedure

Rats were weighed and recorded on the first day of injection and then every three days. Injections were given via the intraperitoneal (IP) route. Rats were brought to the injection/testing room in groups of two in a plastic cage with rat shavings. After injection, the rats were held in the injection/testing room for 30 minutes and then brought back to the animal facility.

Drug Conditions

Rats were randomly assigned in four different groups. Rats in Group 1M received 3mg/kg of morphine for six days. On the seventh day, test day, the rats were administered 1

ml/kg saline. Rats in Group 2M received the same treatment as Group 1M except on test day received morphine. Rats in Group 3S received 3ml/kg of saline based on the weight of the rat for six days. On the seventh day, test day, the rats were not injected. Rats in Group 4S received the same treatment as Group 3S however, on test day the rats received saline.

Stressor Exposure: Elevated-Plus Maze

Based on the subject's group, 30 minutes after the injection, the rat was subjected to the elevated plus-maze (EPM). The elevated-plus maze exposure was conducted in an isolated room with dim lighting to minimize light disturbances. The EPM is a black plastic platform 50cm above ground, consisting of four arms arranged in a plus-sign, with two closed arms with walls (31 x 38cm) on all sides except the center, and two open arms with no walls. The same type arms are across from each other with a center square connecting all four arms.

Each arm was 10 x 38 cm long. On each EPM exposure the rat was individually placed on the EPM center platform facing the open arm. Each rat was left on the elevated plus-maze for 5 minutes. A video camera was mounted above the maze to record and observe the behavior of the rats during the exposure. After each rat exposure, the EPM was cleaned with 50% ethanol solution to eliminate odor influences for the next animal.

Anxiety Measures: Elevated-Plus Maze

All injections 3mg/kg IP morphine or saline were administered 30 minutes prior exposure to the EPM. Rats in Group 1M were given saline, Group 2M morphine, Group 3S no saline, and Group 4S saline. The elevated-plus maze exposure was as mentioned before.

Standard spatiotemporal measures of the numbers of entries onto, and the times spent on, open and closed arms were recorded. Entries were counted when all four paws were on the arm, and the time was counted in a particular arm until all four paws were off the arm. Drug-seeking

behavior was then measured by using the duration spent on the open-arms. Anxiolytic effects were also counted as the amount of time spent on the open arms

In addition, the following behavioral measures, based on those previously reported by Fernandes and File (1996) were recorded: a) stretch attend posture; b) unprotected head dips. Stretch attend postures were defined as forward extensions of head and shoulders followed by retraction without stepping forward with hind paws. Unprotected head dips were defined as scanning over the side of the maze downward while on an open arm.

Tissue Preparation

Rats were euthanized by exposure to a carbon dioxide chamber approximately an hour and a half after exposure to the elevated plus-maze. The rats were perfused transcardially with 50 ml saline for 30 seconds followed by 400-500 ml of 4% paraformaldehyde in 0.1M phosphate buffer. The brains were extracted and post-fixed in 4% paraformaldehyde overnight and then transferred to a 30% sucrose solution. Coronal sections were taken at approximately 40 μ m and were obtained in a -20°C cryostat. The sections were stored in 4°C in cryoprotectant.

Immunohistochemistry

C-Fos

Two sections per animal from each subject were chosen to represent the lateral hypothalamus. Staining for C-Fos was conducted using avidin-biotin-horseradish peroxidase (ABC) method as described by Grahn et al. (1999). Sections were washed three times for 10 minutes each in 0.01M phosphate buffered saline (PBS) prior to 24 hour incubation with a 1:800 dilution of polyclonal rabbit anti-Fos primary antibody (Santa Cruz Lot #E2609) in a blocking solution composed of 1% normal goat serum, 1% bovine serum albumin, and 0.25% Triton-X100 (30%), in 0.01 PBS. After the incubation the tissue was washed for 10 minutes in PBS and

then incubated for 2 hours in biotinylated goat anti-rabbit secondary antibody (Jackson Laboratories Lot #90982) diluted in 1:200 in blocking solution.

After the incubation the sections were washed again three times, 10 minutes each in PBS and incubated for 2 hours with avidin-biotin complexed with horseradish peroxidase (Vectastain Elite ABC kit) in PBS. The sections were then washed three times for 10 minutes each in 0.1M PB and placed in a solution containing diaminobenzidine (DAB), cobalt chloride, ammonium chloride, nickel ammonium sulfate, and glucose oxidase in 0.1PB for 10 minutes. The reaction was started by the addition of β -D-glucose solution. The reaction was allowed to sit for approximately 15 minutes and then the tissue was placed in 0.01M PBS to end the reaction. The tissue was then subjected to orexin staining.

Orexin

The same sections used in c-Fos staining were then stained for orexin by using avidin-biotin-horseradish peroxidase (ABC) method as described by Grahn et al. (1999). Sections were washed three times for 10 minutes each in 0.01M phosphate buffered saline (PBS) prior to 24 hour incubation with a 1:100 dilution of polyclonal orexin anti-goat primary antibody (Santa Cruz Lot #C3110) in a blocking solution composed of 1% bovine serum albumin in 0.01 PBS at 4°C . After the incubation the tissue was washed for 10 minutes in PBS and then incubated for 2 hours in biotinylated donkey anti-goat secondary antibody (Jackson Laboratories Lot #93908) diluted in 1:200 in blocking solution.

After the incubation the sections were washed again three times, 10 minutes each in PBS and incubated for 2 hours with avidin-biotin complexed with horseradish peroxidase (Vectastain Elite ABC kit) in PBS. The sections were then washed three times for 10 minutes each in 0.1M PB and placed in a solution containing diaminobenzidine (DAB) and glucose oxidase in 0.1PB

for 10 minutes. The reaction was started by the addition of β -D-glucose solution. The reaction was allowed to sit for about 15 minutes and then the tissue was placed in 0.01M PBS to end the reaction. The tissue was then washed with 0.01M PBS three times and mounted on slides that were allowed to dry for approximately 24 hours. The slides were then dehydrated in a series of alcohols and defatted with *Histoclear*. The slides were coverslipped with Permount.

Image Analysis

An observer blind to the conditions of the rats analyzed the sections. Each of the sections of the lateral hypothalamus for each rat was assessed for the number of Fos-stained nuclei, orexin stained nuclei, and double-labeled orexin and Fos-stained nuclei. Ovoid particles that were black were counted as Fos-stained nuclei. Ovoid particles that were brown were counted as orexin-stained cells and particles that were brown and black were counted as double-labeled.

Two sections were chosen to represent the lateral hypothalamus (LH) and both sides of the LH were counted. The LH was comparable to that illustrated by Paxinos and Watson (1998) at the anterior-posterior coordinate of 6.44 mm from interaural zero. The area encompassing the LH was estimated using the top of the third ventricle and the optic tract. Particles were counted just medial to the optic tract in an area estimated a little less than one half of the total area between the third ventricle and the optic tract. The LH area was defined by using the same circle-defined shape across all sections.

Statistical Analysis

Behavioral statistical analyses were performed using SPSS 11.0 for *Windows 2000 Professional*. A one-way analysis of variance (ANOVA) was performed for each behavioral measure; stretch-attend postures, head dips and open arm duration as the dependent variable,

with the different conditions as the independent variable. A Post-Hoc analysis (Tukey) revealed specific between group differences.

A one-way analysis of variance (ANOVA) was performed for the number of closed entries as the dependent variable, with the different conditions as the independent variable. A Post-Hoc analysis (Tukey) revealed specific between group differences.

A one-way analysis of variance (ANOVA) was performed for the LH the number of Fos-stained nuclei as the dependent variable, with the different conditions as the independent variable. A Post-Hoc analysis (Tukey) revealed specific between group differences.

A one-way analysis of variance (ANOVA) was performed for the LH analyzing the number of double-labeled nuclei as the dependent variable, with the different conditions as the independent variable. A Post Hoc analysis (Tukey) revealed specific between group differences. The level of significance was defined as $p < 0.05$ in all experiments.

Results

It was hypothesized that rats that received chronic morphine for seven days and then subjected to the elevated plus-maze (2M) would spend more time on the open-arms, an anxiolytic effect. Rats who received chronic morphine for six days and then underwent spontaneous induced-withdrawal anxiety on the seventh day (1M) were expected to exhibit drug-seeking behavior by spending more time on the open arm (Mantsch, Baker, Francis, Katz, Hoks & Serge 2007). A one-way ANOVA was conducted for all test groups 1M, 2M, 3S and 4S for open-arm duration. No significant findings were observed. Interestingly, although not significant, the mean open-arm duration for the 1M group was less than the 2M (Figure 1). This trend shows that spontaneous-induced withdrawal from morphine in rats, may produce more anxiogenic

effects rather than drug seeking behavior which would be defined as more time spent in the open arms.

Other behavioral measurements such as closed entries, stretch-attend postures, and unprotected head dips on the elevated plus-maze were also scored like number of closed entries, stretch-attend postures and unprotected head dips. One-way ANOVAs were conducted for all test groups 1M, 2M, 3S and 4S for the other behavioral measurements. No significant findings were observed in number of closed entries (Figure 2) stretch attend postures (Figure 3) or unprotected head dips (Figure 4).

Cells were double labeled for Fos activation and orexin expression (Figure 5, 6). A one-way between subjects ANOVA was conducted to compare the effect of morphine on the number of double-labeled cells in rats that have been chronically administered morphine for seven days and rats who have chronically received morphine for six days and then underwent spontaneous-induced withdrawal anxiety conditions using the elevated plus-maze.

There was a significant main effect of morphine on the number of double-labeled cells on the test groups at $p < 0.05$ level for the four conditions [$F(3, 26) = 4.664, p = 0.010$] (Figure 7; supplementary images of c-Fos and orexin labeled Figure 7b, c). Post hoc comparisons using the Tukey HSD test indicated that the mean score for rats who did received chronic morphine and morphine on test day ($M = 0.4063, SD = 0.3764$) was significantly different than the rats who received chronic morphine and then saline on test day ($M = 0.7813, SD = 0.7727$), compared to control who did not receive an injection ($M = 0.0625, SD = 0.1767$) and the control saline group ($M = 0.000, SD = 0.000$). This result demonstrates that Fos cell activation, specifically in the LH of orexinergic neurons was greater in rats that were chronically administered morphine and then

received saline after induced spontaneous withdrawal compared to rats who received chronic morphine and control.

Discussion

Drug addiction is a chronic brain disease that has been found to have profound effects on the mesolimbic dopamine system and the release of corticotropin-releasing factor. Research has shown the importance of a different circuitry that innervates both systems during withdrawal. Orexin is considered a major player in the regulation of drug-seeking and drug-taking behaviors as much as other peptide systems like CRF, opioids and cannabinoids (Boutrel, Cannella & de Lecea 2010). Early evidence linking orexin to drug addiction was a report on a decrease in signs of opiate withdrawal of orexin knockout mice (Georgescu et al. 2003). Studies have also shown that Fos expression in the LH occurs in orexin neurons projecting to the ventral tegmental area (VTA). Furthermore, Fos activation increased with elevated morphine preference during protracted withdrawal than non-VTA-projecting orexin neurons. This result indicates that the VTA is an important site of action for orexin's role in reward processing. Orexin has also been shown to reinstate extinguished morphine and cocaine drug-seeking behavior.

Overall, LH orexin neurons have been described as an important input to the VTA for behavioral effects associated with reward-paired stimuli in animals, specifically in observing drug addiction. There has been extensive literature on the relationship between orexin in arousal and stress and the effects of orexin during drug addiction and reward seeking. Specifically, research has focused on paradigms using conditioned-place preference (CPP) and intracranial administration of ORX1 in areas of the brain like HPA axis, NAcc, VTA, LH and other neural projections of the orexin system. However, there has not been much exploration of orexin

activation in rats that have been chronically exposed to morphine and then observed during spontaneous withdrawal on the elevated plus-maze.

The elevated plus-maze (EPM) has been used to study anxiety-related behavior in rodents and assessing the anti-anxiety effects of pharmacological agents and steroid hormones (Pellow, Chopin, File & Brilley 1985), defining brain regions and mechanisms underlying anxiety-related behavior (Pellow, Chopin, File & Brilley 1985; Hogg 1996; Walf & Frye 2007). Activity in the open arms is considered to be a conflict between the rodent's preference for protected areas or closed arms and their innate motivation to explore novel environments. Ethological measures are observed like the number of rears, head dips, freezing or stretched-attend postures (Rodgers & Johnson 1995). Normal exploratory behavior in the elevated plus maze usually favors the closed arms and this tendency to stay in the closed aspects of the maze can be enhanced by drugs, like anxiogenics that increase the aversion towards the anxiety-provoking open arms. In contrast, when subjects are administered anxiolytic compounds, this will reduce the natural aversion to the open arms and promote exploration.

The critical aspect of the EPM is the number of entries made onto the open arm and time spent on the arms. These variables have been correlated with anxiety (Hogg 1996). Locomotor activity is assessed by monitoring the number of total open arm entries or closed arm entries. (File 1992; Lister 1987). The elevated plus-maze has frequently been used in research as a tool to measure anxiety-like behaviors during withdrawal. Subjects who have been repeatedly exposed to drugs of abuse are hypothesized to exhibit withdrawal-like symptoms like drug-seeking behavior. Drug-seeking behavior has been generally characterized as the subject more likely spending time in the closed arms compared to the open arms in the elevated plus maze. However,

other researchers have also defined drug-seeking behavior as more time in the open arms in the EPM as well.

Studies have been able to show the relationship between discrete or contextual cues of previous drug taking and relapse, however the role that drug-withdrawal cues play in relapse has not been successfully determined. Conditioned place preference (CPP) is an animal model used to study the influence of drug-associated stimuli on drug seeking (Bardo and Bevins 2000; Hoffman 1989). CPP paradigm has been a valid measure of characterizing the different stages of drug addiction. Drug-induced conditioned place preference explores the reinforcing effects of drugs of abuse. A distinctive environment is paired repeatedly with administration of a drug and a different environment is repeatedly paired with administration of a vehicle (Bardo & Bevins 2000).

CPP occurs when repeated administration of a drug is paired to an environment and results in the ability of the environment to elicit approach behavior and increased time in the absence of the previously administered drug. This phenomenon is considered as an example of dopamine-mediated incentive learning and that increased time spent by animals in a drug-paired environment can be considered as a measure of drug-seeking behavior and reinforcing effects of drugs (Bardo and Bevins 2000; Le Foll and Goldberg 2009). CPP has been demonstrated for most drugs of abuse, as well as for natural reinforcers such as food. The acquisition of a drug-induced CPP is likely to be correlated with other reinforcing effects of abused drugs, whereas its expression reflects the influence on behavior of environmental stimuli previously associated with a drug's effects (Le Foll & Goldberg 2009). Recent place conditioning studies in rats demonstrate that extinguished CPP can be reinstated by injections of the conditioning drug (Mueller, Perdikaris & Stewart 2002) or stress (Mueller and Stewart 2000). Thus the

reinstatement of CPP in the animal model may provide a simple, noninvasive and rapid procedure for investigating the mechanisms for drug relapse compared to the EPM characterization of drug-seeking.

In order to detect the neural activity of orexin during spontaneous withdrawal, rats were chronically administered morphine for six days, then taken off morphine the seventh day and subjected to the elevated plus-maze. Behavioral measurements were taken during the five minutes on the EPM and then compared to morphine chronically exposed rats of seven days and control. It was hypothesized that rats undergoing spontaneous induced withdrawal would experience anxiogenic effects similar to Buckman, Hodgson, Hofford and Eitan (2009) who reported in mice undergoing both naloxone-precipitated and spontaneous opioid withdrawal exhibited an unexpected outcome where there was an increase time and entries on the EPM on the open arm compared to controls. However, other studies examining cocaine withdrawal, demonstrated that rats that are exposed to chronic administration of cocaine and then one-day withdrawal found an increase in anxiety-like behavior, using the elevated plus-maze (Perrine, Sheikh, Nwaneshiudu, Schroeder and Unterwald 2008).

The present study defined drug-seeking behavior similar to Buckman, Hodgson, Hofford and Eitan (2009), as having a longer duration on the open arm of the EPM. This was hypothesized because of the conditioned-environmental cues as mentioned in the methods; where rats were brought to the testing/holding room at the same time and the same procedural process was done before and after injections. Drug-associated stimuli or “cues” elicit a craving for drugs of abuse as a consequence of repeated pairing with drug use (Childress et al. 1999; See 2002). Cues are hypothesized to motivate drug-seeking behavior and subsequent relapse in abstinent individuals (Childress, McLellan & O’Brien 1986; See 2002; Sinha, Fuse, Aubin,

O'Malley 2000). Conditioning of the procedural process and environmental cues employed in the present study was hypothesized to influence withdrawal-like symptoms. Specifically during an anxiety environment with cues associated with drug withdrawal symptoms that occur when the drug is not available.

Rats who were administered chronic morphine for seven days and then subjected to the EPM were observed. It was hypothesized that compared to the spontaneous withdrawal group, rats chronically exposed to morphine would exhibit an anxiolytic effect dependent on the dose of morphine by spending more time on the open arms. However, the neural activity of chronically exposed rats to morphine would not show, or show fewer orexin activated cells in the lateral hypothalamus compared to the spontaneous induced-withdrawal group and control group.

Interestingly, although not significant, the mean open arm duration of spontaneous withdrawal rats was lower (89.24s, $n=8$) than the chronically morphine exposed rats (121.16s, $n=8$). This was surprising because the literature has suggested that during cocaine and morphine withdrawal, open arm duration increases because of the anxiolytic effects of the substances. There was however, a significant difference in the number of Fos-orexin activated cells in the lateral hypothalamus which may suggest that although the rats undergoing spontaneous withdrawal did not exhibit the drug-seeking behavior as defined, rats were experiencing an anxiogenic effect by spending more time in the closed arms of the EPM. This observation follows the general acceptance in EPM literature that an anxiogenic effect suggests more time spent in the closed arms. Drug-seeking behavior, particularly when using the EPM during drug-withdrawal needs to be defined and accepted throughout the literature. Researchers need to come to a conclusion in what defines drug-seeking behavior and possibly make further behavioral measurements of what entails such behavior in addition to open arm time duration. This

discrepancy makes it difficult to interpret the data from the present study because the mean open arm duration of the spontaneous withdrawal rats can be interpreted as drug-seeking behavior or not.

Alternative measures like using the CPP might give a better indication of drug-seeking behavior because of supporting evidence that an increase in the amount of time the animal spends in the drug-paired environment indicates that the drug-associated contextual stimuli has acquired secondary or conditioned incentive properties, presumably via pairing with the rewarding effects of the drug (See 2002; Hoffman 1989). Pairing the EPM and CPP might give a better indication of withdrawal-like symptoms associated with anxiety, and the CPP may give more information of drug-seeking behavior associated with contextual cues of addiction. Using a place preference relapse model, researchers found that while spontaneous withdrawal and naloxone-precipitated withdrawal did not effectively reinstate extinguished morphine place preference, morphine-withdrawal cues significantly reinstated the morphine CPP. Moreover, activation of the CRF receptor is involved in reinstatement of drug-seeking behavior induced by conditioned withdrawal (Lu et al. 2005).

The number of closed arm entries was also observed to see if there was significance in preference of the closed entries of rats undergoing withdrawal and rats with chronic exposure to morphine. Furthermore, the number of closed entries was hypothesized to see if drug-seeking behavior could be established in this context. No significant findings were observed. However, interestingly the number of closed entries was similar as shown in Figure 4. Rats that were chronically administered morphine and then subjected to spontaneous withdrawal did have the fewest number of entries (3.63), compared to the total mean for four groups, which were 4.10.

The number of closed entries could be used as a behavioral measurement of drug-seeking if the definition of drug-seeking is more time spent in the closed arms of the EPM.

The significant finding of double-labeled Fos-orexin activated cells supports some of the behavioral effects during induced withdrawal. This result demonstrates that double-labeled Fos-orexin activation in the LH was greater in rats undergoing spontaneous withdrawal compared to rats that had chronic exposure to morphine and control. This suggests, that during induced spontaneous withdrawal, orexin neurons are activated when the subject is subjected to the elevated plus-maze compared to control. This finding supports the literature that orexin neurons are activated during morphine withdrawal, specifically when the subject is placed in an anxiety-induced environment. Further studies need to be done in other brain areas that the orexin neurons project to that include the brain's reward circuitry; the dopamine mesolimbic dopamine systems and also the stress activated areas of the brain including the HPA axis and the extended amygdala in order to determine the role of orexin during withdrawal.

Studies examining the effect of orexin in regulating the anxiogenic-like effects of nicotine found that the administration of SB334867 blocked the effects of OXR1. The antagonist SB334867 also prevented the activation of CRF and arginine-vasopressin (AVP) neurons, which also expressed OXR1 (Plaza-Zabala, Martin-Garcia, de Lecea, Maldonado & Berrendero 2010). Another study examining morphine place preference used the OXR1 antagonist SB334867 to observe behavior after morphine-induced behavioral sensitization. Researchers found that OXR1 antagonist prior to CPP did not significantly differ from wild-type controls in locomotor activity following acute or chronic morphine treatments. In contrast, orexin-knockout mice did not differ from wild-type controls in preference for a morphine-paired environment, SB334867 significantly attenuated place preference for morphine but not a cocaine-paired environment

(Sharf, Guarnieri, Taylor & DiLeone 2010). Aston-Jones et al. (2010) examined the OXR1 receptor antagonist SB334867 in self-administration studies where administration of the antagonist blocked cocaine-seeking induced by discrete or contextual cues previously associated with cocaine, but not by priming injection of cocaine. Furthermore, there were no observable effects of OXR1 antagonist on self administration, suggesting that orexin antagonist does not play a role in the reinforcing properties.

Systemic administration of the OXR1 antagonist before CPP significantly attenuates the expression of morphine preference indicating that activation of the LH orexin neuron plays a role in driving the associated preference (Aston-Jones et al. 2010). In order to determine if conditioned activation of orexin neurons is also involved in driving the reinstatement of extinguished drug-seeking, rats were conditioned to morphine CPP then underwent extinction of morphine preference by repeatedly exposing them to the CPP environment without drug reward. When extinction was established, the Y4 receptor agonist rat pancreatic polypeptide (rPP) stimulated Fos induction in orexin neurons when injected into the LH. rPP microinjected into the LH also produced a reinstatement of preference in the morphine CPP (Harris et al. 2005). The reinstatement with LH rPP was further found to be specific for orexin neurotransmission because it completely blocked systemic pretreatment with SB334867. Intracerebroventricular administration of ORX1 was also found to reinstate extinguished cocaine seeking responses in a self-administration paradigm, further suggesting that orexin can drive drug-seeking and relapse (Boutrel et al. 2005).

Other reports have demonstrated that SB334867 decreases both alcohol and nicotine self-administration behaviors (Hollander, Lu, Cameron, Kamenecka & Kenny 2008) compared to administration of ORX directly into the PVN or in the LH which increases ethanol-drinking in

rats without affecting food and water intake (Schneider et al. 2007). Also, blocking the orexin system prevents cue-induced reinstatement of previously extinguished alcohol-drinking behavior (Hollander, Lu, Cameron, Kamenecka & Kenny 2008; Lawrence, Cowen, Yang, Chen & Oldfield 2006). Impact of orexin and opiate intake has not been extensively studied but observations have been made demonstrating a role of orexin in mediating the expression of precipitated morphine withdrawal (Georgescu et al. 2003) as well as aversion for a compartment previously paired with morphine administration in orexin-deficient mice. This suggests that orexin regulates opiate seeking and taking behaviors. Taken together, the administration of ORX1 blockade has been suggested as a drug target in preventing relapse for alcohol, morphine and nicotine seeking (Lawrence, Cowen, Yang, Chen & Oldfield 2006; Harris, Wimmer & Jones 2005).

Originally, the study had a larger experimental design that included the use of the ORX1 antagonist, SB334867. Two more groups (and control) would have been added to the design where rats would be chronically administered morphine for six days and then on the seventh day would not be given morphine but the ORX1 antagonist, SB334867, then subjected to the EPM. The second group would be chronically administered morphine for seven days and also the ORX1 antagonist, SB334867, and then subjected to the EPM. Rats undergoing spontaneous withdrawal and then administered ORX1 antagonist SB334867 on the seventh day instead of morphine would not exhibit drug-seeking behavior as described in the study compared to rats who were exposed to chronic morphine and underwent spontaneous withdrawal. Furthermore, orexin neurons would not be active in the LH. Rats chronically exposed to morphine and then received the ORX1 antagonist SB334867 is hypothesized to behave similar to control where the anxiolytic effects of the morphine are blocked so anxiolytic-like behavior would be attenuated.

(Cason et al. 2010). The addition of these two groups and their controls would further help characterize the role and activation of orexin during spontaneous withdrawal and anxiety.

There were several factors that challenged the results of the current study. For one, time constraints caused the number of brain areas of interest to be limited to only observing the lateral hypothalamus, leaving the VTA and NAcc for future investigation. Although there was care in preventing inconsistency in the quantification of Fos-activated, orexin-activated and double-labeled activated cells, it is possible that some background staining may have been counted as a positive signal. This would cause higher variability between samples, disguising effects of orexin-activated cells. A certain threshold was used to account for orexin-activated cells but this was used at the counter's own discrepancy. Another factor that could have affected the results was the scoring of the behavioral measurements. Again, due to time constraints, behavioral scoring would have been stronger if there were a blind-scorer to the study. Overall, the main factor that challenged the present study was the time constraint and the ultimate goal of the study. Time was used as wisely as possible, therefore parts of the study had to be cut out as described above.

There was a statistically significant difference in Fos-orexin double-labeled cells between groups, which suggests that orexinergic neurons were activated during spontaneous withdrawal from morphine after being subjected to the elevated plus-maze (EPM). There were no other significant differences found, however general trends like the mean number of orexin-activated cells and open-arm duration were interesting insofar that if the experimental design expanded to the use of the OXR antagonist, SB334867, other observations or conclusions could be made. Also the original study was going to examine dopaminergic mesolimbic areas of the brain, and stress activated areas however, this was unachievable due to time constraints. If the study was to

be repeated areas like the VTA, NAcc, extended amygdala and measures of CRF would be measured and analyzed. The activation of the orexin neurons in the LH shows that there's a relationship between withdrawal and anxiety; furthermore the significance indicates that this needs to be further examined and by observing the areas as mentioned before, this would help in determining the orexin's mediating effects of arousal during withdrawal.

As far as we know, there is not an extensive amount of literature of studying the role of orexin during spontaneous morphine withdrawal, particularly by using the elevated plus-maze to measure drug-seeking behavior and anxiety. The present study was the first to observe orexin during spontaneous morphine withdrawal by using the elevated plus-maze. The present study also attempted to characterize drug-seeking behavior that may be characterize better in CPP instead of the EPM. However, coordinating the use of both CPP and EPM in observing drug withdrawal may give insight into the anxiety-like behaviors and drug-seeking behaviors that are associated with addiction with drugs of abuse. Future studies in using the elevated-plus maze, and orexin should be implemented in the growing research in orexin and drug addiction and stress.

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Table 1. *Experimental drug design across all groups, each group was given 3mg/kg of morphine or saline solution based on weight and depending on each group. Every three days, which are labeled *, indicate days where the subjects were weighed, M = morphine, and S = saline. On test day, the rats were exposed to the elevated plus-maze for five minutes and behavioral measurements were taken. n=8 per group.*

	Day 1	Day 2	Day 3*	Day 4	Day 5	Day 6*	Day 7: EPM
Chronic Morphine							
1M : Green	M	M	M	M	M	M	S
2M : Blue	M	M	M	M	M	M	M
Chronic Saline							
3S : Orange	S	S	S	S	S	S	No Injection
4S : Yellow	S	S	S	S	S	S	S

Figure Captions

Figure 1. The mean open-arm duration to the EPM in rats in each group on Day 7, where 1M received saline, 2M received morphine, 3S received no injection and 4S received saline.

Figure 2. The mean number of stretch attend postures during exposure to the EPM on Day 7, where 1M received saline, 2M received morphine, 3S received no injection and 4S received saline.

Figure 3. The mean number of unprotected head dips during exposure to the EPM on Day 7, where 1M received saline, 2M received morphine, 3S received no injection and 4S received saline.

Figure 4. The mean number of closed entries during exposure to the EPM on Day 7, where 1M received saline, 2M received morphine, 3S received no injection and 4S received saline.

Figure 5. The mean number of c-Fos cell activation of both right and left sides of the lateral hypothalamus (LH) of rats after subjected to the EPM on Day 7. Two pieces of stained tissue were used per rat.

Figure 6. The mean number of double-labeled c-Fos and orexin, cell activation of both right and left sides of the lateral hypothalamus (LH) of rats after subjected to the EPM on Day 7. Two pieces of stained tissue were used per rat.

Figure 7. An example of a double-labeled c-Fos/orexin activated cell in the lateral hypothalamus (LH) of rats after subjected to the EPM on Day 7.

Supplementary Figure Captions

Figure 7b. An example of a C-Fos activated cell in the lateral hypothalamus (LH) of rats after subjected to the EPM on Day 7.

Figure 7c. An example of a orexin labeled cell in the lateral hypothalamus (LH) of rats after subjected to the EPM on Day 7.

Figure 1.

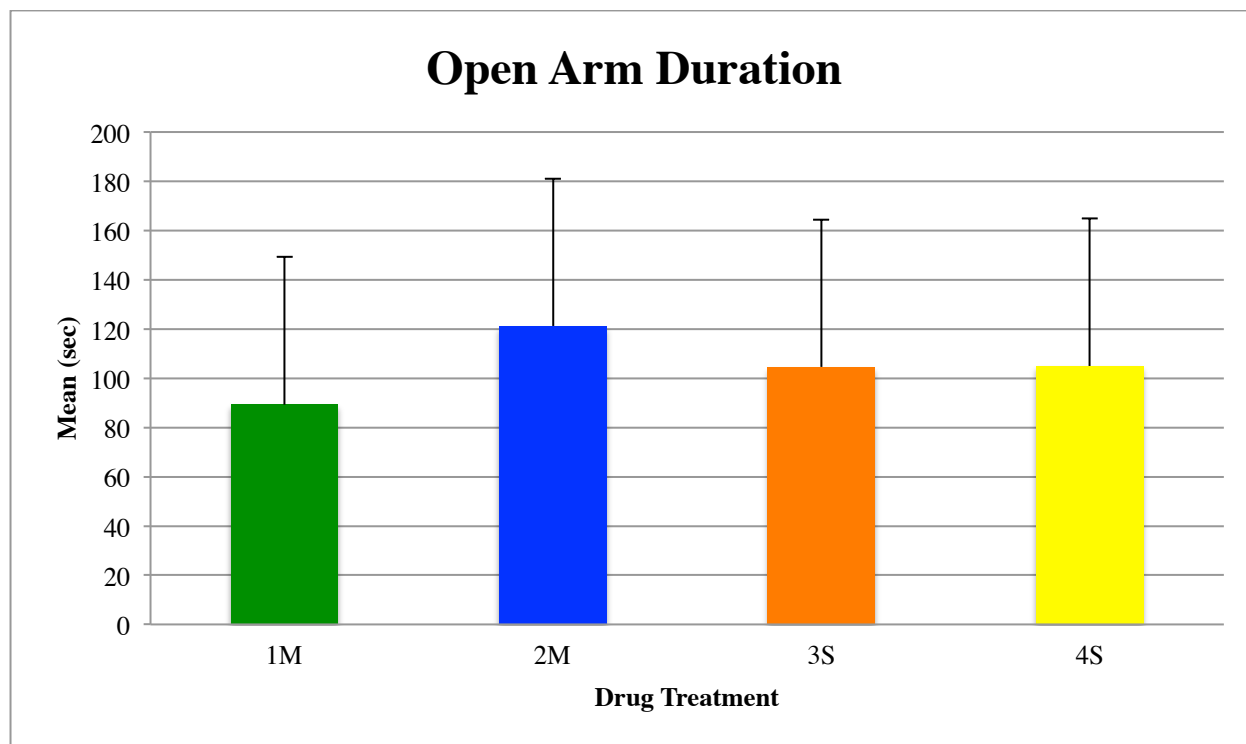


Figure 2.

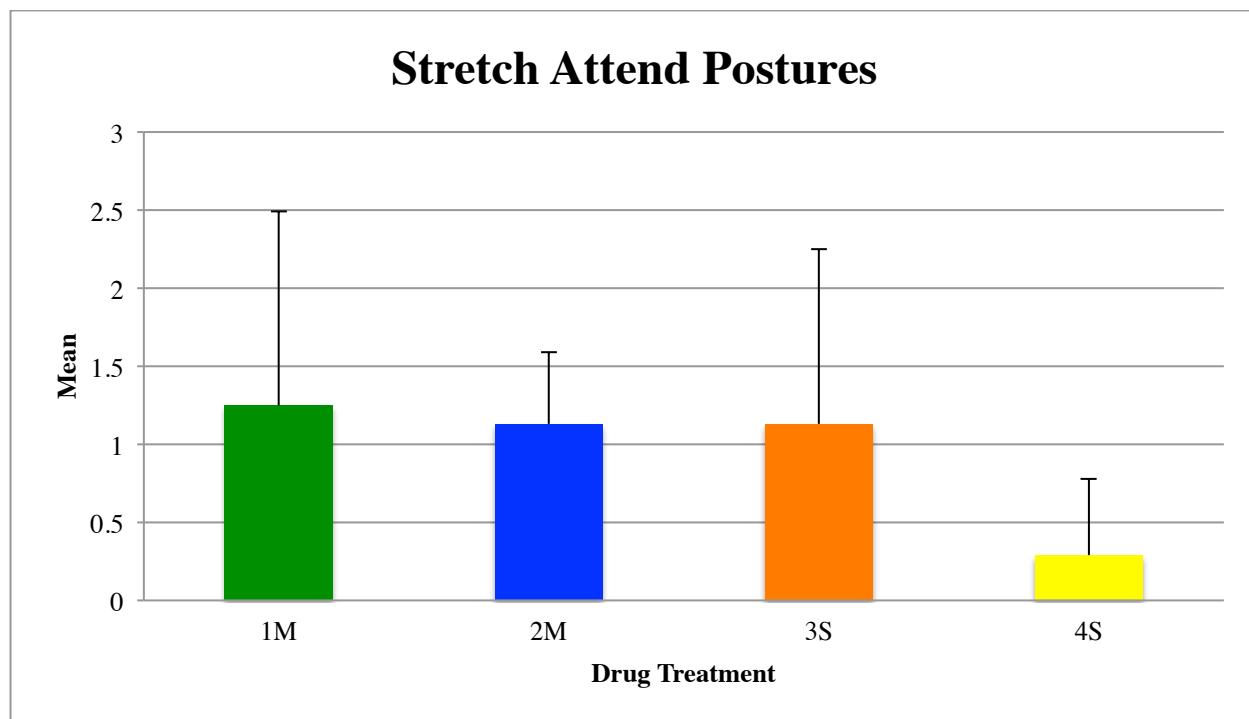


Figure 3.

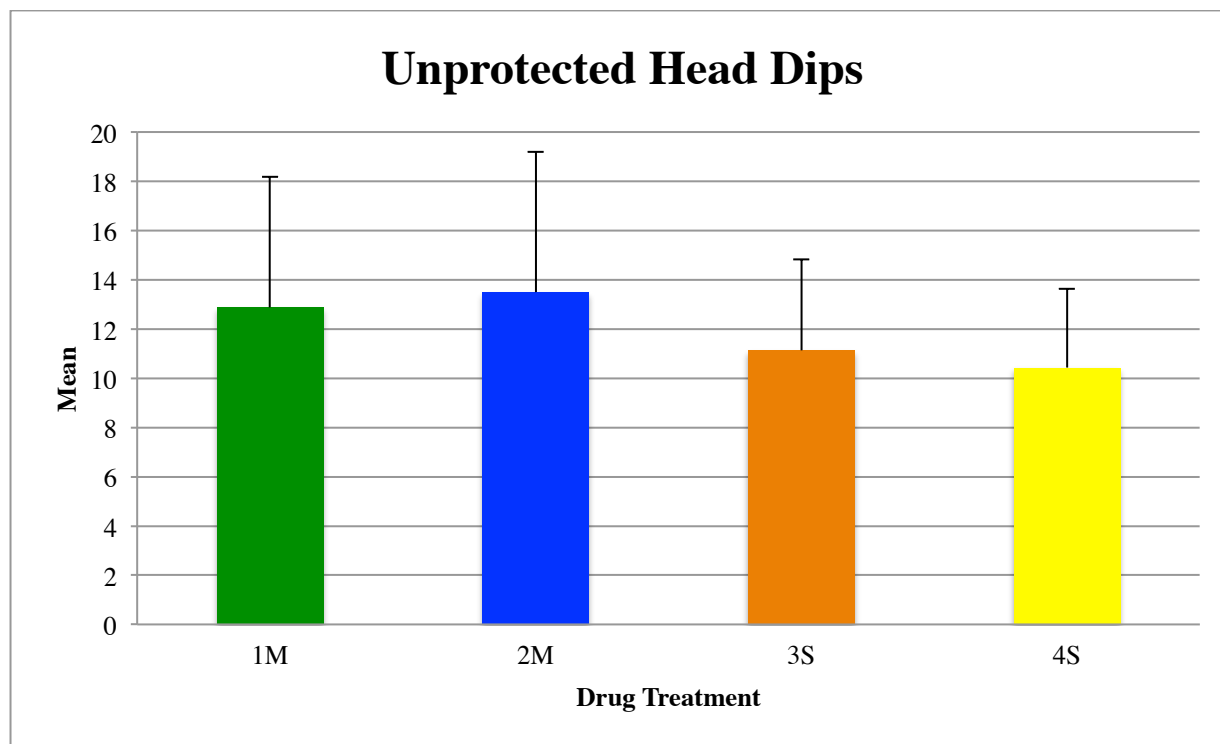


Figure 4.

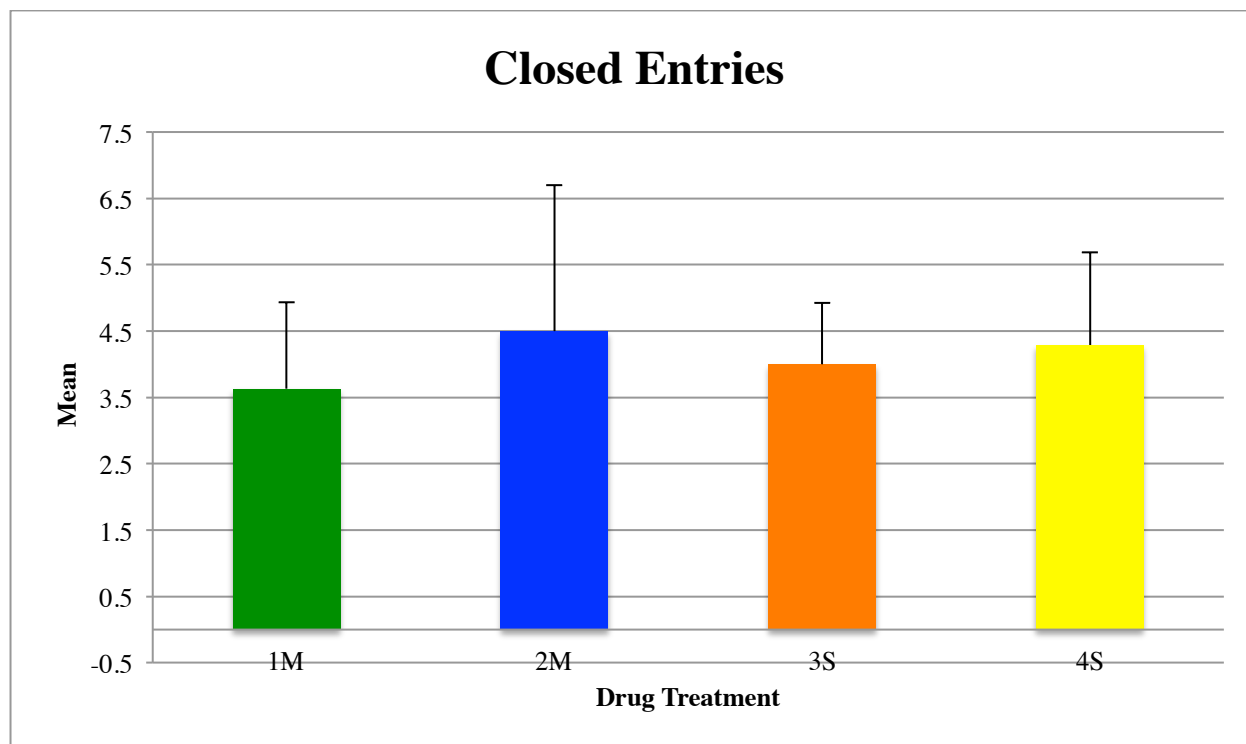


Figure 5.

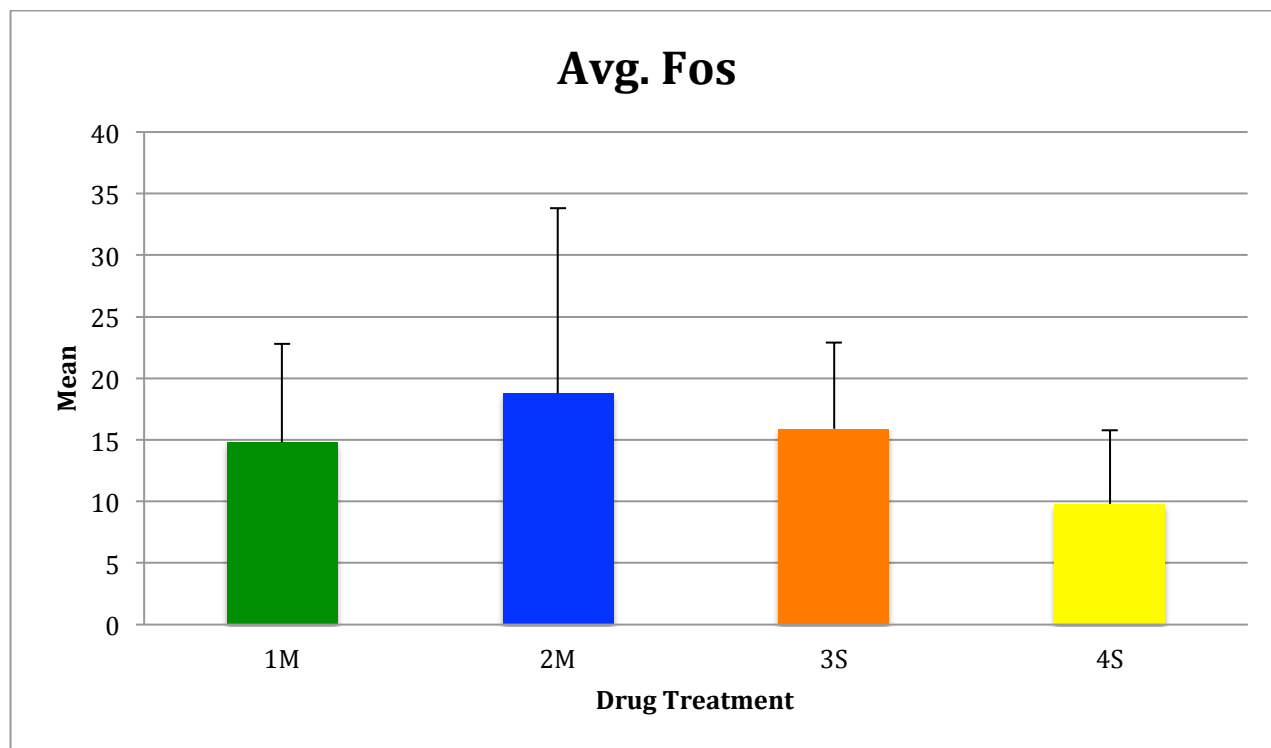


Figure 6.

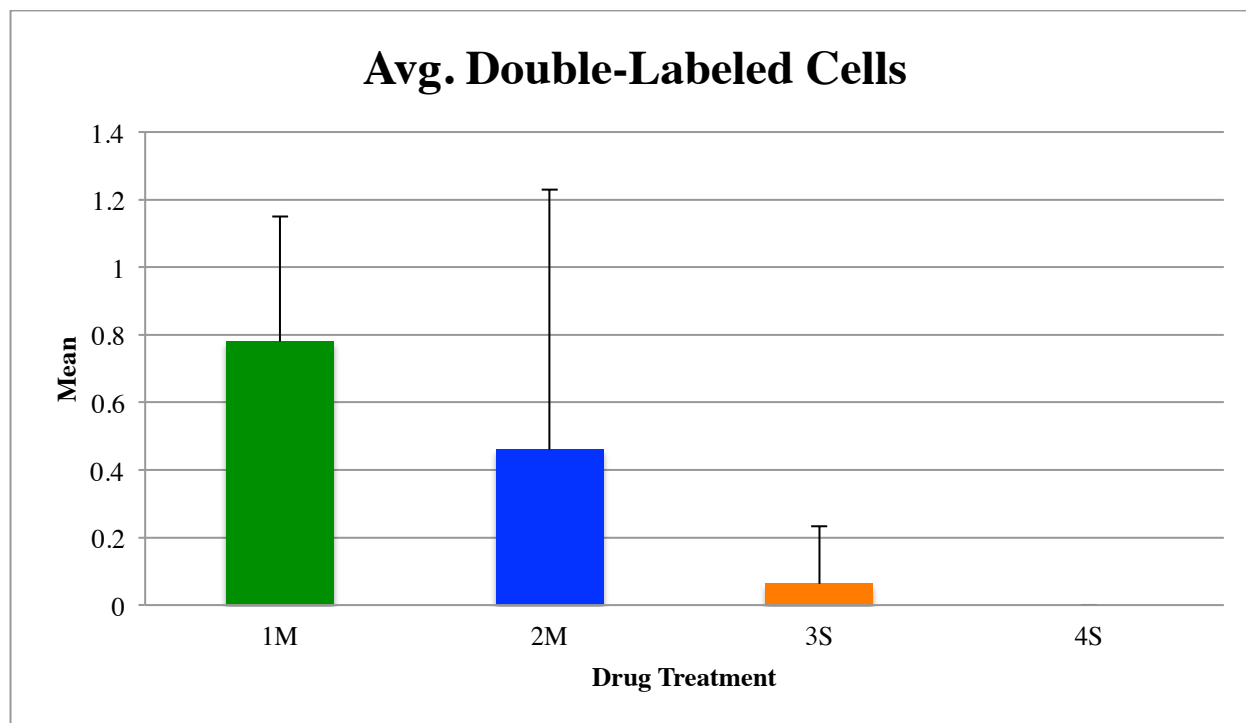


Figure 7.

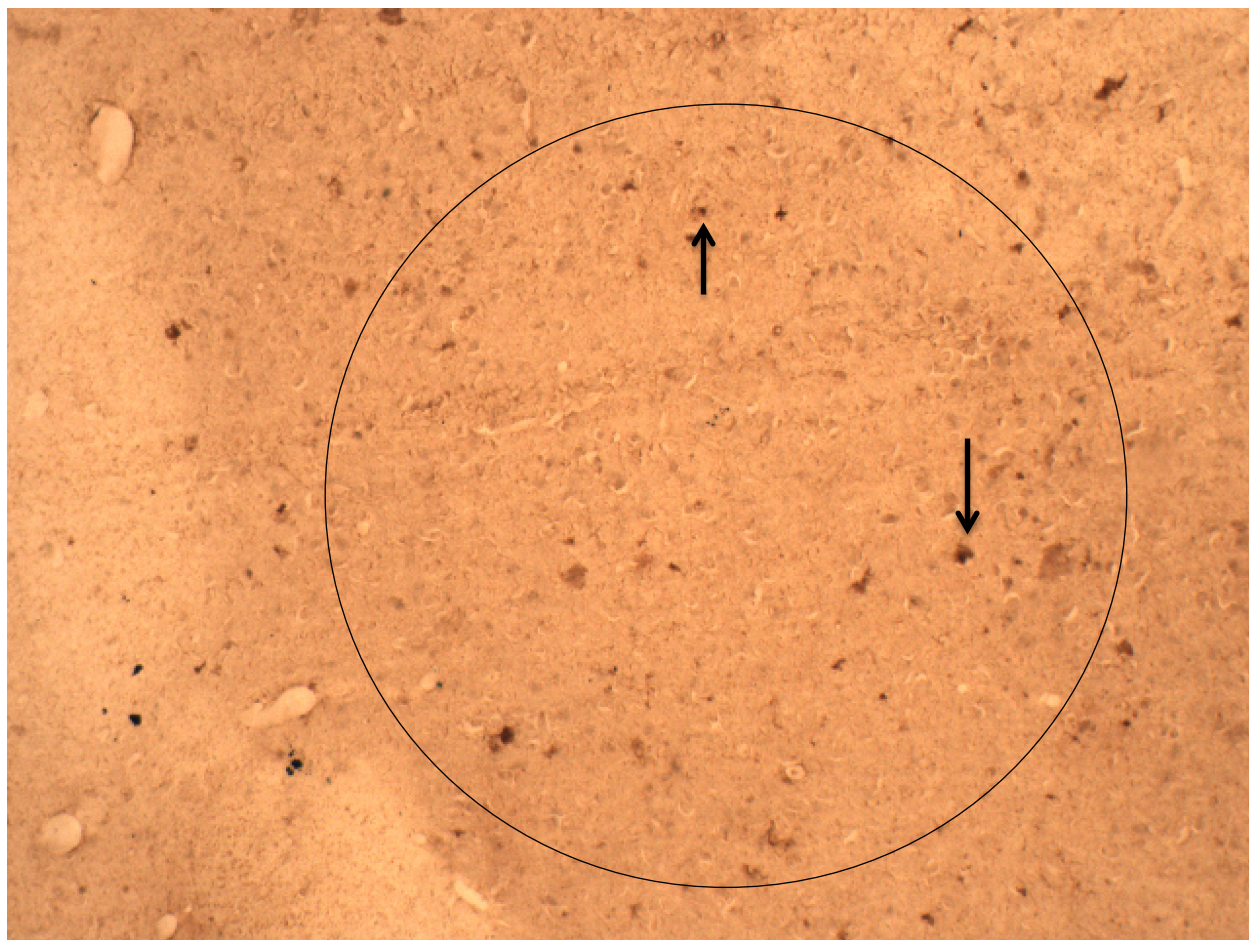


Figure 7b.

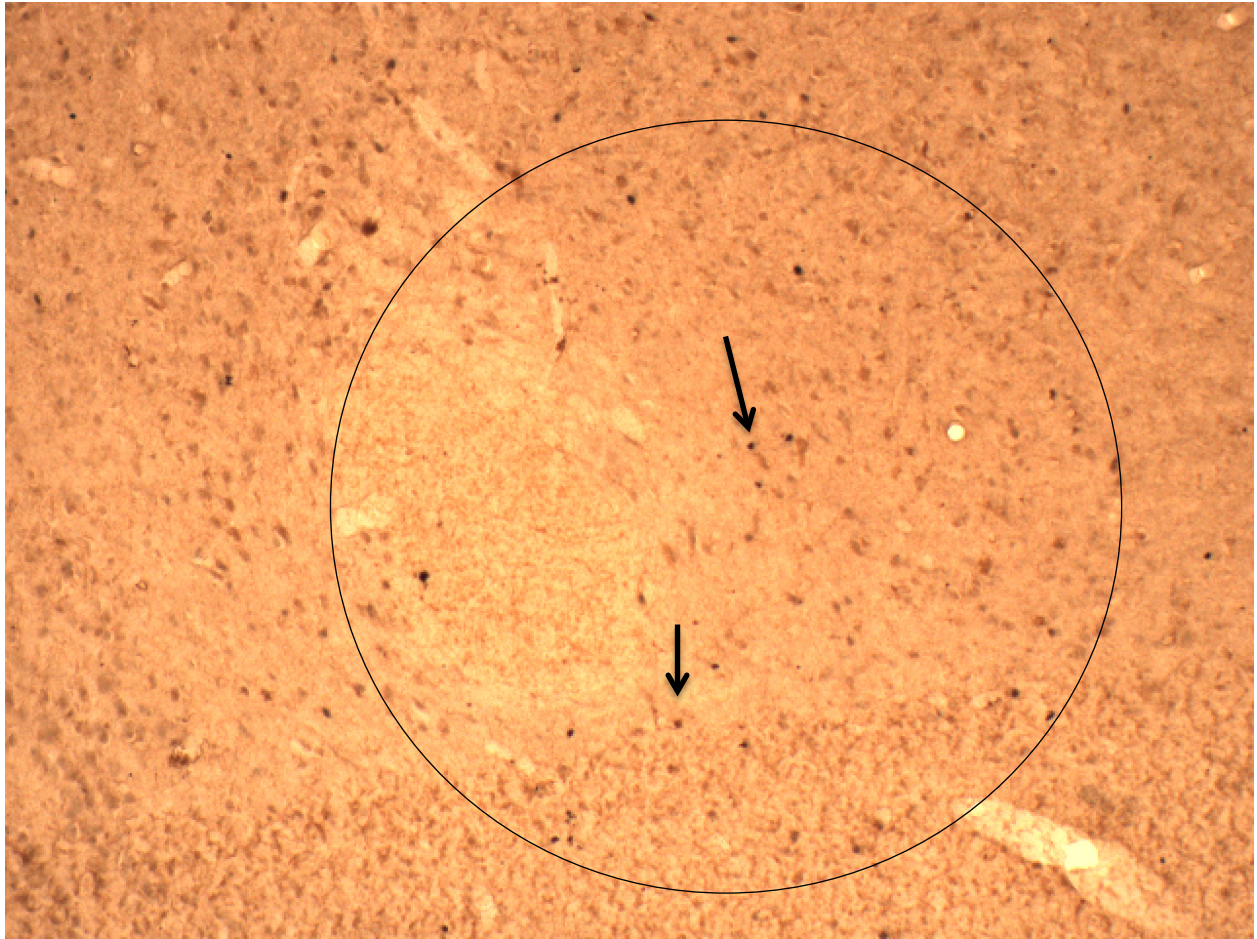


Figure 7c.

