

EXPLORING SEX DIFFERENCES IN MORPHINE WITHDRAWAL-INDUCED
ALTERATIONS OF THE VENTRAL TEGMENTAL AREA

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Samara Morris Bobzean

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Supervising Committee:

Linda Perrotti, Supervising Professor

Carlos Bolanos

Yuan Bo Peng

Qing Lin

Heekyeong Park

UNIVERSITY OF TEXAS AT ARLINGTON

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Dedication

I would like to dedicate this dissertation to a small handful of people in my life that very much deserve their own distinguishably bizarre paragraph. Ms. Hollie Pelloosmaa, you have too much respect for the law for me to actually designate you my getaway driver, but know that you hold a very dear place in my brain. Susanna Latham, I love you too. Mr. Brett Helweg, thanks for putting *Game of Thrones* in the mailbox that night; you changed my life. Finally, Mr. Travis Bradley, you're an asshat. Get your own jokes.

Abstract

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Samara Morris Bobzean

The University of Texas at Arlington, 2016

Supervising Professor: Linda Perrotti, PhD.

Opioid withdrawal syndrome is a feature common to chronic opioid use that often serves as a powerful motivator of continued drug use. GABA-ergic neurons in the tail of the ventral tegmental area (tVTA) are implicated in mediating responses to opioids and opioid withdrawal. The tVTA regulates the effects of opioids on VTA dopamine neurons and a number of earlier studies have shown that alterations in levels of CREB within the tVTA profoundly affect drug-motivated behaviors. Unfortunately, the mechanisms underlying morphine withdrawal have been studied almost exclusively using men and male animals. The objectives of the current study are to investigate sex effects on the expression and duration of spontaneous somatic morphine withdrawal behavior; and to identify the relationship(s) between spontaneous somatic morphine withdrawal behavior and CREB activity in GABAergic neurons of the tVTA in both

males and females. Intact adult, male and female Long Evans rats were made morphine-dependent using twice-daily injections of escalating doses of morphine (2.5-40mg/kg) for 10 days. Spontaneous somatic morphine withdrawal behavior was recorded at 12, 24, 36, 48, 60, and 72 hours after the last morphine administration; all animals were sacrificed via exsanguination after the last behavioral observation (72 hours). The spontaneous withdrawal paradigm used here revealed that while both male and female morphine-dependent rats developed somatic symptoms of withdrawal, males expressed more severe symptoms earlier in withdrawal (within the first 36 hours) compared to females. While, females demonstrated lower overall symptom severity, these symptoms persisted for a longer period of time; as a result, withdrawal symptoms in females were collectively more severe compared to males at the 72 hour time point. CREB activation in tVTA GABAergic cells was significantly higher for morphine-withdrawn females compared to controls 72 hours after the end of treatment. Taken together, these results demonstrate that the timing of the expression of somatic withdrawal is different for males and females. Furthermore, our data suggest that males and females differ in the timing of withdrawal-induced activation of tVTA CREB. These differences in CREB activation likely impact expression of behaviors associated with opioid withdrawal.

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CHAPTER 1: INTRODUCTION

1.1 Introduction to the problem

Opioids are a potent class of drugs that acutely attenuate perceptions of pain and induce pleasurable feelings of euphoria and relaxation in users. However, despite the acute subjective benefits to mood and health, long-term use of both illicit and legal prescription opioids can lead to dependence and/or development of an opioid use disorder (OUD). In 2014, reports from the Substance Abuse and Mental Health Services Administration (SAMSHA), revealed estimates that 1.9 million individuals in the United States met criteria for having an OUD. Furthermore, OUDs accounted for more than 47,000 American substance use related deaths in 2014 according to the Centers for Disease Control. This number has steadily increased since 1999 and is second only to alcohol use disorders.

OUDs often develop over time, are multi-factorial, and therefore very difficult to diagnose and treat. The DSM-V has been organized in such a way to recognize the various criteria that can qualify someone as having an OUD. These criteria include the following: taking more opioid drugs than intended; intense craving for opioid drugs; difficulty reducing opioid intake; failure to fulfill social, work-related, academic, or family obligations; persistent opioid use despite

negative interpersonal and physical consequences; physical symptoms of dependence such as withdrawal when opioids are not taken (Garland, Froeliger, Zeidan, Partin, & Howard, 2013; Zacny et al., 2003). OUDs can present and be defined as mild, moderate, or severe depending on the number of diagnostic criteria met by an individual. Left untreated, OUDs often lead to devastating health, socio-economic, and legal consequences.

A considerable amount of epidemiological research has validated the influence of sex in the physiological and psychological risks for developing an OUD (Back, Payne, Simpson, & Brady, 2010; Fillingim, King, Ribeiro-Dasilva, Rahim-Williams, & Riley, 2009; Green, Grimes Serrano, Licari, Budman, & Butler, 2009). Since 1999, opioid overdose related deaths have increased 265% in men and nearly 400% in women. Although, more men than women admit lifetime and past-year use of all opioids, women are more likely to report the nonmedical use of prescription opioids as their primary drug of abuse (TEDS, 2008). As with other drugs of abuse, the onset and progression of OUD is a multistage process, and sex is a variable that influences each stage of that process. The present paper will review basic opioid pharmacology and examine the evidence for sex differences that ultimately affect the functioning of the brain's natural reward circuit thereby resulting in similarities and differences in the behavioral response to opioid drugs.

1.2 Sex differences in rodent models of opioid reward and withdrawal

Preclinical behavioral studies have been instrumental in elucidating some distinctions between males and females during the acquisition of opioid dependence and addiction. Several preclinical behavioral studies have demonstrated sex differences in opioid reward. Operant conditioning studies demonstrate that female rodents acquire self administration (both oral and intravenous) of opioids faster, at lower doses, and under a wider variety of environmental housing factors than males (Alexander, Coombs, Hadaway, & Va, 1978; Cicero, Aylward, & Meyer, 2003; Lynch & Carroll, 1999). Additionally, females consume greater amounts of opioids and are willing to work harder for an infusion of the drug (achieve higher self-administration breakpoints) than male rats (Cicero et al., 2003). This suggests that females learn faster and find a wider range of doses more rewarding, and will work harder for opioid reinforcement. Studies using a classical conditioning paradigm (conditioned place preference) show that females express preference for environments previously paired with lower doses of morphine than males. Moreover, females demonstrate a preference for environments paired with a wider range of doses of morphine compared to males (Karami & Zarrindast, 2008; Weidemann, SfN, 2014). This suggests that females are more sensitive to environmental cues associated with morphine administration. Taken together, these data from preclinical behavioral studies demonstrate that there are sex differences in the acquisition of opioid use behaviors and reward processing that impact the trajectory of progression of

OUD development; thus, exposure to opioids differentially affects male and female behavior and gender specific treatment options may also be needed.

Most of what is currently known about withdrawal from opioids comes from studies conducted exclusively in men and male animals. Results of the few studies that have been conducted using both male and female subjects show that male rodents express a greater magnitude of withdrawal symptoms compared to females during spontaneous withdrawal from chronic morphine administration (Cicero, Nock, & Meyer, 2002), but not naloxone precipitated withdrawal (Ali, Sharif, & Elkadi, 1995; Cicero et al., 2002). However, caution should be taken when evaluating the methods and results of these experiments and others; at this point in time, as the withdrawal scales and time-points used have been developed using only male animals. Interestingly, in a recent study, heroin-dependent men (but not women) demonstrated exhibit abnormal avoidance and extinction behaviors during a video game cognitive laboratory challenge (Sheynin, Moustafa, Beck, Servatius, Casbolt, Haber, ... & Myers, 2016). At this point in time, there is a paucity of literature that has looked the neurobiological changes associated with the subjective experiences of opioid withdrawal. One recent preliminary study using healthy (male) volunteers identified several brain regions that showed alterations in neural processing following naloxone-precipitated withdrawal (Chu et al., 2015). To our knowledge, this is the only study of its kind. More studies are needed to elucidate the

mechanisms underlying withdrawal related brain functioning, and it is imperative that women participants be included.

At this point, research regarding the biological mechanisms underlying the presently discussed sex differences is nearly nonexistent. Published papers have identified the involvement of the opioid reward and arousal systems in the context of opioid withdrawal; however, it is precarious to assume that these findings can be extrapolated to sex-dependent differences, thus more research is needed, particularly that which investigates the intracellular neurobiological underpinnings of this potentially devastating disorder.

1.3 Introduction to neurobiology of opioid addiction

While the etiology underlying the prevalence of OUD is not completely understood, it is generally accepted that the pathophysiology of this disorder is rooted in the ability of opioid drugs to hijack the brain's natural reward circuit. This complex circuit originates in dopamine (DA) containing cell bodies of the ventral tegmental area (VTA) that project to terminal sites in the forebrain including the prefrontal cortex (PFC), nucleus accumbens (NAcc), dorsal striatum, amygdala, and bed nucleus of stria terminalis (See Figure 1). In addition to dopamine signaling, neural transmission of information through the limbic system is also modulated via GABAergic, glutamatergic, and serotonergic systems. These neurotransmitters in conjunction with opioid peptides, hormones, and other neuromodulators work together within this circuit to mediate a wide

array of motivated behaviors and responses to rewarding and aversive stimuli. While several brain regions and neurotransmitter signaling pathways contribute to this complex reward circuit, experiments presented herein focus on the specific involvement of the VTA.

1.4 Cells of the VTA

The VTA is a heterogeneous brain region that has long been a primary target for the study of opioid dependence. This structure resides in the midbrain, just medial to the substantia nigra and ventral to the red nucleus. The VTA is comprised of predominantly dopaminergic and GABAergic cells, and to lesser extent also contains glutamatergic neurons. Here, I will review the literature related to the contributions of each VTA cell type to development of OUDs.

Currently, the majority of research into VTA-dependent behaviors has almost exclusively focused on DA neurons, the most abundant VTA cell type. First identified during the mid 1960's (Dahlstroem & Fuxe ,1964), DA neurons are produced in the midbrain and account for approximately 60%–65% of all neurons found in the VTA and are identified immunohistochemically by the presence of tyrosine hydroxylase (TH), the rate-limiting enzyme for DA synthesis. Soon after the discovery of these neurons, a series of histochemical and retrograde tracer topographical studies were conducted, and it was identified that these VTA DA neurons have wide-spread projections to areas of the brain that had previously been implicated in studies of motivation and positive reinforcement (Fallon &

Moore, 1978; Swanson, 1982; Ungerstedt, 1971).

To date, the VTA DA neuron population that projects to the NAcc is the most widely studied with regard to substance abuse. Virtually all drugs of abuse acutely elevate extracellular DA levels (Di Chiara & Imperato, 1988). Comprised almost entirely of dense populations of medium spiny neurons (MSNs), the NAcc receives DA input directly from its VTA afferents. MSNs are GABAergic in nature, characterized by large and extensive dendritic branching, and are profoundly sensitive to regulation by drugs, hormones, and endogenous opioid peptides. Although, the specific functional role of opioid drugs and opioid peptides in the NAcc is beyond the scope of this paper, it is worth noting that hormones and opioid peptides collectively regulate dopamine signaling by altering actions of MSN on their extensive projection targets, and these effects are sexually dimorphic (see review by Becker et al., 2012). Overall this area is an extremely crucial contributor to sex differences in emotional processing and goal-directed behavior associated with opioid consumption (Carlezon & Thomas, 2009; Jensen et al., 2003; Mogenson, Jones, & Yim, 1980; Reynolds & Berridge, 2002).

Since the discovery of VTA DA neurons, a large body of research, concerned with the study of opioid drugs has established the meso-accumbens pathway as a crucial substrate for reward acquisition related behaviors (Hyman, Malenka, & Nestler, 2006; Koob & Volkow, 2010; Wise, 2005). Early experiments demonstrate that male rats will learn to lever press for microinjections of opioids

directly into the VTA (Bozarth & Wise, 1981; Devine & Wise, 1994) and develop conditioned place preference for environments associated with direct VTA morphine administration (Phillips & LePiane, 1980). In direct contrast, rats show a decline in motivation to acquire rewards in cases where the VTA has been lesioned or naloxone, a potent opioid antagonist, has been administered (Papp & Bal, 1987). More recent experimental studies have the advantage of access to modern laboratory techniques in order to investigate the effects of activating specific neuronal populations of the VTA. For example, two studies that employed the use of optogenetics, electrophysiology, and confocal microscopy demonstrated that activation of VTA GABA neurons disrupts reward consumatory behaviors and enhances associative learning (Brown, et al. 2012; Zessen, Phillips, Budygin, & Stuber, 2012). Combined, these experiments and others provide evidence establishing a crucial role of the VTA in opioid reward acquisition as well as reward processing that is still a prominent area of study.

Over the past decade evidence has accumulated to support the notion that non-DA neurons are just as important for eliciting opioid-dependence behaviors as DA neurons. Recently, the prominent group of GABAergic neurons in the VTA has become a primary target of study. These cells are located primarily in the posterior “tail of the VTA” (tvTA; Perrotti et al., 2005), also called the “rostromedial tegmental nucleus” (RMTg; Jhou et al., 2009) and are the second most abundant cell type found in the VTA. These GABAergic cells account for approximately 30%–35% of all VTA neurons and nearly all of tvTA neurons

(Nair-Roberts et al., 2008; Sesack & Grace, 2010; Swanson, 1982). GABA cells of the tVTA are connected via gap junctions and contain the rate-limiting enzyme glutamic acid decarboxylase (GAD). They are positioned caudal-laterally to their primary efferent, VTA DA neurons, and receive inputs from numerous areas including the medial PFC, cingulate cortex, optic area, lateral hypothalamus, superior colliculus, periaqueductal gray (PAG), dorsal raphe, and lateral tegmental nucleus, and most notably, the lateral habenula (Figure 2).

Under normal circumstances, tVTA GABA neurons exert strong inhibitory effects on VTA DA cells and therefore tightly regulate neurotransmission to forebrain projection sites (Barrot et al., 2012; Jhou et al., 2009; and Zahm, 2009). However, opioid drugs acutely inhibit VTA GABA modulatory interneurons that share synapses with neighboring dopamine neurons, indirectly activating the meso-accumbens pathway. Consequently, DA neurons become unencumbered by the inhibitory effects of GABA, and DA is allowed to freely transmit to projection sites in the NAcc and other limbic regions. The feelings of intoxication and euphoria that accompany potent opioid doses are largely credited to activation of this meso-accumbens pathway. The neurocircuitry that governs the tVTA has only begun to be elucidated and studied over the past decade.

In contrast to the abundance of dopamine neurons and GABAergic neurons, the VTA is modestly comprised of 2-3% glutamatergic neurons. While small in numbers, recent research has supported a significant role for these

excitatory cells. Jalabert and colleagues recently demonstrated that when glutamate receptor antagonists are infused directly into the VTA, morphine-induced facilitating effects on VTA DA transmission are averted (Jalabert et al., 2011). Though much more research is necessary to draw conclusions concerning the functional role of VTA glutamate neurons, the data from this study suggests that in addition to inhibitory effects of tVTA GABA neurons, opioids require crucial excitatory input to DA neurons before the meso-accumbens circuit can be fully initiated.

While the above discussion of cell types helps in understanding the breadth of cell types involved in the action of opioids, the functional consequences to intracellular signaling can only be understood by reviewing the effects of these drugs on opioid receptor (OR) activation. The next two sections will first provide a review of specific OR subtypes of the VTA/tVTA, their structure, location, and function, and secondly further delve into important opioid effects on intracellular signaling cascades and discuss relevant sex differences.

1. 5 Opioid Receptors

As previously stated, analgesic and rewarding effects of opioids are mediated by actions at ORs located throughout the central and peripheral nervous systems. The VTA/tVTA contains ORs that, when activated by drugs

such as heroin and morphine, as well as endogenous opioids (endorphins, dynorphins, and enkephalins), can alter GABAergic inhibitory control over DA signaling (Bourdy & Barrot, 2012). A growing body of literature suggests that changes to OR activation and function can significantly alter the tVTA/VTA relationship and subsequently have huge consequences to physical and affective states that strongly influence drug-taking behaviors and other reward related behaviors.

ORs are seven-transmembrane proteins that are connected via three intracellular and three extracellular loops, have a C-terminus, and are linked to G-proteins (reviewed by Snyder & Pasternak, 2003). Activation of ORs by opioid ligands stimulates G-protein second messenger systems. This, in turn, triggers the inhibition of adenylyl cyclase activity, stimulates potassium channels to open and voltage gated calcium channels to close (Chieng & Christie, 1994; Nestler, 1996; Hyman and Malenka, 2001). This complex chain of events causes cells affected to hyperpolarize and inhibits cell firing and attenuates the release of neurotransmitters from synaptic terminals.

There are three well established classes of OR subtypes (mu, delta, and kappa). These receptors are localized in distinct populations of cells (Y Chen, Mestek, Liu, Hurley, & Yu, 1993; Yan Chen, Mestek, Liu, & Yu, 1993; Evans, Keith, Morrison, Magendzo, & Edwards, 1992). Activation of these receptors mediates communication between the cortex, limbic system, brain stem, and

peripheral areas of the body about pain and reward (reviewed by Merrer et al., 2009). Fortunately, the availability of a wide variety of opioid agonists and antagonists selective for each subtype has made possible the study of ORs unique roles in opioid analgesia and OUD. Findings from pharmacological studies demonstrate that mu, kappa, or delta receptors elicit a variety of receptor subtype dependent effects.

Abused opioid drugs have the greatest affinity for mu opioid receptors (MORs). MORs have a high affinity for morphine like opioids and endogenous opioid peptides called endorphins; they can be found primarily in the thalamus, PAG, brainstem, spinal cord, and limbic system (Brunton, Chabner, & Knollman, 2010; Martin, 1983). Due to their wide-spread distribution, activation of MORs produces a wide variety of effects including analgesia, euphoria, sedation, decreased gastrointestinal motility, and in extreme cases, respiratory depression. Studies using immunohistochemistry and electron microscopy provide evidence that MORs in the VTA are mostly localized to presynaptic and postsynaptic sites on VTA GABAergic neurons (Garzon & Pickel, 2002). Interestingly, modulation of euphoric and rewarding effects of opioids have predominantly been credited to activation of presynaptic MORs (Chartoff & Connery, 2014; Christie, 2008).

Ligand binding to presynaptic MORs in GABAergic cells of the tVTA causes a complex chain of events that can have large impacts on cell activity and mesocorticolimbic neurotransmission. This occurs via activation of G-protein

subunits which interact with downstream effector systems to inhibit adenylyl cyclase, as well as inwardly rectifying potassium channels, allowing them to open and voltage gated calcium channels to close (Christie, 2008; Nestler, 1996). Consequently, this chain of events leads to hyperpolarization of GABA cells, inhibits cell firing, and attenuates the release of GABA from the synaptic terminals. The net effect of this process is an inhibition of the inhibitory effects of these GABA neurons on neighboring DA neurons of the VTA; thus, indirectly increasing DA transmission to forebrain and limbic projection sites (Bonci & Williams, 1997; Johnson & North, 1992).

MORs are also extremely prevalent in descending brain pathways that regulate pain signaling. This mechanism has been extensively reviewed (see Millan, 2002). Activation of MORs by opioid agonists, particularly in the PAG, suppresses PAG GABAergic interneurons that project to the medulla and locus coeruleus. This leads to activation of the descending analgesic pathway by facilitating the release of norepinephrine (NE) and serotonin (5HT), which ultimately weaken excitability of neurons in the dorsal horn (located in the spinal cord) that project ascending pain signals to the medulla and cortex.

Morphine and other opioids are potent analgesics frequently used to treat symptoms of acute and chronic pain conditions. MOR agonists include endogenous beta-endorphins, illicit opioids such as heroin, and many other opioid analgesic medications including morphine, oxycodone and hydrocodone

(For a more complete list, see Table 1) are largely known for their actions in the opioid-sensitive descending analgesic pathway. Not surprisingly, pain is a cardinal feature of opioid withdrawal syndrome. People and animals previously made dependent on opioids demonstrate varying degrees of unpleasant symptoms including low energy, irritability, anxiety, agitation, insomnia, runny nose, teary eyes, hot and cold flashes, goose bumps, yawning, muscle aches and pains, especially abdominal cramping, nausea, diarrhea (Bradley, 1987). Because most opioid analgesics are potent MOR agonists, and MOR density and activity has been shown to change with repeated intake of these drugs, it is likely that changes in neural substrates that govern these receptors are at least in part responsible for the symptoms that ensue once the drugs are withdrawn.

Compared to MORs, less is known about delta opioid receptors (DORs). They are located in the neocortex, striatum, olfactory areas, substantia nigra, NAcc, and spinal cord and activated by endogenous opioid peptides termed, enkephalins. It should be noted that, enkephalins also bind to MORs and KORs, however, they have higher affinity for DORs. The physiological effects of DOR activation are similar to that of MORs; these effects include analgesia, inhibition of gastrointestinal motility, and respiratory depression (although this is not indicated in all studies).

While MORs and DORs possess many similarities in function, kappa opioid receptors (KORs) are rather different. They reside in the brain stem,

spinal cord, pituitary and limbic areas including the striatum, NAcc, amygdala, hypothalamus, and VTA DA neurons. Activation of KORs is similar in some ways to MORs and DORs, such as possessing analgesic and sedative properties, but vastly dissimilar in other ways. Unlike MORs, KOR activation has some profoundly aversive results after administration of high doses including intense dysphoria, hallucinations, and labored breathing. The pharmacology of KORs is not vastly different from MORs. Activation of both these receptors stimulates G-protein signaling, increasing potassium conductance and decreasing calcium influx, which results in inhibiting vesicular neurotransmitter release. However, because KORs are abundantly located on dopamine neurons, activation of these receptors by endogenous dynorphins or KOR agonists, prevents DA transmission. As such, the utility of KOR agonists as therapeutic analgesics instead of addictive MOR agonists is somewhat thwarted by their unpleasant effects on mesolimbic signaling. Learning to modulate the activity of KORs may be useful for understanding the aversive consequences of opioid administration as well as provide insight into mechanisms underlying withdrawal symptoms.

Sex differences in expression, localization, and trafficking of ORs, especially MORs, have been documented. Human studies using PET imaging have identified sex differences in MOR binding potential in many brain areas including the anterior cingulate, prefrontal cortex, parietal cortex, temporal cortex, amygdala, thalamus, caudate, pons nucleus, and cerebellum (Zubieta, Dannals, & Frost, 1999). Additionally, preclinical studies show that male rodents express

more MOR protein in the PAG and spinal cord compared to female rodents (Kren, Haller, & Welch, 2008; Loyd, Wang, & Murphy, 2008). Furthermore, studies accounting for human menstrual and rodent estrous cycles have demonstrated the influence of cycle phase on hippocampal MOR trafficking and endogenous opioid neurotransmission (Milner et al., 2013). Specifically, the number of MORs in dendritic plasma membrane increases during estrus and to a lesser degree in proestrus. Increased densities of MOR binding sites likewise increase the binding opportunity for endogenous and exogenous opioid ligands and subsequent inhibition of GABAergic cells and augmented excitability of hippocampal cells. Still, more research is needed to validate a comprehensive influence of sex hormones on these organizational differences. Overall, this research suggests that sex differences in opioid analgesia and reward may be linked to interactions between OR receptors and estrogen receptors.

To a lesser extent, sex differences have been identified regarding KORs and DORs. Selective KOR agonists have greater antinociceptive effects in males compared to females (Rasakham & Liu-Chen, 2011). However, some studies show that MOR-KOR mixed agonists are more effective in females (Gordon et al., 1995). Unfortunately, to date, not many studies have examined sex differences in KOR expression, distribution, and localization in the spinal cord and midbrain, which could potentially help explain some of these differences. Of the studies that have been conducted, females express more KORs in the midbrain and more KORs and DORs in the spinal cord than males (Kren et al.,

2008). Though the focus of present experiments is on morphine dependence, differences in midbrain KOR and DOR localization, distribution, and pharmacodynamics properties may contribute to sex differences in opioid reward modulation, and as such, have important implications in the development and expression of physical and psychological dependence and addiction. More studies are needed to clarify the relationship between MORs, KOR, and DORs, gonadal hormones, and drug-receptor interactions. Fortunately, we now know that the behavioral effects of opioids differ depending on the opioid receptor subtype with which they interact.

1.6 The role of cAMP pathway upregulation in opioid action

The role of the cAMP intracellular signaling pathway has been well established in all stages of addiction (reviewed by Carlezon, Duman, & Nestler, 2005). Many studies have focused on the phosphorylated form of cAMP response element (CRE)-binding protein (CREB) and related proteins as indicators of protein kinase A (PKA) activity. Briefly, activation of MORs stimulates G-protein (composed of α , β and γ subunits) to inhibit adenylyl cyclase, which in turn inhibits the conversion of ATP to cAMP which under normal circumstances, acts as a second messenger to stimulate protein kinase A (PKA). PKA phosphorylates many proteins including CREB. Phosphorylated CREB (pCREB) diffuses inside the cell's nucleus where it can bind to regulatory regions specific for the promotion of gene transcription. CREB is a transcription

factor found in all brain cells and is recognized for its ability to regulate gene transcription, a process that contributes to alterations in the functions of neurons, and neuronal circuits, eventually mediating changes in learning, memory, and behavior (Carlezon et al., 2005).

Opioid agonists can induce a variety of effects on intracellular CREB. Studies using male animals have shown that CREB is essential for morphine-induced alterations in gene expression that precipitate both reward and withdrawal associated behavior (Nestler, 2002; Ren et al., 2013; Walters, Kuo, & Blendy, 2003). Acute morphine exposure transiently decreases and then increases CREB phosphorylation (Guitart, Thompson, Mirante, Greenberg, & Nestler, 1992). It is believed that the increase in CREB serves as a negative feedback mechanism to counteract the initial decrease caused by over activation of the opioid receptors by opioid drugs. Additionally, with repeated opioid administration, neurons adapt compensatory homeostatic responses to the persistent opioid disinhibition of the mesolimbic reward pathway. Chronic morphine administration increases cAMP activity in the NAcc and VTA (Olson et al., 2005; Ren et al., 2013; Terwilliger, Beitner-Johnson, Sevarino, Crain, & Nestler, 1991). The net effect is an upregulation of the cAMP pathway that is largely CREB dependent (Lane-ladd et al., 1997). This pathway is even further upregulated or “super-activated” when opioid administration is discontinued or withdrawal is precipitated (Madhavan, He, Stuber, Bonci, & Whistler, 2010).

Therefore, up-regulation of the cAMP pathway is an adaptation to chronic opioid administration.

The functional consequences of up-regulation of the cAMP pathway in various regions of the central nervous system have been thoroughly reviewed (Christie, 2008; Mazei-Robison & Nestler, 2012; Nestler, 2001). The increased excitability of GABAergic neurons effectively strengthens the inhibition of dopamine neurons in the VTA, a potential mechanism for withdrawal and negative reinforcement inspired drug-seeking behavior.

1.7 Potential influence of sex on signaling in the VTA

Several sex difference studies have shown that male and female animals also have important differences in VTA structure and function (reviewed by Gillies, Virdee, McArthur, & Dalley, 2014). For example, retrograde labeling studies have shown that compared to males, females possess higher total cells and proportions of dopamine projecting cells, and more spatial volume in the VTA (McArthur et al., 2007 and Kritzer and Creutz, 2008). The same is true for various dopaminergic projection sites that form the mesocortical pathway, including the PFC, primary motor cortex and premotor cortex (Everitt et al., 2008; O'Connell & Hofmann, 2011; Robbins, 2000). Additionally, female dopamine neurons express more TH, and are more responsive to positive social stimuli (outside of sexual contexts). Taken together, sex differences in the VTA cytoarchitecture suggest that male and female humans and animals may be

endowed with an organizational dimorphisms in reward circuitry that make them different with respect to vulnerability for drug-induced functional changes in DA signaling. Unfortunately, to date, the mechanisms concerning the consequences of changes in CREB signaling on VTA networks remain poorly understood. Most of the studies that explore drug-induced changes to CREB pathways have been done during the acquisition of drug taking behavior. The few studies that have been concerned with withdrawal have been exclusively studied in males despite the fact that biological mechanisms governing sex differences have been uncovered in every phase of addiction (Bobzean et al., 2014 and Perry and Becker, 2012). More research is necessary in order to uncover sex specific differences in alteration in CREB signaling. Further understanding of the mechanisms that govern dysregulation of signaling systems could have profound effects on therapeutic advances not only designed to attenuate withdrawal, but also attenuate drug use acceleration and reduce instances of relapse.

With chronic repeated opioid use, neurons develop changes in cellular, synaptic, and behavioral responsiveness to the persistent drug-induced inhibition of VTA GABAergic holds on DA neurons. Opioid tolerant individuals will need to use increasingly higher doses to achieve desired effects. In a way, opioid tolerance is a compensatory adaptation of the brain to restore homeostasis in instances where persistent elevated opioid driven OR activity disrupts normal organismal functioning. Intriguingly, studies investigating drug and drug abstinence-induced changes in intracellular signaling provide evidence that

reducing certain subtypes of VTA OR activity increases the severity of opioid withdrawal symptoms (Madhavan et al., 2010; Meye, van Zessen, Smidt, Adan, & Ramakers, 2012).

1.8 Project goals

Currently, there is a surprising gap in our current understanding of the molecular mechanisms underlying sex differences in opioid withdrawal behaviors and the specific effects of opioid withdrawal on CREB activity in the VTA of females are virtually unknown. Thus, the main objective of the current study is to examine the molecular signaling adaptations resulting from the interactions between morphine withdrawal, and sex in a rodent model. The explicit goals of the proposed experiments will be to (1) identify and quantify sex differences in morphine abstinence-induced withdrawal symptoms and (2) characterize sex differences in morphine withdrawal-stimulated increases in pCREB in VTA GABAergic neurons. Studying sex differences in drug induced changes in intracellular signaling has potential to further our understanding of psychiatric disorders including OUD and to improve the treatment of affected patients (Lin et al., 2009a). These research experiments are necessary for elucidating important

mechanisms that will aid the development of new pharmacotherapies for treatment of OUDs.

CHAPTER 2: METHODS

2.1 Subjects

Thirty-six experimentally naïve, adult (10-12 weeks old at the start of experiments), male and female, Long Evans rats were double housed with same-sex cage mates in a temperature and humidity-controlled environment under a 12h reversed light/dark cycle with lights on at 7p.m. and off at 7a.m. All animals had free access to food and water throughout the study and were maintained and cared for in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The Institutional Animal Care and Use Committee at the University of Texas at Arlington approved all experiments.

2.2 Determination of the estrous cycle phases

All female rats underwent daily vaginal lavage testing for at least 8 days (the length of two full estrous cycles) prior to beginning experiments. Vaginal secretions were collected by inserting a pipet tip into the vagina of the rat and infusing and extracting 40µl of 0.9% saline. Secretion samples were placed on a slide and observed under a light microscope at 20X magnification. The phase of the cycle (estrus, metestrus, diestrus, proestrus) was determined by the type (cornified cells, nucleated epithelial cells, and leukocytes) and distribution of cells present (See Figure 4).

Note: To control for experimenter contact, male rats were handled on a daily basis for a minimum of 8 days prior to experimentation.

2.3 Dependence induction

All rats were randomly assigned to either morphine or 0.1M phosphate buffered saline (PBS) treatment groups. On each of the 10 consecutive days of treatment, animals received two subcutaneous (s.c.) injections of PBS (1 ml/kg of body weight) or morphine sulfate (2.5, 5, 10, 20, and 40mg/kg; Spectrum, Irvine, CA) dissolved in 0.1M PBS. Morphine dose increased every two days (see Figure 3 for a timeline of procedures and breakdown of experimental groups). All injections were spaced by 12 hours in order to prevent overdose and limit the effects of withdrawal. Rats received all injections in their home cages.

2.4 Somatic Withdrawal Behavioral Observations

Somatic withdrawal signs were observed for 30 min, starting at 8 am on day 11, and every subsequent 12-hour period time point (12, 24, 36, 48, 60, and 72 hours) after the final morphine injection. All observations were scored by one observer (not blind to animal conditions) from videos recordings of each session using a Bell and Howell DNV16HDZ-BL Full Infrared Night Vision Camcorder. The number of wet shakes, writhing movements, hops/darts, paw tremors, facial fasciculation/teeth chatter, and swallowing movements, as well as the presence

of ptosis, piloerection, diarrhea, and a loss of body weight, were recorded for each subject (as described by Seip et al., 2012) in 10 minute time bins. Note that not all behaviors were seen during observation periods and not all symptoms were seen in most animals. A score of one point for each observation was awarded. Overall withdrawal scores were calculated by adding %body weight lost + wet dogs shakes + writhing observations = global withdrawal score. Due to the variability associated with animals falling asleep during the second and third time bins, only behaviors recorded during the first 10 minutes of observations were graphed and used for statistical analyses.

2.5 Double Label Immunofluorescence

Tissue Fixation: Immediately following the last somatic withdrawal assessment (72 hours after withdrawal), animals were deeply anesthetized with a single 1.0ml i.p injection of chloral hydrate (400 mg/ml dissolved in 0.9% saline; Sigma Aldrich, St. Louis, MO) and perfused transcardially with 200ml of ice cold 0.01M PBS followed by 400ml of 4% paraformaldehyde made in 0.01 M PBS. Brains were removed, placed in 4% paraformaldehyde and stored at 4°C overnight. The next day, brains were placed in 20% glycerol for post-fixation for a minimum of 24 hr.

Sectioning and Staining: Brains were sectioned (40 µm thick) on a freezing microtome and coronal sections were stored in 0.01% sodium azide (Sigma Aldrich, St. Louis, MO) dissolved in 1M PBS at 4°C. Brain slices were stained

using immunofluorescence to measure levels of pCREB in the tVTA. To confirm that pCREB expression between male and female rats is consistently embedded within a GABAergic cell population in the tVTA, brains were double-labeled for pCREB and Glutamic Acid Decarboxylase (GAD67). Slices of the VTA were pre-washed in PBS (3 x 10 minutes), followed by a one-hour incubation in a blocking solution (0.5% triton X and 3% donkey serum). Next, an anti-phosphoCREB antibody (1:1500; Millipore Billerica, MA) and an anti-GAD antibody (Millipore MAB5406; dilution of 1:500) were co-incubated with 0.5% Tween 20 and 3% donkey serum in PBS for 20 hours at 4°C. The following day, after washes in PBS (3 x 10 minutes), the tissue was co-incubated in a Cy3-conjugated donkey anti-rabbit secondary antibody (Jackson Immuno 711-165-1152; 1:400) and a Cy2-conjugated donkey anti-mouse secondary antibody (Jackson Immuno 715-485-150; 1:400) in PBS for four hours. The tissue was then washed in PBS (3 x 10 minutes) and subsequently mounted on Fisherbrand Superfrost Plus slides and air-dried. Finally, tissue was dehydrated in graded ethanol solutions and cleared with Fisherbrand Citrisolv and coverslipped.

Cell Counts: Sections of the VTA, located -5.8 to -7.1 mm relative to bregma, were examined bilaterally under a fluorescent light microscope (under 20X magnification) to determine the colocalization of pCREB-positive nuclei embedded within a GAD67-positive cells. Using Image-J software, photos from 3-5 sections (per animal) were counted and averaged for each animal in order to

obtain values used for statistical analyses. Note that 3 animals (2 PBS males and 1 PBS female were not counted due to poor brain slice and staining quality).

2.6 Statistical Analysis

All statistical analyses were performed using SPSS (version 23), with a significance level set at $p < 0.05$. All ANOVA models were followed with post hoc tests or planned comparisons to determine mean differences males and females, treatment groups, and/or across days.

Weight Change

Body weights across morphine exposure (days 1–10) were compared to first injection day weight (day 1) using a 2(Sex) X 2(Treatment) X 10 (Day) mixed ANOVA (day as repeated measure). During withdrawal (i.e., day 11-13), body weights were compared to final injection day weight (day 10) using a 2(sex) X 2(treatment) X 7 (withdrawal hours) mixed ANOVA (withdrawal hours as a repeated measure).

Withdrawal Behaviors

Statistical analyses of collective somatic signs of withdrawal (Withdrawal Score: %body weight lost + wet dog shakes + writhing) were analyzed with 2(Sex) x 2(Treatment) x 6(Time) mixed-factor ANOVAs with 'Time' after morphine cessation being a within subjects factor and sex and condition being between subjects factors. The same ANOVA model was also used to examine

group mean differences for each individual somatic symptom of morphine withdrawal (writhing and wet dog shakes). Significant overall ANOVAs were followed by post hoc comparisons when appropriate.

pCREB expression in the VTA

To better characterize the role of VTA GABAergic neuron CREB activity in morphine withdrawn animals, a 2 (Sex) X 2(Treatment) factorial ANOVA analysis was run. Post hoc tests were used to investigate specific group differences between treatment groups and sexes.

CHAPTER 3: RESULTS

3.1 Weight Change: Chronic Escalating Morphine Exposure

Changes in weight throughout chronic treatment and spontaneous withdrawal periods were measured as a percentage of either the pre-injection weight (Day 1) or pre-withdrawal weight (Day 10). The changes in weight of all animals throughout the study are shown in Figure 5. There was a significant main effect of day $F(9,24) = 19.05, p < 0.001, \text{partial } \eta^2 = .88$], sex \times treatment interaction $F(1,32) = 12.62; p < 0.005, \text{partial } \eta^2 = .28$], and a Day \times Sex \times Treatment interaction $F(9,24) = 4.13; p < 0.005, \text{partial } \eta^2 = .61$] on rats' body weight. Post hoc tests revealed that weights increased significantly every day of treatment ($p < 0.001$) in males treated with PBS, whereas males treated with chronic morphine stopped gaining weight after 3 days of treatment. On the other hand, females treated with PBS demonstrated a more gradual weight gain with significant changes every other day of treatment ($p < 0.05$). Interestingly, PBS treated males gained significantly more weight than females throughout the treatment period ($p < 0.05$), whereas morphine treated males and females did not differ throughout the treatment period ($p > 0.05$).

3.2 Weight Change: Withdrawal from Chronic Morphine Treatment

On day 11-13, after chronic home-cage morphine exposure ended, body weights differed across sex, treatment groups, and time of withdrawal $F(6,27) = 6.74$; $p < 0.001$, partial $\eta^2 = .60$. Significant weight loss was observed after morphine exposure ended in both male and female morphine-treated groups (Figure 5) during the first 36 hours of withdrawal, but not PBS-treated controls, who continued to gradually gain weight. During this time males lost a significantly higher percentage of body weight compared to females $p < 0.001$.

3.3 Wet Dog Shakes

Besides changes in weight, other somatic withdrawal symptoms were also analyzed and compared across all 36 subjects. Spontaneous withdrawal from chronic morphine administration significantly induced wet dog shake symptoms in male and female rats. A 2(Sex) x 2(Treatment) x 6(Time) mixed-factor ANOVA revealed a significant main effect of time: $F(5,28) = 14.78$, $p < 0.001$, partial $\eta^2 = .725$; 'sex': $F(1,32) = 20.943$, $p < 0.001$, partial $\eta^2 = .40$; and 'treatment': $F(1,32) = 72.23$, $p < 0.001$, partial $\eta^2 = .690$. Post hoc tests were performed to examine the main effect of sex; overall males had more wet dog shakes compared to females, the effect of treatment; morphine dependent animals had more wet dog shakes compared to PBS controls. Additionally, and most interestingly, there was a significant 'Time x Sex x Treatment' interaction $F(5,28) = 3.41$; $p < 0.05$, partial $\eta^2 = .379$. Post hoc tests revealed that morphine

dependent male rats had more wet dog shakes compared to females 12, 24, 36 and 60 hours after cessation of morphine treatment; Males and females did not differ in the amount of wet dog shakes 48 or 72 hours after the end of treatment (Figure 6).

3.4 Writhing

Spontaneous withdrawal from chronic morphine administration significantly induced writhing symptoms in male and female rats. A 2(Sex) x 2(Treatment) x 6(Time) mixed-factor ANOVA revealed a significant main effect of time: $F(5,28) = 5.199, p < 0.001, \text{partial } \eta^2 = .399$; and 'treatment': $F(1,32) = 145.546, p < 0.001, \text{partial } \eta^2 = .820$. There was no significant main effect of sex. Overall, morphine dependent animals had more writhing compared to PBS controls. Additionally, and most interestingly, there was a significant 'Time x Sex x Treatment' interaction $F(5,28) = 3.197; p < 0.05, \text{partial } \eta^2 = .363$. Post hoc tests revealed that morphine dependent male rats had more writhing symptoms compared to females 48 hours after cessation of morphine treatment; however, females had more writhing symptoms 60 and 72 hours after the end of treatment. Males and females did not differ in the amount of writhing 12, 24, or 36 hours after the end of treatment. The results of these analyses are depicted in Figure 7.

3.5 Withdrawal Score

Spontaneous withdrawal scores for each animal were calculated in order to examine sex differences from total counted symptoms. The results of our analyses demonstrated treatment and sex dependent effects in male and female Long Evans rats. There was a significant main effect of time: $F(5,28) = 17.13$, $p < 0.001$, partial $\eta^2 = .754$; 'sex': $F(1,32) = 6.526$, $p < 0.05$, partial $\eta^2 = .169$; and 'treatment': $F(1,32) = 380.514$, $p < 0.001$, partial $\eta^2 = .922$. Post hoc tests revealed that overall males had higher withdrawal scores compared to females', morphine dependent animals had higher withdrawal scores compared to PBS controls. Additionally, and most interestingly, there was a significant 'Time x Sex x Treatment' interaction $F(5,28) = 3.05$; $p < 0.05$, partial $\eta^2 = .353$ on Withdrawal Score. Post hoc tests revealed that morphine dependent male rats had higher withdrawal scores compared to females 12, 24, and 36 hours after cessation of morphine treatment; however, females had higher withdrawal after 72 hours. Males and females did not differ 48 or 60 hours after the end of treatment. The results of these analyses are depicted in Figure 8.

3.6 pCREB expression in the VTA

An analysis of mean differences in pCREB/GAD expression between sex and treatment groups was performed in order to characterize the relationship between tVTA pCREB in GABA neurons and morphine withdrawal. A 2 (sex) X 2 (Treatment) factorial ANOVA revealed a marginally significant main effect of for

treatment $F(1,32) = 3.753$, $p = .06$, partial $\eta^2 = .115$ but not sex. Additionally, there was not a significant 'Sex x Treatment' interaction; however, planned comparisons showed that morphine dependent females did have more positive pCREB counts compared to PBS treated females. No significant differences were observed between any other groups. The results of these analyses and representative photomicrographs are depicted in Figure 9.

3.7 VTA pCREB expression is positively correlated with withdrawal score

Pearson's r correlation was computed to determine if number of pCREB+ GABA neurons of the tVTA significantly correlated with withdrawal scores. The results of the correlation indicated that the tVTA pCREB is strongly positively correlated with withdrawal scores ($r = .53$, $p < .001$). The results of these analyses are depicted in Figure 10.

CHAPTER 4: DISCUSSION

4.1 Summary of experiments and study findings

The purpose of the present study was to use a preclinical model of morphine dependence to investigate the effects of sex on the expression and duration of spontaneous somatic morphine withdrawal; and to characterize the relationship between spontaneous somatic withdrawal symptoms and expression of pCREB in GABAergic cells of the VTA in morphine dependent male and female rats. The results of our studies identified that both male and female morphine and PBS treated animals expressed GABAergic cells (as indicated by GAD67 immunofluorescence) primarily in the caudal-lateral region of the VTA (tVTA/RMTg). Overall, male and female rats, previously treated with chronic escalating doses of morphine, expressed more pCREB/GAD67 immunopositive cells in the VTA compared to non-morphine treated rats. However, only females differed statistically from PBS controls; meanwhile, males showed no obvious such differences. Additionally, our data indicate time- and sex-dependent differences in the severity of spontaneous withdrawal symptoms. Lastly, the severity of somatic spontaneous withdrawal symptoms are positively correlated with pCREB/GAD67 expression across all animals.

4.2 Sex differences in withdrawal behaviors

To our knowledge the results from these experiments are only the second

to explore sex differences in spontaneous withdrawal from morphine. To this end, our data confirm the previous finding, which indicates that males express more severe magnitude of opioid withdrawal than females (Cicero et al., 2002). This was indicated in our experiments by the fact that males expressed an extreme plunge in body weight, writhing, and the exhibition of abrupt wet dog (body) shakes. Females on the other hand, had comparatively lower drops in weight and fewer body shakes, but similar instances of writhing. Other symptoms including diarrhea, unkempt fur, paw tremors, piloerection, and abnormal posture were also noted in morphine dependent animals; however, these signs were not scored or compared between sexes due to the subjective nature of scoring and unreliability of presentation during the video observation time period.

In terms of timing, both morphine dependent male and female rats developed somatic symptoms within 12 hours of spontaneous withdrawal from treatment. Males consistently expressed more total scored symptoms during the early stages of withdrawal (the first 36 hours) compared to females. However, to our surprise, females exhibited a slower decline of symptoms compared to males over the 3 day observation period; as a result, withdrawal symptoms in females were collectively more severe compared to males during the late stage of withdrawal (72 hours) after the last morphine treatment. We attribute much of this effect to the slower decline in body weight and persistent writhing symptoms morphine dependent females exhibited at the end of testing.

Our sex difference results are consistent with the previous report with the exception of one subtle but potentially important difference: female withdrawal scores exceeded those of males 72 hours after cessation of morphine treatment. This withdrawal time-point was characterized by fewer wet dog shakes than earlier time points but persistent occurrence of stomach writhing, indicating abdominal discomfort, especially in females. The difference between our findings and those of Cicero, Nock and Meyer are likely due to methodological differences (i.e. symptom weighting factor differences, strain differences, etc.). One such important methodological difference may be that we chose not to use weighting standards when calculating withdrawal scores in the current study, unlike the previous study (Cicero et al., 2002). The use of weighting standards is typically used to obtain an overall measure of symptom severity while putting special emphasis on certain symptoms. However, we must bear in mind that all of the withdrawal measures and thus, the associated weighting standards and values assigned to symptoms were originally developed in a model of morphine withdrawal in male rodents and thus may not necessarily be optimal for studies that incorporate females. Nevertheless, the data here present an interesting finding that suggests that males and females have time dependent differences in somatic withdrawal symptom severity.

Although the findings of the present study are consistent with the one other known study examining sex differences in spontaneous somatic morphine withdrawal, our results differed significantly from numerous other studies of

morphine dependence. Unlike these studies, symptoms including pica, and ptosis were not observed at all in the present study, and other symptoms we *did* see were observed with far less frequency, comparatively. This is not surprising, however, due to the fact that almost all studies of opioid dependence employ the use of naloxone to precipitate withdrawal states; the onset and presentation of spontaneous withdrawal is understandably much more gradual and understated.

4.3 Sex differences in CREB signalling

The second aim of the present study was to investigate the extent to which morphine dependent male and female rats differ in the expression of pCREB in GABAergic cells of the tVTA. Increases in tVTA GABAergic neuron pCREB expression are associated with reduced DA signaling and more severe withdrawal symptoms; thus, we were motivated to see if this could be a potential mechanism to help explain sex differences in the expression of withdrawal symptoms. Overall, morphine dependent male and female rats expressed more pCREB/GAD67⁺ cells in the tVTA compared to non-morphine dependent rats. However, only females differed statistically from controls with respect to the number of pCREB/GAD67⁺ cells expressed. Not surprisingly, the relationship between tVTA CREB activity and withdrawal was somewhat illuminated when pCREB expression was correlated with withdrawal severity score. The more pCREB⁺ GABA cells, the higher the withdrawal score.

Opioid-induced reward and dopamine transmission are modulated by activity of tVTA GABA cells and changes in intracellular CREB phosphorylation can vastly affect gene transcription, cellular function and behavior. To our knowledge, ours is the first study to use a preclinical model of morphine dependence to investigate sex differences in CREB activation in tVTA GABAergic cells. Our data confirm and extend the results of other studies that demonstrate the link between activation of CREB pathways and expression of morphine withdrawal symptoms (Martin et al., 2009). Our results expand on previous studies in such a way that infers a relationship between tVTA CREB activity, withdrawal symptom severity, and sex. In other words, rats that express more CREB in VTA GABAergic have a more severe withdrawal syndrome and this relationship may be stronger in females compared to males in late stages of withdrawal.

Unfortunately, not many studies have investigated the relationship between changes in intracellular signaling and the expression of drug abstinence-induced behaviors. In line with this research, we expected that morphine dependent males would demonstrate more morphine abstinence-induced changes in intracellular signaling in the tVTA, as measured by higher levels of pCREB expression in GABAergic cells. The current study, however, produced somewhat unexpected results. Within the tVTA, morphine dependent females had a greater number of pCREB positive GABAergic cells compared to morphine dependent males. The seemingly perplexing nature of these results is

not completely surprising given the complex nature of the sex dependent differences in timed expression of morphine abstinence –induced withdrawal symptoms. Although males demonstrated overall a higher magnitude of withdrawal symptoms, females had higher overall scores on the day of sacrifice. This circumstance of withdrawal symptom expression may reflect the direction of difference in pCREB expression between male and female morphine dependent rats at the time of sacrifice.

4.4 Possible role for gonadal hormones

With regard to the present study, it is possible that sex differences in both somatic withdrawal behaviors and tVTA CREB activity may be due, in large part, to male and female differences in the fluctuations of gonadal hormones. Studies show that sex differences in opioid analgesia and reward are largely influenced by gonadal hormones (Becker et al., 2012; Frye & Seliga, 2001; Frye, 2001; Frye, 2007). These findings are supported by preclinical studies performed in male and female animals, which suggest that ovarian hormones potentiate symptoms of anxiety and depression, an effect that is in direct contrast with studies demonstrating opposite effects of testosterone. Studies performed in Cheryl Frye's lab, for example, show that testosterone has analgesic effects, reduces anxiety, and enhances cognitive performance in male animals. Unfortunately, not all study results looking at the effects of ovarian hormones on female behaviors are this straightforward. Some studies show that elevations in

estrogen have antidepressant and anxiolytic effects, while others argue the effects are anxiogenic. It appears the difference in findings may be related to hormone dependent changes in OR and estrogen receptor densities throughout (human) female menstrual and (rodent) estrous cycles. Regardless of the hormone dependent mechanisms that alter subjective responses to opioid drugs, these studies definitively highlight the need for continued research to uncover biological underpinning of sex difference with regard to opioids.

One reason for the aforementioned contradictory findings in females may be explained by the ever present shifting milieu of ovarian hormones (Nagaya & Maren, 2015). Women have reported being more likely to experience traits related to mood disturbances during periods of marked hormonal changes (Ahokas, Kaukoranta, Wahlbeck, & Aito, 2001; Douma, Husband, O'Donnell, Barwin, & Woodend, 2005; Parker & Brotchie, 2004; Solomon & Herman, 2009). Surprisingly, obvious disturbances of the estrous cycle were not noted throughout the morphine-treatment and morphine-abstinence phases of the present study. Still, other studies have demonstrated that administration of chronic opioids cause disruptions in female hormonal cycles and these differences may be due to dose, duration and frequency of treatment regimens. Thus, it is not difficult to link the moderating effects of gonadal hormones to the prevalence of mood and anxiety disorders encountered during opioid abstinence-induced withdrawal.

Unfortunately, at this time, few studies have been published concerning the differences between males and females in drug induced alterations in intracellular signaling, and to our knowledge none have been conducted assessing the effects of opioids and opioid withdrawal syndrome. The sheer lack of studies that have been conducted to support the existence of biological mechanisms to account for disparaging differences between male and female animals and humans during withdrawal is unquestionably alarming. It is ultimately likely that these uncovered biological mechanisms interact with additional psychosocial factors to provide a substantial role in determining sex dependent differences in individual vulnerability to developing OUDs.

Interestingly, two studies have been published looking at sex differences in pCREB signaling responsiveness to stress, a risk factor for developing depression and a consequence of abstinence from drug use following long-term use (Lin et al., 2009b; Ter Horst, Wichmann, Gerrits, Westenbroek, & Lin, 2009). The findings were that, first, in male rats, stress reduces pCREB levels in limbic areas of the brain but has no similar effect in females. Secondly, overall changes in limbic pCREB/CREB ratios are more sensitive to stress in males than females (Lin et al., 2009b), however, these changes are influenced by estradiol treatment and changes in environmental factors (Ter Horst et al., 2009). These studies support that the neural mechanisms underlying stress modulation of cell function is different for male and female rats, and these differences may have implications for similar sex differences during periods of opioid withdrawal.

4.5 Limitations

Ultimately, the present study has uncovered important sex differences regarding the structure and function of the VTA. However, more studies will be needed in order to fully investigate sex dependent differences in activation of VTA CREB pathways. At this time, our data suggest that males and females differ in the timing of withdrawal-induced activation of VTA CREB signaling. However, to our knowledge, this is the first study to examine opioid withdrawal-induced CREB activation in females. Our lab is currently in the process of replicating the methods of this study in order to add sacrifice time-points to our histology analysis and further characterize the relationship between CREB and somatic withdrawal symptoms.

4.6 Future direction: Looking beyond physiological withdrawal

Despite the growing body of preclinical and clinical research, the study of sex differences in mechanisms underlying opioid withdrawal has much room to grow. Clearly, morphine withdrawal can be highly aversive; however, it is unknown to what degree that the expression of somatic morphine withdrawal symptoms in this study can be correlated to the negative psychological state associated with withdrawal. At no point during morphine administration or withdrawal were animals in the present study measured specifically for changes in anhedonia, anxiety, or depression like states. Further studies are necessary in

order to determine to what extent physiological symptoms of withdrawal are connected to psychological symptoms of withdrawal.

Psychological withdrawal is, in part, attributed to the salient value of the drug to the user prior to abstinence. Interestingly, previous unpublished work from our lab demonstrates a role for ovarian hormones in the acquisition of morphine dependence. Briefly, twenty-four adult ovariectomized (OVX) female and six male Long Evans rats were used throughout the experiment. Female rats were assigned to one of two groups of hormone treatment (n=12 per group): 0.1ml peanut oil - vehicle (OVX); or 5µg estradiol benzoate (OVX+E). The apparatus used to carry out the CPP consisted of a two large chambers distinct in visual and tactile cues (wall color and floor material) that are connected by a small shuttle chamber. After a preconditioning test (30 min free exploration), each rat was conditioned to associate one compartment with subcutaneous injections of morphine (10mg/kg), while the other compartment was paired with saline injections (saline/morphine conditioning days alternated). After the conditioning phase rats underwent a post-conditioning acquisition test. Time spent in each chamber was automatically recorded for all subsequent comparisons using MedPC (Med Associates, VA) software.

The results of these experiments showed that estradiol treatment to OVX rats attenuates morphine-induced CPP when animals are conditioned with a 10mg/kg dose of morphine (See Figure 11). These data suggest a role for

estradiol in modulating the rewarding properties of morphine and could therefore, lend support to suggest that sex differences in psychological withdrawal do indeed exist.

4.7 Concluding Remarks

Understanding the role of sex in opioid dependence is important due to the high addiction potential of opioids that are widely used in pain management and increasingly by recreational users. The fact that men and women have different susceptibilities for developing opioid dependence and different disease trajectories once a disorder has been cultivated, suggests that the neurobiology governing these differences will have a huge impact on treatment planning for people who develop an OUD. Unfortunately, though much is known about the acute effects of opioids, the mechanisms that regulate symptoms of opioid dependence and withdrawal are poorly understood. In the present study, comparisons of male and female morphine dependent rats revealed sex differences in pCREB expression in GABAergic neurons of the VTA that positively correlated with total somatic withdrawal scores. Future studies are necessary to further characterize time- and sex-dependent patterns in relation to CREB activation and subsequent withdrawal symptoms. Additional paradigms that uniquely and accurately differentiate and characterize distinct features of somatic withdrawal and psychological withdrawal will be crucial for clarification of CREB's role in eliciting specific behaviors and motivational states that incite risk

for relapse. Together this information advances our understanding cellular mechanisms underlying OUDs.

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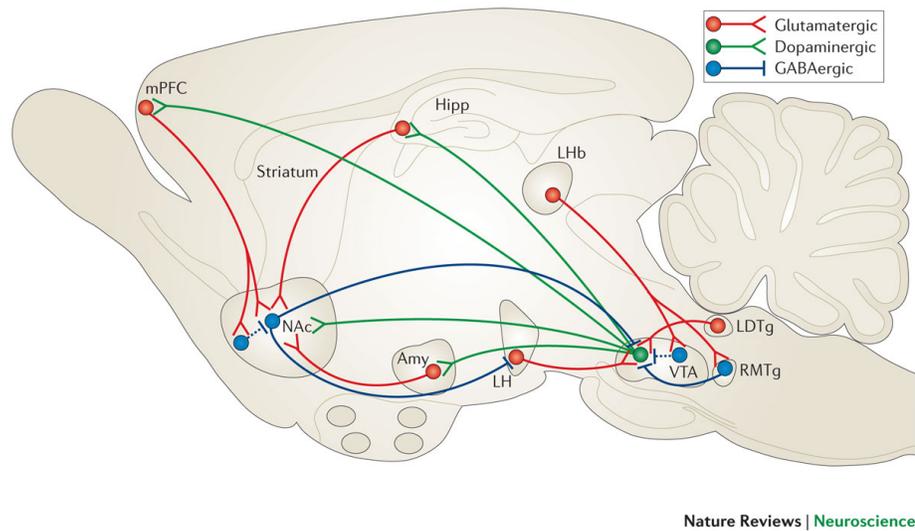


Figure 1. Brain Reward Circuitry. This is a simplified schematic of the major dopaminergic, glutamatergic and GABAergic connections to and from the ventral tegmental area (VTA) and nucleus accumbens (NAcc) in the rodent brain. Reproduced without permission from Russo and Nestler, 2013.

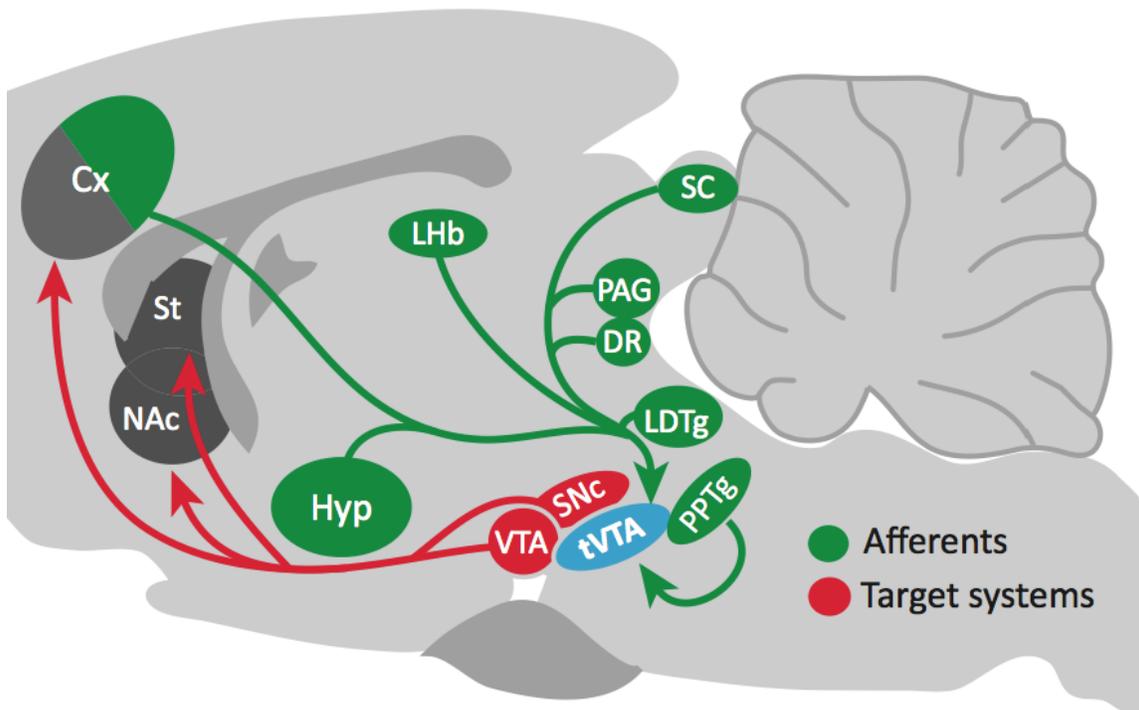


Figure 2. Major afferent and efferent connections of the tVTA. Reprinted without permission from (Bourdy & Barrot, 2012)

Opioid Formulation	μ	κ	δ	Indications	DEA Schedule
AGONISTS					
Natural Opioids					
Morphine	+++	+	+	moderate to severe pain relief	II
Codeine	+	+	+	mild/moderate pain relief, cough suppressant	III
Semisynthetic Opioids					
Diacetylmorphine (heroin)	+++			provides a "rush"	I
Hydrocodone	+			mild/ moderate pain relief	III
Hydromorphone	+++	+		severe pain relief	II
Oxycodone	+++			moderate/severe pain relief	II
Oxymorphone	+++			severe pain relief	II
Synthetic Opioids					
Levorphanol	+++	+	+	moderate/severe pain relief	II
Fentanyl	+++			severe pain (chronic cancer pain), surgical sedation	II
Methadone	+++			moderate/ severe pain relief, opioid dependence	II
AGONIST-ANTAGONISTS					
Semisynthetic Opioids					
Buprenorphine	P+	--		moderate/severe pain relief, opioid dependence	III
Nalbuphine	--	++		moderate/severe pain relief	IV
Synthetic Opioids					
Pentazocine	-	+		moderate/severe pain relief	IV
Butorphanol	P-	+++		moderate/severe pain relief	IV
ANTAGONISTS					
Naloxone	---	--	-	opioid dependence, overdose	N/A
Naltrexone	---	---	-	opioid dependence	N/A

Table 1. Common opioid drugs with their selectivity for different opioid receptors, indications for usage and DEA schedule.

A

Experimental Conditions	(n)
Male Chronic PBS	9
Female Chronic PBS	9
Male Chronic Escalating Morphine	8
Female Chronic Escalating Morphine	10
Total (N)	36

B

Days 1-2	Days 3-4	Days 5-6	Days 7-8	Days 9-10	Day 10-13
2 x 2.5 mg/kg	2 x 5 mg/kg	2 x 10mg/kg	2 x 20 mg/kg	2 x 40 mg/kg	Spontaneous Withdrawal

**Chronic Escalating Morphine
Twice Daily Injections (12hrs apart)**

Figure 3. A. Experimental Groups B. Timeline for experimental procedures.

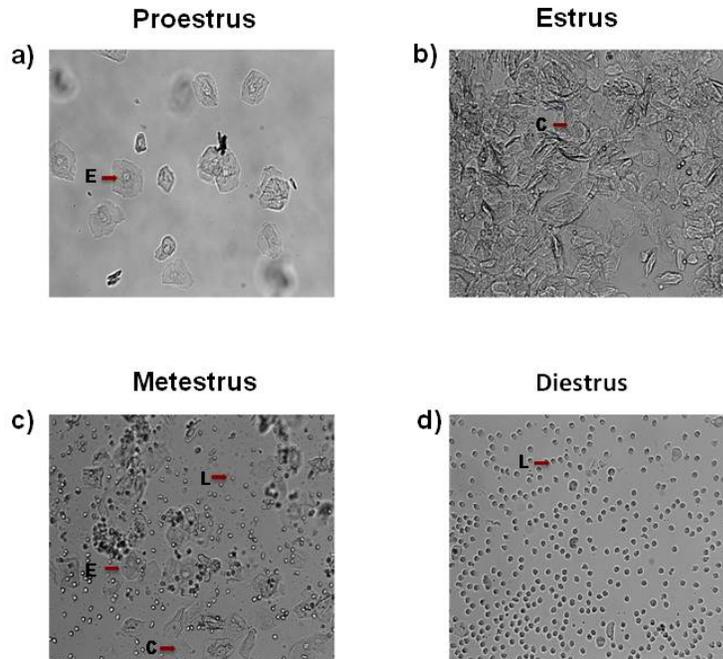


Figure 4. Rat Estrous Cycle. Photomicrographs of unstained native vaginal smear from rats, observed under a light microscope with 20X objective lenses. The proportion of the three types of cells was used for determination of estrous cycle phases. The round and nucleated cells are epithelial cells (E); irregular cells without a nucleus are the cornified cells (C); and the small round cells are the leukocytes (L). A proestrus smear consists a predominance of nucleated epithelial cells (a). An estrus smear consists a predominance of cornified cells (b). A metestrus smear consists of the same proportion of leukocytes, cornified, and nucleated epithelial cells (c); and a diestrus smear primarily consists of a predominance of leukocytes (d).

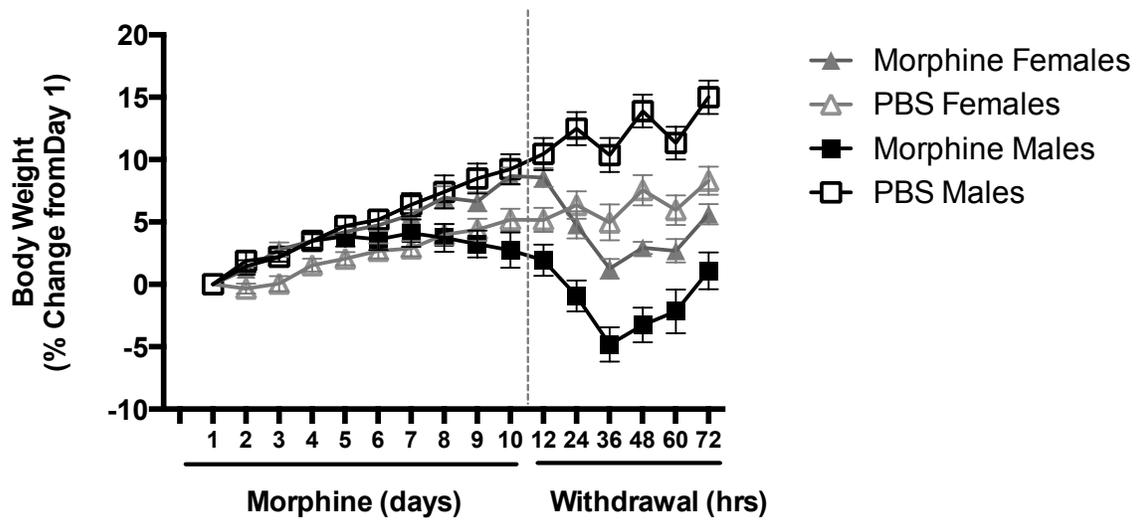


Figure 5. Effects of repeated systemic injections of morphine and withdrawal on body mass. Symbols indicate % change in body weight throughout experimental procedures (morphine females n= 10; PBS females; n=9; morphine males n=8, PBS males n=9).

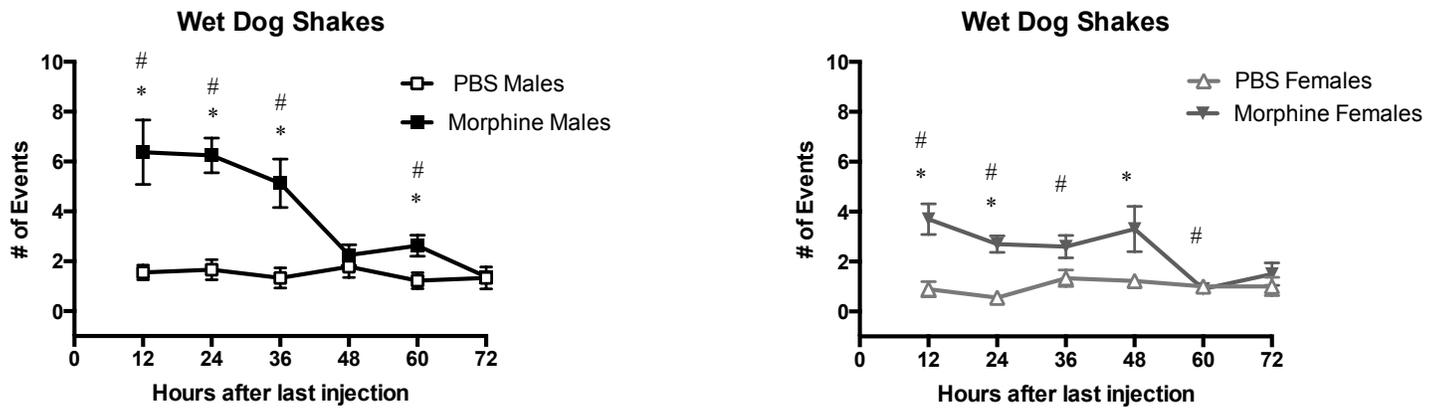


Figure 6. This graph depicts the mean number of wet dog (body) shakes and subsequent to cessation of morphine administration 12, 24, 36, 48, 60, and 72 hours after last injection (morphine females n= 10; PBS females; n=9; morphine males n=8, PBS males n=9). Note that morphine dependent male rats had more wet dog shakes compared to females 12, 24, 36 and 60 hours after cessation of morphine treatment * Represents statistically significant differences between morphine dependent rats and PBS controls ($p<.05$). # Represents statistically significant differences between morphine dependent males and females controls ($p<.05$)

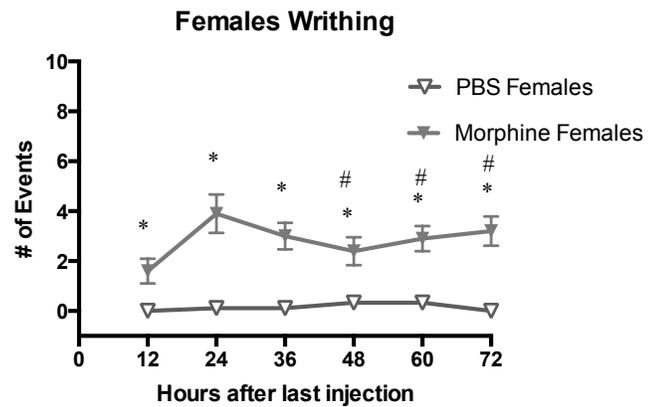
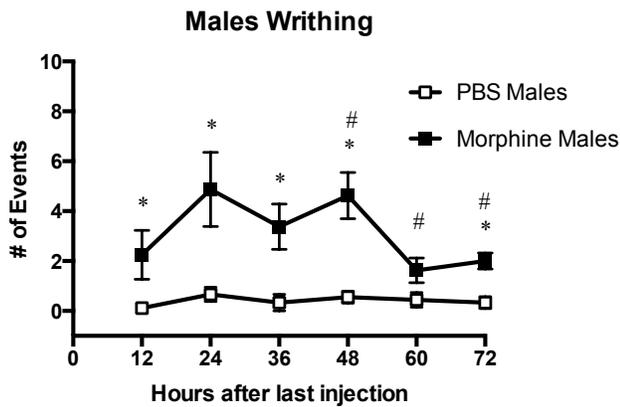


Figure 7. Mean abdominal writhing subsequent to cessation of morphine administration 12, 24, 36, 48, 60, and 72 hours after last injection (morphine females n= 10; PBS females; n=9; morphine males n=8, PBS males n=9). Note that morphine dependent males had more writhing compared to females 48 hours into withdrawal but females had more writhing 60 and 72 hours into withdrawal. * Represents statistically significant differences between morphine dependent rats and PBS controls ($p < .05$). # Represents statistically significant differences between morphine dependent males and females controls ($p < .05$)

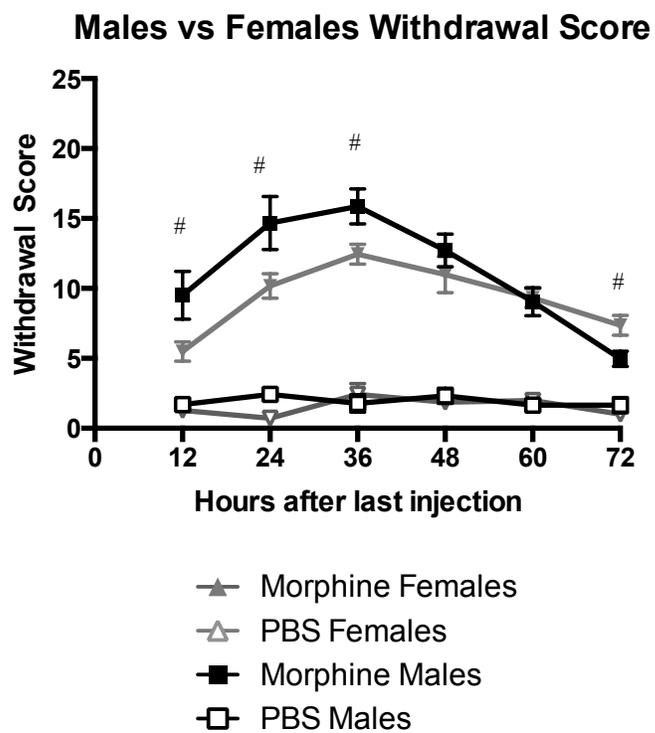


Figure 8. Male and Female Withdrawal Scores. Experiments showing difference in trends of collectively scored withdrawal behaviors subsequent to cessation of morphine administration 12, 24, 36, 48, 60, and 72 hours after last injection (Morphine females n= 10; PBS females; n=9; morphine males n=8, PBS males n=9). # Represents statistically significant differences between morphine dependent males and females controls ($p < .05$).

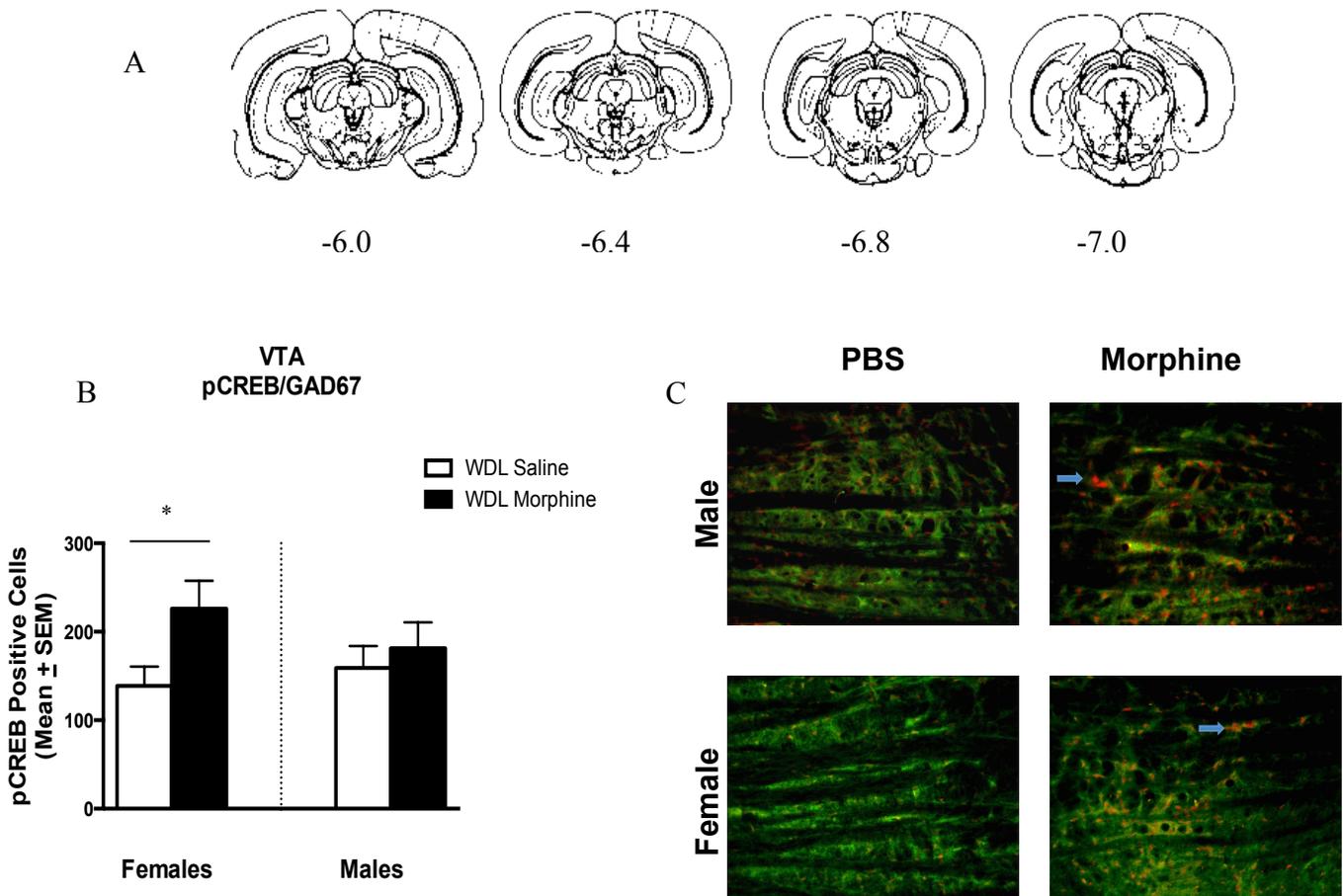


Figure 9. Sex differences in pCREB/GAD67 positive neurons of the VTA. A) Area where cells were quantified; numbers indicate approximate distance from Bregma. B) pCREB /GAD67 positive cell number quantification in animals previously administered saline (white bars) or chronic escalating morphine (black bars). C) 20x Photomicrographs depicting pCREB /GAD67 immunofluorescence.* Represents statistically significant differences between morphine dependent rats and PBS controls ($p < .05$). Arrows show pCREB (red) expressed in GAD67+ cell (green).

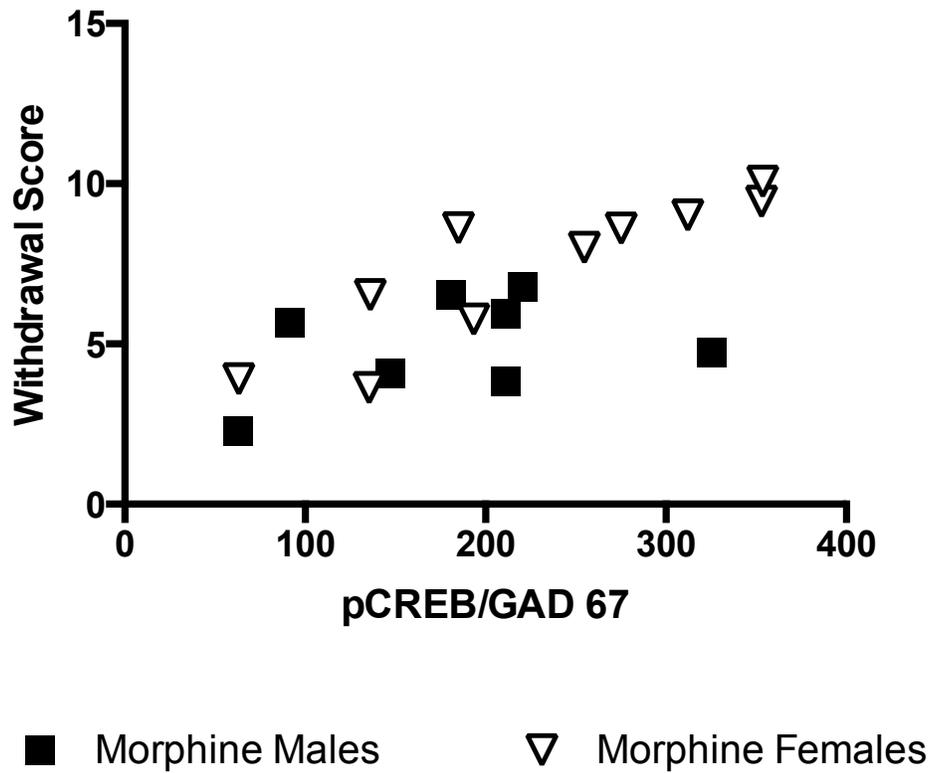


Figure 10. VTA pCREB is related to withdrawal score. The graph above depicts a significant ($p < .05$) relationship between pCREB/GAD67 positive cells and morphine withdrawal scores in morphine dependent animals. Analysis demonstrated that the protein expression of pCREB significantly positively associated with the expression of withdrawal symptoms 72 hours after the last injection.

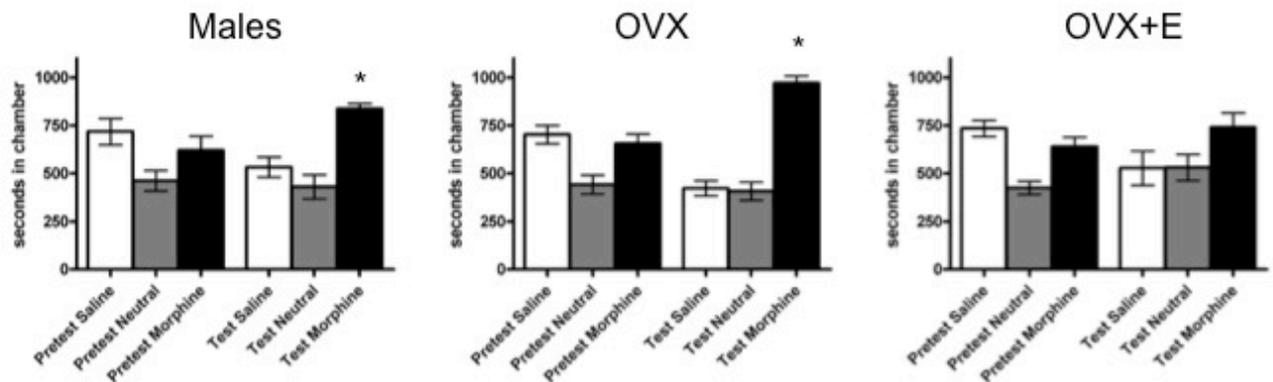


Figure 11. Morphine-induced conditioned place preference in intact male, ovariectomized (OVX), and ovariectomized plus estradiol treated (OVX+E) rats. Data are represented as the mean (\pm SEM) time spent in each chamber (white bar: saline side; grey bar: neutral chamber; black bar: morphine side). * Represents statistically significant differences between time spent in drug paired chamber to saline or neutral chamber ($p < .05$). Male and OVX female rats acquired CPP following conditioning to 10mg/kg of morphine sulfate ($p < .05$), while OVX+E females did not show a significant preference for the morphine chamber compared to the saline chamber. Moreover, OVX females demonstrated a significantly greater preference to the morphine-paired chamber than either intact males or OVX+E females.