Neural Mechanisms of Anxiety during Opiate Withdrawal: Role of the Ventral Tegmental Area and Extended Amygdala

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Abstract

Exposure to addictive drugs alters neural circuits involved in reward and motivation, executive control, habit formation, learning and memory, and negative affect, and all except the last are known to depend on changes in the mesolimbic dopamine system. Negative affective symptoms of withdrawal are common to all drugs of abuse and negatively reinforce drug taking behavior. Using potentiation of the acoustic startle reflex as a measure of anxiety during withdrawal from acute morphine exposure, the experiments detailed in this thesis tested the hypothesis that μ-opioid receptor-mediated activation of VTA dopaminergic neurons is responsible for triggering negative emotional symptoms of withdrawal via recruitment of the extended amygdala. These experiments demonstrate the emergence of a negative affective state that occurs during withdrawal from direct infusion of morphine into the ventral tegmental area (VTA), the origin of the mesolimbic dopamine system. Potentiation of startle during withdrawal from systemic morphine exposure requires a decrease in μ-opioid receptor stimulation in the VTA and can be relieved by systemic or intra-nucleus accumbens administration of a dopamine receptor agonist. Investigation of mechanisms downstream of dopaminergic signaling found a role for type 2 corticotropin-releasing factor receptors following the very first, but not subsequent, opiate exposures. Together these results suggest that transient activation of the VTA mesolimbic dopamine system triggers the expression of anxiety during opiate withdrawal, possibly via direct recruitment of the extended amygdala. This conclusion provides unique insight into the neural mechanisms responsible for negative reinforcement of drug taking during the earliest stages of dependence.

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Chapter 1: Introduction

Abuse of addictive substances is a worldwide phenomenon with major economic and societal costs. Drug addiction is a brain disease characterized by tolerance, withdrawal, and excessive, compulsive drug use even in the face of adverse consequences (Diagnostic and statistical manual of mental disorders: DSM-IV). Exposure to addictive drugs alters neural circuits involved in reward and motivation, executive control, habit formation, learning and memory, and negative affect (Koob and Volkow, 2010). While drugs such as opiates, stimulants, nicotine, alcohol, and others act on diverse molecular targets in the brain, they all share the ability to modulate the output of the mesolimbic dopamine system (Di Chiara and Imperato, 1988; Di Chiara et al., 2004; Nestler, 2005). It is therefore not surprising that the mesolimbic dopamine system is an important component of the neural circuits listed above and that altered dopaminergic signaling is hypothesized to contribute to many of the behavioral changes associated with drug dependence (Volkow et al., 2004; Nestler, 2005; Di Chiara and Bassareo, 2007). Interestingly, although dopaminergic projections terminate in neural structures that are associated with negative emotion and altered by drug exposure, little work has been done to investigate the role that the mesolimbic dopamine system plays in the recruitment of negative emotional brain circuits following drug use. The work described here was designed to test the role of dopaminergic projections from the ventral tegmental area (VTA) to its limbic targets, specifically structures of the extended amygdala, in the expression of a negative emotional state during opiate withdrawal.

Role of negative affect in addictive behavior

Among the many factors that contribute to the development of addiction is the negative reinforcement associated with the withdrawal state. Specifically, although withdrawal from drugs of abuse is characterized by both physical and emotional symptoms, it is the emotional component of withdrawal that is hypothesized to drive the negative reinforcing effects (Koob and Le Moal, 1997). Negative affective signs and symptoms, including anxiety, irritability, anhedonia, and dysphoria, are common to withdrawal from all classes of abused drugs, are manifested after the very first exposure to a drug, and increase in intensity with repeated exposures (Haertzen and Hooks, 1969; Koob and Le Moal, 1997). Because re-exposure to a drug relieves these symptoms (Ternes and O'Brien, 1982), a cycle of negative reinforcement emerges in which individuals administer drug in an attempt to prevent or relieve withdrawal (Wikler, 1961; Solomon and Corbit, 1974; Williams et al., 2001). This type of behavior is reported by human addicts (Chaney and Roszell, 1982; Bradley et al., 1989) and has been observed in animals (Ahmed et al., 2002; Kenny et al., 2006). Negative affective symptoms of withdrawal are therefore hypothesized to contribute to the acquisition and maintenance of drug taking behavior as well as to relapse following periods of prolonged abstinence (Koob and Le Moal, 1997).

Negative reinforcement theories. Both the "opponent process" theory of motivation (Solomon and Corbit, 1974) and its more recent reformulation, the "hedonic allostasis" theory (Koob and Bloom, 1988) formally describe the mechanisms by which the negative emotional component of withdrawal is hypothesized to contribute to addiction.

According to these theories, rewarding experiences engage secondary mechanisms that oppose and constrain positive emotion. Exposure to an addictive drug activates a primary, positive emotional "a process" that is often thought of as drug reward or euphoria. The a process then triggers the emergence of a secondary, negative emotional "b process." While the a process has a rapid onset and offset, the b process is initially slower to begin and slower to terminate. Over repeated drug exposures the b process grows in strength and is activated more rapidly, allowing it to eventually overwhelm the euphoric effects of the drug and cause severe episodes of withdrawal. While drug users may therefore initially take a drug for its euphoric effects, in later stages of addiction drugs are instead taken to achieve a state of normalcy. Hedonic allostasis theory suggests that a change in the hedonic "set-point" occurs so that even after prolonged abstinence individuals experience a dampened emotional state and motivation to seek drugs (Koob and Bloom, 1988).

The importance of these theories to the current work is twofold. First, they highlight the importance of studying the negative emotional component of withdrawal, a critical motivator in the development, maintenance, and relapse to drug abuse. Second, although negative affective states are an intrinsic component of daily drug exposure and are thought to play a primary role in compulsive drug use (Koob and Le Moal, 1997), the neural mechanisms involved in the development of these states have yet to be elucidated. The opponent process view predicts that activation of reward-related circuitry is the first step in the induction of a negative affective withdrawal state following opiate exposure (Koob and Bloom, 1988), suggesting that the origin of negative emotional withdrawal

behaviors may lie in structures responsible for reward and euphoria, namely the VTA mesolimbic dopamine system.

Neural mechanisms of opiate withdrawal: role of the extended amygdala. Despite the predictions of negative reinforcement theories, little work has been done to investigate the role of motivational brain circuits in the expression of withdrawal behaviors. To date, the majority of investigations into the neural mechanisms of negative affective withdrawal states have instead focused on the brain structures known to mediate the expression of negative emotion. These lines of work have discovered a critical role for structures of the extended amygdala in the aversive and anxiety-like aspects of drug withdrawal (Koob and Volkow, 2010).

The "extended amygdala" is a macrostructure in the forebrain comprised of the central (CeA) and medial (MeA) nuclei of the amygdala and the bed nucleus of the stria terminalis (BNST) (de Olmos and Heimer, 1999). The extended amygdala is also continuous and interconnected with the shell of the nucleus accumbens (NAc) and receives input from other limbic structures such as the basolateral amygdala (BLA) and hippocampus (de Olmos and Heimer, 1999). Structures of the extended amygdala are important for the expression of spontaneous and conditioned fear (Walker and Davis, 2008) as well as the emotional component of pain (Neugebauer et al., 2004). The CeA and the BNST directly influence fear-related behaviors through direct projections to motor nuclei in the brainstem (Davis, 1992).

Extended amygdala structures are activated during opiate withdrawal, with the CeA, BNST and shell of the NAc being the most sensitive (Gracy et al., 2001; Frenois et

al., 2002; Shaw Lutchman et al., 2002; Reti and Baraban, 2003; Veinante et al., 2003). Lesions of these structures have also been shown to disrupt negative emotional signs of opiate withdrawal including aversion- (Kelsey and Arnold, 1994; Watanabe et al., 2002a; Watanabe et al., 2002b; Nakagawa et al., 2005) and anxiety-like behaviors (Harris et al., 2006; Cabral et al., 2009). Opiate withdrawal can also be precipitated by infusion of opioid receptor antagonists into the NAc or amygdala of morphine dependent rats (Koob et al., 1989; Criner et al., 2007).

A number of neurotransmitters have been shown to contribute to the negative emotional symptoms of withdrawal through their actions in the extended amygdala and shell of the NAc, including corticotropin-releasing factor (CRF), norepinephrine, and the endogenous κ-opioid receptor agonist dynorphin (Koob and Volkow, 2010). Release of each of these neurotransmitters is increased during opiate withdrawal (Rattan et al., 1992; Turchan et al., 1997; McNally and Akil, 2002; Weiss et al., 2001). Genetic and pharmacological manipulations of CRF receptors suggest that the peptide is involved in the affective signs of withdrawal from chronic opiate exposure (Heinrichs et al., 1995; Stinus et al., 2005) and the maintenance of heroin self-administration (Greenwell et al., 2009). Norepinephrine also contributes, via projections from the A1 and A2 cell groups through the ventral noradrenergic bundle (Delfs et al., 1998; Aston-Jones et al., 1999; Clayton and Williams, 2000), to opiate withdrawal-induced aversion (Aston-Jones et al., 1999) and anxiety (Harris and Gewirtz, 2004; Rothwell et al., 2009). Dynorphin release decreases dopaminergic signaling (Spanagel et al., 1992; Xi et al., 1998) via a negative

feedback mechanism, resulting in aversive effects (McLaughlin et al., 2003; Shippenberg et al., 2007; Land et al., 2008).

The mesolimbic dopamine system

The mesolimbic dopamine system is composed of A10 projection neurons in the midbrain and their limbic targets. Numerous studies have revealed that this neural circuit is a critical contributor to motivated behaviors (Wise, 2004). The dopamine system is also a common pathway for drugs of abuse (Di Chiara et al., 2004; Nestler, 2005) and is believed to be a major contributor to the development of addictive behavior (Volkow et al., 2004; Nestler, 2005; Di Chiara and Bassareo, 2007).

Anatomy and physiology. The dopaminergic neurons of the VTA send efferent projections to a number of limbic structures, but the connection to the NAc is the most widely studied and best understood. The NAc forms the ventral portion of the striatum and is composed of two subregions, the core and the shell, which are characterized by different afferent and efferent projections (Groenewegen et al., 1999). Neurons in the A10 dopaminergic cell group project to the entire NAc, but primarily target the shell (Groenewegen et al., 1999). The majority of the cells in the NAc are GABAergic medium spiny neurons, of which there are two major subpopulations (Nicola et al., 2000; Surmeier et al., 2007). Neurons of the "direct" pathway are excited by dopamine (via binding to D1-like receptors, see below) and project back to the midbrain where they provide negative feedback to dopaminergic cells via release of GABA and dynorphin (Surmeier et al., 2007). Neurons of the "indirect" pathway are inhibited by dopamine

(via binding to D2-like receptors) and through projections to the ventral pallidum and the subthalamic nucleus ultimately cause activation of the thalamus (Surmeier et al., 2007).

On a cellular level, dopamine binds to two classes of metabotropic receptors. D1-like receptors include the D1 and D5 receptor subtypes and D2-like receptors include the D2, D3, and D4 receptor subtypes. Activation of D1-like receptors stimulates the adenylate cyclase signaling pathway and the resulting inhibition of potassium currents increases neuronal excitability (Neve et al., 2004). D2-like receptors, on the other hand, decrease cell excitability by inhibiting adenylate cyclase activity and can therefore control dopamine release when expressed as autoreceptors on dopaminergic cell terminals (Neve et al., 2004). Dopamine receptors can also form heterodimeric signaling complexes (Dziedzicka-Wasylewska et al., 2006; Rashid et al., 2007; Fiorentini et al., 2008). The D1/D2 heterodimer, which is coupled to the phospholipase C signaling cascade, has been identified in the NAc and is hypothesized to exist in the amygdala as well (Rashid et al., 2007).

Opioid mechanisms in the VTA. Opioid peptides activate the brain's reward circuits through disinhibition of mesolimbic dopaminergic neurons in the VTA (Johnson and North, 1992). This disinhibition is accomplished through binding of endogenous opioid peptides such as β-endorphin or exogenous opiates to μ-opioid receptors located on GABAergic neurons. The μ-opioid receptor signals through a G_i protein which results in a number of intracellular changes, including inhibition of adenylate cyclase, inhibition of calcium conductance, decreased neurotransmitter release, and increased conductance through inwardly rectifying potassium channels which results in neuronal

hyperpolarization (Williams et al., 2001). Decreased firing of inhibitory GABAergic cells results in increased phasic (i.e.: burst) firing of dopaminergic neurons (Johnson and North, 1992) which in turn causes dopamine release in target structures such as the NAc (Sombers et al., 2009).

Effects of exogenous opiates. Acute exposure to exogenous opiates increases the spontaneous firing rate of VTA dopamine neurons as well as the frequency of burst firing (Gysling and Wang, 1983; Matthews and German, 1984; Diana et al., 1999; Georges et al., 2006). Basal firing rate increases by about 50% before returning back to baseline (Diana et al., 1999; Georges et al., 2006). Opiate-induced changes in dopaminergic cell firing result in a transient increase in dopamine release in the NAc core and shell (Di Chiara and Imperato, 1988; Spanagel et al., 1992; Acquas and Di Chiara, 1992; Wise et al., 1995; Pontieri et al., 1995) and the BNST (Carboni et al., 2000). This effect has not yet been investigated in the amygdala but it is likely to occur given its similarity to the BNST and NAc shell.

Role in opiate reward. Activation of VTA μ-opioid receptors is an important component of the positive motivational effects of opiate drugs. Rats will self-administer morphine into the VTA (Bozarth and Wise, 1981; Bozarth and Wise, 1984; Devine and Wise, 1994) and intra-VTA infusion of morphine supports conditioned place preference (Phillips and LePiane, 1980; Bozarth, 1987; Bals-Kubik et al., 1993; Olmstead and Franklin, 1997) and locomotor sensitization (Vezina and Stewart, 1984; Shaham et al., 1995). The motivational effects of opiates in the VTA are due at least in part to the corresponding increases in dopamine signaling as dopamine in the NAc is involved in

opiate conditioned place preference (Spyraki et al., 1983) and the maintenance of heroin self-administration (Smith et al., 1985).

Evidence that the mesolimbic dopamine system is also involved in opiate withdrawal

As discussed above, it is well accepted that the mesolimbic dopaminergic system participates in the positive emotional effects of opiates. But what is the evidence that, as predicted by negative reinforcement theories, this system is also important in the negative emotional signs of opiate withdrawal?

Anatomical evidence. Ventral tegmental area dopamine neurons project to structures known to be involved in negative emotional signs of withdrawal. In addition to the well-known projection to the NAc, these neurons also target the BLA and the extended amygdala (Figure 1) (Fallon et al., 1978; Hasue and Shammah-Lagnado, 2002; Meloni et al., 2006). The existence of these connections raises the possibility that they play a role in triggering activity in extended amygdala structures during drug withdrawal, a hypothesis that is further supported by evidence that cellular activity in the CeA and lateral BNST during opiate withdrawal is dependent on D1-like receptors (Valjent et al., 2004).

Electrophysiological and neurochemical evidence. Electrophysiological studies of VTA dopamine neurons have revealed that withdrawal from chronic, escalating morphine injections (i.e.: intermittent) causes a decrease in firing rate below baseline for up to seven days (Diana et al., 1999), though this effect is not seen after a chronic, continuous opiate exposure regimen (Georges et al., 2006). In agreement with these

electrophysiological results, dopamine release in target structures drops below baseline during withdrawal from chronic, intermittent (Acquas et al., 1991; Crippens and Robinson, 1994) but not chronic, continuous opiate exposure (Leri et al., 2003). Dopamine levels below baseline can also be observed following administration of an opioid receptor antagonist such as naloxone (Pothos et al., 1991; Rossetti et al., 1992; Spanagel et al., 1994). These findings suggest that the negative emotional symptoms of withdrawal may depend on reduced activation of the mesolimbic dopamine system.

Behavioral evidence. Limited evidence suggests that μ-opioid receptors in the VTA may be critical for the expression of emotional (Stinus et al., 1990), but not physical (Bozarth and Wise, 1984), signs of withdrawal. There is also evidence that dopamine signaling contributes to emotional signs of opiate withdrawal. Manipulation of dopaminergic signaling attenuates the aversive component of opiate withdrawal (Bechara et al., 1995; Laviolette et al., 2002; Chartoff et al., 2006; but see Caillé et al., 2003) as well as withdrawal-induced increases in aggressive behavior (Rodríguez-Arias et al., 1999). VTA dopamine neurons (Liu et al., 2008, Mileykovskiy and Morales, 2011) and the NAc shell (Fenu et al., 2001; Barrot et al., 2002; Barrot et al., 2005; Carlezon and Thomas, 2009) have also been shown to be involved in negative emotional behavioral responses to non-drug related stimuli, suggesting a more general role for this system in the generation of negative affect (Carlezon and Thomas, 2009).

Interactions with the extended amygdala. Additional support for the VTA mesolimbic dopamine system's involvement in withdrawal comes from its interactions with extended amygdala neurotransmitter systems known to contribute to withdrawal

behaviors. Changes in the dopaminergic system may be responsible for the recruitment of CRF, providing a mechanism for the reward system's involvement in the production of negative emotional behaviors. Dopamine terminals appose CRF containing neurons in the CeA (Eliava et al., 2003) and dorsolateral division of the BNST (dlBNST) (Meloni et al., 2006) and mesolimbic dopaminergic projections regulate CRF production in both of these nuclei (Smialowska et al., 1999; Day et al., 2002; Stewart et al., 2008). Intriguingly, dopamine signaling through D1- and D2-like receptors in the BNST results in a short-term potentiation of glutamatergic transmission, and this is dependent on activation of the CRF type 1 receptor (Kash et al., 2008). Opiate-induced release of dopamine in the CeA and BNST may therefore result in increased CRF signaling, a known mediator of negative affect during opiate withdrawal.

Behavioral evidence also supports the idea that the mesolimbic dopamine system recruits structures of the extended amygdala during opiate withdrawal. Dopamine is involved in the production of negative affective behavioral responses to non-drug related stimuli. Dopamine release in the amygdala and NAc is triggered by stress (Herman et al., 1982; Inglis and Moghaddam, 1999) and dopamine release in these structures is involved in the acquisition and retrieval of conditioned fear (Greba and Kokkinidis, 2000; Pezze and Feldon, 2004). The dopaminergic system also appears to have a role in unconditioned fear responses, including increased anxiety-like behavior mediated by CRF receptors in the BNST (Meloni et al., 2006; Rezayof et al., 2009).

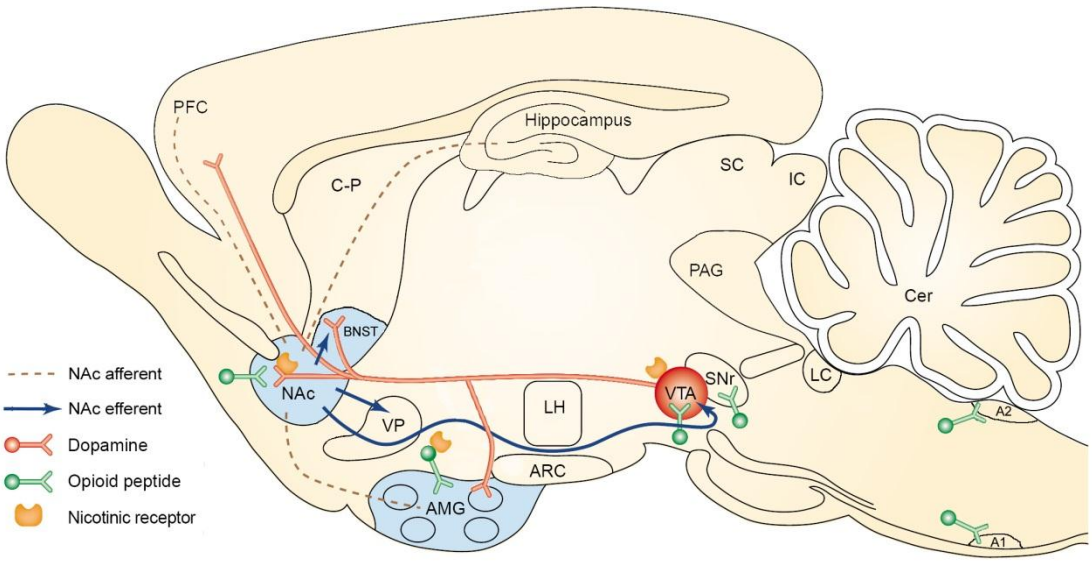

Figure 1. Relevant projections of the mesolimbic dopamine system. Mesolimbic dopamine neurons originate in the ventral tegmental area and project rostrally to the nucleus accumbens, amygdala, bed nucleus of the stria terminalis, and prefrontal cortex. Many of the structures of the mesolimbic system also contain receptors for opioid peptides and nicotinic acetylcholine receptors. Abbreviations: A1 and A2 (noradrenergic A1 and A2 cell groups) AMG (amygdala), ARC (arcuate nucleus), BNST (bed nucleus of the stria terminalis), Cer (cerebellum), C-P (caudate and putamen), IC (inferior colliculus), LC (locus coeruleus), LH (lateral hypothalamus), NAc (nucleus accumbens), PAG (periaqueductal gray), PFC (prefrontal cortex), SC (superior colliculus), SNr (substantia nigra reticulata), VP (ventral pallidum). Adapted from Nestler, 2001.

Acute opiate dependence

While many studies utilize chronic dependence paradigms that expose animals to an opiate continuously for days, an acute opiate dependence paradigm was best suited for these experiments for a number of reasons. First, the study of acute dependence is important because it can help elucidate the mechanisms responsible for the early stages of drug taking. In order to paint a full picture of drug abuse it is important to understand the neural changes that develop during the acquisition of dependence (Harris and Gewirtz, 2005). Because human drug use is invariably disrupted (e.g.: by periods of drug unavailability or sleep), acute dependence paradigms also more accurately model addictive behavior (Dole et al., 1966; Kreek, 2000). Second, in order to directly assess the role of the mesolimbic dopamine system in the negative emotional component of opiate withdrawal, these studies utilized intra-cerebral drug infusion. Methodological constraints associated with this procedure precluded multiple days of treatment and long-term survival of the animals. The use of smaller and shorter drug exposures also avoids other issues, such as toxicity, that occur following chronic, continuous opiate administration (Harris and Gewirtz, 2005).

Despite these important differences, acute dependence is a relatively unexplored field. Most studies to date have found that acute and chronic dependence paradigms largely involve similar mechanisms. Much like chronic paradigms, withdrawal from acute opiate exposure produces affective symptoms such as conditioned place aversion (Parker and Joshi, 1998; Azar et al., 2003), an elevation of intracranial self-stimulation thresholds (Easterling and Holtzman, 1997; Liu and Schulteis, 2004), an increase in

ultrasonic vocalizations (Kalinichev and Holtzman, 2003; Harris and Gewirtz, 2004), suppression of operant responding (Easterling and Holtzman, 1997; Schulteis et al., 1997), and anxiety-like behavior (Zhang and Schulteis, 2008). The neural mechanisms of negative emotional signs of acute and chronic opiate dependence also appear to be similar. Both phenomena are μ-opioid receptor mediated, involve structures of the extended amygdala, and require neurotransmitters, such as norepinephrine, that are important in the expression of negative affect (Harris and Gewirtz, 2005).

The acoustic startle reflex and withdrawal-potentiated startle

The acoustic startle reflex is a reliable and well characterized measure of anxiety-like behavior in rodents and humans. The startle reflex is elevated in the presence of anxiety-provoking stimuli such as footshock and discrete cues paired with footshock. The latter phenomenon ("fear-potentiated startle") is mediated through direct and indirect projections from the CeA to the caudal pontine reticular nucleus (PnC) (Figure 2) (Davis, 2006). Other anxiety-provoking stimuli capable of potentiating startle in rodents include intracerebroventricular (ICV) infusion of the stress peptide CRF and exposure to a bright light. These effects are dependent on the BNST (Swerdlow et al., 1986; Walker and Davis, 1997) and its projections to the PnC.

In rodents and humans, potentiation of the startle reflex has also been demonstrated to occur during withdrawal from drugs of abuse (Krystal et al., 1997; Kalinichev and Holtzman, 2003; Harris and Gewirtz, 2004; Harris et al., 2004). This "withdrawal-potentiated startle" effect likely represents the anxiogenic effects of

withdrawal. The finding that withdrawal-potentiated startle can be blocked by the anxiolytic drugs chlordiazepoxide, clonidine, and propranolol supports this conclusion (Harris and Gewirtz, 2004; Rothwell et al., 2009).

Potentiated startle can be elicited in rats during withdrawal from morphine (Kalinichev and Holtzman, 2003; Harris and Gewirtz, 2004; Harris et al., 2004; Rothwell et al., 2009; Cabral et al., 2009), nicotine (Engelmann et al., 2009), cocaine (unpublished observations), and ethanol (Rassnick et al., 1992) and from nicotine (Engelmann and Cuthbert, 2008; Hogel et al., 2010) and ethanol (Krystal et al., 1997) in humans. It has also been shown to escalate in severity with repeated drug exposures (Krystal et al., 1997; Harris and Gewirtz, 2004). Finally, chemical inactivation of the CeA, BLA, BNST, or shell of the NAc blocks potentiation of the startle reflex during withdrawal from acute opiate exposure (Harris et al., 2006).

There are a number of advantages to using the withdrawal-potentiated startle model. As stated above, acoustic startle is a well characterized model of anxiety and the neural circuitry of the startle reflex is well defined (Lee et al., 1996; Koch, 1999). Additionally, because startle can be measured multiple times in the same animal, the time course of withdrawal can be tracked (Rothwell et al., 2009). The ability to precisely identify the onset of withdrawal allows the experimenter to administer treatments before withdrawal onset. Finally, potentiated startle can be observed after only a single exposure to morphine, making it particularly useful in the study of acute dependence.

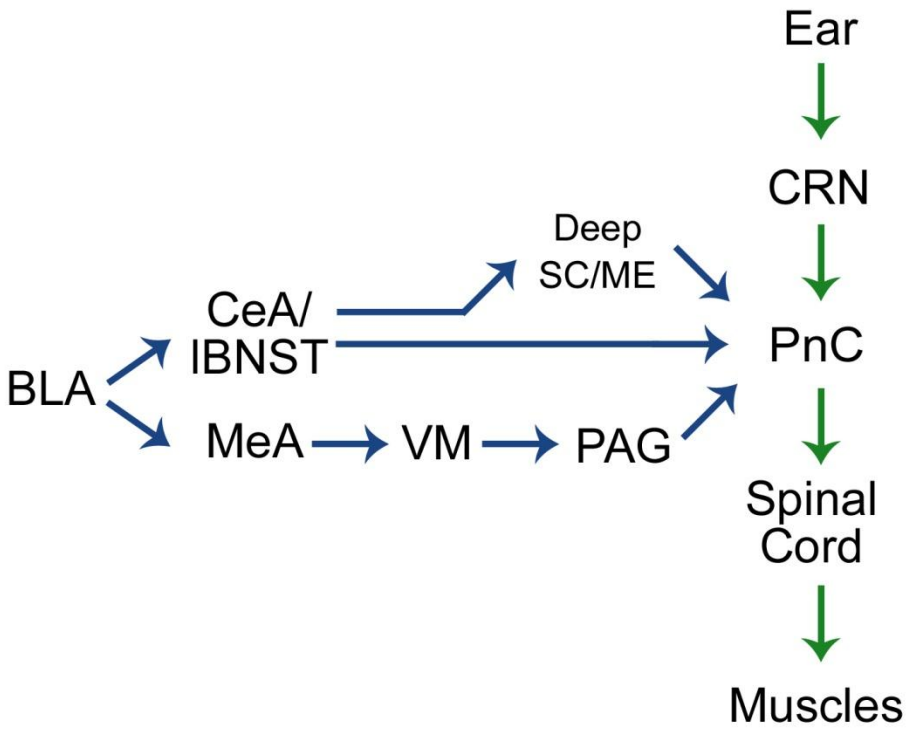

Figure 2. Neural pathways known to mediate potentiation of the acoustic startle reflex. Projections of the acoustic startle reflex pathway are represented by green arrows. Auditory information is relayed through the PnC to motor neurons in the facial motor nucleus or spinal cord. Circuits that project to the PnC and participate in potentiation of startle are represented by blue arrows. The CeA is responsible for potentiation to discrete cues paired with fearful stimuli. The lBNST mediates potentiation to long-duration stimuli and intracerebroventricular CRF infusion. Dopamine receptor agonists cause startle potentiation via their actions in the deep layers of the SC/ME. Abbreviations: BLA (basolateral nucleus of the amygdala), CeA (central nucleus of the amygdala), CRN (cochlear root neurons), deep SC/ME (deep white layers of the superior colliculus/deep mesencephalic reticular nucleus), lBNST (lateral bed nucleus of the stria terminalis), MeA (medial nucleus of the amygdala), PAG (periaqueductal gray), PnC (caudal pontine reticular nucleus), VM (ventromedial hypothalamus). Adapted from Davis, 2006.

Outline of Experiments

The following experiments were designed to test the hypothesis that anxiety during withdrawal from acute opiate exposure begins with μ-opioid receptor activation in the VTA and depends on the subsequent activation of dopamine and CRF systems in structures of the extended amygdala. Chapter 3 details the role of μ-opioid receptors in the VTA in the production of an anxiogenic withdrawal state following acute, intra-VTA exposure to morphine: decreased opioid receptor stimulation is shown to be sufficient and necessary for morphine withdrawal-potentiated startle. Chapter 4 investigates whether dopaminergic signaling in target structures of the VTA is important for withdrawal. Here it was found that morphine withdrawal-potentiated startle requires a decrease in dopaminergic activity at both D1- and D2-like receptors in the shell of the NAc, but not other extended amygdala nuclei. Finally, Chapter 5 investigates the role of the CRF system in withdrawal from acute morphine exposure and concludes that the development of morphine withdrawal-potentiated startle involves the CRF-R2 receptor.

Chapter 2: Methods

Subjects

Male Sprague Dawley rats (Harlan, Indianapolis, IN), weighing between 225 and 400 g at the start of the experiment, were housed in groups of four in metal cages with a 12 h light-dark cycle and free access to food and water, except during testing. Animals were acclimated to housing conditions for two weeks and then gently handled for two consecutive days. Rats that underwent intracranial cannulation surgery were subsequently housed individually in metal cages. All procedures conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of Minnesota Institutional Animal Care and Use Committee.

Drugs

Morphine sulfate was purchased from Mallinckrodt (Hazelwood, MO). Naloxone hydrochloride, R,S-propranolol hydrochloride, (−)-nicotine hydrogen tartrate salt, SKF 82958, and quinpirole were purchased from Sigma-Aldrich (St. Louis, MO). Apomorphine, antisauvagine-30 and CP-154,526 were purchased from Tocris (Ellisville, MO). Systemically administered drugs were dissolved in 0.9% saline and injected subcutaneously (s.c.), except propranolol which was dissolved in deionized water and given intraperitoneally (i.p.). Drugs infused intracerebrally were dissolved in sterile saline (morphine), 25% DMSO in sterile saline (apomorphine), or deionized water (antisauvagine-30 and CP-154,526). The nicotine solution was titrated to a pH of approximately 7.1 using sodium hydroxide. Throughout the text, 0 mg/kg and 0 µg

denote groups given vehicle injection or infusion, respectively. All drug doses are expressed as the weight of the salt, except nicotine which is expressed as the weight of the base.

Intracranial Cannulation and Infusion

Animals were anesthetized with Nembutal (sodium pentobarbital, 75 mg/kg, i.p.), injected with atropine (1 mg/kg, s.c.), and secured in a Kopf stereotaxic instrument. Following exposure of the skull, 22-gauge guide cannulae (Plastics One Products, Roanoke, VA) were lowered to the appropriate stereotaxic coordinates measured in mm from Bregma. Jeweler screws were anchored to the skull, and the entire assembly was cemented into place using Loctite 444 Tak Pak Instant Adhesive (Henkel Corporation, Düsseldorf, Germany) and Perm Reline & Repair Resin (Hygenic Corporation, Akron, OH). "Dummy" cannulae (model C232DC or C313DC; Plastics One Products) were inserted to maintain patency, with the tips flush with the end of the guide cannulae. When necessary, dust caps (model 303DC/1; Plastics One Products) were secured over the dummy cannulae to prevent their removal.

Infusions were made over the course of 2 min through 28-gauge infusion cannulae (Plastics One Products) with tips that extended 1 mm past the end of the guide. Infusion cannulae were attached with polyethylene tubing to a 5 µL Hamilton microsyringe and were left in place for 1 min following infusions. Infusions were given in a room distinct from the colony and behavioral testing rooms.

Acoustic Startle

Acoustic startle was tested in four identical plastic cages (17 x 8.5 x 11 cm) resting on compression springs and located within individual ventilated sound-attenuating chambers. Cage movement resulted in displacement of a piezoelectronic accelerometer (Model ACH-01, Measurement Specialties, Valley Forge, PA) attached to each cage. Voltage output from the accelerometer was filtered and amplified by a custom-built signal processor, digitized on a scale of arbitrary units ranging from 0-1000 (National Instruments SCB100 and PCI-6071E boards), and recorded using Matlab (The MathWorks, Natick, MA). Startle amplitude was defined as the peak accelerometer voltage during the first 200 ms after onset of the startle stimulus. High frequency 23 speakers (Radio Shack Supertweeters, range = 5-40 kHz) located 10 cm beside each cage delivered the startle stimuli, which were 50 ms bursts of filtered white noise (low pass: 22 kHz, rise-decay <5 ms) at intensities of 95 or 105 dB. Ventilating fans elevated background noise to approximately 60 dB.

Each startle test session consisted of a 5 min acclimation period followed by presentation of 40 startle stimuli (20 each at 95 or 105 dB in semi-random order) with a 30 s fixed inter-stimulus interval. Activity levels were also monitored throughout the session. For each experiment, acoustic startle was first tested on 2 consecutive drug-free days. After the second day, average startle amplitudes were used to match animals into groups with similar overall mean startle amplitude (Gutman et al., 2008). Each test day began with a pre-drug exposure, baseline startle session (pretest) and concluded with a final post-drug exposure startle session (posttest).

Locomotor Activity

Locomotor activity was monitored in clear plastic cages (8.5 x 17.5 x 9 in) with a central insert (2.5 x 9 x 9 in) ground corncob bedding on the floor. Each cage was placed in a metal frame containing five sets of infrared photobeams, which traversed the short axis of the cage 2 in above the ground. A computer running custom software (Applied Concepts, Ann Arbor, MI) monitored the number of "crossovers," defined by successive interruption of beams on opposite ends of the cage. Crossovers were analyzed in 10 min bins and also summed across the entire experimental session. One day prior to drug exposure, animals were habituated to activity boxes for 1 h.

Histology

Animals were deeply anesthetized with Beuthanasia (sodium pentobarbital 390 mg/kg, i.p.) and perfused intracardially with 0.9% saline followed by 10% formalin. Brains were subsequently removed and immersed in a 30% sucrose-formalin solution for at least three days. Coronal sections (30 μm) from the relevant brain regions were cut, mounted onto gelatin-coated slides, stained with cresyl violet, and scored for correct cannulae placement by an observer who was blind to group assignments.

Data Analysis

Throughout the text and figures all data are expressed as mean ± SEM. Startle data were collapsed across both intensities (95/105 dB) before further statistical analysis, as the magnitude of withdrawal-potentiated startle does not depend on startle stimulus

intensity (Harris and Gewirtz, 2004). In each experiment, one-way analysis of variance (ANOVA) was conducted to verify similar baseline startle amplitude between experimental groups (Tables 1, 3, 5, and 7). Changes in startle or activity after experimental treatment were calculated as percent change from baseline on the same day, that is percent change = ((test-baseline)/baseline) x 100 (Harris and Gewirtz, 2004). Data were evaluated for outliers with the Grubb's extreme studentized deviate test (GraphPad Software; http://www.graphpad.com/quickcalcs/Grubbs1.cfm) with a significance level of $\alpha = 0.01$.

Data from Experiments 1 and 11 were analyzed using factorial ANOVA, with repeated measures on within-subject factors. For main effects or interactions involving repeated measures, the Huynh–Feldt correction was applied to control for violations of the sphericity assumption. Between-subjects effects were further analyzed with one-way ANOVA. Planned comparisons (paired t-tests or polynomial trend analysis, as appropriate) were used to test within-subjects effects in Experiments 2-5, 7-10, and 12. In Experiment 6 the effects of individual doses of SKF82958 and quinpirole were analyzed with Dunnett's tests. All statistical analyses were conducted using SPSS (version 17.0) with a Type I error rate of $\alpha = 0.05$ (two-tailed). Results of statistical tests are reported in the text as well as in Tables 2, 4, 6, and 8.

Chapter 3: Ventral Tegmental Area Opioid Receptor Activity is Necessary and Sufficient for the Expression of Opiate Withdrawal-Induced Anxiety

Introduction

Although negative emotional symptoms of withdrawal are an intrinsic component of daily drug exposure and likely contribute to the development of dependence (Koob and Le Moal, 1997; Baker et al., 2004; Rothwell et al., 2009), the neural mechanisms involved in the development of these states have yet to be elucidated. The opponent process view predicts that activation of reward-related circuitry is the first step in the induction of a negative affective withdrawal state following opiate exposure (Koob and Bloom, 1988; Koob and Le Moal, 1997; Vargas-Perez et al., 2009). Opiates activate the brain's reward circuits through disinhibition of mesolimbic dopaminergic neurons in the VTA (Johnson and North, 1992). The hypothesis that anxiety during opiate withdrawal occurs in response to reduced activation of VTA μ-opioid receptors was therefore tested. Anxiety-like behavior was assessed in rats using the acoustic startle reflex. The experiments described below provide direct support for the opponent process theory of opiate withdrawal by demonstrating that morphine's actions in the rodent VTA are both sufficient and necessary to induce anxiety-like behavior during withdrawal from acute opiate exposure.

Materials and Methods

Intracranial Cannulation and Infusion

22-gauge guide cannulae (model C232G-2.0; Plastics One Products, Roanoke, VA) were implanted bilaterally into the VTA (AP: -5.3 mm, ML: ±1.0 mm, DV: -7.2 mm from Bregma). "Dummy" cannulae (model C232DC; Plastics One Products) were inserted to maintain patency, with the tips flush with the end of the guide cannulae. Infusions of 0.5 µL per hemisphere were made over the course of 2 min through 28-gauge infusion cannulae (model C232I-2.0; Plastics One Products) with tips that extended 1 mm past the end of the guide.

Experimental Design

Experiment 1: Intra-VTA morphine infusion.

On each test day animals were infused with 0, 1, or 5 µg morphine sulfate per hemisphere at 0 h. Locomotor activity was monitored for 2 h immediately after the infusion. Startle was assessed at one of four post-morphine infusion time points (2, 4, 6, and 8 h). A Latin Square design was used so that each rat was tested once at each of the four post-infusion time points over a series of four days. Rats received a total of four test days, each separated by two intervening days to prevent tissue damage (Vezina and Stewart, 1984). Of the rats implanted with cannulae in the VTA, seven were removed from analysis due to misplaced cannulae (located in the lateral hypothalamus, LH) or significant lesions at the infusion site, leaving final sample sizes of 17 (0 µg), 14 (1 µg), and 14 (5 µg). In a separate control experiment animals with cannulae located 1 mm

dorsal to the VTA in the interstitial nucleus of the medial longitudinal fasciculus (IMLF) were infused with 0 µg or 1 µg of morphine and tested as described above. Of these animals, eight were removed from analysis due to misplaced cannulae, leaving final sample sizes of 9 (0 µg) and 7 (1 µg).

Experiment 2: Intra-VTA morphine infusion during withdrawal from systemic morphine.

Rats were injected with either 0 or 10 mg/kg of morphine at 0 h and received an intra-VTA infusion of morphine (0 or 1 µg per side) 3 h later. Startle was tested at 4 h. A crossover design was used so that each rat was infused with the two doses of intra-VTA morphine in a random order over two consecutive test days. One animal was removed from analysis due to problems with the infusion on the second test day and six animals were removed due to misplaced cannulae, leaving final sample sizes of 12 (0 mg/kg) and 11 (10 mg/kg).

Morphine-injected animals in this experiment had previously been tested for intra-VTA methylnaloxonium-precipitated withdrawal (Stinus et al., 1990; Maldonado et al., 1992) following systemic morphine exposure (no significant startle potentiation, data not shown), and had therefore previously received one 10 mg/kg injection of morphine and two 500 ng intra-VTA infusions of methylnaloxonium. The lack of startle potentiation following methylnaloxonium infusion was likely due to the anxiolytic effects of morphine in brain regions outside of the VTA (Cabral et al., 2009).

Experiment 3: Intra-VTA naloxone infusion before intra-VTA morphine.

Rats were infused with 0, 3, or 10 µg of naloxone hydrochloride followed by 0 or 1 µg of morphine per side 20 min later. Startle was tested at 4 h. Each rat was infused with the three doses of intra-VTA naloxone in a random order over three test days, separated by two intervening days. One animal was removed from analysis due to misplaced cannulae and one was removed as an outlier, leaving final sample sizes of 11 (0 µg) and 8 (1 µg).

Experiment 4: Systemic propranolol injection during withdrawal from intra-VTA morphine.

Rats were infused with 0 or 1 µg per side of morphine followed by an injection of the β-adrenergic receptor antagonist propranolol (0 or 10 mg/kg) 3 h 30 min later. Startle was tested at 4 h. Each rat was administered the four treatments in a random order over four test days, separated by two intervening days. Two animals were removed from analysis due to misplaced cannulae, leaving a final sample size of 6.

Results

Experiment 1: Intra-VTA morphine infusion.

To test whether the VTA is involved in the induction of anxiety following acute opiate exposure, morphine sulfate (0, 1, or 5 µg per side) was microinfused through chronically, indwelling cannulae aimed bilaterally at the VTA (Figure 3A). Startle was measured 2, 4, 6 or 8 h after the infusion over four test days (Figure 4A). A significant

main effect of time ($F_{3,126} = 3.555$, $p = 0.016$) and a significant time x group interaction ($F_{6,126} = 3.194$, $p = 0.006$) were observed. Compared with the 0 µg group at the same time point, significant potentiation of the startle reflex was observed only in the 1 µg group 4 h after infusion ($F_{1,30} = 4.402$, $p = 0.045$) and in the 5 µg group 8 h after infusion ($F_{1,30} = 6.034$, $p = 0.020$) (Figure 4B-C). The increase in startle at 4 h was also found to escalate in magnitude over the four test days in the 1 µg group (Day 1: 22.7% ± 9.2%, Day 2: 35.8% ± 9.2%, Day 3: 43.9% ± 10.8%, Day 4: 68.8% ± 11.1%), resulting in a significant linear effect of day ($F_{1,13} = 8.550$, $p = 0.015$). This effect was not observed in the 0 µg group ($F_{1,16} = 0.015$, $p = 0.904$). Data from animals with misplaced cannulae (N=5) were run against the 0 µg group in a separate analysis and no significant effects were observed (Figure 4D) (Table 2). Analysis of data from animals with cannulae aimed 1 mm dorsal to the VTA (Figure 3B) also yielded no significant effects (Figure 4E) (Table 2), indicating the effect of VTA morphine infusion is not a consequence of dorsal diffusion to the periaqueductal gray (Bozarth and Wise, 1984).

To verify that the infusion protocol activated mesolimbic dopamine circuitry, locomotor activity was measured for 2 h following morphine infusion. Analysis of locomotor activity revealed significant main effects of day ($F_{2.444,102.627} = 10.424$, $p < 0.001$) and group ($F_{2,42} = 3.786$, $p = 0.031$). Sensitization of the locomotor response was observed only in 1 µg- (linear effect of day $F_{1,13} = 11.503$, $p = 0.005$) and 5 µg-infused animals (linear effect of day $F_{1,13} = 5.500$, $p = 0.036$) (Figure 5A-D). Analysis of data from animals with cannulae aimed 1 mm dorsal to the VTA (0 µg group, data not shown; 1 µg group, included in Figure 5D) revealed no significant effects (Table 2).

Table 1. Mean raw startle output for Experiment 1.

	0 µg Morphine	1 µg Morphine	5 µg Morphine	Miss (LH)	Dorsal 0 µg	Dorsal 1 µg
2 hours	49.3 ± 3.4	47.8 ± 4.5	45.1 ± 4.1	46.7 ± 2.4	48.6 ± 8.3	49.3 ± 7.6
4 hours	54.4 ± 5.5	43.5 ± 3.8	40.5 ± 2.8	35.5 ± 8.0	60.7 ± 9.5	52.8 ± 7.1
6 hours	48.6 ± 3.8	50.1 ± 4.9	45.0 ± 4.3	46.8 ± 4.0	49.0 ± 10.0	55.7 ± 10.7
8 hours	47.8 ± 3.8	47.2 ± 5.2	39.5 ± 3.2	49.3 ± 7.4	48.7 ± 6.0	43.9 ± 6.1

Data are average startle values of animals before intra-VTA morphine infusion.

Table 2. Results of statistical tests for Experiment 1.

	Test	Groups	Statistics
Startle			
VTA morphine 0, 1, and 5 µg	RM ANOVA	Time point (main effect)	$F_{3,126} = 3.555$, **p = 0.016***
		Time point x Group (interaction)	$F_{6,126} = 3.194$, **p = 0.006***
		Group (main effect)	$F_{2,42} = 1.411$, p = 0.255
	One-way ANOVA	0 vs. 1 µg at 2 h	$F_{1,30} = 2.386$, p = 0.133
	One-way ANOVA	0 vs. 1 µg at 4 h	$F_{1,30} = 4.402$, **p = 0.045***
	One-way ANOVA	0 vs. 1 µg at 6 h	$F_{1,30} = 0.568$, p = 0.457
	One-way ANOVA	0 vs. 1 µg at 8 h	$F_{1,30} = 0.000$, p = 0.985
	One-way ANOVA	0 vs. 5 µg at 2 h	$F_{1,30} = 1.719$, p = 0.200
	One-way ANOVA	0 vs. 5 µg at 4 h	$F_{1,30} = 0.104$, p = 0.749
	One-way ANOVA	0 vs. 5 µg at 6 h	$F_{1,30} = 3.560$, p = 0.069
	One-way ANOVA	0 vs. 5 µg at 8 h	$F_{1,30} = 6.034$, **p = 0.020***
0 µg and Miss	RM ANOVA	Time point (main effect)	$F_{3,60} = 0.138$, p = 0.937
		Time point x Group (interaction)	$F_{3,60} = 0.387$, p = 0.762
		Group (main effect)	$F_{1,20} = 0.668$, p = 0.668
	One-way ANOVA	0 vs. Miss at 2 h	$F_{1,21} = 1.983$, p = 0.174
	One-way ANOVA	0 vs. Miss at 4 h	$F_{1,21} = 0.088$, p = 0.769
	One-way ANOVA	0 vs. Miss at 6 h	$F_{1,21} = 0.037$, p = 0.849
	One-way ANOVA	0 vs. Miss at 8 h	$F_{1,21} = 0.602$, p = 0.447
Dorsal morphine 0 and 1 µg	RM ANOVA	Time point (main effect)	$F_{2.419, 41.120} = 0.018$, p = 0.991
		Time point x Group (interaction)	$F_{2.419, 41.120} = 0.083$, p = 0.946
		Group (main effect)	$F_{1,17} = 0.083$, p = 0.102
	One-way ANOVA	0 vs. 1 µg at 2 h	$F_{1,18} = 1.194$, p = 0.290
	One-way ANOVA	0 vs. 1 µg at 4 h	$F_{1,18} = 1.908$, p = 0.185
	One-way ANOVA	0 vs. 1 µg at 6 h	$F_{1,18} = 0.588$, p = 0.454
	One-way ANOVA	0 vs. 1 µg at 8 h	$F_{1,18} = 1.599$, p = 0.223
Activity			
VTA morphine 0, 1, and 5 µg	RM ANOVA	Time point (main effect)	$F_{2.444,102.627} = 10.424$, **p < 0.001***
		Time point x Group (interaction)	$F_{4.887, 102.627} = 1.831$, p = 0.115
		Group (main effect)	$F_{2,42} = 3.786$, **p = 0.031***
		Linear effect 0 µg morphine	$F_{1,16} = 4.286$, p = 0.055
		Linear effect 1 µg morphine	$F_{1,13} = 11.503$, **p = 0.005***
		Linear effect 5 µg morphine	$F_{1,13} = 5.500$, **p = 0.036***
Dorsal morphine		Linear effect 1 µg morphine	$F_{1,9} = 0.869$, p = 0.376

* Indicates significant result.

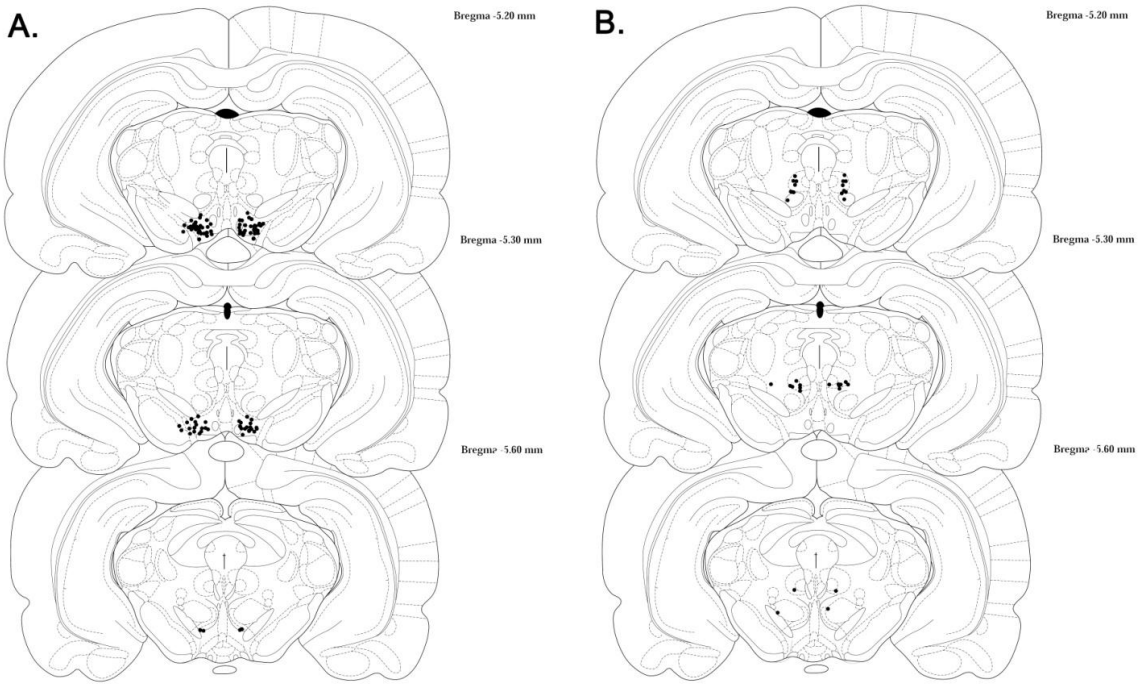

Figure 3. Cannula tip placements for Experiment 1. Tip locations for animals with correct placements are indicated with black circles. Animals were bilaterally implanted with chronically indwelling cannulae aimed A) at the VTA or B) 1 mm dorsal to the VTA (in the IMLF).

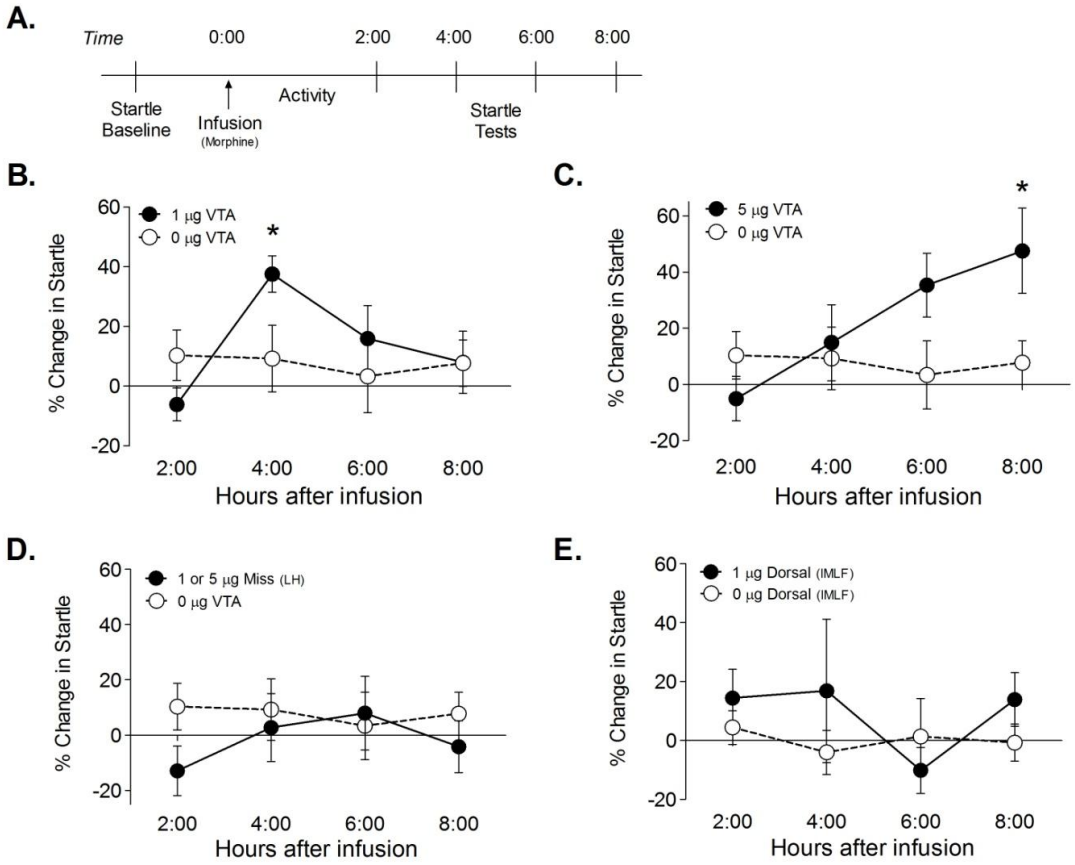

Figure 4. Startle time course during spontaneous withdrawal from intra-VTA morphine infusion. A) Timeline of experimental test day for Experiment 1. B) Startle was significantly potentiated 4 h after infusion in the 1 µg group vs. the 0 µg group. C) Startle was significantly potentiated 8 h after infusion in the 5 µg group vs. 0 µg group. D) Animals with misplaced cannulae, located anterior to the VTA in the LH, failed to show significant startle potentiation at any time point. For purposes of comparison, the same 0 µg control group is presented in panels B-D. E) Animals with cannulae aimed 1 mm dorsal to the VTA, located in the IMLF, failed to show significant startle potentiation at any time point. *$p < 0.05$ compared to 0-µg group at that time point.

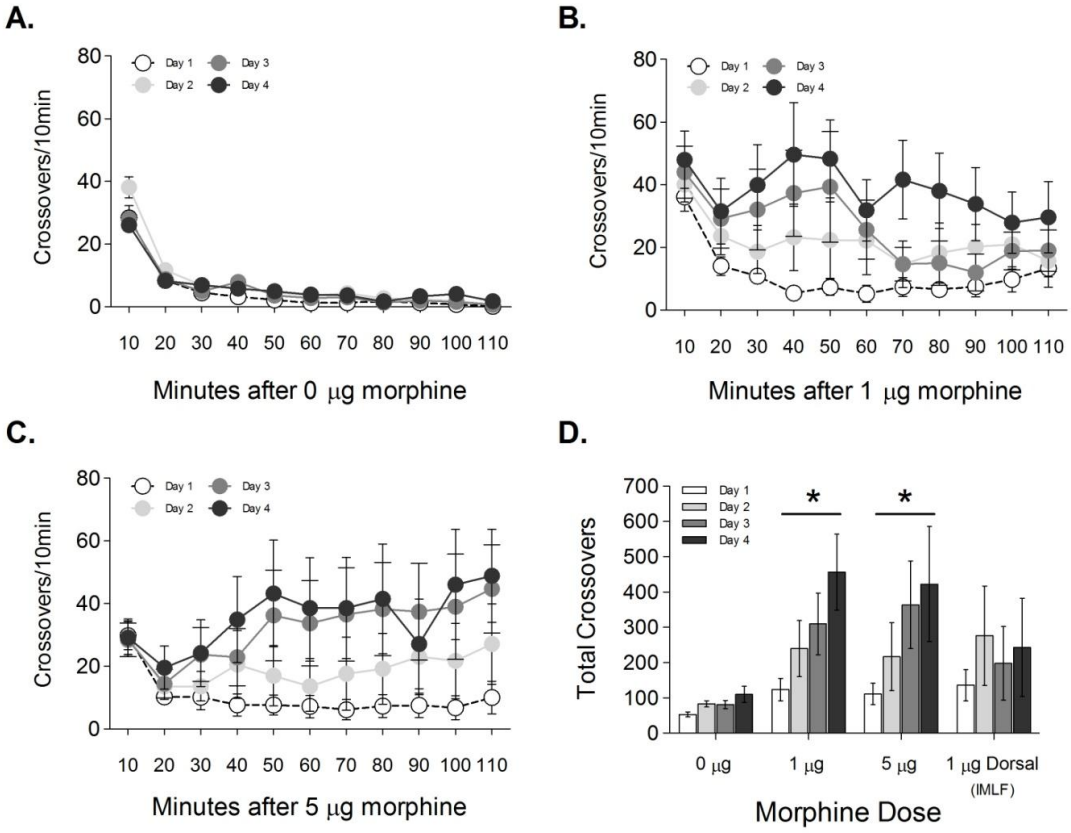

Figure 5. Locomotor activity following intra-VTA morphine infusion. Time course of activity across four days of testing for the A) 0 µg group, B) 1 µg group, and C) 5 µg group. D) Total locomotor activity across four days of testing. Activity in the 1 µg and 5 µg morphine groups significantly increased over days, as evidenced by significant linear trends in both groups (*$p < 0.05$). No significant effects were found in the 0 µg or dorsal control groups.

Experiment 2: Intra-VTA morphine infusion during withdrawal from systemic morphine.

To test whether the expression of opiate withdrawal-induced anxiety requires a reduction in VTA opioid receptor stimulation, rats were systemically injected with morphine sulfate (0 or 10 mg/kg) followed by intra-VTA infusion of morphine (0 or 1 µg per side) 3 h later (Figure 6A). Therefore, in animals given systemic morphine followed by 1 µg of intra-VTA morphine, drug levels would be expected to be reduced in all brain regions except the VTA. These animals showed a significantly lower level of withdrawal-potentiated startle than the 0 µg group ($t_{11} = 2.331$, $p = 0.040$) (Figure 6B). No significant differences were seen between the two 0 mg/kg groups ($t_9 = 1.115$, $p = 0.294$).

Experiment 3: Intra-VTA naloxone infusion before intra-VTA morphine.

To verify that the effects of intra-VTA morphine observed in Experiment 1 were the result of opioid receptor activation, rats received an intra-VTA infusion of naloxone hydrochloride (0, 3, or 10 µg per side) followed by intra-VTA morphine (0 or 1 µg per side) 20 min later (Figure 6C). Animals given 3 or 10 µg of naloxone before 1 µg of morphine had a significantly lower level of withdrawal-potentiated startle than animals given 0 µg naloxone (linear effect of dose $F_{1,23} = 5.635$, $p = 0.027$) (Figure 6D). This effect was not observed in the three groups infused with 0 µg morphine ($F_{1,32} = 1.205$, $p = 0.281$).

Table 3. Mean raw startle output for Experiments 2-4.

	0 mg/kg Morphine	10 mg/kg Morphine
Experiment 2		
0 µg morphine	48.1 ± 5.7	38.4 ± 6.1
1 µg morphine	49.3 ± 5.2	36.4 ± 4.0
Experiment 3		
0 µg naloxone	45.2 ± 6.5	39.5 ± 6.1
3 µg naloxone	43.9 ± 5.3	40.6 ± 4.8
10 µg naloxone	46.1 ± 6.7	41.7 ± 4.1
Experiment 4		
0mg/kg propranolol	43.8 ± 7.3	45.4 ± 7.0
10mg/kg propranolol	49.9 ± 8.1	42.0 ± 6.7

Data are average startle values of animals before morphine injection.

Table 4. Results of statistical tests for Experiments 2-4.

	Test	**Groups**	**Statistics**
Experiment 2			
0 mg/kg morphine	Paired T-test	0 vs. 1 µg morphine	$t_9 = 1.115$, $p = 0.294$
10 mg/kg morphine	Paired T-test	0 vs. 1 µg morphine	$t_{11} = 2.331$, **$p = 0.040$***
Experiment 3			
	Linear effect	0 mg/kg morphine	$F_{1,32} = 1.205$, $p = 0.281$
	Linear effect	10 mg/kg morphine	$F_{1,23} = 5.635$, **$p = 0.027$***
Experiment 4			
0 mg/kg morphine	Paired T-test	0 vs. 10 mg/kg propranolol	$t_5 = 1.268$, $p = 0.261$
10 mg/kg morphine	Paired T-test	0 vs. 10 mg/kg propranolol	$t_5 = 0.340$, $p = 0.748$

* Indicates significant result.

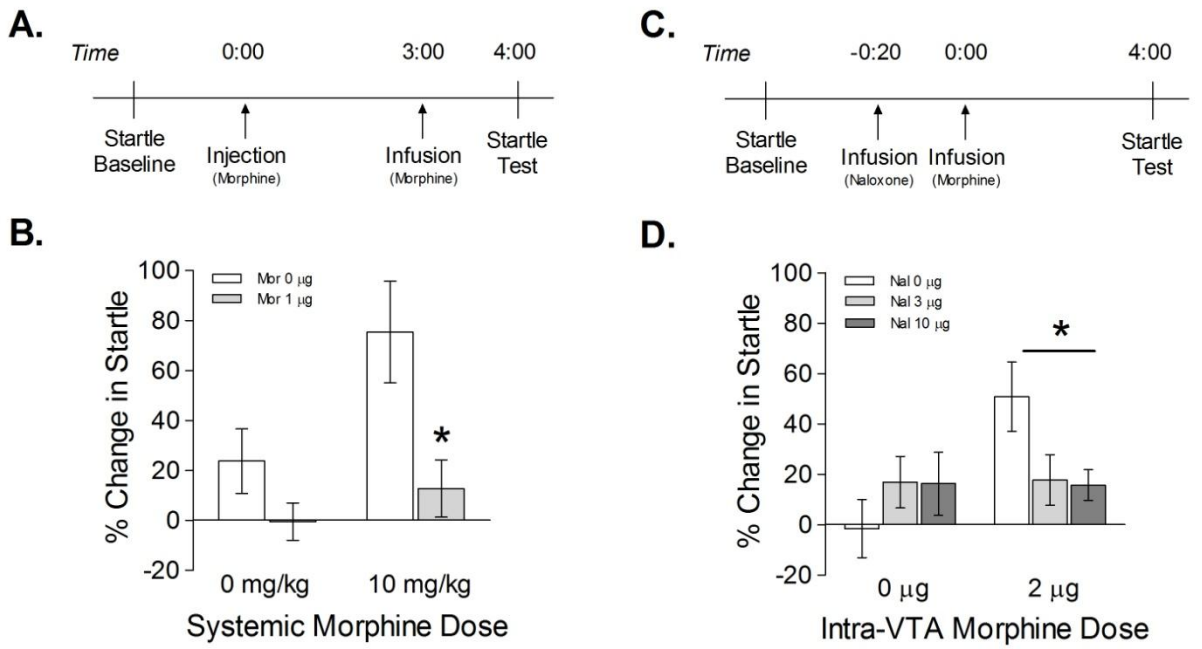

Figure 6. Withdrawal from intra-VTA morphine is dependent on decreased activity at VTA opioid receptors. A) Timeline of test day for Experiment 2. B) Intra-VTA morphine significantly attenuated startle potentiation (*$p < 0.05$ compared to 0 µg group). C) Timeline of test day for Experiment 3. D) Intra-VTA naloxone significantly attenuated startle potentiation, as evidenced by a significant linear trend (*$p < 0.05$).

Experiment 4: Systemic propranolol injection during withdrawal from intra-VTA morphine.

To test whether noradrenergic signaling is involved in anxiety during withdrawal from intra-VTA morphine, rats received an intra-VTA infusion of morphine (0 or 1 µg) followed 3 h and 30 min later by a systemic injection of propranolol (0 or 10 mg/kg) (Figure 7A). No significant differences were observed between groups (Figure 7B) (Table 4).

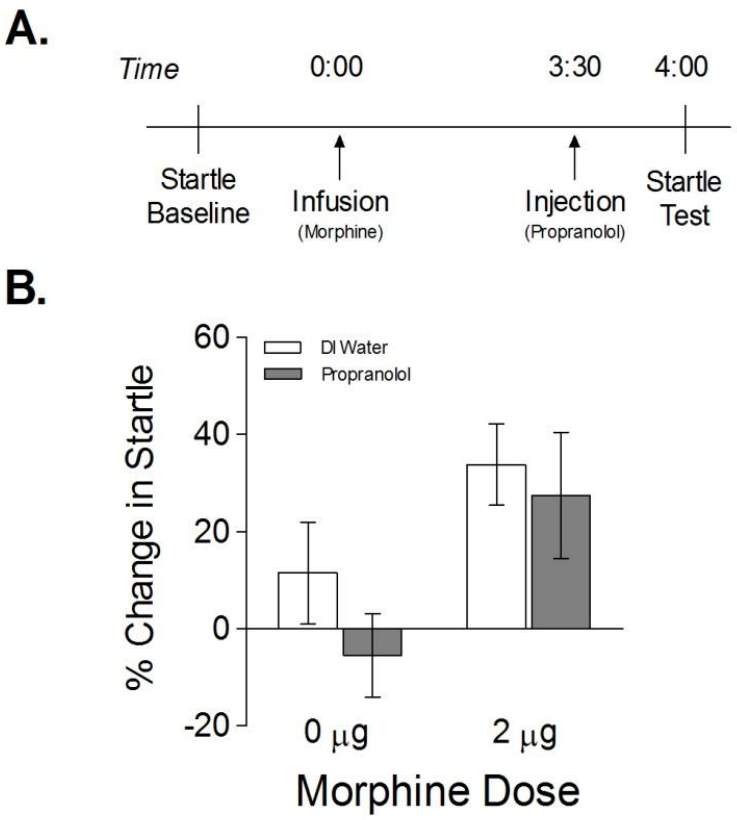

Figure 7. Withdrawal from intra-VTA morphine does not involve β-noradrenergic receptors. A) Timeline of test day for Experiment 4. B) Propranolol had no effect on startle potentiation.

Discussion

Negative affective symptoms, such as anxiety and dysphoria, are a common consequence of withdrawal from drugs of abuse that occur after each drug exposure. They are therefore an intrinsic component of drug taking (Koob and Le Moal, 1997; Baker et al., 2004). The current experiments show that withdrawal-induced anxiety develops following activation of the same neural circuitry that mediates the rewarding aspects of drugs. Specifically, they demonstrate that the expression of anxiety during opiate withdrawal results from a drop in activity at VTA opioid receptors. Direct microinfusion of morphine into the VTA produces startle potentiation four hours later, a time course that is remarkably similar to previously published studies with systemic opiate administration (Harris and Gewirtz, 2004; Rothwell et al., 2009). This response is specific to the VTA and occurs at a longer latency following administration of a higher dose of morphine, supporting the conclusion that the behavioral results are a withdrawal effect, and not merely a side effect of morphine exposure. Both potentiated startle and locomotor activity increased in magnitude over repeated testing, further suggesting that they rely on shared mechanisms (Rothwell et al., 2010). Finally, startle potentiation four hours after systemic opiate exposure is alleviated by a second intra-VTA microinfusion of morphine, demonstrating that a reduction in activity at VTA opioid receptors is necessary for the expression of withdrawal.

The findings presented here offer strong and direct support for opponent process theory, which posits that positive motivational stimuli activate two emotional processes: an initial rewarding process and a secondary negative withdrawal process that is

dependent on the first and grows over time (Solomon and Corbit, 1974; Koob and Bloom, 1988). In the case of opiate drugs, the former process is dependent on VTA opioid receptors and the subsequent release of dopamine in target structures (Bozarth and Wise, 1984; Olmstead and Franklin, 1997; Fenu et al., 2006). As predicted by opponent process theory, these data demonstrate that anxiety-like behavior during opiate withdrawal is initiated by prior activation of the same circuitry and escalates over repeated exposures. It was also found that the degree of psychomotor activation produced by VTA morphine infusion is enhanced with repeated exposure, consistent with previous reports (Vezina and Stewart, 1984; Shaham et al., 1995) and other evidence that intermittent drug exposure causes sensitization of the mesolimbic dopamine system (Robinson and Berridge, 2003). The similarities between drug-induced sensitization and withdrawal further support the conclusion that these behaviors depend on shared neural circuitry.

The induction of anxiety following opiate exposure is likely due to changes in firing of VTA dopaminergic neurons. Subpopulations of these neurons have been found to be either inhibited (Schultz and Romo, 1987; Ungless et al., 2004; Liu et al., 2008; Brischoux et al., 2009; Mileykovskiy and Morales, 2011) or excited (Brischoux et al., 2009; Valenti et al., 2011) by negatively valenced stimuli. The current experiments demonstrate that expression of anxiety requires both the initial activation of VTA opioid receptors (Experiment 3) as well as the subsequent loss of receptor occupancy as the effects of the drug subside (Experiment 2). These results suggest that relative changes in dopaminergic cell firing (i.e.: an increase in firing followed by a decrease) are involved in

triggering opiate withdrawal. The transient nature of drug exposure may therefore mimic the effects of aversive and anxiogenic stimuli on both of the subpopulations of neurons described above.

Interestingly, antagonism of β-adrenergic receptors with propranolol did not attenuate withdrawal following intra-VTA morphine. In contrast, this treatment does block withdrawal-potentiated startle following systemic morphine exposure (Rothwell et al., 2009) as well as the aversive effects of withdrawal from chronic morphine (Delfs et al., 2000). These results suggest that withdrawal from systemic drug exposure involves activation of additional neural circuits outside of the mesolimbic dopamine system. This fact may account for the slightly smaller and more variable behavioral effect observed after intra-VTA as opposed to systemic morphine administration.

In contrast to the current findings, intra-VTA morphine infusion does not produce physical signs of dependence (Bozarth and Wise, 1984). This is not surprising given the abundant evidence that physical and emotional aspects of withdrawal are mediated by distinct mechanisms (Koob et al., 1992; Higgins and Sellers, 1994). It will be important for future work to determine whether other emotional facets of the withdrawal syndrome, such as anhedonia and dysphoria, involve the same mechanisms described here. It will also be interesting to see if similar increases in anxiety-like behavior are seen with models of anxiety that involve a suppression instead of a potentiation of behavior. Such an outcome is likely given that potentiated startle correlates highly with freezing behavior, another common measure of anxiety in rodents (Leaton and Borszcz, 1985).

Taken as a whole, these findings demonstrate that the induction of a negative affective opponent process following acute opiate exposure is dependent on a reduction in activation of the neural circuitry responsible for the rewarding effects of the drug. Since the negative emotional component of withdrawal is thought to play a strong motivational role in drug taking behavior (Koob and Le Moal, 1997; Baker et al., 2004), our findings may be particularly relevant to the study of the development and escalating severity of drug dependence.

Chapter 4: Transient Nucleus Accumbens Dopamine Receptor Activity Contributes to Opiate Withdrawal-Induced Anxiety

Introduction

Exposure to exogenous opiates increases the firing rate of VTA dopaminergic neurons (Gysling and Wang, 1983; Matthews and German, 1984; Diana et al., 1999; Georges et al., 2006) and produces a corresponding increase in dopamine levels in structures of the extended amygdala (Di Chiara and Imperato, 1988; Spanagel et al., 1992; Acquas and Di Chiara, 1992; Wise et al., 1995; Carboni et al., 2000). As drug levels decrease, and VTA μ-opioid receptor activity is reduced, dopamine signaling also returns to baseline levels (Di Chiara and Imperato, 1988; Diana et al., 1999; Carboni et al., 2000; Georges et al., 2006). It was therefore hypothesized that the next step in triggering anxiety-like behavior following intra-VTA morphine infusion is decreased dopamine receptor activity in the extended amygdala. To test this hypothesis, the general dopamine receptor agonist apomorphine was first administered systemically just prior to testing for withdrawal from systemic morphine exposure. The contribution of D1- and D2-like dopamine receptors was then evaluated by injecting subtype specific dopamine receptor agonists alone and in combination. These two classes of dopamine receptors have opposite effects on cell excitability (Neve et al., 2004), but have both been shown to contribute to aversive and withdrawal behaviors (Carlezon and Thomas, 2009).

Dopaminergic neurons in the VTA project to a number of limbic targets, including nuclei of the extended amygdala (Figure 1) (Fallon et al., 1978; Hasue and Shammah-

Lagnado, 2002; Meloni et al., 2006). In order to determine whether dopamine receptors within the extended amygdala are involved in the production of anxiety, apomorphine was locally infused into the shell of the NAc, the BNST, and the CeA. All of these structures are activated during opiate withdrawal (Gracy et al., 2001; Frenois et al., 2002; Shaw Lutchman et al., 2002; Reti and Baraban, 2003; Veinante et al., 2003) and dopamine levels increase in the NAc shell and BNST following morphine exposure (Di Chiara and Imperato, 1988; Acquas and Di Chiara, 1992; Carboni et al., 2000). The dorsolateral component of the BNST (dlBNST) was specifically targeted because this region contains the most dense distribution of dopaminergic fibers (Freedman and Cassell, 1994).

The results of these studies demonstrate that reduced dopamine signaling contributes to anxiety during opiate withdrawal. This effect seems to be dependent on both D1- and D2-like receptors and is localized to the shell of the NAc. Finally, an experiment in which apomorphine was administered prior to testing for nicotine withdrawal-potentiated startle demonstrates that similar mechanisms contribute to nicotine withdrawal.

Materials and Methods

Intracranial Cannulation and Infusion

22-gauge guide cannulae (model C313G; Plastics One Products, Roanoke, VA) were implanted unilaterally into the NAc shell (AP: 1.7 mm, ML: ±1.5 mm, DV: -7.2 mm from Bregma), dlBNST (AP: -0.4 mm, ML: ±3.7 mm, DV: -4.8 mm from Bregma,

inserted at a 15 degree angle), CeA (AP: -2.2 mm, ML: ±4.0 mm, DV: -6.4 mm from Bregma). Infusions of 0.3 µL were made over the course of 2 min through 28-gauge infusion cannulae (model C313I; Plastics One Products) with tips that extended 1 mm past the end of the guide.

Experimental Design

Experiment 5: Apomorphine injection during withdrawal from systemic morphine.

Rats were injected with morphine or saline at 0 h followed by 0 (N=14 per group), 50 (N=10 per group), or 100 µg/kg (N=10 per group) apomorphine hydrochloride 3 h and 50 min later. This higher dose of apomorphine has been shown to cause cellular changes mediated by activation of postsynaptic dopamine receptors (Bergstrom et al., 1982; Carlson et al., 1987; Rosenkranz and Grace, 1999). Startle was tested at 4 h.

Experiment 6: SKF82958 or quinpirole injection during withdrawal from systemic morphine.

Rats were injected with either 0 or 10 mg/kg of morphine at 0 h followed by vehicle (N=12) or 10 (N=8) or 50 µg/kg (N=8) of the D1-like receptor agonist SKF82958 or 10 (N=11) or 50 µg/kg (N=8) of the D2-like receptor agonist quinpirole 3 h and 30-50 min later. Startle was tested at 4 h. A crossover design was used so that each rat was injected with the two doses of morphine in a random order over two consecutive test days.

Experiment 7: SKF82958 and quinpirole cocktail injection during withdrawal from systemic morphine.

Rats were injected with either 0 (N=12) or 10 mg/kg (N=11) of morphine at 0 h and received an injection of a cocktail of the D1-like receptor agonist SKF82958 and the D2-like receptor agonist quinpirole (0, 10, or 50 µg/kg of each agonist) 3 h and 30 min later. Startle was tested at 4 h. A Latin Square design was used so that each rat was injected with the three doses of the dopamine receptor agonist cocktail in a random order over three consecutive test days.

Experiment 8: Apomorphine infusion in local brain structures during withdrawal from systemic morphine.

Following bilateral implantation of cannulae into the shell of the NAc, dlBNST, or CeA, rats were injected with either 0 or 10 mg/kg of morphine at 0 h and received an infusion of apomorphine (0, 1, or 5 µg per side; Willner et al., 1985; Hull et al., 1986) 3 h and 40 min later. Startle was tested at 4 h. A Latin Square design was used so that each rat was infused with the three doses of apomorphine in a random order over three test days. Rats received a total of three test days, each separated by two intervening days to prevent tissue damage. In the NAc, 3 animals were removed for misplaced cannulae or large lesions at the infusion site and 1 animal was removed as an outlier, leaving final sample sizes of 7 (0 mg/kg) and 11 (10 mg/kg). In the dlBNST, 10 animals were removed for misplaced cannulae or large lesions at the infusion site and 1 animal was removed because of a blocked cannula, leaving final sample sizes of 8 (0 mg/kg) and 8

(10 mg/kg). In the CeA, 8 animals were removed for misplaced cannulae or large lesions at the infusion site and 1 animal was removed as an outlier, leaving final sample sizes of 7 (0 mg/kg) and 11 (10 mg/kg).

Experiment 9: Apomorphine injection during withdrawal from systemic nicotine.

Rats were injected with 0 (N=10) or 0.25 mg/kg (N=10) nicotine for seven days. Because withdrawal from nicotine is less robust, seven days of nicotine exposure are necessary to observe withdrawal-potentiated startle (Engelmann et al., 2009). On days 8, 9, and 10 animals were injected with nicotine or saline at 0 h followed by 0, 50, or 100 µg/kg apomorphine hydrochloride 1 h and 50 min later. Startle was tested at 2 h. A Latin Square design was used so that rats received each dose of apomorphine once over a series of three consecutive test days.

Results

Experiment 5: Apomorphine injection during withdrawal from systemic morphine.

As drug levels spontaneously begin to fall in the VTA after acute opiate exposure, the release of dopamine in target structures also decreases (Spanagel et al., 1992; Acquas and Di Chiara, 1992; Wise et al., 1995; Carboni et al., 2000). To test the hypothesis that the expression of anxiety during opiate withdrawal involves a loss of dopaminergic tone rats were systemically injected with morphine (0 or 10 mg/kg) followed by the dopamine receptor agonist apomorphine 3 h and 50 min later (0, 50, or 100 µg/kg). Startle was tested at 4 h (Figure 8A). Animals given 10 mg/kg of morphine followed by

apomorphine showed a significant dose-dependent decrease in potentiated startle (linear effect of dose $F_{1,29} = 9.09$, $p = 0.006$) (Figure 8B). This effect was not observed in the 0 mg/kg groups ($F_{1,29} = 1.453$, $p = 0.239$).

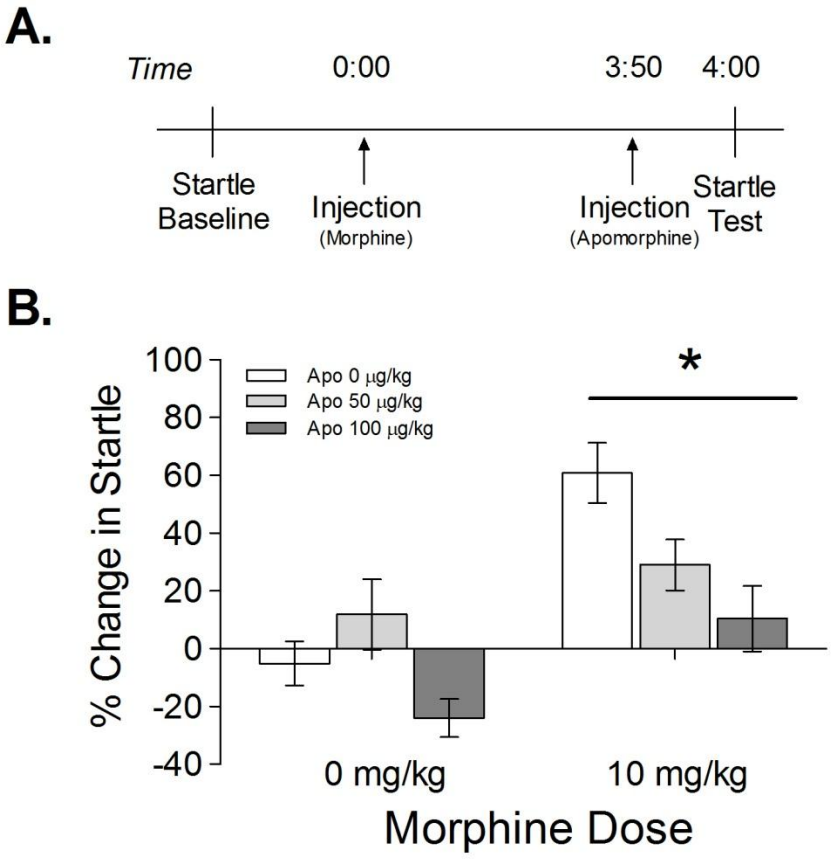

Figure 8. Withdrawal from intra-VTA morphine is dependent on dopaminergic signaling. A) Timeline of test day for Experiment 5. B) Startle potentiation in the morphine group decreased with apomorphine dose, as evidenced by a significant linear trend (*$p < 0.05$).

Experiment 6: SKF82958 or quinpirole injection during withdrawal from systemic morphine.

To determine whether the effect of apomorphine seen in Experiment 4 was mediated by D1- or D2-like dopamine receptors rats were systemically injected with 0 or 10 mg/kg of morphine. Animals received a systemic injection of the D1-like receptor agonist SKF82958 or the D2-like receptor agonist quinpirole (0, 10, or 50 µg/kg) and startle was tested 10-30 min later (Figure 9A). SKF82958 or quinpirole did not significantly decrease morphine withdrawal-potentiated startle at any dose tested (Figure 9B) (Table 6). Both drugs did, however, significantly reduce startle amplitude on their own (SKF 82958 50 µg/kg: Dunnett's test $p = 0.033$; quinpirole 50 µg/kg: Dunnett's test $p = 0.024$).

Experiment 7: SKF82958 and quinpirole cocktail injection during withdrawal from systemic morphine.

Because neither SKF82958 nor quinpirole were able to attenuate withdrawal-potentiated startle on their own, the hypothesis that activation of both D1- and D2-like receptors is necessary for dopamine's anxiolytic effects during withdrawal was tested. Rats were systemically injected with 0 or 10 mg/kg of morphine. The animals received a systemic injection of a cocktail of both SKF82958 and quinpirole (0, 10, or 50 µg/kg of each) 3 h and 30 min later. Startle responding was tested at 4 h (Figure 9A). Animals given 10 mg/kg of morphine followed by the agonist cocktail showed a significant dose-dependent decrease in potentiated startle (linear effect of dose $F_{1,10} = 9.408$, $p = 0.012$)

(Figure 9C). Although the 50 µg/kg dose of the cocktail also slightly decreased startle in animals given 0 mg/kg morphine, this effect was not significant (Table 6).

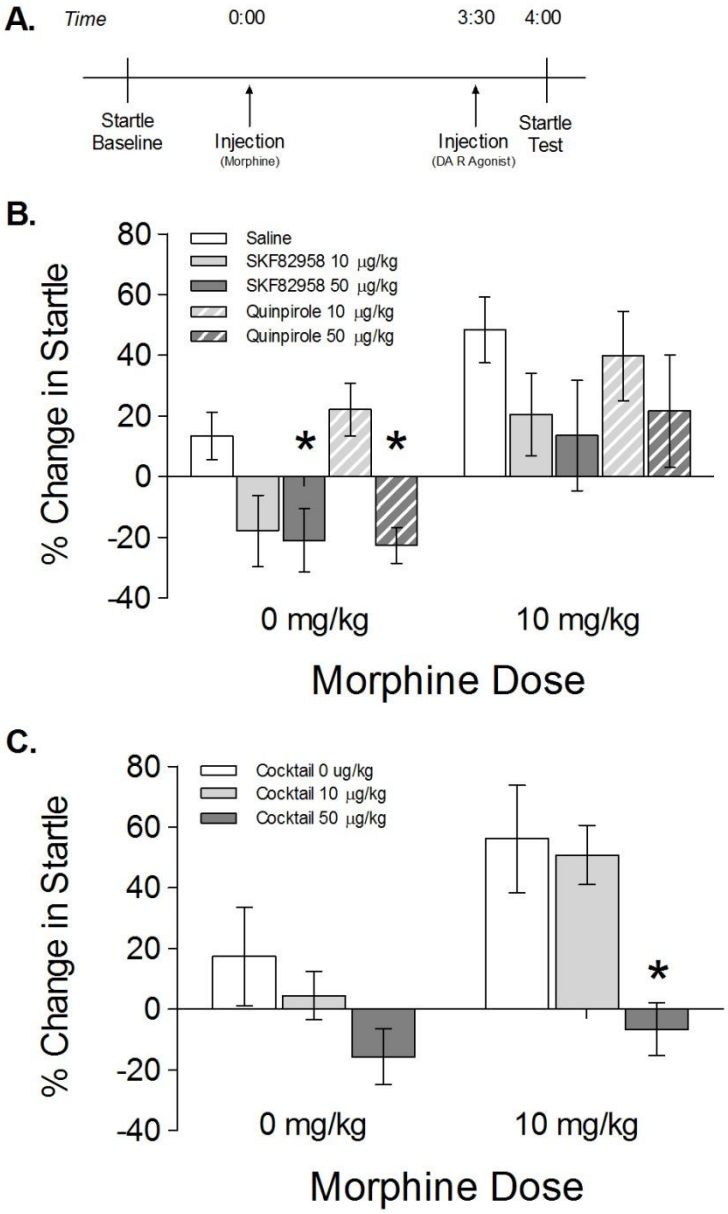

Figure 9. Activation of both D1- and D2-like receptors relieves withdrawal from systemic morphine. A) Timeline of test day for Experiments 6 and 7. B) Withdrawal-potentiated startle was not significantly reduced by SKF82958 or quinpirole treatment, although these treatments did have effects on baseline startle. C) A cocktail of SKF82958 and quinpirole significantly reduced startle potentiation (*$p < 0.05$).

Experiment 8: Apomorphine infusion in local brain structures during withdrawal from systemic morphine.

Experiments 5-7 demonstrated that activation of D1- and D2-like receptors attenuates opiate withdrawal-induced anxiety. To identify the location of the receptors involved in mediating this effect rats were bilaterally implanted with chronically indwelling cannulae targeted at the NAc shell, dlBNST, or CeA. On the test day, animals were injected with 0 or 10 mg/kg morphine and 3 h and 40 min later infused with apomorphine (0, 1, or 5 µg per side) (Figure 10A). Animals that received 10 mg/kg of morphine followed by apomorphine in the NAc showed a significant dose-dependent decrease in potentiated startle ($F_{1,32} = 15.52$, $p < 0.001$) (Figure 10B). No significant effects were observed following apomorphine infusion into the dlBNST or CeA (Figure 10C-D) (Table 6).

Because dopamine is also involved in production of motor behaviors, activity levels were measured during each startle session. Changes in activity after agonist infusion were calculated as percent change from baseline on the same day. The 5 µg dose of apomorphine increased activity levels following intra-NAc shell infusion. This effect was apparent in both saline- (19.9% ± 12.2%) and morphine-treated (33.8% ± 12.9%) animals. No increases in activity were observed following infusion of 5 µg apomorphine into the dlBNST (saline: -21.8% ± 13.2%; morphine: -21.3% ± 6.1%) or CeA (saline: -16.6% ± 13.2%; morphine: -22.4% ± 4.1%).

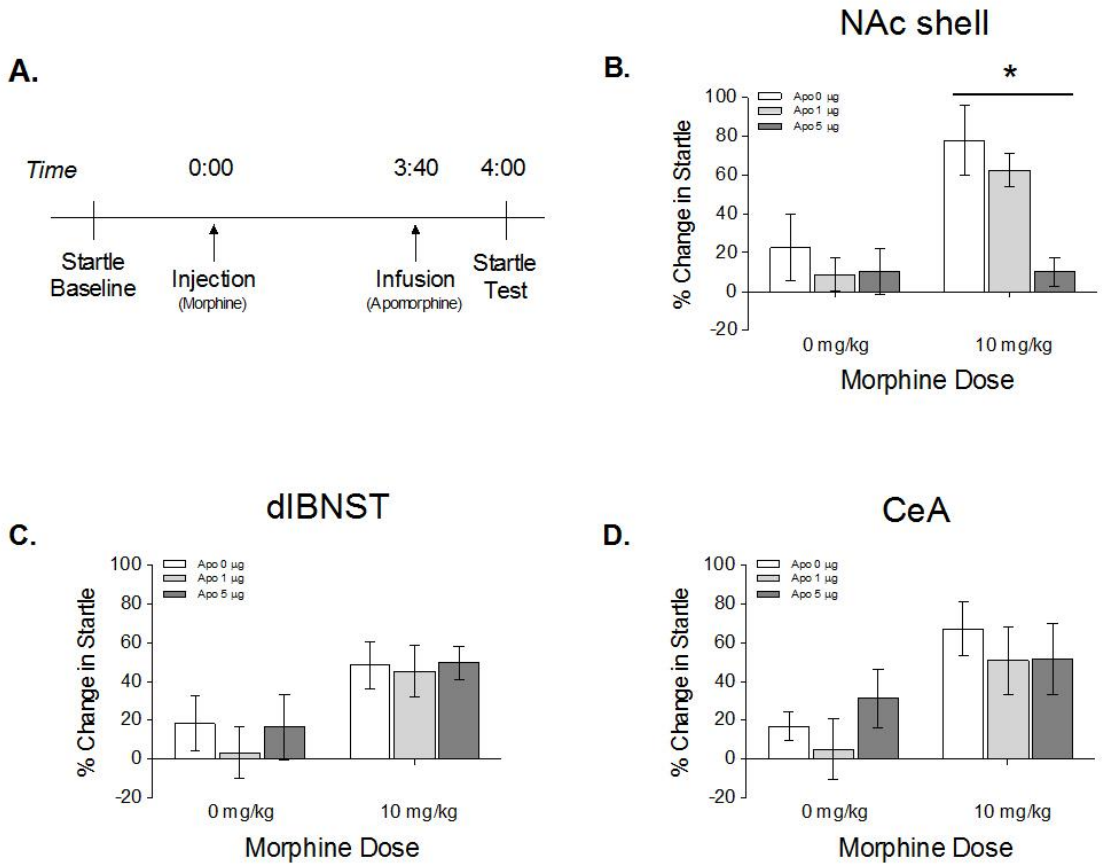

Figure 10. Withdrawal from systemic morphine is dependent on dopaminergic signaling in the shell of the nucleus accumbens. A) Timeline of test day for Experiment 8. B) Startle potentiation was attenuated by apomorphine infusion into the NAc shell, as evidenced by a significant linear trend (*$p < 0.05$). C) Startle potentiation was not reduced by apomorphine infusion into the dlBNST. D) Startle potentiation was not reduced by apomorphine infusion into the CeA.

Experiment 9: Apomorphine injection during withdrawal from systemic nicotine.

Experiment 5 demonstrated that the expression of anxiety during opiate withdrawal involves a loss of dopaminergic tone. To test whether this is also true of withdrawal from another drug of abuse, rats were systemically injected with 0 or 0.25 mg/kg of nicotine for seven days. On the test day, animals received nicotine followed by a systemic injection of apomorphine hydrochloride (0, 50, or 100 µg/kg) 1 h and 50 min later and startle was tested at 2 h (Figure 11A). Animals given 0.25 mg/kg of nicotine showed startle potentiation which was significantly reduced by injection of apomorphine ($F_{1,29} = 9.255$, $p = 0.005$) (Figure 11B). Startle was decreased in the 0 mg/kg nicotine group as well ($F_{1,29} = 6.430$, $p = 0.017$). Removal of the 100µg/kg dose of apomorphine from analysis revealed a significant effect of apomorphine in the 0.25 mg/kg nicotine group ($F_{1,19} = 8.170$, $p = 0.010$) but not the 0 mg/kg nicotine group ($F_{1,19} = 0.671$, $p = 0.424$), demonstrating that the lower dose of apomorphine attenuated withdrawal-potentiated startle without affecting baseline startle.

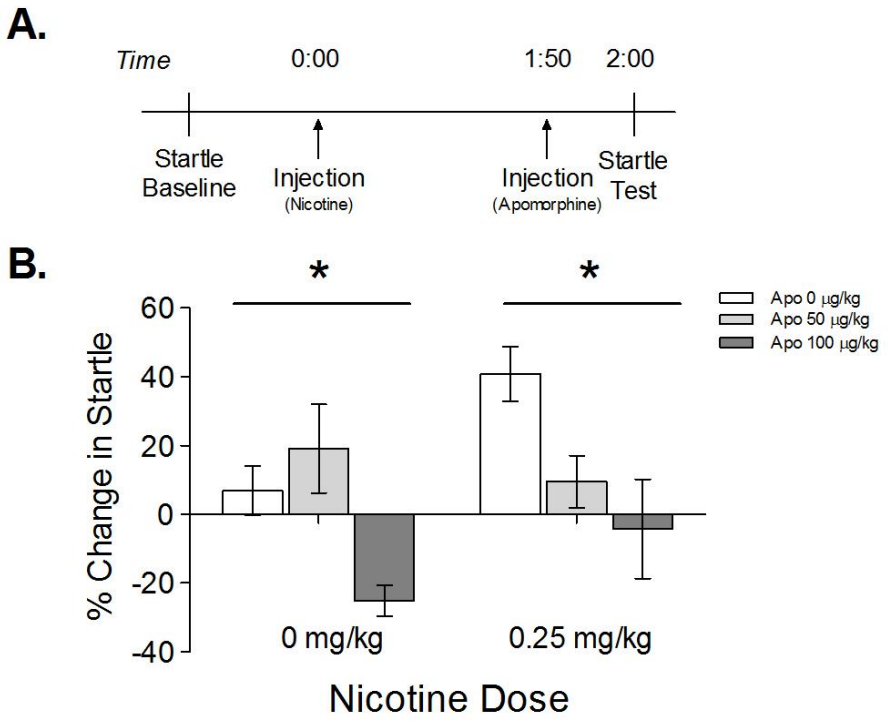

Figure 11. Withdrawal from systemic nicotine is dependent on dopaminergic signaling. A) Timeline of test day for Experiment 9. B) Startle potentiation was attenuated by apomorphine injection, as evidenced by a significant linear trend (*$p < 0.05$). See text for discussion of significant effects in 0 mg/kg group.

Table 5. Mean raw startle output for Experiments 5-9.

	0 mg/kg Morphine	10 mg/kg Morphine
Experiment 5		
0 µg/kg apomorphine	47.4 ± 7.6	43.6 ± 6.3
50 µg/kg apomorphine	31.2 ± 3.8	32.1 ± 3.1
100 µg/kg apomorphine	46.0 ± 7.0	49.2 ± 6.6
Experiment 6		
Saline	45.4 ± 3.5	42.7 ± 4.2
10 µg/kg SKF82958	43.3 ± 6.2	42.5 ± 5.6
50 µg/kg SKF82958	46.7 ± 3.3	43.6 ± 4.6
10 µg/kg quinpirole	33.8 ± 3.9	42.0 ± 7.0
50 µg/kg quinpirole	34.7 ± 3.6	36.5 ± 3.5
Experiment 7		
0 µg/kg cocktail	38.6 ± 4.7	41.2 ± 4.0
10 µg/kg cocktail	40.4 ± 4.1	42.0 ± 4.2
50 µg/kg cocktail	47.5 ± 6.2	45.3 ± 4.3
Experiment 8		
NAc shell		
0 µg apomorphine	33.5 ± 4.0	38.1 ± 4.8
1 µg apomorphine	34.8 ± 4.3	40.4 ± 5.3
5 µg apomorphine	32.1 ± 3.7	37.9 ± 4.9
dlBNST		
0 µg apomorphine	38.5 ± 3.7	29.2 ± 2.2
1 µg apomorphine	37.8 ± 4.3	26.0 ± 2.6
5 µg apomorphine	31.5 ± 5.5	27.1 ± 3.6
CeA		
0 µg apomorphine	38.3 ± 8.5	33.9 ± 3.6
1 µg apomorphine	38.3 ± 4.7	35.9 ± 3.5
5 µg apomorphine	36.5 ± 9.5	36.3 ± 3.0

	0 mg/kg Nicotine	0.25 mg/kg Nicotine
Experiment 9		
0 µg/kg apomorphine	49.6 ± 5.7	46.1 ± 6.8
50 µg/kg apomorphine	51.8 ± 4.7	51.9 ± 9.6
100 µg/kg apomorphine	49.7 ± 6.1	52.0 ± 11.3

Data are average startle values of animals before drug injection.

Table 6. Results of statistical tests for Experiments 5-9.

	Test	Groups	Statistics
Experiment 5			
	Linear effect	0 mg/kg morphine	$F_{1,29} = 1.453$, $p = 0.239$
	Linear effect	10 mg/kg morphine	$F_{1,29} = 9.092$, **$p = 0.006$***
Experiment 6			
0 mg/kg morphine	Dunnett's	0 vs. 10 µg/kg SKF82958	$p = 0.060$
	Dunnett's	0 vs. 50 µg/kg SKF82958	**$p = 0.033$***
	Dunnett's	0 vs. 10 µg/kg quinpirole	$p = 0.873$
	Dunnett's	0 vs. 50 µg/kg quinpirole	**$p = 0.024$***
10 mg/kg morphine	Dunnett's	0 vs. 10 µg/kg SKF82958	$p = 0.500$
	Dunnett's	0 vs. 50 µg/kg SKF82958	$p = 0.303$
	Dunnett's	0 vs. 10 µg/kg quinpirole	$p = 0.978$
	Dunnett's	0 vs. 50 µg/kg quinpirole	$p = 0.536$
Experiment 7			
	Linear effect	0 mg/kg morphine	$F_{1,11} = 3.819$, $p = 0.077$
	Linear effect	10 mg/kg morphine	$F_{1,10} = 9.408$, **$p = 0.012$***
Experiment 8			
NAc shell	Linear effect	0 mg/kg morphine	$F_{1,19} = 0.442$, $p = 0.515$
	Linear effect	10 mg/kg morphine	$F_{1,32} = 15.52$, **$p < 0.001$***
dlBNST	Linear effect	0 mg/kg morphine	$F_{1,23} = 0.009$, $p = 0.925$
	Linear effect	10 mg/kg morphine	$F_{1,23} = 0.006$, $p = 0.941$
CeA	Linear effect	0 mg/kg morphine	$F_{1,20} = 0.278$, $p = 0.816$
	Linear effect	10 mg/kg morphine	$F_{1,32} = 0.437$, $p = 0.541$
Experiment 9			
	Linear effect	0 mg/kg nicotine	$F_{1,29} = 6.430$, **$p = 0.017$***
	Linear effect	10 mg/kg nicotine	$F_{1,29} = 9.255$, **$p = 0.005$***
Without 100 µg group	Linear effect	0 mg/kg nicotine	$F_{1,19} = 0.671$, $p = 0.424$
	Linear effect	10 mg/kg nicotine	$F_{1,19} = 8.170$, **$p = 0.010$***

* Indicates significant result.

Discussion

Experiments 1-3 demonstrated that anxiety during withdrawal from acute opiate exposure is dependent on reduced opioid receptor activity in the VTA. The experiments described in this chapter support the hypothesis that a drop in dopamine receptor activation is the necessary next step in this process. Although a unique contribution of either dopamine receptor subtype alone cannot be ruled out, due to effects on baseline startle, the ability of the general dopamine receptor agonist apomorphine and a cocktail of SKF82958 and quinpirole to completely prevent the expression of opiate withdrawal-potentiated startle suggests that anxiety is dependent on reduced activity at *both* D1- and D2-like receptors. Local infusion of apomorphine into the shell of the NAc also attenuated withdrawal-potentiated startle. This result suggests that transient dopaminergic signaling in the shell of the NAc is necessary for the expression of withdrawal following systemic morphine exposure. Apomorphine infusion into the dlBNST or the CeA, on the other hand, did not affect withdrawal-potentiated startle. Although this finding suggests that reduced dopamine receptor activity in these structures is not *necessary* for the expression of withdrawal-induced anxiety, it does not preclude dopaminergic signaling in either of these structures from contributing to the generation of negative affect, a point which will be discussed in greater detail below. Finally, the ability of apomorphine to attenuate nicotine withdrawal-potentiated startle raises the possibility that changes in dopaminergic activity may represent a shared mechanism involved in the withdrawal syndromes of other classes of abused drugs.

Systemic administration of dopamine receptor agonists significantly decreased baseline startle in Experiments 6 and 9. This effect was surprising as previous reports have shown dopamine receptor agonists increase startle (Davis and Aghajanian, 1976; Meloni and Davis, 1999; Meloni and Davis, 2000). Decreases in baseline cannot account for the results of Experiment 6, since the dopamine receptor agonists did not attenuate morphine withdrawal-potentiated startle anyway, or of Experiment 9, since the effect of apomorphine on nicotine withdrawal-potentiated startle held even when the highest dose of apomorphine was removed from analysis. Additionally, the tendency of the dopamine receptor agonists to decrease baseline startle was modest when compared to the decrease in potentiated startle observed in the morphine/nicotine animals and this difference was not likely due to a floor effect, since other treatments (e.g.: presentation of a "prepulse") can cause much greater inhibition of startle than was observed here. In Experiment 8, infusion of apomorphine into the NAc shell increased locomotor activity, an effect that was not seen in the dlBNST or CeA. This increase in locomotor activity cannot explain the decreased startle potentiation seen in these animals, since treatments that increase locomotor activity in saline-injected animals to a similar or greater degree (e.g.: apomorphine in the current experiment or intra-VTA morphine infusion in Experiment 1) do not cause significant decreases in startle.

The results presented here suggest that expression of anxiety during opiate withdrawal likely coincides with a relative decrease in activation of the mesolimbic dopamine system. Decreased dopaminergic activity has been shown to contribute to the production of negative emotional states (Stinus et al., 1990; Nestler and Carlezon, 2006;

Liu et al., 2008) and manipulation of dopaminergic signaling attenuates signs of opiate withdrawal (Harris and Aston-Jones, 1994; Bechara et al., 1995; Rodríguez-Arias et al., 1999; Laviolette et al., 2002; Chartoff et al., 2006). Importantly, the initial increase in NAc dopamine release following acute exposure to 10 mg/kg of morphine would have largely returned to baseline at the time at which spontaneous withdrawal-potentiated startle is observed (Di Chiara and Imperato, 1988). The current finding that apomorphine infusion into the NAc shell prevents the expression of withdrawal-induced anxiety also agrees with the idea that reduced activity within the VTA to NAc dopamine projection contributes to negative emotional withdrawal behaviors.

The results of the SKF82958 and quinpirole studies support the conclusion that reduced activity at both D1- and D2-like receptors, probably in the NAc shell, is involved in the expression of withdrawal. Both of these receptor subtypes are found in the NAc, though D1-like receptors are more abundant than D2-like receptors (Boyson et al., 1986). While investigations of the contributions of D1- vs. D2-like receptors are limited, there is evidence that activation of either receptor subtype can attenuate the somatic signs of opiate withdrawal (Harris and Aston-Jones, 1994; Walters et al., 2000; Chartoff et al., 2006). D1-like receptor agonists also prevent opiate withdrawal-induced aggression (Tidey and Miczek, 1992; Rodríguez-Arias et al., 1999) and conditioned place aversion (Chartoff et al., 2006), though this latter effect appears to be mediated by receptors in the VTA (Chartoff et al., 2009). One intriguing possibility is that the effects of D1- and D2-like receptor agonists on opiate withdrawal are mediated by a heterodimeric D1-D2 dopamine receptor signaling complex. A D1-D2 heterodimer found in the NAc requires

activation of both receptors to stimulate intracellular signaling (Rashid et al., 2007), which could explain why only the dopamine receptor agonist cocktail completely blocked withdrawal-potentiated startle.

In addition to changes within the mesolimbic dopamine system, the development of an opponent process during drug withdrawal involves "between-systems" adaptations in structures responsible for the expression of negative affect (Koob and Bloom, 1988; Stinus et al., 1990; Harris et al., 2006; Smith and Aston-Jones, 2008). The initial activation of the mesolimbic dopamine system by morphine may be the first step in the recruitment of between-systems adaptations that produce anxiety. Although infusion of a dopamine receptor agonist into the CeA and dlBNST did not attenuate opiate withdrawal-potentiated startle, these results only demonstrate that a reduction in dopaminergic activity is not necessary for anxiety. It is therefore possible that increased dopamine release in one or both of these structures following morphine exposure is responsible for their recruitment during opiate withdrawal (Stinus et al., 1990; Nakagawa et al., 2005; Harris et al., 2006). Dopaminergic signaling in the BLA, which is necessary for the acquisition of fear-potentiated startle (Nader and LeDoux, 1999; Greba and Kokkinidis, 2000; Greba et al., 2001; Fadok et al., 2009), may also trigger extended amygdala activity. Transient release of dopamine in portions of the extended amygdala (Di Chiara and Imperato, 1988; Spanagel et al., 1992; Acquas and Di Chiara, 1992; Wise et al., 1995; Carboni et al., 2000) may trigger increased release of corticotropin-releasing factor and norepinephrine (Guiard et al., 2008; Kash et al., 2008). Additionally, dopaminergic regulation of CRF synthesis in the extended amygdala (Smialowska et al.,

1999; Day et al., 2002; Stewart et al., 2008) could contribute to allostatic changes that mediate increased withdrawal severity following repeated opiate exposure.

Alternatively, the NAc may be responsible for the recruitment of the amygdala and the BNST during opiate withdrawal. Medium spiny neurons of the NAc shell project to the BNST (Nauta et al., 1978; Usuda et al., 1998), and changes in activity there may trigger activity throughout the extended amygdala. The effects on startle potentiation may also occur through a more indirect pathway: the NAc shell projects to the ventral pallidum which in turn projects to the amygdala (Nauta et al., 1978; Haber et al., 1985; Usuda et al., 1998). At least one study has demonstrated that electrical stimulation of the ventral pallidum can modulate the amplitude of the acoustic startle reflex (Li et al., 1999). The NAc shell could also lead to startle potentiation, independent of the amygdala and BNST, via its projections to the pedunculopontine tegmental nucleus (PPTg). The PPTg projects to the startle circuit and has been shown to play a role in spontaneous morphine withdrawal (Koch et al., 1993; Vargas-Perez et al., 2009). Clearly, further research is necessary to determine exactly how the dopaminergic link between the VTA and the extended amygdala is involved in the production of anxiety following acute opiate exposure.

Chapter 5: Corticotropin-Releasing Factor Type 2 Receptors are Involved in Opiate Withdrawal-Induced Anxiety

Introduction

CRF is a 41-amino acid neuropeptide that was initially identified as a crucial component of the hypothalamic-pituitary-adrenal (HPA) axis stress response (Vale et al., 1981; Rivier et al., 1983), but it also has extra-hypothalamic actions that contribute to stress and anxiety behaviors (Heilig et al., 1994). For example, the CRF system is known to be involved in the negative emotional component of withdrawal from chronic opiate exposure (Heinrichs et al., 1995; Stinus et al., 2005) as well as other classes of abused drugs (Sarnyai et al., 1995; Basso et al., 1999; Bruijnzeel et al., 2007), though the mechanisms responsible for increased CRF release in the extended amygdala have yet to be discovered. The experiments described above demonstrate that the dopaminergic neurons of the VTA are important contributors to the expression of opiate withdrawal-induced anxiety. Because these neurons project to extended amygdala structures (Fallon et al., 1978; Hasue and Shammah-Lagnado, 2002; Meloni et al., 2006) and directly appose CRF containing neurons in the CeA (Eliava et al., 2003) and dlBNST (Meloni et al., 2006), it was hypothesized that CRF's actions during opiate withdrawal are triggered by changes in activation of the mesolimbic dopamine system.

Before testing this hypothesis it was necessary to determine whether, as is the case with chronic opiate exposure, the CRF system is involved in anxiety during withdrawal from acute opiate administration. The following experiments investigate the role of the

CRF receptor subtypes, CRF-R1 and CRF-R2, in opiate withdrawal-potentiated startle. These experiments use the μ-opioid receptor antagonist naloxone to precipitate withdrawal two hours after morphine injection (Harris et al., 2004), allowing the timing of withdrawal to be controlled and simplifying the decision about when to administer experimental treatments.

Materials and Methods

Intracranial Cannulation and Infusion

22-gauge guide cannulae (model C313G; Plastics One Products, Roanoke, VA) were implanted unilaterally into the lateral ventricle (AP: 0.0 mm, ML: ±1.2 mm, DV: -3.5 mm from Bregma). Infusions of 2 μL (Swerdlow et al., 1986; Lu et al., 2000) were made over the course of 2 min through 28-gauge infusion cannulae (model C313I; Plastics One Products) with tips that extended 1 mm past the end of the guide.

Experimental Design

Experiment 10: Effects of CRF receptor antagonist infusion on baseline startle.

Rats were injected with saline at 0 h and received an ICV infusion of deionized water vehicle or the CRF-R1 antagonist CP-154,526 (5 μg) or the CRF-R2 antagonist antisauvagine-30 (5 μg) 1 h and 40 min later (Lu et al., 2000; de Groote et al., 2005; Moffett and Goeders, 2007). Startle was tested at 2 h. A Latin Square design was used so that each rat was infused once with each of the three antagonists in a random order over three consecutive test days. Two animals were removed for misplaced or blocked

cannulae, leaving a final sample size of 10.

Experiment 11: CRF receptor antagonist infusion prior to precipitated withdrawal from acute, systemic morphine.

Rats were injected with 10 mg/kg of morphine at 0 h and received an ICV infusion of CP-154,526 or antisauvagine-30 (0 or 5 µg) 1 hr and 40 min later. Naloxone hydrochloride (2.5 mg/kg) was injected 10 min later. Startle was tested at 2 h. A crossover design was used so that each rat was infused with the two doses of each antagonist in a random order over two consecutive test days. Each animal was only tested with one CRF receptor antagonist. In the CP-154,526 experiment, one animal was removed for a misplaced cannula, leaving a final sample size of 7. In the antisauvagine-30 experiment, six animals were removed for misplaced or blocked cannulae, leaving a final sample size of 8.

Experiment 12: CRF receptor antagonist infusion prior to precipitated withdrawal from repeated, systemic morphine.

Rats were injected with 10 mg/kg of morphine for six days. On the seventh day animals were injected with 10 mg/kg of morphine at 0 h and received an ICV infusion of deionized water 1 hr and 40 min later. Naloxone hydrochloride (2.5 mg/kg) was injected 10 min later. Startle was tested at 2 h. On the eighth day the same procedure was followed except animals received an ICV infusion of CP-154,526 or antisauvagine-30 (5 µg). In the CP-154,526 experiment, two animals were removed for misplaced cannulae,

leaving a final sample size of 7. In the antisauvagine-30 experiment, three animals were removed for misplaced or blocked cannulae, leaving a final sample size of 6.

Results

Experiment 10: Effects of CRF receptor antagonist infusion on baseline startle.

To control for the effects of the CRF receptor antagonists CP-154,526 and antisauvagine-30 on baseline startle rats were injected with saline and then infused with the CRF antagonists 1 h and 40 min later (Figure 12A). Startle was tested at 2 h. No significant effects of the antagonists were found (Figure 12B) (Table 8).

Experiment 11: CRF receptor antagonist infusion prior to precipitated withdrawal from acute, systemic morphine.

To test if CRF receptors are involved in withdrawal from acute opiate exposure, rats were injected with 10 mg/kg of morphine. One hour and 40 min later the animals were infused with the CRF-R1 antagonist CP-154,526 or the CRF-R2 antagonist antisauvagine-30. Withdrawal was precipitated with a systemic injection of naloxone hydrochloride (2.5 mg/kg) 10 min later (Figure 13A). Startle was tested at 2 h. CP-154,526 infusion had no significant effects on morphine withdrawal-potentiated startle (Figure 13B) (Table 8). Antisauvagine-30 infusion attenuated potentiated startle after the first ($F_{1,15} = 5.592$, $p = 0.030$), but not the second ($F_{1,15} = 0.556$, $p = 0.468$), morphine exposure (Figure 13C).

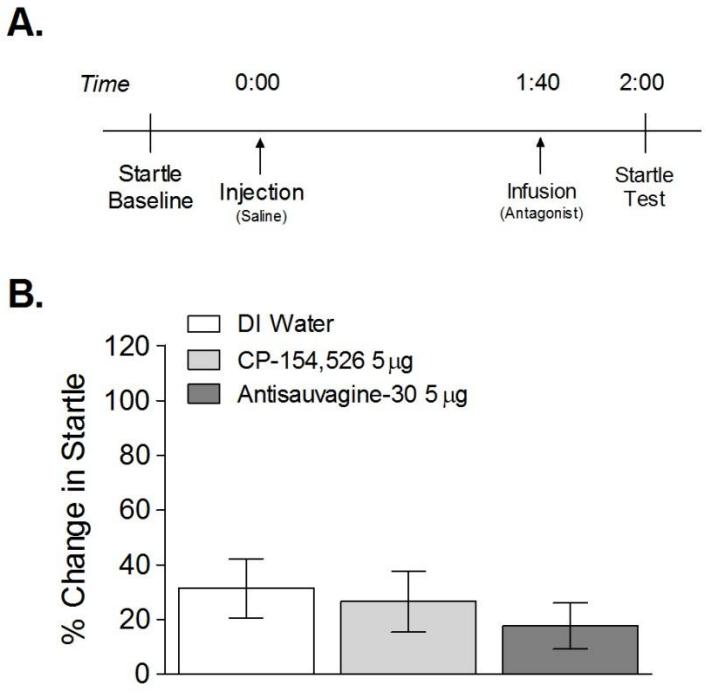

Figure 12. CRF receptor antagonists do not affect baseline startle. A) Timeline of test day for Experiment 10. B) CRF receptor antagonists had no effect on baseline startle.

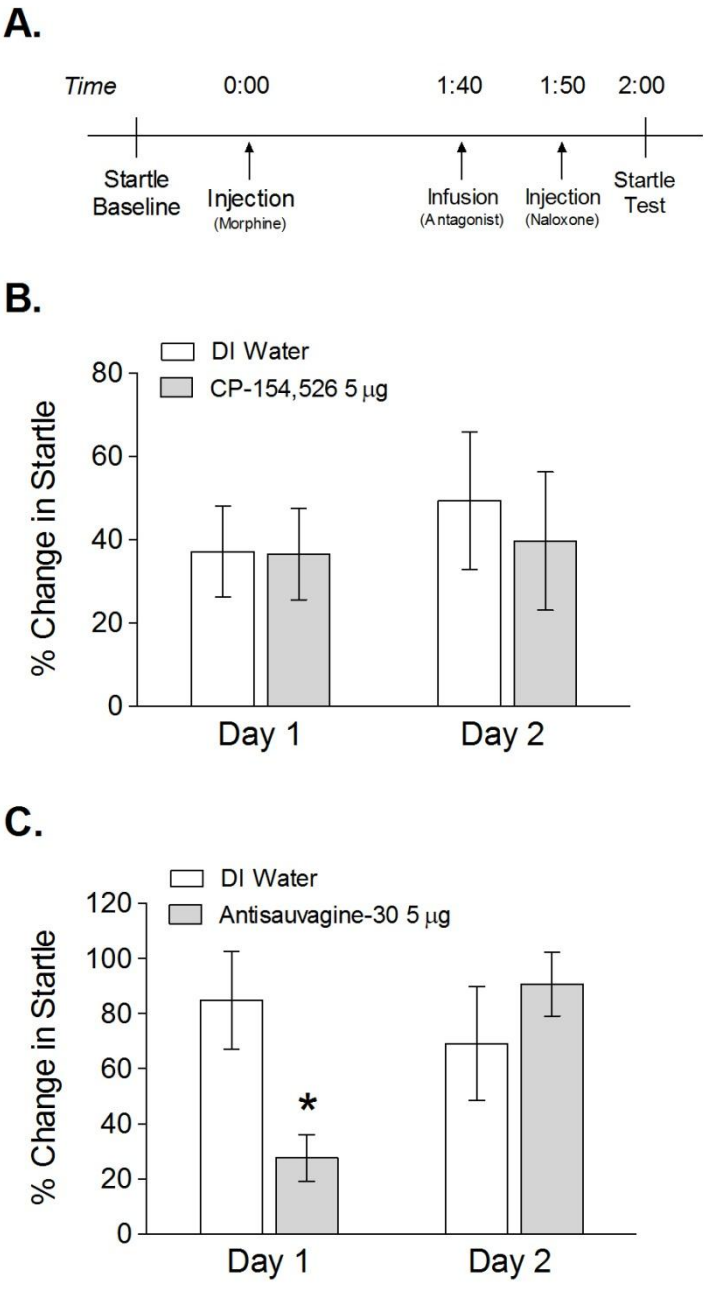

Figure 13. CRF type 2 receptors are involved in withdrawal from acute morphine exposure. A) Timeline of test day for Experiment 11. B) The CRF-R1 antagonist CP-154,526 had no effect on startle potentiation. C) The CRF-R2 antagonist antisauvagine-30 significantly attenuated startle potentiation (*$p < 0.05$).

Experiment 12: CRF receptor antagonist infusion prior to precipitated withdrawal from repeated, systemic morphine.

Because the results of the above studies suggested that there may be a difference in the effects of CRF receptor antagonism after acute versus repeated morphine, the effects of CP-154,526 and antisauvagine-30 following repeated morphine exposure were tested. Rats were injected with 10 mg/kg of morphine for six days and then tested for morphine withdrawal-potentiated startle on the seventh and eighth days (Figure 14A). Potentiated startle following deionized water infusion did not significantly differ between animals treated with CP-154,526 or antisauvagine-30 ($F_{1,12} = 1.033$, $p = 0.331$) and are therefore presented together in Figure 14. Neither CP-154,526 nor antisauvagine-30 infusion had significant effects on morphine withdrawal-potentiated startle (Figure 14B) (Table 8).

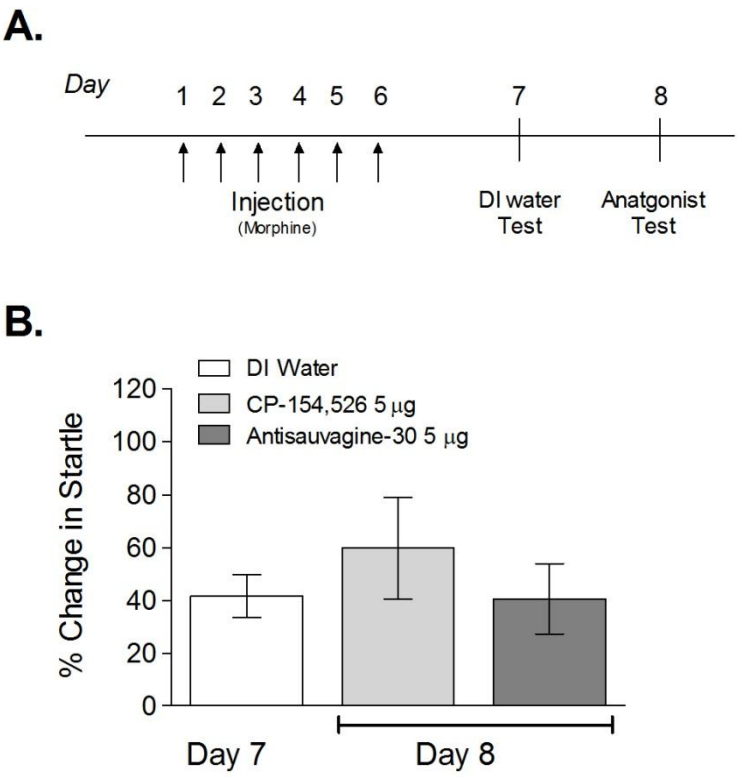

Figure 14. CRF signaling is not involved in withdrawal from repeated morphine exposure. A) Timeline of test day for Experiment 12. B) CRF receptor antagonists had no effect on startle potentiation.

Table 7. Mean raw startle output for Experiments 10-12.

	0 mg/kg Morphine	
Experiment 10		
0 µg antagonist	41.8 ± 4.9	--
5 µg CP-154,526	47.4 ± 7.7	--
5 µg antisauvagine-30	43.9 ± 7.2	--
	Day 1	**Day 2**
Experiment 11		
0 µg CP-154,526	41.1 ± 3.9	37.9 ± 5.4
5 µg CP-154,526	46.9 ± 6.1	36.4 ± 3.8
0 µg antisauvagine-30	35.8 ± 5.4	43.9 ± 10.3
5 µg antisauvagine-30	42.0 ± 7.7	32.4 ± 2.5
		10 mg/kg Morphine
Experiment 12		
0 µg CP-154,526	--	36.6 ± 4.1
5 µg CP-154,526	--	33.8 ± 3.2
0 µg antisauvagine-30	--	38.0 ±10.2
5 µg antisauvagine-30	--	31.6 ± 4.8

Data are average startle values of animals before morphine injection.

Table 8. Results of statistical tests for Experiments 10-12.

	Test	**Groups**	**Statistics**
Experiment 10			
0 mg/kg morphine	Paired t-test	0 vs. 5 µg CP-154,526	$t_9 = 0.313$, $p = 0.761$
	Paired t-test	0 vs. 5 µg antisauvagine-30	$t_9 = 0.962$, $p = 0.361$
Experiment 11			
CP-154,526	RM ANOVA	Day (main effect)	$F_{1,14} = 0.365$, $p = 0.555$
		Day x Infusion (interaction)	$F_{1,14} = 0.582$, $p = 0.458$
		Infusion (main effect)	$F_{1,14} = 0.413$, $p = 0.531$
	One-way ANOVA	0 vs. 5 µg on Day 1	$F_{1,15} = 0.050$, $p = 0.826$
	One-way ANOVA	0 vs. 5 µg on Day 2	$F_{1,15} = 0.719$, $p = 0.411$
Antisauvagine-30	RM ANOVA	Day (main effect)	$F_{1,14} = 3.853$, $p = 0.070$
		Day x Infusion (interaction)	$F_{1,14} = 5.378$, **$p = 0.036$***
		Infusion (main effect)	$F_{1,14} = 1.202$, $p = 0.291$
	One-way ANOVA	0 vs. 5 µg on Day 1	$F_{1,15} = 5.592$, **$p = 0.030$***
	One-way ANOVA	0 vs. 5 µg on Day 2	$F_{1,15} = 0.556$, $p = 0.468$
Experiment 12			
	Paired t-test	0 vs. 5 µg CP-154,526	$t_6 = 0.771$, $p = 0.470$
	Paired t-test	0 vs. 5 µg antisauvagine-30	$t_5 = 0.770$, $p = 0.476$

* Indicates significant result.

Discussion

Experiments 10-12 tested the role of the CRF-R1 and CRF-R2 receptors in the generation of anxiety during withdrawal from acute opiate exposure. CRF-R2 was found to be necessary for the expression of withdrawal-potentiated startle following the first, but not subsequent, morphine exposures. While these results cannot directly support the hypothesis that opiate withdrawal-induced anxiety involves dopaminergic recruitment of CRF systems, they leave open the possibility that, at least early on, this may be one mechanism by which withdrawal behaviors are triggered.

The findings presented here differ from the literature on the CRF system's role in aversive behavior during withdrawal from chronic, continuous morphine exposure, which has found CRF-R1 to be critical (Heinrichs et al., 1995; Stinus et al., 2005). In light of these reports, the inability of either receptor antagonist to reduce startle potentiation after two or more morphine exposures is also surprising. Lack of agreement between previous studies and the current one may point to a fundamental difference between anxiety and aversion or between acute and chronic opiate exposure. The possibility that CRF-R1 receptors are only recruited after drug exposures much more extensive than those administered here seems the most likely, given CRF's and CRF-R1's known role in anxiety behaviors in general and potentiation of the startle reflex in particular (Swerdlow et al., 1986; Heilig et al., 1994; Schulz et al., 1996; Risbrough et al., 2003).

The observation of reduced anxiety after CRF-R2 but not CRF-R1 blockade is also in conflict with the fact that CRF has a 10-fold higher affinity for CRF-R1 (Bale and Vale, 2004). Because CRF released during opiate withdrawal would bind preferentially

to type 1 receptors, it seems unlikely that antisauvagine-30 would attenuate startle potentiation while CP-154,526 would have no effect. The CRF-like peptides urocortin and urocortin II (Ucn and Ucn II), which are endogenous CRF-R2 agonists that produce anxiety-like behaviors in rodents (Sajdyk et al., 1999; Hsu and Hsueh, 2001; Reyes et al., 2001; Pelleymounter et al., 2004), may therefore be preferentially activated during the early stages of opiate dependence. Future studies could investigate this possibility using immunohistochemical techniques or by measuring withdrawal-potentiated startle in Ucn or Ucn II knockout mice.

The identified role of CRF-R2 in acute opiate withdrawal-potentiated startle is consistent with studies that have found this receptor to be involved in the production of anxiety (Ho et al., 2001; Takahashi et al., 2001; Pelleymounter et al., 2002; Hammack et al., 2003; Land et al., 2008), but the fact that antisauvagine-30 only partially attenuates startle potentiation is noteworthy. A higher dose of antisauvagine-30 may have been needed for a complete blockade. The partial potentiation of startle may also reflect the contribution of other neural systems to the induction of anxiety during opiate withdrawal, independent of CRF-R2 receptor signaling. One likely candidate is noradrenergic signaling, which previous work has shown to contribute to opiate withdrawal-potentiated startle (Harris and Gewirtz, 2004; Rothwell et al., 2009).

Although the CRF receptor antagonists were not infused locally, it is likely that the effect observed after ICV antisauvagine-30 infusion is mediated by the lateral BNST. Intracerebroventricular infusion of CRF precipitates anxiety behaviors such as potentiation of the acoustic startle reflex (Swerdlow et al., 1986; Schulz et al., 1996;

Risbrough et al., 2003), and this effect is dependent on the lateral BNST (Lee and Davis, 1997). Additionally, unlike other structures involved in the expression of negative emotion such as the CeA and the NAc, CRF-R2 is expressed in the BNST (Van Pett et al., 2000). Portions of the BNST also lie quite close to the lateral ventricle, which means it would have been affected by the infusion even if other, more lateral structures (e.g.: the CeA or BLA) were not. Release of CRF in the BNST likely causes cellular activation through potentiation of glutamatergic transmission or suppression of inhibitory neurons (Nie et al., 2004; Rainnie et al., 2004; Kash et al., 2008; Giesbrecht et al., 2010).

CRF projections terminating in the BNST originate in the CeA (Erb et al., 2001), which could explain why functional lesions of either of these structures before morphine withdrawal attenuates anxiety (Harris et al., 2006). Interestingly, inactivation of the CeA and BNST was also effective in blocking withdrawal-potentiated startle only on the first, but not the second, day of morphine exposure (Harris et al., 2006). Both the Harris et al. (2006) study and the current one therefore suggest that a single morphine exposure causes plasticity in the neural circuits responsible for anxiety during withdrawal. Such plasticity may be key in producing the behavioral changes associated with the development of drug dependence.

In summary, the experiments presented in this chapter demonstrate that CRF-R2 plays a role in the generation of anxiety during the earliest stages of opiate dependence. In view of other findings on the role of the BNST in anxiety in general and startle potentiation in particular, this effect is most likely dependent on the lateral BNST. The lack of a CRF-R1 effect or of any effect following multiple opiate exposures suggests

that, unlike withdrawal from chronic opiates, withdrawal from acute opiate exposure may, for the most part, occur independently of CRF receptor signaling.

Chapter 6: Conclusions

The work presented here was designed to investigate the neural origins of a negative emotional withdrawal state induced by acute exposure to opiates. Despite the widespread acknowledgement that dopaminergic mechanisms contribute to the recruitment of a number of neural circuits involved in the development and maintenance of addiction – including those mediating reward and motivation, executive control, habit formation, as well as learning and memory – little work has been done to determine whether dopamine is also involved in the recruitment of negative emotional circuits involved in withdrawal (Volkow et al., 2004; Nestler, 2005; Di Chiara and Bassareo, 2007). The twelve experiments detailed above therefore tested the hypothesis that μ-opioid receptor-mediated activation of VTA dopaminergic neurons is responsible for the recruitment of extended amygdala structures involved in the expression of anxiety.

Negative reinforcement theories of drug dependence, such as Solomon and Corbit's opponent process theory and Koob's hedonic allostasis theory, postulate that the negative emotional component of withdrawal contributes to addiction by creating a cycle of negative reinforcement in which individuals administer drug in an attempt to prevent or relieve their symptoms (Solomon and Corbit, 1974; Koob and Bloom, 1988). These theories predict that activation of negative emotional brain circuitry develops in response to activity in reward-related circuits (Solomon and Corbit, 1974; Koob and Bloom, 1988). The experiments described in Chapter 3 tested this prediction by examining whether μ-opioid receptor activity in the VTA alone could produce an anxious withdrawal state, measured as a potentiation of the acoustic startle reflex.

The withdrawal state that emerged following intra-VTA morphine was characteristic of an opponent process, as described by negative reinforcement theories (Solomon and Corbit, 1974; Koob and Bloom, 1988). Anxiety during withdrawal from intra-VTA morphine occurred after the very first morphine exposure, had a delayed onset, and escalated in strength over repeated exposures. This effect was also shown to be dependent on μ-opioid receptor activity and relieved by a second intra-VTA morphine exposure. These results therefore directly support the predictions of the negative reinforcement theories of dependence and begin to elucidate the mechanisms by which opponent hedonic processes are recruited by drug exposure.

In addition to the predictions of negative reinforcement theories, a number of previous anatomical, electrophysiological, neurochemical, and behavioral studies suggest that dopamine neurons in the VTA contribute to the emotional component of opiate withdrawal (Fallon et al., 1978; Acquas et al., 1991; Bechara et al., 1995; Diana et al., 1999; Laviolette et al., 2002; Chartoff et al., 2006; Meloni et al., 2006). The experiments described in Chapter 4 investigated the role of dopamine in opiate, as well as nicotine, withdrawal. Collectively, the results of these experiments demonstrate that reduced dopaminergic signaling in the NAc contributes to the expression of opiate withdrawal-induced anxiety and that this effect seems to require both D1- and D2-like receptor subtypes. A final experiment also demonstrated that dopaminergic mechanisms contribute to anxiety during withdrawal from nicotine.

This last finding raises the possibility that the mesolimbic dopamine system may play an important role in the emotional component of withdrawal from all drugs of abuse,

a hypothesis that is supported by a number of findings in the literature. Activation of the mesolimbic dopamine system is a feature shared by all classes of abused drugs (Di Chiara and Imperato, 1988) and, much like opiate withdrawal, the electrophysiological activity and neurochemical output of dopamine neurons is reduced during withdrawal from ethanol (Rossetti et al., 1991; Rossetti et al., 1992; Diana et al., 1993; Shen, 2003; Rada et al., 2004), nicotine (Hildebrand et al., 1998; Rada et al., 2001; Liu and Jin, 2004), and other stimulants (Parsons et al., 1991; Robertson et al., 1991; Rossetti et al., 1992). Behavioral studies of nicotine have also found that reduced dopaminergic signaling in the NAc is associated with emotional symptoms of withdrawal (Hildebrand et al., 1999; Cryan et al., 2003; Paterson et al., 2007). While future work will be necessary to confirm whether decreased dopaminergic signaling is a common mechanism in the manifestation of withdrawal from all addictive drugs, such a conclusion seems likely given that reduced mesolimbic dopamine system activity seems to be responsible for aversive behavior in general (Acquas et al., 1989; Mark et al., 1991; Calcagnetti and Schechter, 1991; Schechter and Meechan, 1994; Pothos et al., 1995; Liu et al., 2008).

The inability of a dopamine receptor agonist to attenuate withdrawal when infused into the CeA or dlBNST was intriguing, given the large body of work implicating each of these structures in negative affect during opiate withdrawal (Stinus et al., 1990; Kelsey and Arnold, 1994; Gracy et al., 2001; Frenois et al., 2002; Shaw Lutchman et al., 2002; Watanabe et al., 2002a; Watanabe et al., 2002b; Nakagawa et al., 2005; Harris et al., 2006). In light of these previous studies and the fact that both the CeA and dlBNST are key structures regulating potentiation of startle to other fear- and anxiety-provoking

cues (Davis, 2006), it seems likely that these structures play some role in withdrawal-potentiated startle. Then again, Harris et al. (2006) found that inactivation of either the CeA or the BNST did not prevent withdrawal-potentiated startle following a second morphine exposure. Such results are possible if both the CeA and BNST exert similar and redundant effects on startle during opiate withdrawal, but they may also indicate that these structures are not involved in anxiety during the early stages of dependence. Confirmation of this latter possibility would be a surprising discovery and warrants further investigation.

Because withdrawal-induced negative affect involves CRF and noradrenergic signaling in the extended amygdala (Koob and Volkow, 2010) the current studies also sought to determine whether dopamine is responsible for recruiting these mechanisms. Experiment 4 suggests that norepinephrine is not involved in the development of anxiety following intra-VTA morphine. Because most previous studies of opiate withdrawal utilize a chronic, continuous dependence paradigm, the experiments described in Chapter 5 investigated whether the CRF system is recruited during withdrawal from acute opiate exposure. While antagonism of the CRF type 2 receptor attenuated anxiety following the first morphine exposure, no other role for CRF receptor signaling was found. Activation of CRF receptors during withdrawal may therefore only occur after prolonged periods of drug exposure. Future research should investigate whether dopaminergic mechanisms are involved in recruiting CRF systems during withdrawal from chronic, continuous drug exposure.

The finding that the β-adrenergic receptor antagonist propranolol, which blocks withdrawal-potentiated startle following systemic morphine (Rothwell et al., 2009), did not affect withdrawal from intra-VTA morphine suggests that dopaminergic signaling in the extended amygdala is not the only mechanism involved in the expression of anxiety during the early stages of drug dependence. Noradrenergic cells express μ-opioid receptors and are consequently inhibited during opiate exposure (Maldonado, 1997) independent of any changes in the mesolimbic dopamine system. The neurons in the lateral tegmental field (A1 and A2 cell groups) project via the ventral noradrenergic bundle to the BNST and the shell of the NAc (Delfs et al., 1998; Aston-Jones et al., 1999; Delfs et al., 2000) and lesion of these fibers or blockade of β-adrenergic receptors in the BNST attenuates opiate withdrawal-induced aversion (Delfs et al., 2000) and stress-induced reinstatement of cocaine seeking (Shaham et al., 2000; Leri et al., 2002). Based on this evidence and the results of Experiment 4, it is likely that noradrenergic and dopaminergic mechanisms work in parallel to produce emotional signs of withdrawal. Future studies could evaluate whether the noradrenergic system is recruited directly by opiates by assessing withdrawal following morphine infusion into the lateral tegmental field.

Another potential mediator of withdrawal-induced anxiety that was not investigated in the present experiments is the κ-opioid receptor and its endogenous agonist dynorphin. Production of dynorphin by NAc medium spiny neurons is increased during opiate withdrawal (Turchan et al., 1997) and activation of κ-opioid receptors reduces dopamine release in the NAc (Spanagel et al., 1992; Xi et al., 1998).

Additionally, activation of κ-opioid receptors is anxiogenic in a number of paradigms (Marin et al., 2003; Narita et al., 2006; Knoll et al., 2007; Bruchas et al., 2009; Wittmann et al., 2009; Carr and Lucki, 2010; Knoll et al., 2011). Decreases in dopaminergic signaling responsible for withdrawal-induced anxiety may therefore be mediated by activity at κ-opioid receptors. Recruitment of κ-opioid receptors may also occur downstream of the NAc shell, as they are expressed in the BNST and the amygdala (Mansour et al., 1994). Given the limited involvement of both norepinephrine and CRF in producing withdrawal-potentiated startle following intra-VTA morphine demonstrated here, the dynorphin/κ-opioid receptor system may be a promising avenue for future investigations into the downstream mediators of the dopamine system's involvement in withdrawal.

The behavioral protocol used here differentiates these studies from previous investigations of the negative emotional component of withdrawal. Drug exposure involves intrinsic withdrawal episodes which likely contribute to the development of dependence and these "daily" withdrawals occur spontaneously after every drug exposure (Dole et al., 1966; Koob and Le Moal, 1997; Kreek, 2000; Baker et al., 2004). The current experiments simulated these conditions by administering small, acute doses of morphine and allowing withdrawal to occur spontaneously. These studies therefore offer insight into the neural mechanisms of the type of withdrawal states that participate in the acquisition and maintenance of addictive behavior. The finding that anxiety during withdrawal is dependent on changes in dopamine signaling suggests that negative reinforcement of drug dependence is initiated by the same circuitry as the positive

reinforcing effects of drugs. The results of Experiment 2, in which withdrawal was relieved by a subsequent intra-VTA morphine exposure, also suggest that a mechanism for supporting negative reinforcement exists within the mesolimbic dopamine system. These results therefore imply – contrary to received wisdom – that self-administration of drugs into the mesolimbic dopamine system may be driven by negative reinforcement (Bozarth and Wise, 1981; Hoebel et al., 1983; Devine and Wise, 1994; Gatto et al., 1994; David and Cazala, 1994; Carlezon et al., 1995). Future studies could further investigate whether positive and negative drug-induced emotional states rely on shared circuitry by correlating drug self-administration with emotional symptoms of withdrawal.

Another unique feature of the current experiments is the use of the acoustic startle reflex to investigate anxiety-like behavior during withdrawal. The majority of previous studies have utilized conditioned place aversion to study the aversive, or dysphoric, symptoms of the negative emotional component of withdrawal. As discussed throughout this thesis, the experimental findings of studies using conditioned place aversion largely agree with the results presented here: manipulations of the dopamine system prevent withdrawal-induced aversion (Bechara et al., 1995; Laviolette et al., 2002; Chartoff et al., 2006; Chartoff et al., 2009) and reduced dopamine transmission causes place aversion in drug naïve subjects (Acquas et al., 1989; Mark et al., 1991; Calcagnetti and Schechter, 1991; Schechter and Meechan, 1994; Pothos et al., 1995; Liu et al., 2008). It should be noted, though, that withdrawal-potentiated startle and conditioned place aversion may not measure the same underlying emotional construct. Indeed, it has previously been shown that startle potentiation following systemic morphine exposure occurs at a time when

rodents still demonstrate a conditioned place *preference* (Rothwell et al., 2009). Place aversion during spontaneous withdrawal seems to be induced only by long-term opiate exposures that would produce prolonged decreases in dopamine signaling below baseline (Acquas et al., 1991; Crippens and Robinson, 1994). Spontaneous potentiation of the startle reflex, on the other hand, occurs following opiate exposures that result in smaller, more transient changes in which dopamine levels rise and then return back to baseline (Di Chiara and Imperato, 1988). Startle potentiation may therefore represent an earlier, more modest version of the emotional withdrawal state captured by place aversion. The ability to detect such small changes in an animal's hedonic state make withdrawal-potentiated startle an ideal paradigm for investigating the neural mechanisms of the early stages of drug dependence.

The current experiments are some of the first to investigate the role of the mesolimbic dopamine system in anxiety during drug withdrawal, and while they are a good starting point, they consequently cannot on their own completely validate the hypotheses made in this thesis. To determine whether dopaminergic signaling is involved in withdrawal from long-term drug use, future experiments should manipulate the regimen of drug exposure (e.g.: chronic, continuous). Examining withdrawal following periods of protracted abstinence will also reveal whether the same mechanisms described here contribute to relapse. Furthermore, manipulating the class of drug administered as well as the withdrawal behaviors assessed will help determine whether dopaminergic recruitment of the extended amygdala is a general feature of drug withdrawal. It will also be important to uncover the mechanisms by which dopaminergic

activity in the shell of the NAc leads to recruitment of other extended amygdala structures and how these mechanisms differ in naïve vs. dependent subjects.

Finally, drugs of abuse target brain circuits involved in natural, adaptive behaviors. The ability of morphine to trigger negative emotional states through activation of the mesolimbic dopamine system therefore suggests that this system plays a larger role in the generation of negative affect. This conclusion is supported by a growing body of literature demonstrating the role of the mesolimbic system in fear conditioning (Borowski and Kokkinidis, 1996; Nader and LeDoux, 1999; Pezze and Feldon, 2004; Fadok et al., 2009; Muschamp et al., 2011; Mileykovskiy and Morales, 2011), anxiety (Fride and Weinstock, 1988; Barrot et al., 2002; Barrot et al., 2005; Meloni et al., 2006; Rezayof et al., 2009), conditioned taste aversion (Mark et al., 1991; Fenu et al., 2001), responses to stress (Geyer and Segal, 1974; Herman et al., 1982; Inglis and Moghaddam, 1999; Belda and Armario, 2009), and nociception (Gear et al., 1999; Becerra et al., 2001; Barrot et al., 2002). Dopamine is hypothesized to make neutral stimuli motivationally relevant (Berridge and Robinson, 1998; Wise, 2004) and these studies emphasize that this is true regardless of whether a stimulus carries a positive or negative valence. While the neural circuits responsible for positive and negative affect are largely studied separately, experiments like the ones described herein are a reminder that these circuits are inexorably intertwined. This point is further highlighted by findings from the human literature that variations in the D2 dopamine receptor are associated with increased incidences of anxiety disorders (Peroutka et al., 1998; Schneier et al., 2000; Lawford et al., 2006) and that Parkinson patients receiving dopamine

replacement therapy can develop a withdrawal syndrome characterized by symptoms of anxiety, dysphoria, and depression (Rabinak and Nirenberg, 2010). Continued study of how positive and negative emotional circuits complement and promote one another will therefore be necessary for a full understanding of emotional and motivational behaviors and treatment of disorders ranging from anxiety and depression to Parkinson disease to drug addiction.

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