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Opiate-Induced Neuroplastic Alterations to Dopamine Signaling in the Basolateral Amygdala-Prefrontal Cortical Pathway

Laura G. Rosen
The University of Western Ontario

Supervisor
Steven Laviolette
The University of Western Ontario Co-Supervisor
Walter Rushlow
The University of Western Ontario

Graduate Program in Neuroscience
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Abstract

Opiate addiction is a chronic disorder with high rates of relapse. The failure to maintain sobriety after prolonged abstinence is believed to be due in part to the persistence of potent memories associated with the drug-taking experience. Activation of these memories by re-exposure to drug-related cues can trigger craving in many individuals. Thus, understanding the neurobiological processes underlying the formation of these memories may provide insight into the persistence of addiction. The mammalian basolateral amygdala (BLA) and medial prefrontal cortex (mPFC) comprise a functionally interconnected circuit that is critical for processing opiate-related associative memories. There is some evidence that chronic opiate exposure results in alterations to the function of dopamine (DA) D1 and D2 receptors and their intracellular targets within the BLA, but critical questions remain in regards to these effects within the BLA-mPFC circuit. For instance, opiate-induced alterations to intra-mPFC DA signaling in the context of associative opiate memories has not yet been explored. Furthermore, the role of the DA D3 receptor has not yet been investigated. Finally, there is little understanding of the temporal dynamics underlying these changes in DAergic signaling. Using behavioural models of associative memory formation (conditioned place preference and conditioned place aversion) paired with analyses of protein expression, we further characterized how chronic opiate exposure results in neuroplastic changes to DA receptor expression and signaling in the BLA-mPFC pathway. Here, we report that chronic opiate exposure results in a series of alterations to D1, D2 and D3 signaling within the BLA-mPFC circuit in the context of both opiate reward and withdrawal aversion memories. Specifically, we highlighted the importance of D2 and CaMKII α signaling within the mPFC, identified the role of intra-BLA D3-Cdk5-calcineurin signaling in reward and aversion memory formation, and temporally mapped opiate-induced alterations to intra-BLA memory molecules. Together, these results provide a more complete understanding of how opiate exposure profoundly alters DA signaling between the dependent and non-dependent states. Interestingly, we found that many of the changes induced by chronic opiate exposure are not only transient, but may be functionally reversible, thus providing an avenue for future development of pharmacological interventions for opiate addiction.

Keywords

addiction, associative memory, aversion learning, basolateral amygdala, conditioned place aversion, conditioned place preference, D1 receptor, D2 receptor, D3 receptor, dopamine, medial prefrontal cortex, opiate addiction, opiates, reward learning, western blotting

Co-Authorship Statement

Chapter 2:

Entitled “Opiate Exposure State Controls a D2-CaMKII α -Dependent Memory Switch in the Amygdala-Prefrontal Cortical Circuit” was written by Laura G. Rosen with inputs from Steven R. Laviolette and Walter J. Rushlow. Experimental procedures and data analysis were performed by Laura G. Rosen with assistance from Jordan Zunder, Justine Renard, Jennifer Fu and Walter Rushlow. Steven R. Laviolette and Walter J. Rushlow provided intellectual input.

Chapter 3:

Entitled “Opiate exposure state controls dopamine D3 receptor and cdk5/calcineurin signaling in the basolateral amygdala during reward and withdrawal aversion memory formation” was written by Laura G. Rosen with inputs from Steven R. Laviolette and Walter J. Rushlow. Experimental procedures and data analysis were performed by Laura G. Rosen and Walter J. Rushlow. Steven R. Laviolette and Walter J. Rushlow provided intellectual input.

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List of Abbreviations

AC	Adenylyl cyclase
ANOVA	Analysis of variance
BLA	Basolateral amygdala
cAMP	Cyclic adenosine monophosphate
CaMKII	Ca ²⁺ /calmodulin-dependent protein kinase II
Cdk5	Cyclin-dependent kinase 5
CPA	Conditioned place aversion
CPP	Conditioned place preference
DA	Dopamine
DARPP-32	Dopamine- and cAMP-regulated phosphoprotein, Mr 32 kDa
ERK1/2	Extracellular signal-regulated kinases 1/2
GABA	γ -Aminobutyric acid
mPFC	Medial prefrontal cortex
NAc	Nucleus accumbens
NMDA	<i>N</i> -Methyl-D-aspartate
VTA	Ventral tegmental area

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Chapter 1

1 General Introduction

1.1 INTRODUCTION TO OPIATE ADDICTION

1.1.1 Epidemiology

Opiate addiction is a serious and growing health crisis, due in large part to the over-prescription of opiates for pain and other health problems (Rudd et al., 2016). As of 2010, an estimated 15.5 million people worldwide are dependent on opioid drugs (Degenhardt et al., 2014). The picture in Canada is even more alarming. Canada leads the world in prescription opiate consumption per-capita (Moore et al., 2017), and Ontario is recognized world-wide as being in a prescription-opiate crisis (Gomes et al., 2011; Lynas, 2013). Opiate overdoses have become the leading cause of death for Ontarians aged 18-35 (Gomes et al., 2014).

1.1.2 Opiate Addiction

The opiate class of drugs can act on any of the body's three major opiate receptor classes in the central and peripheral nervous system: δ , κ , and μ . Opiates such as morphine, heroin and synthetic opiate analogues, act preferentially on the μ opiate receptor system. Opiates can include extracts from the poppy seed (e.g. morphine) as well as semi-synthetic (e.g. heroin) and synthetic (e.g. oxycodone, fentanyl) drugs.

Opiates are highly effective in treating chronic pain, but also possess high abuse and addiction potential due to their ability to elicit intense feelings of pleasure or euphoria (Fields and Margolis, 2015). Repeated use and abuse of opiates leads to neuroadaptations that result in both physical and psychological dependence and, in turn, continued drug use (Koob and Le Moal, 2001). Opiate dependence can increase the risk for other serious health problems such as HIV/AIDS and hepatitis C as a result of injection drug use (Nabipour et al., 2014). Those who abuse opiates are also more prone to social marginalization (Raketic et al., 2013) and illegal activity, as users turn to whatever means possible to obtain the drug (Fischer et al., 2005).

With repeated use, individuals can rapidly develop tolerance and require escalating doses of the drug to experience the desired effects. This is accompanied by dependence, which

is typically discovered in individuals after the cessation of drug use (Le Merrer et al., 2009). The resulting withdrawal state is reflected by severe withdrawal symptoms, such as nausea, pain, feelings of unease and drug craving (Koob, 2009).

The characteristics of the addictive process can be broadly divided into three stages: binge/intoxication, withdrawal/negative affect, and preoccupation/anticipation (Koob and Volkow, 2016). Perhaps most notably, addiction results in continued and compulsive drug use despite negative social, economic and/or health consequences.

1.1.3 Treatment

There is no known cure for addiction (Kalivas and Volkow, 2005). There are, however, many pharmacological treatments available to help minimize withdrawal symptoms and ease the transition away from opiate dependence.

Opiate agonist treatments are perhaps the most common of these medications, and include methadone and buprenorphine. Opiate agonist treatments are widely regarded as effective for reducing withdrawal symptoms, opioid craving, and criminal activity associated with drug seeking (Zanis and Woody, 1998; Kleber, 2008; Huhn et al., 2014). However, they are costly, carry abuse potential and are not completely effective in preventing relapse (Mitchell et al., 2009; Cotton et al., 2017).

1.1.4 Relapse: A Moral Weakness?

Rates of relapse are enormously high for people that have abused opiates, regardless of the length of time spent abstinent. Even following rehabilitation and detoxification, relapse to heroin can be as high as 85% following successful completion of treatment programs (Hunt et al., 1971). Anxiety, dysphoria and stress are common factors that can result in drug craving and relapse to drug use. For some individuals, relapses can occur years beyond the cessation of drug use and the experience of withdrawal (McLellan et al.,

2000). As a result, scientists and clinicians have expressed the need for a different approach to treating addiction (Fischer et al., 2016).

One particularly powerful trigger for craving is exposure to opiate-related stimuli. Due to their highly rewarding nature, the use of opiate drugs results in the formation of potent, euphoric memories of stimuli associated with the drug-taking experience. These can range from paraphernalia to people, locations and even olfactory cues (Hone-Blanchet et al., 2014). High rates of relapse, failed treatment programs and an inability for people to “choose” to stop drug use have led to a strong perception of addiction as a moral failing or personal weakness (Harding, 1986). However, researchers have uncovered a more compelling explanation for the persistence of addiction, even in the most well-intentioned individuals.

1.2 CONCEPTUALIZING ADDICTION: CLINICAL AND NEUROBIOLOGICAL MODELS

1.2.1 Disease Model of Addiction

With the development of neuroscience research, theories behind the persistence of addiction moved away from placing blame on maladaptive personality traits, and towards viewing addiction as a brain disease with underlying physiological abnormalities (Leshner, 1997; Wise, 2004; Hyman, 2005). There is a considerable body of research that has investigated this hypothesis in an effort to uncover the precise mechanisms of the brain disease model of addiction.

Evidence has emerged that the addicted and the non-addicted brain differ in specific, measurable ways such as alterations to receptor availability, gene expression and responses to environmental stimuli (Self, 2004; Thomas et al., 2008; Oever et al., 2012). In response to these scientific advances, a variety of hypotheses have further been proposed in an effort to explain the development and persistence of opiate addiction.

1.2.2 The Dopamine Reward Hypothesis of Addiction

The dopamine (DA) reward hypothesis was developed by Roy Wise in the 1980s, and posits that DA within the brain is critical for subjective pleasure gleaned from positive rewards (Wise, 2008). The hypothesis is based on the assumption that behaviour can be controlled by reward and punishment. Early support for a biological basis of this notion came from studies that humans would both work for, and gain pleasure from, electrical stimulation of targeted forebrain regions, among them the lateral hypothalamus (Sem-Jacobsen, 1959; Heath, 1963). Wise and Stein (1969) shortly thereafter identified DA as the neurotransmitter responsible for transmitting the reward signals that influence behaviour.

The DA reward hypothesis states that DA release initiated by exposure to rewarding stimuli is crucial for the habit-forming properties of addictive drugs (Wise and Bozarth, 1982). Wise proposes that users seek out drugs to experience a degree of pleasure (i.e. a release of DA) far greater than what is provided by natural stimuli such as food. Following the initial exposure, reward-related stimuli can then incite a motivational drive, where users are driven to further seek out the rewarding stimulus. Repeated DA-release by subsequent exposures to the stimulus further “stamp-in” the motivational importance of the stimulus (Wise, 2004). However, the eventual finding that aspects of morphine and nicotine reward could be DA-independent suggested that the hypothesis was an incomplete model for the development of opiate addiction.

1.2.3 The Opponent-Process Model of Addiction: Addiction as Hedonic Allostasis

The opponent-process model of addiction places heavy weight on the role of emotional states in the development of addiction. Drugs of abuse can elicit a range of emotions from potent euphoria to overwhelming dysphoria. George Koob proposes that the experience of such emotional extremes disrupts emotional homeostasis, and results in the emergence of an allostatic state.

Both positive reinforcement (i.e. euphoria experienced by drug taking) and negative reinforcement (i.e. experience of withdrawal) are hypothesized to contribute to allostatic changes to both stress and reward systems within the brain (Koob, 2015). As an individual transitions from impulsive drug use to compulsive drug use, the motivation underlying drug seeking and drug taking is proposed to be driven less by positive reinforcement, and increasingly by negative reinforcement.

The mechanism underlying this transition is believed to be a decrease in the responsiveness of the brain's reward system and an overactivation of anti-reward systems (Ahmed and Koob, 2005). The neurobiological underpinnings of this hypothesis have been identified as multiple counter-adaptive opponent processes (Koob and Le Moal, 2001). The dysfunction of reward has been linked to the ventral striatum, and the emergence of stress systems has been localized to the amygdala. The combination of decreased reward transmission paired with activation of the stress systems is believed to be a major motivation for the re-emergence of drug seeking and drug taking behaviours (Koob et al., 2004).

1.2.4 The Incentive Saliency Model of Addiction

The incentive saliency model of addiction posits that addiction is a result of an over-attribution of the incentive saliency of drugs resulting from drug-induced sensitization of reward pathways.

In vulnerable individuals, repeated exposure to potentially addictive drugs can alter the brain circuits that regulate the attribution of incentive saliency to stimuli. As a result, these circuits are rendered hypersensitive (Robinson and Berridge, 1993). This hypersensitivity results in pathologically high levels of incentive saliency being attributed to the drug and their related cues (Robinson and Berridge, 2008).

In this model, the incentive sensitization can result in the persistence of desire for drugs for years, a phenomenon which can persist even long after drug use has ceased. Both unconscious desire for the drug and the conscious cravings are proposed to be

manifestations of sensitized incentive salience. The neurobiological underpinnings of the incentive salience model are situated within the mesocorticolimbic system (Berridge and Valenstein, 1991).

1.2.5 Addiction as a Disorder of Learning and Memory

Disorders of memory are most commonly associated with those of memory loss, with Alzheimer's disease and other dementias representing the most salient examples. There is, however, the possibility that memory may also be disordered by forming associations that are too powerful, leaving individuals unable to forget certain memories. This can be particularly disruptive in people who have formed associations that are maladaptive (Hyman, 2005).

Opiate abuse can result in the formation of euphoric memories related to the drug-taking experience. These highly potent memories are easily triggered by drug-related cues, are able to produce strong subjective craving and can therefore be a major factor in driving drug use. More specifically, the drug-taking experience can become motivationally significant as a result of association with environmental cues via Pavlovian conditioning (Gawin and Kleber, 1986; O'Brien et al., 1998). To users, drug-related cues possess the ability to predict drug availability, trigger memories of the drug's effects, generate cravings (even after long periods of abstinence) and prompt drug-seeking behaviours (Wikler, 1984; Grant et al., 1996; Childress and Mozley, 1999; Garavan et al., 2000; Robbins and Everitt, 2002; Volkow et al., 2012). These cues can include both internal (i.e. somatic sensations, including withdrawal) and external (i.e. environments, drug paraphernalia and even people) stimuli associated with drug use (Hyman et al., 2006).

This idea has been corroborated by evidence that addicts tend to relapse in settings associated with prior drug use, and have an easier time remaining abstinent in contexts without a previous association with drug use (Lüscher, 2016). For instance, heroin-addicted Vietnam veterans had much higher success rates staying abstinent upon return to the US as compared to local addicts (see Robins, 1993 for review).

Research has suggested that just as drugs of abuse act on normal mechanisms of reward processing, they also potently activate normal mechanisms of reward learning (Hyman et al., 2006). It is therefore possible that just as opiates over-activate reward systems, they also trigger the formation of highly potent reward memories that are stronger than those formed for natural reinforcers (Robbins and Everitt, 1999; Everitt and Robbins, 2005; Belin et al., 2013).

In their model of the development of addiction, Koob and Le Moal (2001) emphasize the importance of learning and reinforcement in the transition from voluntary drug use to loss of control and compulsive use (i.e. addiction). They describe addiction as an endpoint of the transition from occasional voluntary use to the loss of control, and underscore drug seeking as being representative of a state where addicts are driven by the activation of drug-related memories by drug cues in a stimulus-response manner (Robbins and Everitt, 1999; Everitt and Robbins, 2005; Belin et al., 2013; Everitt, 2014).

Specifically, they highlight the ability of even initial drug seeking and self-administration to drive incentive learning, and increase the desire for reward derived from external stimuli (Robinson and Berridge, 2008; Berridge et al., 2009). From there, controlled and, eventually habitual, drug use provides the opportunity for conditioned reinforcement between drug-induced euphoria and drug-related stimuli. Heavy intoxication and binges are proposed as mechanisms for further disrupting cognition, and eventually lead to the failure of executive control and, ultimately, addiction. Opiate drugs, given their particularly powerful ability to elicit euphoria, can result in an especially rapid progression to the addictive state.

1.2.6 Testing Reward Learning in Animal Models

In order to test the validity of this hypothesis of addiction and understand its cellular and molecular mechanisms, the use of animal models of addiction is vital. Two of the most common preclinical ways to probe the formation and expression of associative drug

memories in animal models are the conditioned place preference (CPP) and conditioned place aversion (CPA) paradigms.

CPP involves training an animal in either a drug- or a vehicle- paired environment, then testing whether the animal forms a preference for the drug-paired environment, as demonstrated by the expression of drug-seeking behaviour when placed into the drug-paired environment in a drug-free state (Bechara and van der Kooy, 1989; Nader and van der Kooy, 1997; Bechara et al., 1998). The CPP paradigm is the most popular tool for assessing the learning processes involved in the development and expression of context-drug associations (Bardo and Bevins, 2000), and is based on the assumption that animals will spend more time in an environment where they previously received drugs than in one where they received only a vehicle solution (Bardo and Bevins, 2000; Hyman et al., 2006).

Conversely, CPA measures the negative affective properties of subjective opiate withdrawal (Stinus et al., 2000; Azar et al., 2003; Wang et al., 2012). Here, animals are trained in one environment in a state of withdrawal. Upon testing, animals will typically spend less time in the withdrawal environment than an environment with no previous association.

Indeed, preclinical evidence has found that chronic exposure to drugs of abuse results in alterations to the neural mechanisms underlying both CPP and CPA processing (reviewed in Tzschentke, 2007; see section 1.6). One of the major areas of research surrounding how drug addiction alters learning and memory processing involves studies of DAergic function (Berke and Hyman, 2000). Given its role in the processing of reward, motivation, and cognition, the mesocorticolimbic DA system has thus become a major target of addiction research.

1.3 THE NEUROANATOMICAL BASIS OF OPIATE ADDICTION: THE MESOCORTICOLIMBIC PATHWAY

All drugs of abuse act on the mesocorticolimbic pathway in some way. The mesocorticolimbic system is a major DA circuit that consists of a number of interconnected regions (See Fig 1.1) Together, this system processes subjective pleasure, motivation, reward learning and associated behavioural responses. Unsurprisingly, dysfunction of the mesocorticolimbic system has been found both in addicted humans and in preclinical models of addiction.

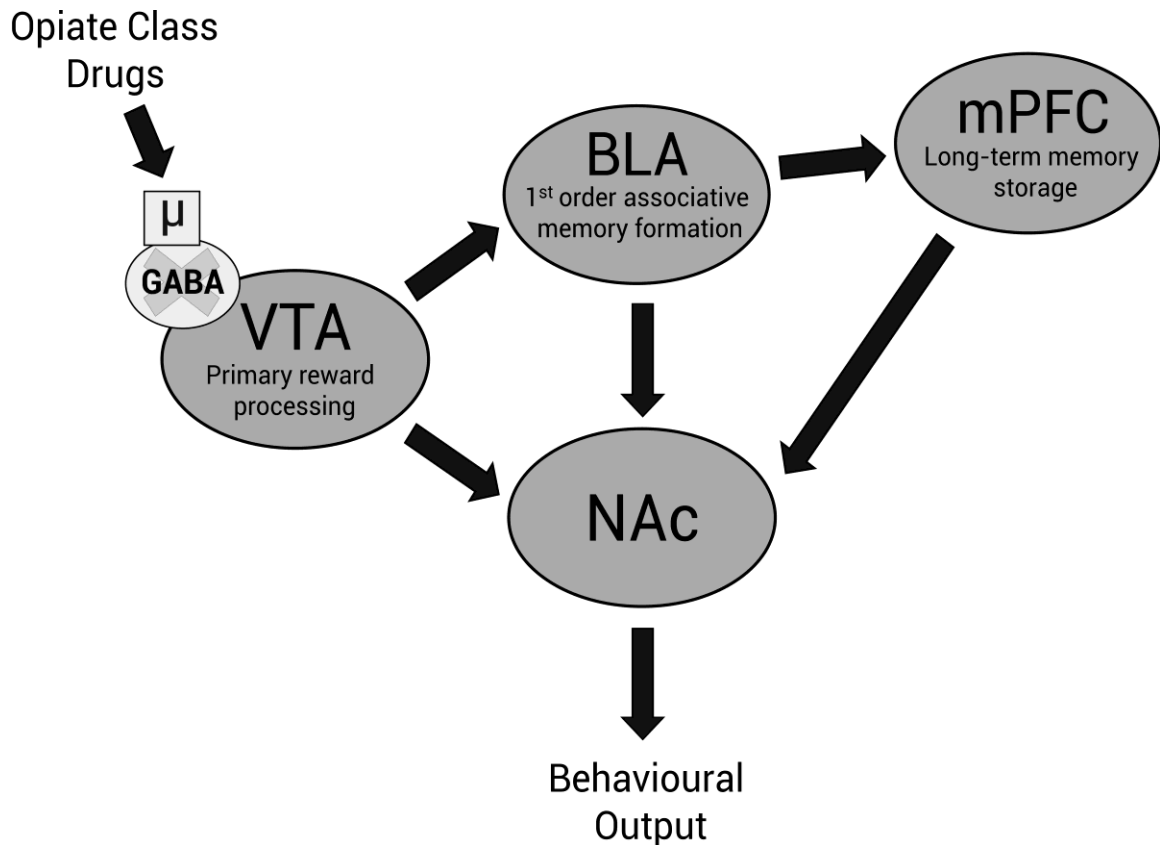


Figure 1.1 The mesocorticolimbic system

This schematic represents a theoretical framework for how opiate reward is processed in the mesocorticolimbic system. By acting on μ opioid receptors in the VTA, opiate drugs remove GABAergic inhibition, and allow for DA signals to the BLA. Here, integration with sensory regions allows for the processing of first-order associative memories. Over time, associative memories transition to the mPFC for further processing and storage, and alterations are made within the BLA-mPFC pathway. Recall of opiate memories may be triggered by the exposure to drug-related cues, and the resulting behavioural output is processed in the NAc.

1.3.1 Ventral Tegmental Area

The ventral tegmental area (VTA) is the primary site of opiate reward processing (Breiter et al., 1997). Direct infusion of morphine into the VTA promotes drug-seeking behaviours, whereas infusion into other areas of the mesocorticolimbic system fail to produce such effects (Olmstead and Franklin, 1997). Opiate class drugs act on μ opioid receptors within the VTA to disinhibit GABAergic inputs to DA interneurons, thus allowing for the processing of reward (Johnson and North, 1992; Klitenick et al., 1992). Activation of μ opioid receptors within the VTA also increases DA transmission to connected areas, increasing DA concentration (De Vries and Shippenberg, 2002; Adinoff, 2004; Ford et al., 2006). There is ample evidence that activation of the VTA by drugs of abuse results in this increase in DA release in the amygdala, hippocampus, prefrontal cortex and nucleus accumbens.

1.3.2 Nucleus Accumbens

The nucleus accumbens (NAc) integrates signals from connected mesocorticolimbic areas into signals that drive behavioural responses to stimuli, representing the “limbic-motor interface” (Ambroggi et al., 2009). In the context of drug-related stimuli, the shell division of the NAc facilitates drug-seeking behaviour following the activation of an associative drug memory (Bassareo et al., 2002). Importantly, DAergic projections from the VTA to the NAc are considered to be the chief pathway for processing the acute rewarding effects of opiate drugs (Wise, 1989; Nader and van der Kooy, 1997).

1.3.3 Basolateral Amygdala

The basolateral nucleus of the amygdala (BLA) is involved in both appetitive and aversive Pavlovian conditioning (Baxter and Murray, 2002). The BLA receives a wide variety of sensory inputs from the thalamus, frontal cortex, and, relevant to opiate reward processing, the VTA (Pitkänen et al., 2000). In turn, the BLA projects to the PFC, NAc and hippocampus, among others.

The BLA receives direct DAergic inputs from the VTA (Ford et al., 2006), however it is not involved in the direct perception of opiate euphoria itself (Olmstead and Franklin, 1997). Instead, the BLA acts as a hub to integrate VTA DAergic input with sensory and associative information (Grace and Rosenkranz, 2002; Rosenkranz and Grace, 2003). Indeed, lesions of the BLA will impair heroin-seeking behaviour, and pharmacological inhibition of BLA signaling prevents the reinstatement of cue-induced heroin seeking (Alderson et al., 2001; Fuchs and See, 2002).

In addition to reward memory processing, the BLA also processes aversion memories related to opiate withdrawal. BLA activation, as measured by c-fos expression, has been found to increase in response to associative memory processing. This is contrasted by adjacent neurons in the central amygdala being activated only in response to acute opiate withdrawal, rather than memory processing (Frenois, 2005).

1.3.4 Medial Prefrontal Cortex

The medial prefrontal cortex (mPFC) regulates cognition and, via reciprocal connections with the BLA, emotion (Little and Carter, 2013). Specifically, neuronal populations within the mPFC encode opiate-related memories, as demonstrated by increased firing in response to the recall of such memories (Sun et al., 2011).

Neuroimaging studies have suggested that dysfunctional activation of the mPFC in humans results in impaired executive function and increased impulsivity. These results have further been correlated with the compulsive drug-seeking and inability to suppress maladaptive behaviours seen in opiate addicts (Volkow and Fowler, 2000; Goldstein and Volkow, 2002; Kaufman et al., 2003; Paulus et al., 2005).

Both preclinical and clinical studies have implicated the mPFC in the expression of cue-induced associative opiate memory activation. This memory activation has been linked to opiate seeking and consumption in a variety of human imaging and animal studies (Daglish and Weinstein, 2001; Volkow et al., 2004; Langleben et al., 2008).

Blocking DA transmission within the mesolimbic DA system, either via lesion or application of DA antagonists, has been demonstrated to inhibit the development of a preference to environments associated with opiate drugs (Wise, 1989; Wise and Rompre, 1989). Further behavioural studies have corroborated this link, as well. Sun et al. (2011) found that neurons in the mPFC, specifically within the prelimbic cortex, increased firing and bursting activity in response to re-exposure to environmental cues that had been previously paired with morphine-induced reward. Chang et al. (1997) also demonstrated that neuronal response patterns within the mPFC varied depending on the phase of opiate-related drug seeking behaviour. The involvement of the mPFC in the processing of opiate related memories is also evidenced at the genetic level, influencing immediate early gene markers. Cue-induced reinstatement of heroin seeking has been found to increase both c-fos and zif268 in prelimbic neurons, even up to 3 weeks following heroin extinction (Shalev et al., 2003; Schmidt et al., 2005)

Most importantly, bidirectional connections between the BLA and mPFC, particularly when associated with DAergic signaling within the mesocorticolimbic system, control the acquisition and processing of opiate associative memories (Gholizadeh et al., 2013).

1.4 THE BLA-mPFC PATHWAY PROCESSES OPIATE-RELATED ASSOCIATIVE MEMORIES

Although a great deal of research has focused on how the mammalian brain processes reward, the neural circuitry of reward processing is distinct from the substrates that encode environmental and associative drug-related cues. Understanding this distinction allows for a more direct examination of the phenomena that contribute to the persistence of addictions, even after long periods of abstinence. The BLA-mPFC pathway is largely responsible for the higher-order processes involved in the formation of associative opiate memories, and both of these regions are believed to play critical roles in reward valuation and the acquisition of reward memories (Everitt, 2003; Kalivas et al., 2005).

The functional connection between the BLA and mPFC in heroin-dependent individuals is not well studied, but evidence of disordered resting state activity has been found (Zhang et al., 2011, 2015). Xie and colleagues (2011) have also reported a correlation between impulsivity in heroin-abstinent addicts and hyperactivity in the amygdala-frontal cortical circuit.

In animal models, connectivity between the BLA and mPFC has been implicated in the acquisition, consolidation and expression of associative opiate reward memories. For example, pharmacological inactivation of the BLA has been found to block the potentiation of opiate reward memory formation that can be induced via NMDA receptor antagonism within the PFC (Bishop et al., 2011). Inputs from the BLA are also necessary for consolidation of opiate reward memory within the mPFC. Gholizadeh and colleagues (2013) found that inhibition of protein synthesis within the BLA prevents the consolidation of opiate memories in the mPFC when administered up to 3 hours following behavioural conditioning. However, the consolidation of opiate reward memories transfers to a mPFC-dependent mechanism between 6 and 12 hours following conditioning, indicating that the consolidation of opiate reward memories follows a temporal gradient between BLA and mPFC in the early- and late-stages following behavioural conditioning, respectively. The BLA also impacts the role of the mPFC in later consolidation and recall of opiate associative reward memories. For instance, Sun and Laviolette (2012) found that inactivation of the BLA during reward learning results in later facilitated extinction of both opiate reward and aversion memories, as reflected by neuronal activity within the PFC.

1.5 OPIATE REWARD MEMORY LEARNING: INVOLVEMENT OF DOPAMINE

The rewarding effects of drugs of abuse are believed to result from an increase in DA concentration within the mesolimbic system (Di Chiara and Imperato, 1988). For instance, morphine, methadone, ethanol, nicotine, amphetamine and cocaine all increase DA within the mesocorticolimbic system (Di Chiara and Imperato, 1988). Some

researchers even posit that dysfunctional DA signaling can predispose individuals to drug use and abuse in an effort to overcome their natural DA deficiency (Blum et al., 2011, 2012a, 2012b).

DA was once believed to directly represent the expression of pleasure within the brain, but evidence that animals can demonstrate DA-independent hedonic responses has changed this viewpoint. Animals have expressed preference for drugs in studies that blocked DA pharmacologically, via lesioning, or by genetic inactivation of the rate-limiting enzyme in DA synthesis, tyrosine hydroxylase (Berridge and Robinson, 1998; Cannon and Palmiter, 2003; Robinson et al., 2005). Thus, a more complex role for DA than representing a direct reward signal was proposed. Beyond the processing of acute reward, the mesocorticolimbic pathway is considered the primary circuit for encoding drug-related associative reward or withdrawal aversion memories (Wise, 1989; Koob and Volkow, 2010). DA signaling is believed to play a central role in reward learning by assigning incentive salience to reward-related cues, thus allowing for those cues to trigger a “wanting” state with future exposure (Schultz et al., 1997; Berridge and Robinson, 1998; Schultz, 2006).

Extending this notion, researchers developed the hypothesis that DA encodes the prediction of reward, rather than reward itself (Ikemoto, 2010). Specifically, DA is believed to encode the history of reward, and this information allows individuals to form predictions and act in a way that maximizes the receipt of a reward (Sutton and Barto, 1999; Montague et al., 2004). Schultz and colleagues tested this hypothesis in awake monkeys during a classical conditioning task. They found that the delivery of an unexpected reward resulted in a phasic increase in DA firing within the VTA. Once the conditioned stimulus was acquired, reward delivery no longer increased firing of DAergic neurons (Schultz et al., 1993, 1997; Hollerman and Schultz, 1998; Schultz, 1998, 2006). Bayer & Glimcher (2005) extended this idea further and found that the firing of midbrain DA neurons fired most reliably to rewards that were “better than expected”. Interestingly, the application of DA’s role in reward prediction in computational models has provided evidence that due to their ability to potently increase synaptic DA, drugs of addiction will consistently produce a “better than expected” signal. Thus, future behaviour becomes

geared towards the acquisition of drugs over natural stimuli that cannot produce as potent a reward signal (Montague et al., 2004; Redish, 2004). Given its role in reward processing, the study of DAergic signaling is therefore a promising avenue for understanding how drugs of abuse exert their effects on the addicted brain.

1.5.1 Dopamine Receptor Signaling

DA receptors are G-protein-coupled receptors that fall into two categories, the D1 and D2 receptor families, based on their ability to modulate adenylyl cyclase (AC) and cyclic AMP (cAMP) activity (Kebabian and Calne, 1979; Andersen et al., 1990; Sibley and Monsma, 1992). Further genetic analyses have revealed a total of five DA receptors, which are further classified by their structural and pharmacological properties. Specifically, the D1 family (D1, D5) and the D2 family (D2, D3, D4) were defined (Andersen et al., 1990; Tiberi et al., 1991; Sibley and Monsma, 1992; Sokoloff et al., 1992). In addition to their differing action on AC, DA receptors demonstrate differences in their distribution, sensitivity to DA, and their intracellular signaling targets. These distinctions will be discussed below, with a particular focus on DA D1, D2 and D3 receptors for their role in reward and reinforcement processing.

1.5.2 D1 Receptors

The D1 family of receptors activate the $G_{\alpha_{s/olf}}$ family of G proteins, and stimulate cAMP production via AC. They are located exclusively on post-synaptic DA cells, including striatal GABAergic medium spiny neurons (MSNs) (Beaulieu and Gainetdinov, 2011). D1 receptors are highly expressed in mesocorticolimbic regions (Missale et al., 1998; Vallone et al., 2000; Beaulieu and Gainetdinov, 2011). For instance, the BLA specifically sends projections to MSNs that express D1 receptors (Floresco et al., 2001; Wall et al., 2013; Wassum and Izquierdo, 2015).

The D1 receptor is critical for learning and memory (Goldman-Rakic et al., 2004). For instance, behavioural conditioning has been reported to require phasic DA activation via

D1 receptors (Kauer and Malenka, 2007; Zweifel et al., 2009). The D1 receptor also plays a role in drug reward processing. For example, D1 knockouts will not self-administer cocaine (Caine et al., 2007). Increased D1 signaling is associated with the emergence of a sensitized response to drug administration (Bertran-Gonzalez et al., 2008). Furthermore, D1 has been implicated in conditioning for drug reward (Volkow et al., 2012).

With respect to intracellular signaling, D1 receptors regulate a variety of pathways, one of which is ERK1/2. Interestingly, activation of D1, but not D2, has been reported to increase the activity of ERK1/2 in the NAc and mPFC (Xue et al., 2015). ERK1/2 has also been linked to D1 signaling in reward conditioning studies. Kirschmann et al. (2014) found that a D1-NMDA mechanism was necessary for conditioned reward stimuli to activate ERK1/2 signaling in the NAc. Furthermore, reports of psychostimulant-induced ERK1/2 activation has been reported to occur exclusively in striatal neurons expressing D1 (Bertran-Gonzalez et al., 2008). Finally, both intra-BLA D1 and ERK1/2 have been implicated in the formation of opiate reward memory. Following chronic exposure to opiates, however, the expression of ERK1/2 within the BLA becomes downregulated, and opiate reward memory formation becomes independent of a D1-ERK1/2 mechanism (Lintas et al., 2011; Lyons et al., 2013).

1.5.3 D2 Receptors

Unlike D1 receptors, the D2 family of receptors are coupled to $G\alpha_{i/o}$ G-proteins, allowing them to inhibit the production of cAMP (Beaulieu and Gainetdinov, 2011). D2 receptors can be found either pre- or post-synaptically, making their function within the brain more dynamic than that of D1 (Sokoloff et al., 2006; Rondou et al., 2010; Beaulieu and Gainetdinov, 2011). D2 receptors are found predominantly in the striatum and NAc, but are also present in the amygdala and other cortical regions (Missale et al., 1998; Vallone et al., 2000; Beaulieu and Gainetdinov, 2011).

Like the D1 receptor, D2 is involved in learning and memory mechanisms (Xu et al., 2009). D2 has a higher affinity for DA than D1, making it more likely to be easily activated following drug-induced DA release and thus influence reward processing (Durieux et al., 2009).

In the context of drug addiction, dysfunction of the D2 receptor has been linked with impulsivity, and may be able to predict later development of addiction (Trifilieff and Martinez, 2014). Decreases in D2 signaling have been associated with a preference for immediate reward and compulsive intake (Perez et al., 2011; Trifilieff and Martinez, 2014). Conversely, increased D2 activation is linked with a willingness to exert greater effort in order to achieve a reward (Martinez et al., 2012; Trifilieff and Martinez, 2014).

One of the known intracellular signaling functions of the D2 receptor is to regulate calcium signaling. Neuronal calcium sensor-1, for instance, is involved in the desensitization of D2 receptors (Kabbani et al., 2002; Woll et al., 2011). Furthermore, CaMKII has been reported to directly modulate D2R function (Liu et al., 2009), and its activation has been linked to the reinstatement of morphine-seeking behaviors (Liu et al., 2012a, 2012b).

Finally, intra-BLA CaMKII α has demonstrated a dramatic downregulation following chronic exposure to heroin. Interestingly, this decrease was associated with the emergence of a D2/CaMKII α -sensitive substrate underlying the acquisition of opiate reward memories (Lintas et al., 2011; Lyons et al., 2013).

1.5.4 D3 Receptors

The D3 receptor is a member of the D2 family, and has a unique distribution pattern in the central nervous system. It is expressed mostly in limbic regions such as the NAc shell region (Sokoloff et al., 1992; Missale et al., 1998; Beaulieu and Gainetdinov, 2011). The localization of the D3R within the limbic system makes it a promising target for addiction research (Goodman, 2008).

Reports of D3 receptor function highlight its critical role in drug-induced reward learning (Di Chiara and Imperato, 1988; Nestler, 2005; Stuber et al., 2005; Hyman et al., 2006; Schultz, 2010). As a result, the D3 receptor has become a major target of pharmacological therapies aimed at treating addictive disorders (Parsons et al., 1996; Pilla et al., 1999; Heidbreder et al., 2005; Micheli and Heidbreder, 2008; Heidbreder and Newman, 2010).

Intracellular targets of the D3 receptor include cyclin-dependent kinase 5 (Cdk5) and calcineurin, among others that overlap with intracellular D2 targets (Chergui et al., 2004; Chen et al., 2009; Liu et al., 2009; Avalos-Fuentes et al., 2013). Intracellular action resulting from D3 signaling has been previously linked to synaptic plasticity mechanisms and memory formation for conditioned reward (Mansuy et al., 1998; Biala et al., 2005; Baumgärtel and Mansuy, 2012; Lyons et al., 2013).

1.6 EVIDENCE OF A MOLECULAR SWITCHING PHENOMENON IN THE OPIATE ADDICTED BRAIN

The notion that opiate exposure results in long-term changes to the biochemical signaling properties of the mammalian brain has been evaluated for decades. One of the earliest examinations of this hypothesis was performed by Nestler and Aghajanian (1997). They reported that acute opiate exposure inhibits cAMP-dependent protein phosphorylation within the locus coeruleus. With chronic opiate administration, this inhibition recovered to baseline level, but then rebounded to much higher than normal levels in the presence of an opioid receptor antagonist, thereafter remaining elevated.

More recently, it has become increasingly evident that prior history of opiate exposure must be factored into studies of the neural mechanisms underlying opiate processing. Opiate use in particular over other drugs of abuse can rapidly lead to dependence and withdrawal, even after a single exposure (Larcher et al., 1998; Kawasaki et al., 2011; Rothwell et al., 2012).

There is further evidence that previous history of opiate exposure can alter future processing of opiate reward following an opiate-induced “switch” in reward processing substrates. Bechara et al. (reviewed in 1998) pioneered research into the opiate switch phenomenon. Their work uncovered two, distinct motivational pathways responsible for processing opiate reward as a function of opiate exposure within the pedunculopontine tegmental nucleus (PPT). They found that lesions of the PPT blocked the acquisition of a morphine CPP only in rats with no previous exposure to opiates. Animals that were opiate-dependent and in a state of withdrawal, however, were still able to express a CPP for a morphine environment following PPT lesions. This switch between opiate naïve and dependent/withdrawn states was attributed to a functional dissociation within the VTA. Specifically, processing of morphine reward within the VTA was found to rely on a DA-independent PPT mechanism in the opiate naïve state, but opiate dependence and withdrawal resulted in a transition to a DA-dependent system (Nader and van der Kooy, 1997; Bechara et al., 1998; Laviolette et al., 2002).

Further research into the opiate exposure state switching mechanism uncovered the role of GABA_A receptors within the VTA. Blockade of GABA_A within the VTA of opiate naïve rats resulted in the disinhibition of DA-independent reward signaling pathways via the PPT (Laviolette and van der Kooy, 2004). In opiate-dependent rats in a state of withdrawal, however, intra-VTA GABA_A receptors mediated an excitatory signal, and activated a DA-dependent reward signal via the mesolimbic DA circuit (Laviolette and van der Kooy, 2003, 2004; Laviolette et al., 2004).

With a clear foundation for how opiate exposure can result in a functional switch in the brain’s processing of the primary rewarding effects of opiates, the question remained how opiate exposure may influence higher-order processing of opiate reward. For instance, does opiate exposure also result in a similar functional shift in how opiate-related memories are formed, and could such a switch be involved in the formation of the highly potent drug-related memories that are evident in addicted individuals? Given its role in the processing of opiate-related memories, the BLA was a prime candidate to seek further evidence of the opiate-induced molecular switching mechanism.

With the direct microinfusion of D1 and D2-specific antagonists into the rat brain, Lintas et al. (2011) found that intra-BLA DA receptors mediate the formation of opiate reward CPP as a function of opiate exposure. In animals with no prior exposure to opiates, intra-BLA D1 antagonism blocked the formation of a morphine CPP, but not in opiate-dependent/withdrawn animals. Antagonism of the intra-BLA D2 receptor, however, successfully blocked the formation of a morphine CPP in opiate dependent animals, but not opiate naïve animals.

To understand the molecular underpinnings of this double-dissociation of DA receptor function in the BLA, targets of the intracellular D1 and D2 receptors were probed in a similar manner. Lyons et al. (2013) tested the involvement of ERK1/2 in D1-dependent opiate reward memory formation and CaMKII α in D2-dependent memory formation. They found that intra-BLA inhibition of ERK1/2 blocked the acquisition of a morphine CPP memory in opiate naïve animals, but not in opiate-dependent animals. In contrast, intra-BLA CaMKII α antagonism prevented the formation of a morphine CPP in opiate-dependent/withdrawn animals, but not opiate naïve animals. Furthermore, the ability for D1 or D2 activation within the BLA to potentiate the salience of opiate reward was found to rely on intra-BLA ERK1/2 and CaMKII α signaling, respectively. Lyons et al. (2013) further explored how the expression of ERK1/2 and CaMKII α may be altered by chronic heroin exposure, and found dramatic downregulations of CaMKII α and a significant decrease in the expression of ERK1/2. Together, these results suggested that chronic opiate exposure leads to changes in the expression of intracellular DA signaling targets that result in an opiate exposure state-dependent switch in the behavioural significance of these signaling cascades in the formation of associative opiate reward memories.

1.7 SUMMARY AND RATIONALE

Long-term addiction is due in part to the persistence of opiate associative memories for reward and aversion. While the above discussion has pointed out existing knowledge pertaining to the neurobiological mechanisms by which opiates produce their rewarding and addictive properties, much less is understood concerning how brain mechanisms

responsible for opiate-related associative memories may contribute to the intractability of opiate addiction. In conjunction with the formation of maladaptive memory formation, opiate use results in physiological adaptations to create dependence and addiction, specifically within the BLA-mPFC pathway of the mesocorticolimbic system.

Understanding acute vs. long-term opiate-induced changes to DA D1, D2 and D3 receptors and their downstream molecular memory signaling pathways within the BLA-mPFC circuitry will help us to uncover the neurophysiological underpinnings involved in the acquisition and persistence of opiate associative memories. Ultimately, this will provide the preclinical research needed to understand, and hopefully treat, long-term opiate addiction by revealing novel pharmacotherapeutic targets aimed at disrupting, preventing and/or reversing neuropathological brain adaptations linked to the persistence of opiate-related addiction memories.

1.7.1 General Hypothesis

My overarching hypothesis is that chronic heroin exposure results in a series of alterations to both the function and expression of DA D1, D2 and D3 receptors and their associated intracellular signaling targets within the BLA-mPFC pathway, with functional consequences for reward and aversive associative drug memory formation during distinct phases of the opiate addiction process. My thesis addresses this general hypothesis with the following three specific research aims:

1. Investigate opiate exposure state-dependent alterations to D1 and D2 signaling mechanisms within the BLA-mPFC circuit in the context of opiate reward memory formation using a combination of conditioned place preference behavioural analyses and molecular analyses of downstream molecular memory signaling pathways involving the ERK 1/2 and CaMKII α protein expression patterns in the BLA-mPFC circuit.

2. Investigate the specific role of D3 receptor signaling and its associated downstream molecular signaling pathways within the basolateral amygdala in the context of opiate reward and withdrawal aversion memory acquisition as a function of opiate exposure state.

3. Explore the short vs. long-term temporal dynamics of how specific memory signaling molecules involved in associative drug memory formation in the BLA are altered following chronic heroin exposure, dependence and withdrawal.

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Chapter 2

2 Opiate Exposure State Controls a D2-CaMKII α -Dependent Memory Switch in the Amygdala-Prefrontal Cortical Circuit

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2.1 INTRODUCTION

The mammalian basolateral amygdala (BLA) and medial prefrontal cortex (mPFC) form a functionally interconnected circuit that is critical for the formation of memories linked to the rewarding properties of opiates (Bishop et al, 2011; Sun and Laviolette, 2012; Sun et al, 2011). Intra-BLA processing of opiate-related memories is mediated via dopamine (DA) D1 receptor (D1R) and D2R signaling as a function of opiate exposure state. Specifically, D1R transmission is required for acute opiate memory formation in the previously drug-naïve state, whereas D2R signaling is necessary for opiate memory formation during states of chronic opiate exposure and spontaneous withdrawal (Lintas et al, 2011, 2012).

Extracellular signal-related kinase 1/2 (ERK1/2) and the Ca²⁺/calmodulin-dependent protein kinase type II α isoform (CaMKII α) are linked to DAergic activity and the formation of drug-related memories (Lyons et al, 2013). ERK signaling is associated with opiate reward and spontaneous withdrawal phenomena in mesocorticolimbic regions (Morón et al, 2010; Valjent et al, 2004). Furthermore, chronic opiate exposure leads to alterations in ERK signaling (Bilecki et al, 2005; Lyons et al, 2013). Activity of CaMKII is involved in reinstatement of morphine-seeking behaviors (Liu et al, 2012a, b), and modifications to CaMKII expression are linked to opiate exposure differentially between acute and chronic treatments (Andersen et al, 2012; Lu et al, 2000; Lyons et al, 2013). Although both molecules interact with D1R and D2R, ERK signaling is related more closely to D1R-mediated effects (Fricks -Gleason and Marshall, 2011; Lai et al, 2008), whereas CaMKII directly modulates D2R function (Liu et al, 2009). Opiate exposure not only alters levels of ERK1/2 and CaMKII in the BLA, but ERK1/2 signaling is required for acute opiate memory formation in rats with no history of opiate exposure. In contrast, CaMKII-mediated signaling is necessary for memory formation following chronic opiate exposure and spontaneous withdrawal (Lyons et al, 2013).

The mPFC is involved in processing opiate-related memories and in generating neuronal responses to drug cues during relapse and drug seeking (Bossert et al, 2012; Doherty et al, 2013; Lucas et al, 2012; Pelloux et al, 2013; Sun et al, 2011). The BLA-mPFC circuit operates as an integrative, temporally mediated pathway during opiate-related associative

memory processing (Bishop et al, 2011; Gholizadeh et al, 2013; Sun and Laviolette, 2012; Sun et al, 2011) and thus represents a crucial target for understanding the processing of opiate reward memories (Cardinal et al, 2002). We hypothesized that opiate-related molecular adaptations within the BLA-mPFC circuit may underlie the transition between opiate-naïve vs chronically exposed states, during the formation of opiate reward memories.

Using a combination of behavioral pharmacological and molecular analyses, we investigated the role of intra-mPFC CaMKII, ERK1/2, and DAergic signaling during the formation of opiate reward memories as a function of opiate exposure state. Remarkably, we report that chronic opiate exposure and spontaneous withdrawal induces a functional switch in the signaling pathways involved in the formation of opiate associative memories within the mPFC that are functionally opposite to those observed in the BLA. Thus, chronic opiate exposure and spontaneous withdrawal serves as a dissociative boundary between distinct DAergic and CaMKII/ERK memory substrates within the BLA-mPFC circuit.

2.2 MATERIALS AND METHODS

2.2.1 Surgical Procedures

All procedures were performed in accordance with the Canadian Council on Animal Care and approved by the Western University Council on Animal Care. Male Sprague-Dawley rats (350–400 g; Charles River) were anesthetized with ketamine/xylazine (80 mg and 6 mg/kg, respectively), given 1 mg/kg of meloxicam (non-steroidal anti-inflammatory analgesic) and placed into a stereotaxic device. Stainless steel guide cannulae (22 gauge; PlasticsOne) were bilaterally implanted into the brain regions of interest based on anatomical boundaries defined by Paxinos and Watson (2005) as follows: mPFC (15° angle), from bregma, anteroposterior (AP) – 2.9, mediolateral (ML) – 1.9, from the dural surface, dorso-ventral (DV) – 3.0; BLA (no angle), from bregma, AP – 2.6, ML +5.0, DV – 7.2. Rats in the mPFC-BLA disconnection groups received a single unilateral PFC

cannulation and contralateral BLA cannulation. The hemispheres for unilateral cannulations were counter-balanced within groups to control for laterality.

2.2.2 Drug Treatments

The selective CaMKII inhibitor autocamtide-2-related inhibitory peptide (AIP; Tocris Bioscience), the D2/D3 antagonist eticlopride (eticlopride hydrochloride; Tocris Bioscience), morphine (morphine hydrochloride, MacFarlane Smith), and heroin (diacetyl-morphine, MacFarlane Smith) were dissolved in physiological saline, pH adjusted to 7.4. The selective ERK1 inhibitor (PD334581; Tocris Bioscience) was dissolved in a solution of 50% dimethyl sulfoxide (DMSO) and 50% physiological saline, pH adjusted to 7.4, and prepared at room temperature. Microinjections into mPFC or BLA (0.5 μ l of volume per infusion) were delivered via plastic tubing connected to a 1 μ l Hamilton microsyringe over 1 min. Injectors were left *in situ* for an additional 1 min to ensure diffusion from the injector tip. For all groups, microinfusions were performed immediately before injections of morphine or saline (i.p.) and subsequent placement in the conditioning environment. The dose of morphine used for conditioned place preference (CPP; 5 mg/kg, i.p.) is a supra-reward threshold conditioning dose, and produces robust behavioral CPP (Bishop et al, 2011; Lyons et al, 2013; Sun et al, 2011).

2.2.3 Chronic Opiate Exposure and Spontaneous Withdrawal

For rats in the chronic opiate exposure/spontaneous withdrawal condition, opiate exposure and spontaneous withdrawal was induced as previously described (De Jaeger et al, 2013; Lintas et al, 2011; Lyons et al, 2013). This regimen produces aversive motivational effects (conditioned place aversion), which are qualitatively similar to those produced following a 3-week morphine administration regimen (Nader et al, 1994; Bechara et al, 1995; Laviolette and van der Kooy, 2004). Rats received daily subcutaneous injections of 0.5 mg/kg heroin in their home cage starting 7 days before behavioral conditioning, and the first conditioning session began 21 h following their last

heroin injection. Maintenance doses of heroin were administered during the conditioning phase 2.5 h following the end of each conditioning session, for a total of 15 injections over the course of an experiment. Rats in the opiate-naïve condition were yoked to chronic exposure/withdrawn animals with saline injections corresponding to heroin injections.

2.2.4 Place Preference Conditioning

A fully counterbalanced, unbiased CPP paradigm was used as described previously (Lintas et al, 2011; Lyons et al, 2013). Following surgical recovery, rats were pre-conditioned for 20 min in a motivationally neutral gray box and randomly assigned to an experimental group. The 8-day conditioning procedure commenced the following day. One conditioning environment was black with a Plexiglas floor that is wiped down with 0.25 ml of 2% acetic acid immediately before placing the rat into it. The alternating environment was white with a wire mesh and woodchip floor, so the environments differed in color, floor texture, and odor. These environments elicit no baseline preference in rats, as reported previously (Laviolette and van der Kooy, 2003). Rats received an equal number of morphine-paired and saline-paired conditioning sessions, each 30 min in duration (i.e., four morphine environment pairings and four saline environment pairings over the 8-day procedure). Testing occurred 3–5 days following the final conditioning session in a drug-free state. The test environment contains both conditioning environments separated by a narrow, neutral gray zone. At the start of the 10-min test session, rats are placed in the neutral zone, and time spent in each environment is recorded and scored separately for each rat.

2.2.5 Histology

Following the completion of behavioral experiments, rats were deeply anesthetized with euthanyl (sodium pentobarbital, 240 mg/kg, i.p.) and transcardially perfused with 0.9% physiological saline, followed by 10% formalin. Once extracted, brains were refrigerated

at 4°C in a 25% sucrose in formalin solution for a minimum of 48 h before sectioning at 40 µm. Brain slices were stained with Cresyl Violet for verification of BLA and mPFC cannulae placements. Subjects with placements outside the anatomical boundaries ($n = 25$ out of total $n = 152$) as defined by Paxinos and Watson (2005) were excluded from analysis.

2.2.6 Western Blot Procedure

Two groups of rats received 15 daily injections of physiological saline or heroin (0.5 mg/kg, s.c.), equivalent to those received during behavioral conditioning. Tissue was extracted at 21 h following the final injection, and western blotting procedures were performed as outlined in Lyons et al, 2013. Primary antibody dilutions were as follows: α -tubulin (1 : 120 000; Sigma-Aldrich), phosphorylated ERK1/2 [T202/Y204] (pERK1/2; 1 : 2000; Cell Signaling Technology), total ERK1/2 (tERK1/2; 1 : 5000, Cell Signaling Technology), phosphorylated CaMKII α [T286] (pCaMKII α ; 1 : 10 000; Cell Signaling Technology), total CaMKII α (tCaMKII α ; 1 : 5000; Cell Signaling Technology), total CaMKII β (1:100; Santa Cruz Biotechnology), Ca²⁺/calmodulin-dependent protein kinase type IV (CaMKIV; 1:50 000; Sigma-Aldrich), calcineurin A (CnA; 1:100 000; Sigma-Aldrich), D1R (1:100; Santa Cruz Biotechnology), and D2R (1:100; Santa Cruz Biotechnology). Secondary antibodies (Thermo Scientific) were all used at a dilution of 1:20 000. Blots were incubated in a TBS-T solution with either 5% non-fat dried milk (Carnation) or 2.5% Bovine Serum Albumin Fraction V (Calbiochem) as recommended by the manufacturer.

2.2.7 Data Analysis

CPP data were analyzed with two-way analysis of variance (ANOVA) with a between-subjects factor of drug treatment, and a within-subjects factor of conditioning environment. Post-hoc analyses were performed with Bonferroni corrections where

appropriate. Densitometry values for western blots were obtained with Kodak digital analysis software and analyzed with two-tailed t-tests.

2.3 RESULTS

2.3.1 Effects of Chronic Heroin Exposure and Spontaneous Withdrawal on Calcium-Related Signaling Molecules in the mPFC

Given previous findings showing a profound reduction in intra-BLA CaMKII α expression levels following chronic heroin exposure (Lyons et al, 2013), we first examined the protein expression profile of the CaMKII α isoform in the mPFC as a function of opiate exposure state. Sample western blots presented in Figure 2.1a show representative phosphorylated and total CaMKII α levels in opiate-naïve vs chronic exposure states. Rats chronically exposed to opiates and in spontaneous withdrawal at the time of tissue processing ($n = 8$) showed no change in CaMKII α phosphorylation levels compared with vehicle-treated rats (Figure 2.1b; $n = 8$; $t_{(14)} = -0.446$, $p = .662$), but displayed significantly increased total CaMKII α expression ($t_{(14)} = -3.152$, $p < .01$). As a result, the ratio of phosphorylated to total CaMKII α was significantly decreased (Figure 2.1c; $t_{(14)} = 3.850$, $p < .01$).

In order to investigate potential changes to other isoforms of CaMKII, we next tested the CaMKII β isoform. A representative western blot is presented in Figure 2.1d. Analysis revealed no significant increase in total CaMKII β expression in chronic opiate exposure/withdrawn rats ($n = 8$) compared with vehicle controls ($n = 8$; $t_{(14)} = -1.383$, $p = .188$; Figure 2.1e). To determine if opiate exposure targets CaMKII α specifically or Ca²⁺ (or Ca²⁺/calmodulin) more generally, we examined potential alterations to either CaMKIV (Figure 2.1f and g) or calcineurin A (CnA) (Figure 2.1h and i). There was no observed difference in the expression of CaMKIV or CnA between opiate-naïve vs chronically exposed/withdrawn rats ($t_{(13)} = 0.153$, $p = .881$; $t_{(12)} = -0.075$, $p = .941$, respectively).

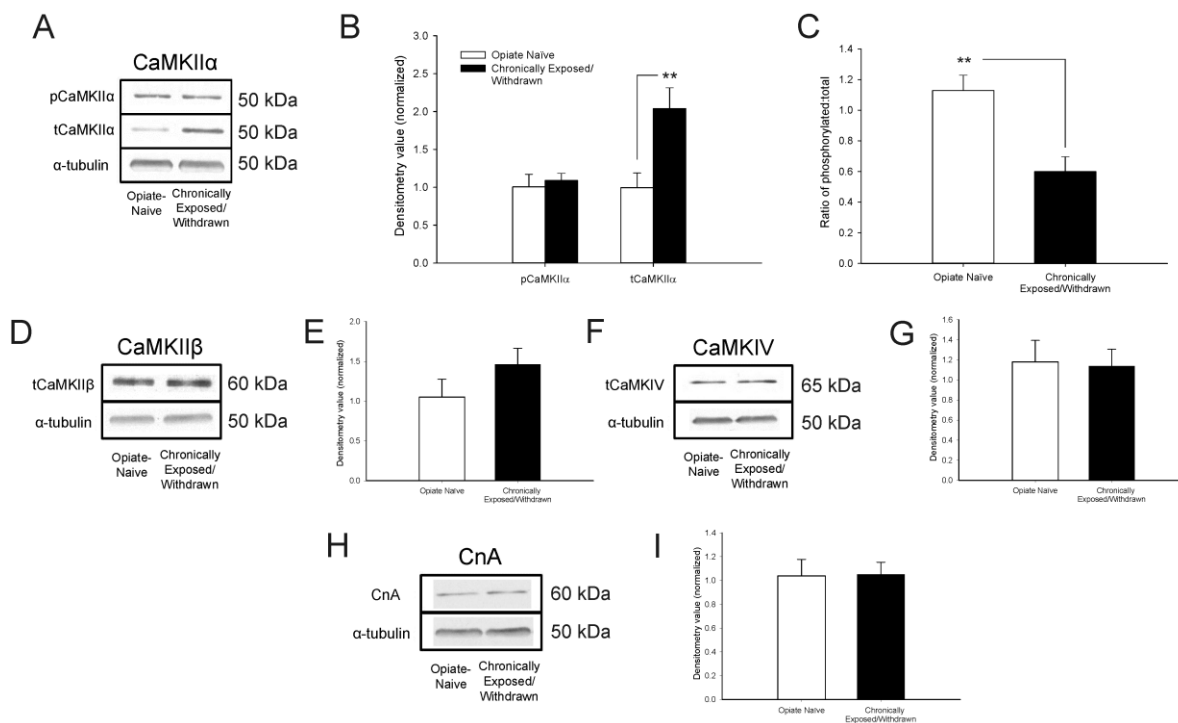


Figure 2.1 Western blot analysis of calcium-related signaling molecules in the mPFC

Chronic heroin exposure results in a state-dependent alteration in the expression of signaling molecules in the mPFC. (a) Representative western blot for phosphorylated and total CaMKII α expression. (b) Densitometry analysis revealed no significant changes to phosphorylated CaMKII α , but an increase in total CaMKII α in opiate chronic exposure/withdrawn tissue. (c) The ratio of phosphorylated to total CaMKII α is decreased in opiate chronic exposure/withdrawn animals relative to saline-treated animals. (d) Representative western blot for total CaMKII β . (e) Expression levels of CaMKII β are unchanged following chronic heroin exposure. (f) Representative western blot for total CaMKIV. (g) Levels of CaMKIV expression are not altered by chronic heroin exposure. (h) Representative western blot for CnA. (i) Expression levels of CnA are not changed by chronic heroin exposure (**p < 0.01; error bars represent SEM).

2.3.2 Histological Analysis for CPP Experiments

Histological analysis of intra-BLA and intra-mPFC cannula placements revealed microinjector tips within the anatomical boundaries of BLA and mPFC as outlined in Paxinos and Watson (2005). Figure 2 presents microphotographs and schematics of both intra-BLA (Figure 2.2a and b) and intra-mPFC (Figure 2.2c and d) placements of representative experimental groups. Histological analysis revealed intra-mPFC

placements to be localized to the pre-limbic (PLC) subregion of the mPFC. Rats found to have placements outside the anatomical boundaries of the mPFC or BLA as defined by Paxinos and Watson (2005) were excluded from analyses.

2.3.3 Opiate Exposure State Controls the Functional Role of Intra-mPFC CaMKII Signaling during Opiate Reward Memory Formation

Given our observed changes in intra-mPFC CaMKII α expression following chronic opiate exposure and spontaneous withdrawal, we next evaluated the behavioral significance of intra-mPFC CaMKII signaling across opiate exposure states. Here, we used a previously tested behaviorally effective dose range of the highly selective CaMKII inhibitor AIP (5–500 ng/0.5 μ l) for bilateral intra-mPFC microinfusions (Lyons et al, 2013). First, using opiate-naïve experimental groups, intra-mPFC inhibition of CaMKII dose-dependently blocked the acquisition of morphine (5 mg/kg; i.p.) CPP (Figure 2.2e). Two-way ANOVA revealed a significant main effect of treatment ($F_{(3, 25)} = 10.93, p < .01$), main effect of environment ($F_{(1, 26)} = 14.55, p < .01$) and treatment by environment interaction ($F_{(3, 22)} = 3.05, p < .05$). Post-hoc analyses revealed that whereas intra-mPFC vehicle ($n = 7$) or the lowest dose of 5 ng AIP ($n = 6$) were ineffective at blocking morphine CPP (p -values $< .01$), both 50 ng ($n = 6$) and 500 ng ($n = 7$) doses blocked the formation of morphine CPP, with rats demonstrating no significant preference for morphine vs saline-paired environments (p -values $> .05$).

We next examined the effects of intra-mPFC CaMKII inhibition on opiate reward behaviors in chronically exposed/withdrawn experimental groups (see section 2.2). Using the previously determined highest behaviorally effective dose of AIP (500 ng/0.5 μ l; see Figure 2.2e), we found that intra-mPFC blockade of CaMKII failed to block morphine CPP. Although statistical analyses revealed a significant effect of group ($F_{(1, 14)} = 7.21, p < .05$) and conditioning environment ($F_{(1, 15)} = 68.44, p < .01$), both the groups receiving

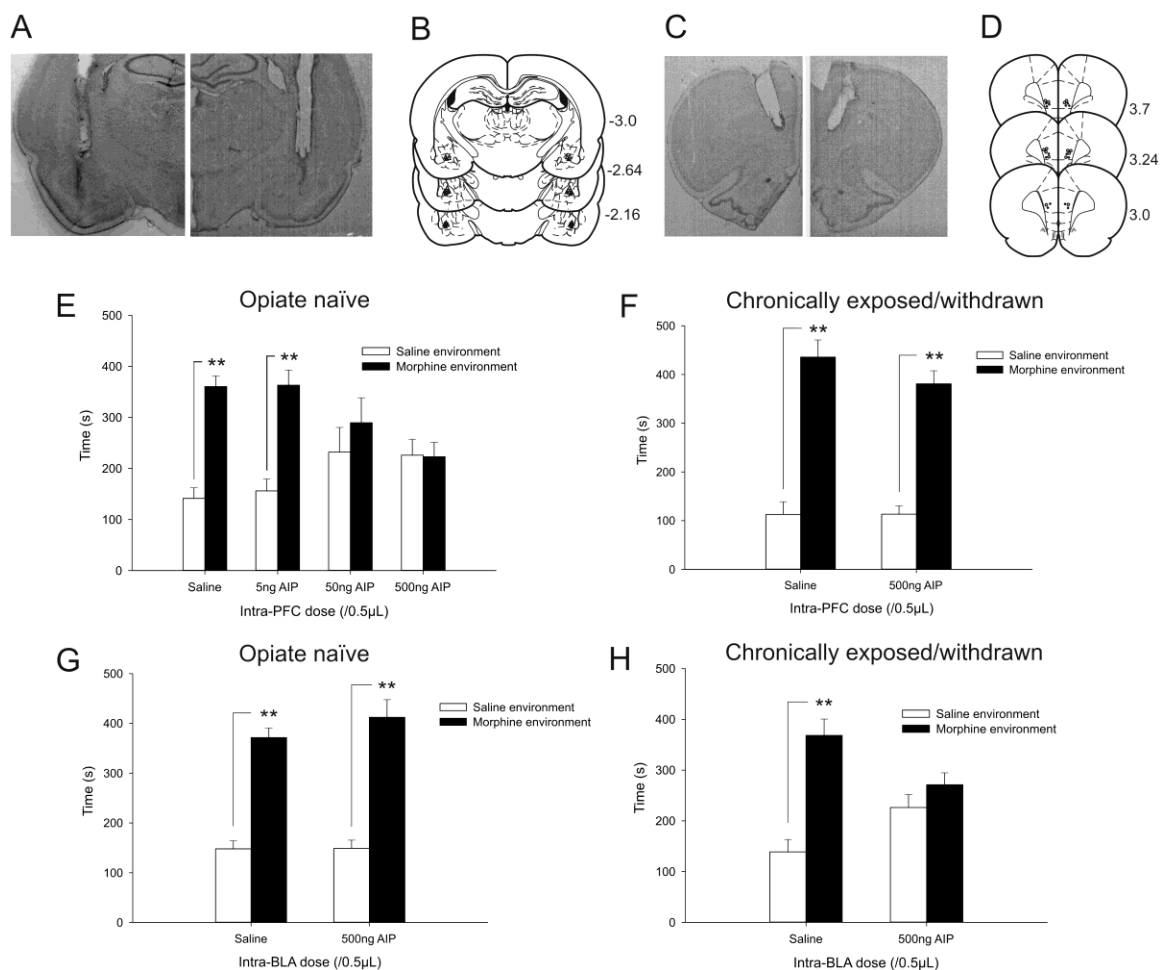


Figure 2.2 Histology and analysis of CPP experiments evaluating the role of intra-mPFC and intra-BLA CaMKII activity across opiate exposure states

Intra-mPFC and intra-BLA CaMKII signaling is functionally controlled by opiate exposure state. (a) Representative microphotograph of intra-BLA guide cannulae and microinjection tip placement. (b) Schematic of bilateral intra-BLA placements. Open triangles: 500 ng AIP in the opiate-naïve state; closed triangles 500 ng AIP in the chronic exposure/ spontaneous withdrawal state. (c) Representative microphotograph of intra-mPFC guide cannulae and microinjection tip placement. (d) Schematic of bilateral intra-mPFC placements. Open circles: 500 ng AIP in the opiate-naïve state; closed circles: 1 µg UO126 in the opiate chronic exposure/withdrawn state. (e) Intra-mPFC microinfusion of AIP dose-dependently blocked the acquisition of a morphine (0.5 mg/kg) CPP in a range from 5 ng to 500 ng in the opiate-naïve state. (f) Intra-mPFC microinfusion of 500 ng AIP did not block the acquisition of a morphine CPP in the opiate chronic exposure/withdrawn state. (g) In opposition to the mPFC, intra-BLA microinfusion of 500 ng AIP did not block the acquisition of a morphine CPP in rats trained in a previously opiate-naïve state; (h) however, intra-BLA microinfusion of 500 ng AIP blocked the acquisition of a morphine CPP in the opiate chronic exposure/withdrawn state (** $p < 0.01$; error bars represent SEM).

intra-mPFC AIP ($n = 7$) and vehicle ($n = 8$) demonstrated robust morphine CPP at testing (Figure 2.2f; p -values $< .01$). In contrast to these findings in the mPFC, we have previously reported that intra-BLA CaMKII activity is not required for the acquisition of morphine CPP in the opiate-naïve state but, is necessary during states of chronic opiate exposure/spontaneous withdrawal (Lyons et al, 2013). Thus, to confirm the role of intra-BLA CaMKII signaling across opiate exposure states, we next examined the effects of intra-BLA CaMKII inhibition on opiate reward memory acquisition. Consistent with previous findings (Lyons et al, 2013), intra-BLA CaMKII inhibition in the opiate-naïve state failed to block morphine CPP relative to vehicle controls. Two-way ANOVA revealed a significant effect of conditioning environment (Figure 2.2g; $F_{(1, 12)} = 86.59$, $p < .01$), and both the vehicle ($n = 7$) and 500 ng AIP ($n = 5$) groups showed significant place preference for the morphine-paired environment following behavioral conditioning (p -values $< .01$). Inhibition of intra-BLA CaMKII, however, blocked the acquisition of morphine CPP in chronically exposed/withdrawn rats. Statistical analyses revealed a significant interaction of treatment group by environment between intra-BLA AIP and vehicle-treated groups (Figure 2.2h; $F_{(1, 13)} = 6.441$, $p = .02$). Rats receiving 500 ng of AIP ($n = 8$) showed no preference for morphine vs saline-paired environments ($p > .05$) vs those receiving intra-BLA vehicle ($n = 7$), who spent significantly more time in the morphine environment ($p < .01$). Thus, within the BLA-mPFC circuit, blockade of CaMKII signaling reveals a behavioral double dissociation as a function of opiate exposure state: CaMKII signaling is required for intra-BLA opiate reward memory acquisition in the chronic opiate exposure/withdrawn state, but not naïve states. In contrast, intra-mPFC CaMKII signaling is required for opiate reward memory acquisition in the opiate-naïve, but not chronic exposure/withdrawn states.

2.3.4 Chronic Opiate Exposure and Spontaneous Withdrawal Alters the Expression and Function of Intra-mPFC D2R Receptor Transmission

Our present results show that intra-mPFC CaMKII signaling is required for opiate memory formation exclusively in the opiate-naïve state (Figure 2.2e) and that intra-mPFC

CaMKII α levels are markedly elevated in the opiate exposed and withdrawn state (Figure 1a and b). Remarkably, these results are in the opposite direction to those observed previously in the BLA wherein intra-BLA CaMKII α signaling is functionally linked to D2R transmission only in the chronic opiate exposure/withdrawn state, concomitant with a significant downregulation in CaMKII α levels (Lyons et al, 2013). Thus, we hypothesized that upregulation of CaMKII α levels in the mPFC may be linked to opposing roles for intra-mPFC D2R transmission as a function of opiate exposure state. Accordingly, we next examined the effects of D2R blockade directly in the mPFC, comparing the opiate-naïve with chronic exposure/withdrawn states. Consistent with our hypothesis, intra-mPFC D2R blockade with the selective D2R antagonist, eticlopride (1 μ g/0.5 μ l; $n = 7$) blocked acquisition of morphine CPP in the naïve, but not in the chronic exposure/withdrawn states (Figure 2.3a and b). For opiate-naïve groups, two-way ANOVA revealed a significant effect of conditioning environment ($F_{(1, 14)} = 4.76$, $p < .05$), but no significant group effect ($F_{(1, 13)} = 2.20$, $p = .16$; Figure 2.3a). However, post-hoc analyses revealed that rats treated with eticlopride ($n = 7$) exhibited no preference for the morphine-paired environment ($p > .05$), whereas vehicle controls ($n = 7$) demonstrated significant CPP ($p < .05$). In contrast, in chronically opiate exposed/withdrawn rats, intra-mPFC D2R inhibition failed to block morphine CPP. Two-way ANOVA revealed a significant main effect of conditioning environment ($F_{(1, 12)} = 38.43$, $p < .01$; Figure 2.3b). Post-hoc analyses demonstrated that both vehicle-treated ($n = 7$) and eticlopride-treated ($n = 5$) groups exhibited significant preferences for morphine-paired environments (p -values $< .01$, $< .05$, respectively). Thus, blockade of D2R transmission in the mPFC selectively blocks morphine reward CPP in the opiate-naïve state, in direct contrast to the role of intra-BLA D2R transmission during opiate reward memory formation (Lyons et al, 2013). Given our observed behavioral dissociation in the role of intra-mPFC D2R transmission as a function of opiate exposure state, we next measured levels of intra-mPFC D2R protein expression between the two exposure states. A representative western blot of D2R expression levels is pictured in Figure 2.3c. Interestingly, levels of D2R expression showed a significant increase in chronically exposed/withdrawn rats ($n = 8$) as compared with opiate-naïve ($n = 8$) groups (Figure 2.3d; $t_{(14)} = -2.659$, $p = .01$), demonstrating that chronic opiate exposure/spontaneous

withdrawal triggers a compensatory upregulation of D2R expression within the mPFC, concomitant with a lack of sensitivity to D2R blockade during the acquisition of opiate reward memories, relative to the opiate-naïve state.

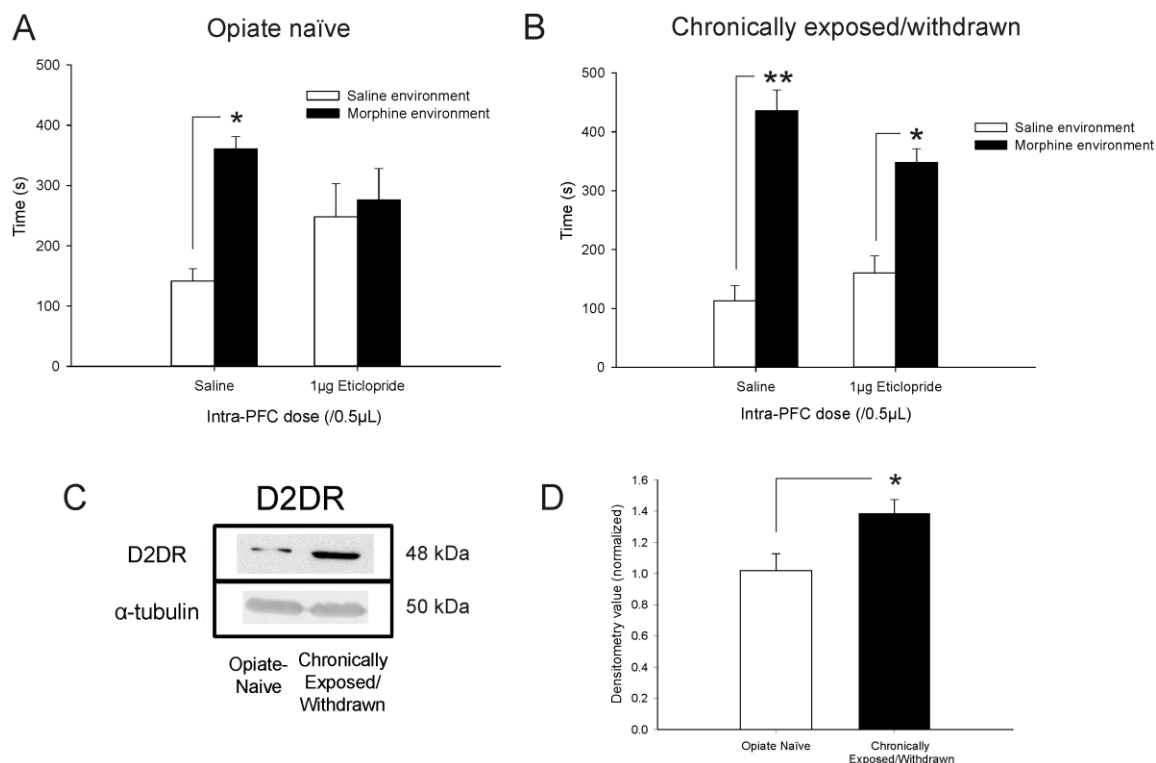


Figure 2.3 Assessment of intra-mPFC D2 function and expression in animals following chronic opiate exposure

Chronic heroin exposure alters function and expression of intra-mPFC D2R transmission. (a) Intra-mPFC microinfusion of $1\mu\text{g}$ eticlopride blocked the acquisition of a morphine (5 mg/kg) CPP in the opiate-naïve state. (b) In the opiate chronic exposure/withdrawn state, a $1\mu\text{g}$ eticlopride infusion into the mPFC did not block the acquisition of a 5 mg/kg morphine CPP. (c) Representative western blot of D2R expression in the mPFC between the opiate-naïve and chronic exposure/withdrawn states. (d) D2R expression is increased in the mPFC of opiate chronic exposure/withdrawn animals (* $p < 0.05$, ** $p < 0.01$; error bars represent SEM).

2.3.5 Chronic Opiate Exposure Alters ERK1 Phosphorylation in the mPFC

Previous studies have demonstrated a state-dependent switch in the roles of the intra-BLA D1-ERK1/2 signaling pathway in the formation of opiate reward memories (Lintas et al, 2011; Lyons et al, 2013). Accordingly, we next tested for changes in protein expression and behavioral functions of the intra-mPFC D1-ERK1/2 pathway between the opiate-naïve and chronic exposure/withdrawn states. Representative western blots of phosphorylated and total ERK1/2 are presented in Figure 2.4a. Examining expression levels of intra-mPFC ERK isoforms across opiate exposure states revealed a significant increase in the expression of ERK1 phosphorylation between the opiate-naïve ($n = 7$) and chronic exposure/withdrawn states ($n = 7$; $t_{(12)} = -2.231$, $p < .05$; Figure 2.4b), but no change to total ERK1 levels (tERK1: $t_{(12)} = -0.650$, $p = .361$; Figure 2.4b). Consequently, the ratio of phosphorylated to total ERK1 expression was significantly increased in favor of pERK1 following chronic heroin exposure ($t_{(12)} = -2.646$, $p < 0.05$; Figure 2.4c). Levels of phosphorylated and total ERK2 in the chronic exposure/withdrawn state were not statistically different from the naïve state (Figure 2.4d; pERK2: $t_{(12)} = -1.232$, $p = .241$; tERK2: $t_{(12)} = 0.110$, $p = .914$), and thus no change was seen in the ratio of phosphorylated to total ERK2 (Figure 2.4e; $t_{(12)} = -1.592$, $p = .137$). Given prior evidence demonstrating a functional relationship between alterations in intra-BLA ERK1/2 expression levels with the function of D1R transmission during opiate reward memory formation (Lyons et al, 2013), we next tested expression levels of the D1R directly within the mPFC. Western blots comparing the expression of DA D1Rs showed no difference in the expression of D1R between saline control and opiate chronic exposure/withdrawn rats (Figure 2.4f and g; $t_{(12)} = -0.317$, $p = .756$), demonstrating that, in contrast to the BLA, intra-mPFC alterations in ERK1 expression are not concomitant with changes in expression levels of the D1R.

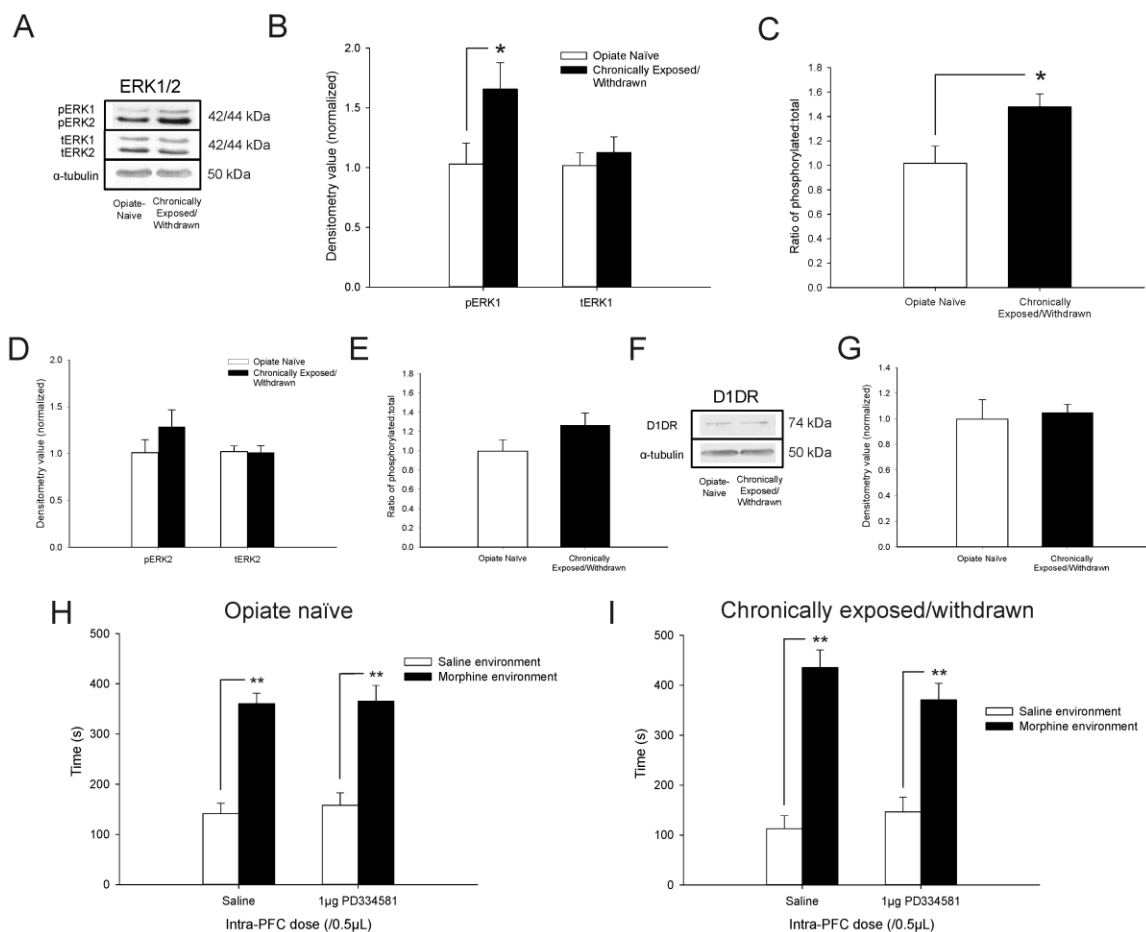


Figure 2.4 Intra-mPFC function and expression of D1-ERK1/2 function and expression in animals following chronic opiate exposure

Effect of chronic opiate exposure on the function and expression of ERK1/2 and expression of D1R in the mPFC. Panel (a) illustrates a representative western blot of phosphorylated ERK1/2 and total ERK 1/2. (b) Western blot analysis revealed a significant increase in phosphorylated ERK1, but not total ERK1 following chronic heroin exposure. (c) The ratio of phosphorylated to total ERK1 was significantly increased in opiate chronic exposure/ withdrawn animals. (d) Levels of phosphorylated ERK2 and total ERK2 were not significantly altered in opiate chronic exposure/withdrawn groups. (e) The ratio of phosphorylated to total ERK2 was unchanged in opiate exposure/withdrawn animals. (f) Representative western blot of D1R expression in the mPFC. (g) Chronic heroin exposure does not alter the expression of D1R protein. Intra-mPFC microinfusion of 1 μ g PD334581 did not block the acquisition of a morphine CPP in either the opiate-naïve state (h) or in the opiate chronic exposure/withdrawn state (i) (* $p < 0.05$, ** $p < 0.01$; error bars represent SEM).

2.3.6 Intra-mPFC ERK1 Signaling is not Required for Opiate Memory Formation in Either the Opiate Naïve or the Opiate Chronic Exposure/Withdrawn States

To examine the potential behavioral significance of the observed alterations in pERK1 in chronic exposure/ withdrawn mPFC tissue, we next tested the effect of ERK inhibition on the acquisition of a morphine CPP. Here, we used a pharmacologically selective ERK1 inhibitor, PD334581 (1 μ g/0.5 μ l), to directly examine the potential functional role of changes to pERK1 levels in opiate chronic exposure/withdrawn rats, given our findings that ERK1, but not ERK2 phosphorylation levels are altered as a function of chronic heroin exposure.

Two-way ANOVA revealed that selective inhibition of ERK1 in the mPFC had no effect on the acquisition of morphine CPP in the opiate-naïve state. There was no observed effect of treatment group (Figure 2.4h; $F_{(1, 13)} = 2.08$, $p = .17$), but we did observe an effect of conditioning environment ($F_{(1, 14)} = 39.45$, $p < .01$). Post-hoc analyses revealed that rats showed a significant morphine CPP following either intra-mPFC vehicle ($n = 7$; $p < .01$, as above) or PD334581 ($n = 7$; $p < .01$). Furthermore, inhibition of ERK1 in the mPFC had no effect on morphine CPP in the opiate chronic exposure/withdrawn state (Figure 4i). Statistical analyses revealed no effect of drug treatment ($F_{(1, 13)} = 3.54$, $p = .08$) and a significant effect of conditioning environment ($F_{(1, 14)} = 41.24$, $p < .01$). Groups receiving intra-mPFC microinfusion of vehicle ($n = 7$) or 1 μ g PD334581 ($n = 7$) both demonstrated a significant preference for the morphine-paired environment (p -values $< .01$).

2.3.7 Integrated ERK and CaMKII Signaling in the BLA-mPFC Circuit is Required for Opiate Reward Memory Acquisition

Thus far, the present results show that in the opiate-naïve state, intra-mPFC CaMKII (but not ERK) signaling is necessary for opiate reward memory formation. In contrast, in the BLA, opiate reward memory acquisition requires ERK (but not CaMKII) signaling specifically in the opiate-naïve state (Lyons et al, 2013). Given this BLA-mPFC

dissociation and previous evidence suggesting that the processing of opiate reward memory requires functional connectivity between the BLA and mPFC (Gholizadeh et al, 2013; Sun and Laviolette, 2012), we next examined if integrated activity involving intra-BLA ERK and intra-mPFC CaMKII signaling is necessary for the acquisition of morphine reward memories. We tested this hypothesis by functionally disconnecting the effects of intra-BLA ERK signaling from intra-mPFC CaMKII signaling by simultaneously blocking intra-BLA ERK signaling in one hemisphere (U0126; 1 μ g/ 0.5 μ l) and intra-mPFC CaMKII signaling (AIP; 0.5 μ g/0.5 μ l) in the contralateral hemisphere ($n = 5$) before morphine CPP conditioning (see Materials and methods section). In contrast, control groups received either intra-BLA combined with intra-mPFC vehicle infusions ($n = 7$), intra-BLA ERK inhibition (U0126, 1 μ g/0.5 μ l, $n = 7$) paired with intra-mPFC vehicle infusions, or intra-mPFC CaMKII inhibition (AIP; 0.5 μ g/0.5 μ l) paired with intra-BLA vehicle infusions ($n = 6$). A schematic representation of these experimental conditions is presented in Figure 2.5a.

Two-way ANOVA revealed a significant interaction between conditioning environment and treatment ($F_{(3, 21)} = 5.61, p < .01$; Figure 2.5b). Post-hoc analyses revealed that intra-BLA/intra-PFC vehicle microinfusions, unilateral blockade of intra-BLA ERK activity, and unilateral inhibition of intra-mPFC CaMKII activity all left the acquisition of a morphine CPP intact (p -values $< .01$). Unilateral intra-BLA ERK blockade paired with contralateral mPFC CaMKII inhibition completely blocked the formation of morphine CPP, with rats showing no preference for morphine vs saline-paired environments at testing ($p > .05$). Thus, contralateral disconnection of intra-BLA ERK from intra-mPFC CaMKII signaling during morphine CPP acquisition is sufficient to prevent morphine CPP reward memory formation.

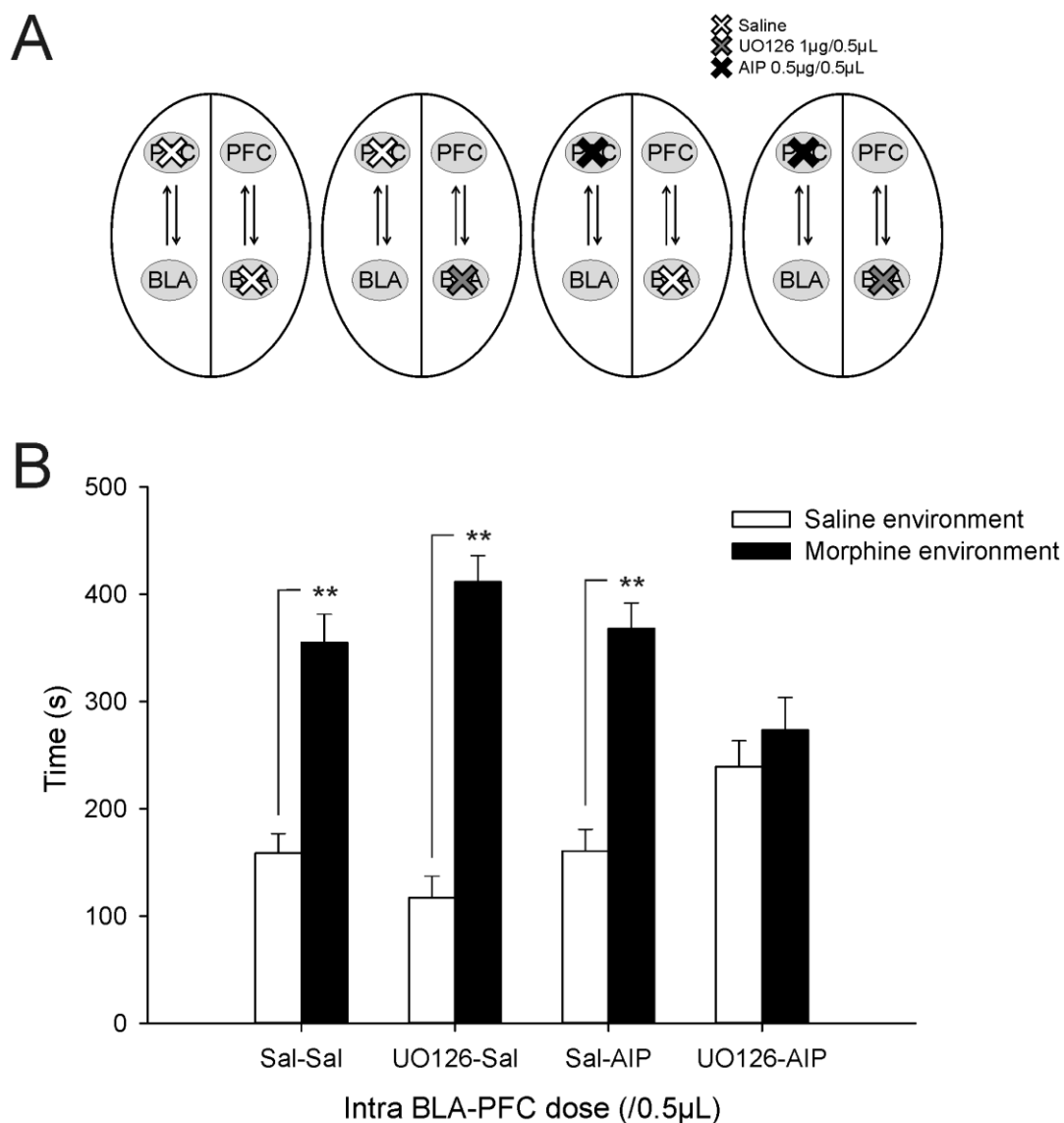


Figure 2.5 The BLA-mPFC circuit is required for opiate reward memory acquisition

Functional dissociation of the BLA-mPFC pathway in morphine CPP acquisition. (a) Schematic representation of experimental protocol for blocking the signaling molecules necessary for acquisition of a morphine CPP in the opiate-naïve state. (b) Unilateral intra-BLA microinfusion of 1 μ g UO126 is insufficient to block the acquisition of a morphine (0.5 mg/kg) CPP, whereas unilateral intra-BLA microinfusion of 1 μ g UO126 and contralateral intra-mPFC microinfusion of 500 ng AIP blocks the acquisition of a morphine CPP (** $p < 0.01$; error bars represent SEM).

2.4 DISCUSSION

The BLA-mPFC circuit is critical for the processing of opiate-related reward information (Lintas et al, 2011, 2012; Lyons et al, 2013; Sun and Laviolette, 2012; Sun et al, 2011). Importantly, bi-directional connections between the BLA and mPFC have been demonstrated to strongly modulate opiate-related reward memory and learning at the behavioral, neuronal, and temporal levels of analysis. For example, at the cortical level, the activity patterns of mPFC neuronal sub-populations are correlated with the encoding, recall, and extinction of opiate-related reward memory (Sun and Laviolette, 2012; Sun et al, 2011). Functional connections between the BLA and mPFC are required for the temporal transfer of associative opiate reward memories and inactivation of the BLA modulates the neuronal activity dynamics of mPFC, and regulates the acquisition and extinction of these memories (Gholizadeh et al, 2013; Sun and Laviolette, 2012). Nevertheless, the molecular mechanisms involved in BLA-mPFC circuit dynamics during the acquisition of opiate reward memories are not well understood.

Previous reports have demonstrated that opiate exposure state functions as a dynamic functional boundary between the roles of both the D1R and D2R systems and downstream molecular memory signaling molecules, including the ERK1/2 and CaMKII α pathways, specifically within the BLA (Lintas et al, 2011; Lyons et al, 2013). Consequently, in the opiate-naïve state, acquisition of opiate reward memory requires ERK1/2 and D1R-dependent substrates within the BLA. However, following chronic opiate exposure and spontaneous withdrawal, intra-BLA opiate reward memory formation switches to a D2R, CaMKII α -dependent signaling substrate (Lyons et al, 2013). In this study, we report that the role of the mPFC in the acquisition of opiate reward memories follows the opposite molecular memory pattern as a function of exposure state; that is, D2R-CaMKII-dependent signaling is required only in the opiate-naïve state, but not in the chronic exposure/withdrawn state, and both D2R and CaMKII α expression levels are strongly increased as a function of chronic opiate exposure and spontaneous withdrawal. In stark contrast, in the BLA, chronic opiate exposure/spontaneous withdrawal induces a marked down-regulation of CaMKII α and ERK1/2 expression patterns (Lyons et al, 2013). Interestingly, although inhibition of

ERK1/2 signaling in the BLA was shown previously to block opiate reward memory formation specifically in the opiate-naïve state (Lyons et al, 2013), in this study, inhibition of intra-mPFC ERK signaling was not sufficient to impair opiate reward memory formation, regardless of exposure state. This was the case despite the observation that chronic opiate exposure and spontaneous withdrawal induced an upregulation specifically in the ERK1 isoform within mPFC tissue. It is important to note that the intra-mPFC modulation of ERK1 levels shown here was substantially lower in magnitude to changes in intra-BLA ERK1/2 levels observed previously (Lyons et al, 2013) and, similar to CaMKII α levels, was in the opposite direction (increased vs decreased expression levels as a function of opiate exposure state). Although beyond the scope of this study, an alternative possibility is that other signaling pathways may be able to compensate for ERK1 activity in the presence of ERK1 antagonism.

Forebrain CaMKII α is necessary for contextual learning, specifically at the time of memory acquisition (Achterberg et al, 2014), consistent with our findings that intra-mPFC CaMKII inhibition during reward conditioning results in the blockade of morphine CPP acquisition. Furthermore, we have reported previously that intra-mPFC CaMKII signaling is necessary for the consolidation of acute opiate reward memories (Gholizadeh et al, 2013). At the molecular level, the observed increase in mPFC CaMKII expression is consistent with similar studies of region-specific increases of CaMKII following chronic morphine exposure (Chen et al, 2008; Narita et al, 2004). One study investigating potential biomarkers of chronic opiate exposure also found increased levels of Ca²⁺ in the serum of active heroin users (Divsalar et al, 2014). Interestingly, we observed a highly specific alteration in the total levels of the CaMKII α isoform within the mPFC following chronic opiate exposure and spontaneous withdrawal, which was not observed in either the CaMKII β or CaMKIV isoforms. Furthermore, no significant alterations were observed in mPFC expression levels of calcineurin A. The present results show alterations in the functional role of the D2-CaMKII pathway, with CaMKII and D2 inhibition preventing the formation of a morphine reward memory in the naïve, but not the chronic exposure/ withdrawn opiate exposure states. Whether chronic opiate exposure and spontaneous withdrawal results in alterations to D2 directly to affect downstream CaMKII expression, or whether CaMKII levels influence the expression of D2 is

unknown. It has, however, been suggested that alterations in D2 receptor trafficking reduces the expression of intra-mPFC CaMKII (Papaleo et al, 2012) and not vice versa.

CaMKII α signaling is functionally linked to D2R function. For example, previous reports have found that CaMKII α is bound to an intracellular domain of the D2R both in vitro and in vivo, within the striatum (Zhang et al, 2014). Modulation of CaMKII activity controls D4 receptor function within the mPFC during the acquisition of fear-related memories (Lauzon et al, 2012) and CaMKII α has been reported to control activity-dependent function of striatal D3 receptors by increasing their phosphorylation, thereby controlling D3 receptor-mediated responses to the psychostimulant effects of cocaine (Liu et al, 2009). Although the modulatory role of CaMKII α on prefrontal cortical D2 receptor substrates is not currently understood, the present evidence demonstrates for the first time, convergent increases in both total levels of CaMKII α and increased D2 receptor expression following chronic opiate exposure. Furthermore, these molecular alterations corresponded to a loss in sensitivity of D2R receptor function in the modulation of opiate reward memory formation, dependent upon drug exposure state, consistent with previous reports linking CaMKII α with D2R functional parameters, both in vitro and in vivo, in subcortical regions.

In addition, it is possible that increased intra-mPFC D2R and/or tCaMKII α expression in the opiate chronic exposure/ withdrawn state renders D2-CaMKII-dependent memory mechanisms resistant to antagonism by pharmacological manipulation. Increased expression of tCaMKII α may also reflect the formation of a stronger, more stable opiate reward memory, as cortical CaMKII α expression has been associated with the consolidation and permanence of classical conditioning memories (Frankland et al, 2001; Naskar et al, 2014). Increased expression of tCaMKII α may also indicate a sensitized response to the exposure of an opiate-related cue (ie, the morphine environment). Given our findings of increased cortical D2R expression following chronic opiate exposure, it is of interest that previous reports have demonstrated decreased D2R function in subcortical brain regions involved in drug-reward processing. For example, heroin addicts display downregulated D2R expression levels in the striatum (Martinez et al, 2012; Wang et al, 1997), suggesting a hypo-function of mesolimbic inputs to striatal regions. Although we

are not aware of any imaging studies demonstrating increased prefrontal cortical D2R expression levels in the opiate chronic exposure state, our findings may suggest that subcortical vs cortical DAergic transmission patterns are differentially altered in states of spontaneous withdrawal. Indeed, given that intra-BLA molecular and pharmacological DA function is opposite to that observed in the mPFC as a function of opiate exposure state, the present findings suggest important functional differences between cortical vs subcortical regulation of DA receptor function as a function of opiate exposure state.

The observed increase in mPFC pERK1 expression is consistent with previously reported increases in prefrontal cortical pERK following spontaneous withdrawal from chronic heroin self-administration (Edwards et al, 2009). Although intra-BLA ERK signaling has previously been shown to be necessary for opiate reward memory formation in the drug-naïve state (Lyons et al, 2013), the lack of behavioral effect of ERK inhibition in the mPFC remains consistent with data from Gholizadeh et al (2013), wherein acute opiate memory consolidation was shown to be ERK dependent in the BLA, but ERK independent in the mPFC. Whether changes to ERK activation or expression demonstrates a behaviorally relevant role in intra-mPFC opiate reward memory processing at other stages of memory processing (ie, later stage consolidation or retrieval) is still unknown. Alternatively, a significant increase in pERK1 activation following chronic heroin administration could render antagonism of ERK1 more difficult to achieve, and thus masking the behavioral effects of intra-mPFC ERK blockade. Finally, given that ERK1/2 is involved with many other molecular pathways, it is possible that the observed increase in pERK1 following chronic heroin exposure was mediated by an upstream change that does not involve the D1R, further suggested by our findings that increased expression in ERK1 levels did not correspond to any observable changes in the expression levels of intra-mPFC D1R.

Consistent with previous reports demonstrating a functional interplay between the BLA and mPFC during opiate reward memory processing (Gholizadeh et al, 2013; Sun and Laviolette, 2012), we found that simultaneous contralateral disconnection of intra-BLA ERK from intra-mPFC CaMKII signaling was sufficient to block opiate reward memory formation. In contrast, unilateral inhibition of ERK signaling was not sufficient for this

effect. Given that we observed a functional switch in the roles of both the D2R and CaMKII signaling as a function of opiate exposure state, which occurred in the opposite direction to that reported previously in the BLA (Lyons et al, 2013), future studies are required to more fully characterize how intra-BLA ERK activity may regulate associative neuronal activity in the mPFC and vice versa. Furthermore, an investigation into how these functional switches in the role of D2R and CaMKII signaling may impact the functioning of circuitry related to the formation of more naturalistic rewards, such as sucrose, would be an interesting extension of these results. Nevertheless, the present findings further implicate the functional importance of BLA-mPFC interconnectedness, both in terms of DAergic transmission and molecular substrates linked to memory processing during the opiate addiction process.

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Chapter 3

3 OPIATE EXPOSURE STATE CONTROLS DOPAMINE D3 RECEPTOR AND CDK5/CALCINEURIN SIGNALING IN THE BASOLATERAL AMYGDALA DURING REWARD AND WITHDRAWAL AVERSION MEMORY

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3.1 INTRODUCTION

Chronic exposure to opiates can induce plastic alterations in dopamine (DA) receptor function in brain regions involved in associative memory formation (Lintas et al., 2011, 2012). One such area, the basolateral nucleus of the amygdala (BLA), contains high levels of the dopamine (DA) D3 receptor (D3R; Gurevich and Joyce, 1999; Vorel et al., 2002; Beaulieu and Gainetdinov, 2011; Avalos-Fuentes et al., 2013), a member of the D2 receptor family that is critically involved in addiction-related memory processing (Yan et al., 2013). Indeed, D3R transmission in the amygdala influences the acquisition and expression of appetitive Pavlovian behaviors and opiate-related associative memory cues (Hitchcott and Phillips, 1998; Galaj et al., 2015). Chronic exposure to drugs of abuse has been shown to induce plastic adaptations in the D3R system as demonstrated in post-mortem tissue from cocaine (Staley and Mash, 1996; Segal et al., 1997) and opiate-dependent individuals (Cosgrove, 2010). Furthermore, D3R-related genetic polymorphisms have been observed in individuals expressing early-onset heroin dependence (Kuo et al., 2014) and in those with a history of heroin addiction (Levrant et al., 2014). Nevertheless, the precise functional role of intra-BLA D3R transmission in the processing of opiate-related learning and memory is not well understood.

Associative memory processing in the BLA involving select DA receptor subtypes and their downstream signaling cascades have been identified as critical players in opiate-related learning and memory (Lintas et al., 2011). For example, previous evidence has identified an intra-BLA, dopamine (DA) receptor-mediated, switching mechanism controlling the acquisition of opiate reward memories as a function of previous opiate exposure history and withdrawal state (Lyons et al., 2013). Specifically, intra-BLA D1 receptor (D1R) transmission and downstream modulation of the extracellular signal-related kinase (ERK) pathway, is critical for opiate reward memory formation during the early, non-dependent phase of opiate-related memory processing. However, following chronic opiate exposure and dependence, intra-BLA opiate-memory formation functionally switches to a D2 receptor (D2R) substrate associated with the calcium/calmodulin-dependent kinase II (CaMKII) memory signaling pathway (Lintas et

al., 2011). Thus, functional and molecular alterations in the D2R family represent important amygdala adaptations during the opiate addiction process.

Given its relatively high concentration in the BLA and its established role in drug-related learning and memory, the D3R is a potential candidate for processing opiate-related associative memory in the BLA. The D3R is linked both to the cyclin-dependent kinase 5 (Cdk5) pathway and calcineurin downstream molecular pathways (Chergui et al., 2004; Chen et al., 2009; Liu et al., 2009; Avalos-Fuentes et al., 2013), both of which play important roles in synaptic plasticity and the formation of conditioned reward memory (Mansuy et al., 1998; Biala et al., 2005; Baumgärtel and Mansuy, 2012; Lyons et al., 2013). However, how chronic opiate exposure may alter the functional activity state of the D3R within the BLA and its downstream molecular pathways is not currently understood, especially in the context of opiate-related memory formation.

The aim of the present study was to examine how intra-BLA D3R transmission may regulate the formation of both opiate-related reward and withdrawal aversion-related associative memory formation. In addition, we examined how chronic opiate exposure may alter the functional role and expression levels of intra-BLA D3R and its downstream effects on the Cdk5/calcineurin signaling pathways. Using both reward and withdrawal aversion place conditioning procedures combined with intra-BLA molecular protein analyses in rats, we report that chronic opiate exposure and withdrawal causes profound changes in intra-BLA D3R expression and function during the formation of both opiate reward and withdrawal aversion associative memories. Furthermore, these opiate-related alterations are dependent upon concomitant alterations in the function and expression of the D3R-linked signaling pathways, calcineurin and Cdk5.

3.2 MATERIALS AND METHODS

3.2.1 Surgical procedures

All procedures were performed in accordance with the Canadian Council on Animal Care and approved by the Western University Council on Animal Care. Male Sprague-Dawley

rats (350–400 g; Charles River) were anesthetized with ketamine/xylazine (80 mg and 6 mg/kg, respectively), given 1 mg/kg of meloxicam (non-steroidal anti-inflammatory analgesic) and placed into a stereotaxic device. Stainless steel guide cannulae (22 gauge; PlasticsOne) were bilaterally implanted into the BLA based on anatomical boundaries defined by Paxinos and Watson (2005) as follows: (no angle), from bregma, AP -2.6 , ML $+5.0$, DV -7.2 .

3.2.2 Drug treatments

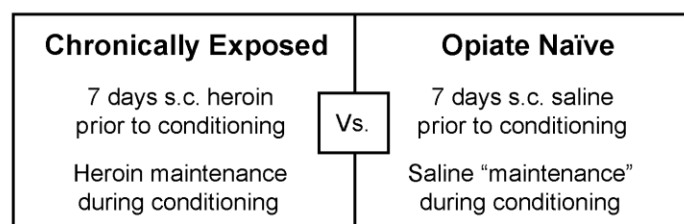
The selective D3R antagonist, PG01037 dihydrochloride (PG01037; 133-fold selective over D2R; Tocris Bioscience), Calcineurin auto-inhibitory peptide (calAIP; Tocris Bioscience), morphine (morphine hydrochloride, MacFarlane Smith) and heroin (diacetyl-morphine, MacFarlane Smith) were dissolved in physiological vehicle (0.9% NaCl; saline), pH adjusted to 7.4. The competitive Cdk5 antagonist Roscovitine (Sigma-Aldrich) was dissolved in a 50% DMSO and physiological vehicle solution. All intra-BLA drug or vehicle microinjections (0.5 μ L per infusion) were delivered via tubing connected to a 1 μ L Hamilton microsyringe over 1 min. Injectors were left *in situ* for an additional 1 min to ensure diffusion from the injector tip. For all groups, micro-infusions were performed immediately prior to injections of morphine or vehicle (i.p.) and subsequent placement in the conditioning environment. The dose of morphine used for CPP (5 mg/kg, i.p.) is a supra-reward threshold conditioning dose, and produces robust behavioural CPP (Sun et al., 2011; Lyons et al., 2013).

3.2.3 Comparison of opiate exposure states

Experimental groups consisted of non-dependent (i.e. rats that were previously naïve to opiates at the beginning of behavioural or molecular experiments) or chronically exposed and in withdrawal (i.e. rats that were chronically opiate exposed and in a state of withdrawal at the time of behavioural or molecular experiments), as previously described (Lintas et al., 2011; De Jaeger et al., 2013; Lyons et al., 2013; Rosen et al., 2015). For

chronic opiate exposure/withdrawal groups, rats were pretreated with once-daily injections of 0.5 mg/kg heroin (s.c.) in their home cage starting 7 days prior to behavioural conditioning. The first conditioning session began 21 h following the last heroin injection. During the conditioning procedure, maintenance doses of heroin were administered 2.5 to 3 h after the end of each conditioning session, for a total of 15 injections over the course of an experiment. Non-dependent rats received vehicle injections over the same time course, in place of heroin. This regimen produces aversive motivational effects (CPA) similar to those produced by a 3-weeks of morphine administration (Bechara et al., 1995; Laviolette and van der Kooy, 2004), thus indicating a state of potent opiate dependence. An outline of exposure states is illustrated in Fig. 3.1A.

A Treatment Conditions



B Experimental Timeline

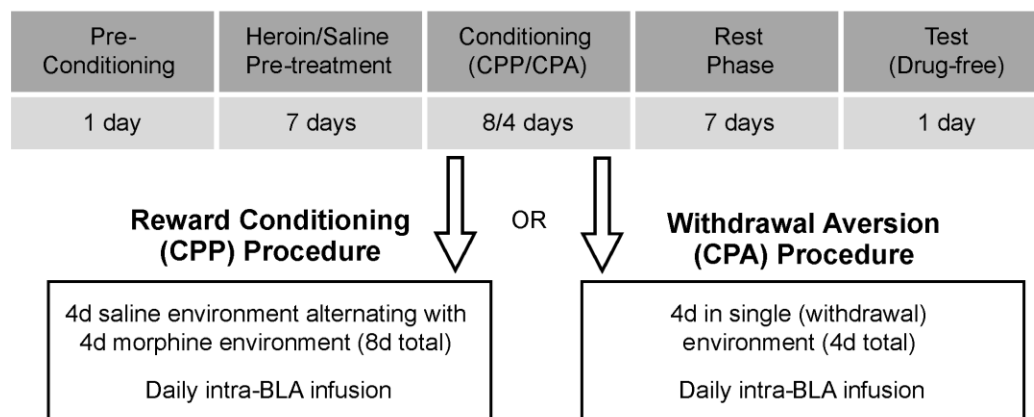


Figure 3.1 Outline of treatment conditions and experimental timeline

Experimental design and timeline. (A) Outline of two opiate exposure conditions. (B) Schematic outlining experimental timeline for conditioned place preference (CPP) and conditioned place aversion (CPA) paradigms.

3.2.4 Place preference conditioning

An unbiased, fully counterbalanced place conditioning paradigm was used, as described previously (Lintas et al., 2011; Lyons et al., 2013). All rats ($n = 117$) were given one week to recover from surgery before habituation for 20 min in a motivationally neutral grey box. Rats were then randomly assigned to an experimental group, and the conditioning procedure began the following day. One conditioning environment was white with a woodchip and wire mesh floor, and the alternate environment was black with a Plexiglas floor, wiped down with 0.25 mL of 2% acetic acid. Thus, the two conditioning environments differed in terms of texture, odor and colour. As described previously, these two conditioning environments elicit no baseline preference in rats (Laviolette and van der Kooy, 2003). Rats spent an equal number of 30-min conditioning sessions in morphine-paired and vehicle-paired environments (i.e. four morphine-environment pairings and four vehicle-environment pairings over the 8-day procedure). Testing was carried out in a drug-free state 7 days following the final conditioning session. The test environment consisted of both conditioning environments separated by a neutral grey zone. Rats were placed onto the neutral zone at the start of the test and allowed to freely explore the arena for 10 min while time spent in each environment was independently recorded.

3.2.5 Opiate withdrawal conditioned place aversion procedure

To study the effects of the D3R and its downstream targets on the acquisition of a CPA to environments paired with opiate withdrawal, an adapted CPA paradigm was used (Laviolette et al., 2002). In the single-side opiate withdrawal procedure, rats are exposed to only one of the two conditioning environments described above. Opiate dependent/withdrawn rats are placed into the environment immediately following microinfusion 21 h following their previous heroin injection (i.e. during withdrawal), and receive a maintenance dose of heroin 2.5 to 3 h following removal from the box, as per the CPP paradigm. Withdrawal conditioning environments were randomly assigned and counter-

balanced across groups. Rats ($n = 40$) received four exposures to the conditioning environment. As per the CPP procedure, rats were given a 7-day recovery period before testing in a drug-free state. Upon testing, rats will typically spend significantly less time in the environment in which they experienced aversive effects of withdrawal (Bechara et al., 1995), i.e. they form a CPA to the withdrawal environment. Previous studies have found this paradigm to be sensitive to both spontaneous and naloxone-precipitated withdrawal, and to not confounded by any novelty effects of the non-withdrawal environment upon testing (Bechara et al., 1995; Laviolette et al., 2002). An outline of both place preference and withdrawal aversion paradigms is presented in Fig. 3.1B.

3.2.6 Western blot procedure

Rats received 15 daily injections of either physiological vehicle (non-dependent group) or heroin (chronic exposure group; 0.5 mg/kg, s.c.), equivalent to the injections received by the experimental groups (opiate naïve vs. heroin exposed/withdrawn, $n = 8$ per group) rats used for behavioural pharmacology experiments. The tissue extraction and western blot procedure was performed as outlined in Lyons et al., 2013. Primary antibody dilutions were as follows: D1R (1:100; sc-14,001; Santa Cruz Biotechnology), D3R (1:100; sc-9114; Santa Cruz Bio-technology), phosphorylated Cdk5 [Tyr 15] (pCdk5; 1:100; Santa Cruz Biotechnology), Cdk5 (1:200; Santa Cruz Biotechnology), Calcineurin A (CnA; 1:100,000; Sigma-Aldrich) and α -tubulin (1:120,000; Sigma-Aldrich). All secondary antibodies (Thermo Scientific) were used at a 1:20,000 dilution. Blots were incubated in a TBS-T solution with either 2.5% Bovine Serum Albumin Fraction V (Calbiochem) or 5% non-fat dried milk (Carnation) as recommended by the manufacturer.

3.2.7 Histology

Upon the conclusion of behavioural experiments, rats were anesthetized with Euthanyl (sodium pentobarbital, 240 mg/kg, i.p.) and transcardially perfused with 0.9%

physiological vehicle and 10% formalin. Brains were extracted and refrigerated in a 25% sucrose in formalin solution at 4 °C for a minimum of 48 h. Brains were then sliced at 60 μm and stained with Cresyl Violet to allow for verification of cannula placements. Subjects with placements outside the anatomical boundaries ($n = 20$ out of 137 total) of the BLA as defined by Paxinos and Watson (2005) were excluded from analysis.

3.2.8 Data analysis

Conditioned place preference and conditioned place aversion data were analyzed with two-way analysis of variance (ANOVA), and post hoc analyses were performed with Fisher's least significant difference (LSD) test where appropriate. Densitometry values for western blots were obtained with Kodak digital analysis software and analyzed with two-tailed t -tests.

3.3 RESULTS

3.3.1 Chronic opiate exposure and withdrawal selectively downregulates intra-BLA D3R expression

While previous reports have demonstrated that chronic opiate exposure and withdrawal leads to a selective decrease in intra-BLA D2-like receptor expression (Rosen et al., 2015; Lyons et al., 2013), the precise D2R subtype involved in these effects is not known. Thus, to determine the potential involvement of intra-BLA D3R subtype plasticity across opiate exposure states, we first measured and compared expression levels of intra-BLA D3R vs. D1R levels in either non-dependent or chronically exposed and withdrawn rats (see Section 3.2.3). Example western blots illustrate the expression levels of D3R and D1R between a opiate exposure states in Fig. 3.2A and B, respectively. Interestingly, rats that have been exposed to chronic opiates express a selective downregulation of the D3R (Fig. 3.2C, $n = 7$, $t_{(12)} = 2.276$, $p = .042$), but not the D1R (Fig. 3.2D, $n = 8$, $t_{(14)} = 0.984$, $p = .342$).

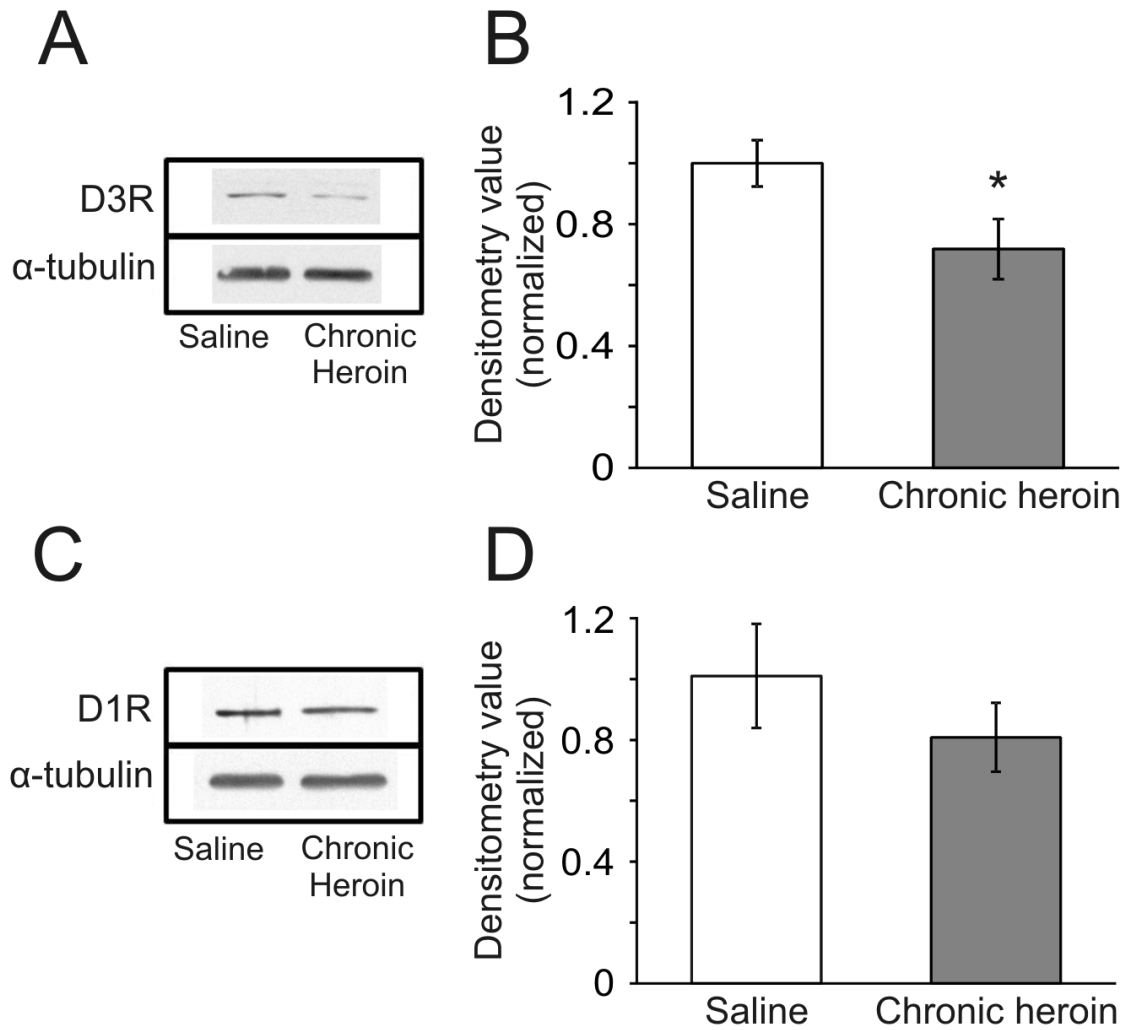


Figure 3.2 Expression of intra-BLA D1R and D3R following chronic heroin exposure

Chronic heroin differentially alters the expression of dopamine receptor subtypes in the BLA. (A) Representative western blot for D3R expression. (B) Densitometry analysis revealed a significant downregulation of the D3R in response to chronic heroin exposure (saline $n = 7$, heroin $n = 7$). (C) Representative western blot for D1R expression. (D) Densitometry analyses revealed that chronic heroin does not significantly alter the expression of the D1R. (saline $n = 8$, heroin $n = 8$). Asterisks represent $p < .05$.

3.3.2 Effects of intra-BLA D3R blockade on morphine reward CPP in the opiate-naïve state

We first examined the potential role of intra-BLA D3R blockade on the formation of a morphine reward CPP (5 mg/kg; i.p.) in rats with no previous history of opiate exposure (see Section 3.2.4). Histological analyses confirmed the placements of microinjector tips within the anatomical boundaries of the BLA as defined by Paxinos and Watson (2005). A microphotograph and schematic of intra-BLA placement (Fig. 3.3A, B) presents placements of representative experimental groups. We first tested three intra-BLA challenge doses of the selective D3R antagonist, PG01037 (10 ng, 100 ng and 1000 ng/0.5 μ L) (Fig. 3.3C). Two-way ANOVA revealed no treatment effect of the D3R antagonist relative to rats receiving intra-BLA vehicle ($F_{(3,27)} = 2.830, p = .156$), but a main effect of conditioning environment ($F_{(1,28)} = 40.93 p < .001$). Post-hoc analyses revealed that rats receiving intra-BLA vehicle ($n = 6$), 10 ng PG01037 ($n = 8$) 100 ng PG01037 ($n = 7$) or 1000 ng PG01037 ($n = 7$), spent significantly greater amounts of time in morphine-paired environments (p 's all $< .05$), thus indicating that intra-BLA D3R antagonism had no effect on the acquisition of a morphine CPP in non-opiate-exposed, non-dependent rats.

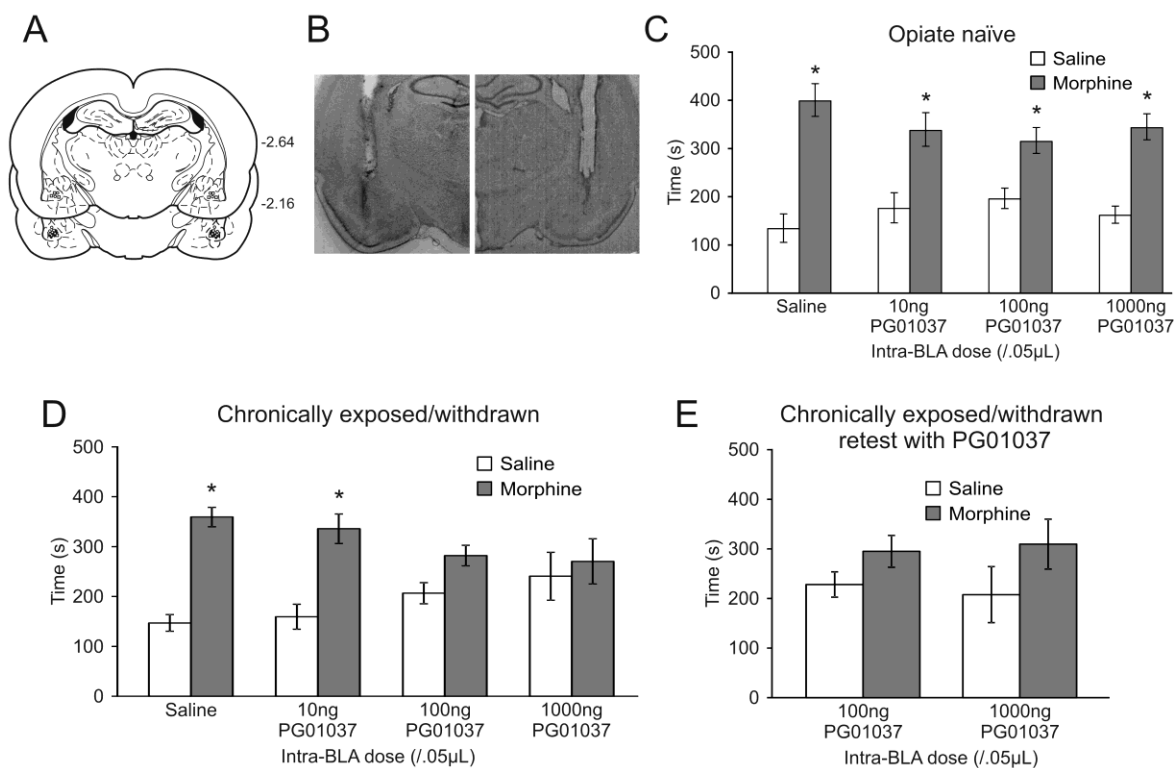


Figure 3.3 Assessment of intra-BLA D3R blockade on the acquisition of morphine CPP across opiate exposure states

Antagonism of the D3R affects the acquisition of a morphine CPP in a state-dependent manner. (A) Schematic of bilateral intra-BLA cannula placements. Open squares: intra-BLA vehicle in the opiate naïve state; open circles: 100 ng intra-BLA PG01037 in the opiate naïve state; closed circles: 1000 ng PG01037 in the chronic exposure/withdrawal state. (B) Representative microphotograph of intra-BLA guide cannula and microinjection tip placement. (C) Microinfusion of PG01037 in the opiate naïve state does not inhibit the acquisition of a morphine CPP. Group *n*'s: saline *n* = 7, 10 ng PG01037 *n* = 8, 100 ng PG01037 *n* = 7, 1000 ng PG01037 *n* = 7. (D) Microinfusion of PG01037 dose-dependently blocks the acquisition of a morphine CPP in the chronic exposure/withdrawal state. Group *n*'s: saline *n* = 8, 10 ng PG01037 *n* = 9, 100 ng PG01037 *n* = 8, 1000 ng PG01037 *n* = 6. (E) Microinfusion of PG01037 immediately before testing does not result in a recovered morphine CPP. Group *n*'s: 100 ng PG01037 *n* = 8, 1000 ng PG01037 *n* = 6. Time (s) represents time spent in morphine- or saline-paired environment on testing day. Asterisks represent *p* < .05.

3.3.3 Intra-BLA D3R antagonism blocks opiate reward memory formation in chronically exposed and withdrawn rats

Next, we examined the effect of intra-BLA D3R antagonism in rats chronically exposed to opiates and in withdrawal. We tested three doses of intra-BLA PG01037 during conditioning to create a full dose-response curve (Fig. 3.3D). Two-way ANOVA revealed a significant effect of conditioning environment ($F_{(1,31)} = 19.513, p < .001$). Post-hoc analyses revealed that groups receiving 100 ng ($n = 8$) or 1000 ng of intra-BLA PG01037 ($n = 6$) failed to express a morphine CPP (p 's $> .05$), whereas groups treated with a lower dose of intra-BLA PG01037 (10 ng; $n = 9$) or vehicle ($n = 8$) formed a significant preference for the morphine-paired environment (p 's < 0.05). Thus, in contrast to results in opiate-naïve rats, intra-BLA D3 blockade prevented the acquisition of morphine reward CPP following chronic opiate exposure and withdrawal.

Although we observed a block of CPP following conditioning with the D3R antagonist PG01037, previous studies have reported that a failure to acquire a CPP when a DA antagonist was applied during conditioning could be reversed by administering the antagonist upon testing (Ting-A-Kee and Mercuriano, 2013). To rule out the possibility of state-dependent recall of morphine CPP, we tested two groups of rats immediately after infusion of PG01037. We used the two effective doses from the initial dose response experiment in chronically treated rats (100 ng and 1000 ng) as illustrated in Fig. 3.3E. Two-way ANOVA revealed no effect of treatment or conditioning environment ($F_{(1,13)} = .170, p = 0.687, F_{(1, 14)} = 2.236, p = .161$, respectively). Rats in both the 100 ng group ($n = 8$) and 1000 ng group ($n = 8$) failed to demonstrate morphine CPP, thus indicating that the block of CPP by the D3R antagonist PG01037 in opiate exposed and withdrawn rats is not simply due to a block of state-dependent learning.

3.3.4 The downstream molecular targets of D3R, Cdk5 and calcineurin, are altered following chronic opiate exposure and withdrawal

Given our findings that chronic opiate exposure/withdrawal downregulates BLA D3R expression and increases sensitivity to D3R blockade during morphine CPP conditioning (Figs. 3.2, 3.3), we next explored whether these functional alterations in BLA D3R function may be mechanistically linked to downstream changes in the Cdk5/calcineurin signaling pathways. First, we compared the activation and expression of Cdk5 in the BLA as a function of opiate exposure state. Fig. 3.4A shows representative western blots of both phosphorylated and total expression levels of Cdk5 (pCdk5 and tCdk5, respectively) across opiate exposure states. Rats chronically exposed to opiates and in withdrawal at the time of tissue processing ($n = 7$) expressed a significant increase in pCdk5 compared to vehicle-treated rats ($n = 7$), indicating an increase in Cdk5 activation (Fig. 3.4B, $t_{(12)} = -2.303$, $p < .05$). There was no observed difference in tCdk5, however, indicating no change to cellular tCdk5 stores (vehicle $n = 7$, heroin $n = 7$; $t_{(12)} = -0.960$, $p = .356$). Finally, as a result of the change in phosphorylated, but not total, levels of Cdk5, we examined the ratio of phosphorylated (active) to the total cellular pool of Cdk5 and found a trending, though nonsignificant, change in ratio between vehicle- and heroin-treated tissue ($t_{(12)} = -2.021$, $p = .066$).

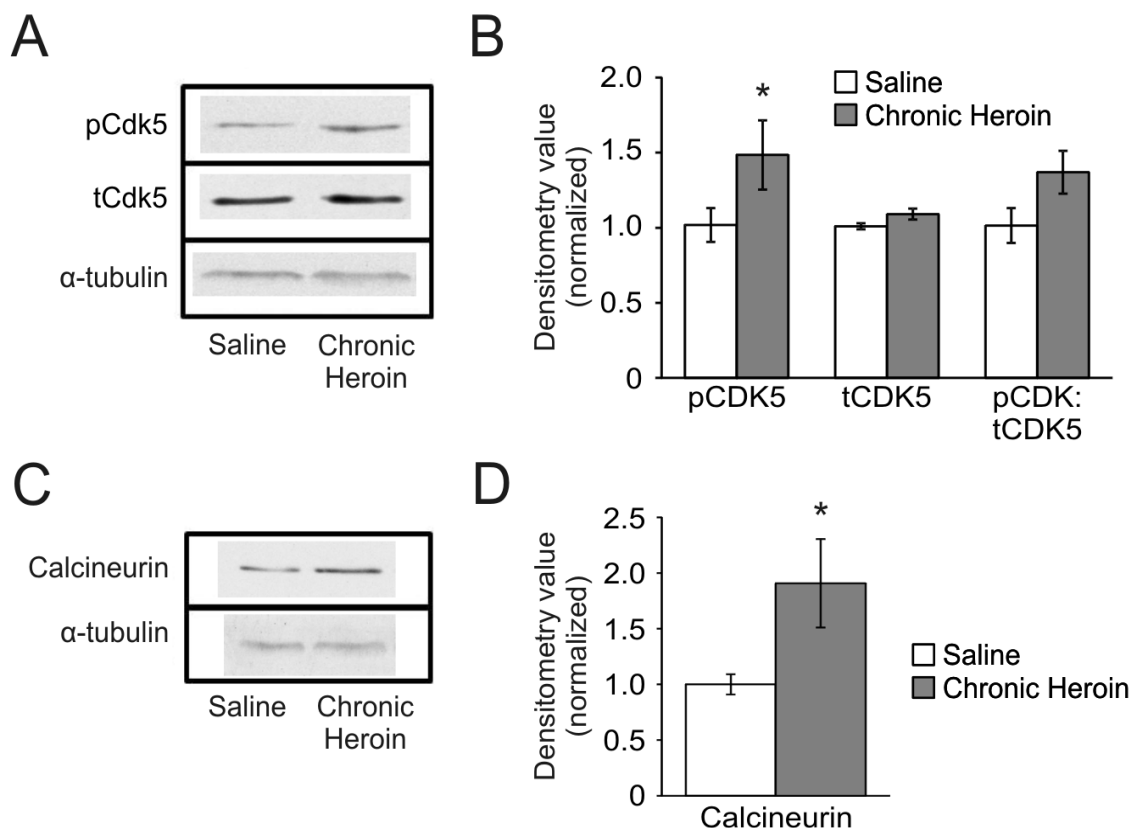


Figure 3.4 Intra-BLA Cdk5 and calcineurin expression following chronic opiate exposure

Chronic heroin affects the expression of signaling molecules downstream of the D3R. (A) Representative western blot for pCdk5 and tCdk5. (B) Densitometry analysis revealed a significant upregulation of pCdk5 (saline $n = 7$, heroin $n = 7$), but not tCdk5 (saline $n = 7$, heroin $n = 7$) following chronic heroin exposure. (C) Representative western blot for calcineurin expression. (D) Densitometry analysis revealed a significant increase of calcineurin following chronic heroin exposure (saline $n = 7$, heroin $n = 7$). Asterisks represent $p < .05$.

In addition to Cdk5, the D3R also affects downstream calcineurin signaling. We therefore compared the expression of intra-BLA calcineurin between vehicle- and heroin-treated rats, as shown in the representative western blot in Fig. 3.4C. An independent-samples t -test revealed a significant increase in the expression of calcineurin in rats that had been treated with heroin and were in a state of spontaneous withdrawal at the time of tissue processing (Fig. 3.4D, vehicle $n = 7$, heroin $n = 7$; $t_{(12)} = -2.215$, $p < .05$).

3.3.5 The D3R-dependent BLA memory switch is reversed by inhibition of intra-BLA Cdk5 and calcineurin signaling

In order to test whether the observed upregulations in intra-BLA pCdk5 and calcineurin expression following chronic opiate exposure and withdrawal are mechanistically linked to observed changes in BLA D3R sensitivity, we next challenged the effects of intra-BLA D3R blockade with co-administration of selective Cdk5 and calcineurin inhibitors. We hypothesized that if opiate-induced alterations to Cdk5 and calcineurin were underlying the ability of a D3R antagonist to block CPP acquisition in the dependent/withdrawn opiate exposure state, then the co-infusion of the D3R antagonist PG01037 with either Cdk5 or calcineurin inhibitors would reverse the effects of intra-BLA D3R blockade in the chronically exposed and withdrawn state. Here, we used two doses (100 ng and 200 ng/0.5 μ L) of the Cdk5 antagonist, roscovitine, and two doses of calAIP (50 ng and 500 ng/0.5 μ L).

Two-way ANOVA revealed a significant effect of conditioning environment ($F_{(1,40)} = 25.606, p < .001$) on times spent in vehicle vs. morphine paired environments (Fig. 3.5). Post-hoc analyses confirmed that the 1000 ng dose of intra-BLA PG01037 blocked the formation of a morphine CPP, as rats spent equal amounts of time in vehicle vs. morphine-paired environments ($p > .05$), whereas rats receiving intra-BLA vehicle spent significantly more time in morphine-paired environments ($p < .05$). Interestingly, co-infusion of the Cdk5 antagonist with intra-BLA PG01037 dose-dependently reversed the block of morphine CPP. Thus, whereas rats receiving 1000 ng PG01037 co-infused with a lower dose of ros (100 ng; $n = 7$) still demonstrated a block of morphine CPP ($p > .05$), co-infusion of PG01037 with a higher dose of roscovitine (200 ng; $n = 6$), reversed the effects of D3R blockade, with rats showing a significant morphine CPP ($p < .05$; Fig. 3.5). Similarly, co-infusion of calAIP with PG01037 dose-dependently reversed the effects of intra-BLA D3R blockade on morphine CPP. Thus, whereas rats receiving a lower dose of calAIP (50 ng) with PG01037 ($n = 6$) still demonstrated a block of morphine CPP ($p > .05$), rats receiving a higher dose of calAIP (500 ng) with PG01037 ($n = 7$) demonstrated a significant morphine CPP ($p < .01$; Fig. 3.5).

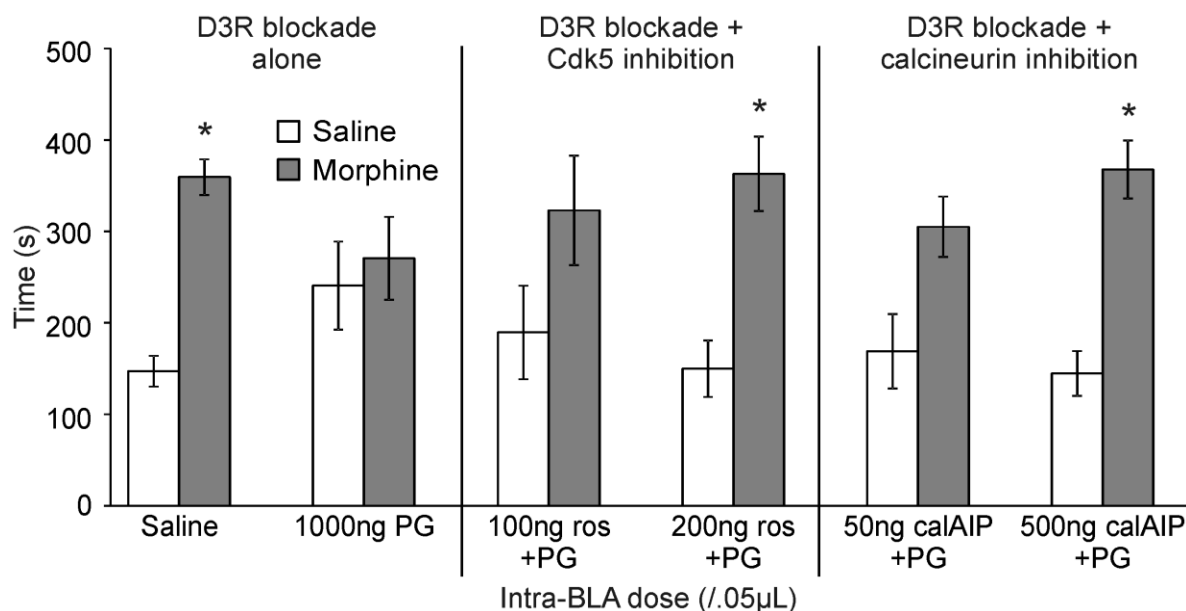


Figure 3.5 Reversal of intra-BLA D3-mediated block of CPP acquisition by administration of Cdk5 and calcineurin inhibitors

Pharmacological blockade of heroin-induced increases to Cdk5 and calcineurin restores the D3R-dependent block of CPP acquisition in chronically exposed/withdrawn animals. Co-infusion of Cdk5 inhibitor roscovitine (ros) dose-dependently recovers the block of CPP acquisition by 1000 ng PG01037 (PG) in chronically exposed/withdrawn animals. Co-infusion of calcineurin AIP (calAIP) also dose-dependently recovers the block of CPP acquisition by 1000 ng PG01037 in chronically exposed/withdrawn animals. Data in the D3R blockade alone section is presented again from Fig. 3.3D for ease of comparison. Group n 's: saline $n = 8$, 100 ng PG01037 $n = 6$, 100 ng ros + PG01037 $n = 7$, 200 ng ros + PG01037 $n = 6$, 50 ng calAIP + PG01037 $n = 6$, 500 ng calAIP + PG01037 $n = 7$. Time (s) represents time spent in morphine- or saline- paired environment on testing day. Asterisks represent $p < .05$.

3.3.6 Opiate withdrawal aversion memory formation depends upon intra-BLA D3R and downstream calcineurin and Cdk5 signaling

In addition to the formation of associative reward memories, the BLA is critical for the formation of associative opiate aversion memories related to withdrawal (Frenois, 2005). Accordingly, we next examined if intra-BLA D3R transmission may be involved in the acquisition of aversive opiate withdrawal memories. We ran six groups of rats in a conditioned place (withdrawal) aversion procedure (see Section 3.2.5). The results of

opiate withdrawal CPA memory experiments are presented in Fig. 3.6. (A) Two-way ANOVA revealed a significant treatment by environment interaction on times spent in opiate-withdrawal paired environments during testing ($F_{(5, 34)} = 3.352, p = .014$). Post-hoc analysis revealed that rats receiving intra-BLA vehicle ($n = 7$) spent significantly less time ($p < .05$) in withdrawal paired environments. In contrast, rats ($n = 7$) receiving the previously established effective dose of intra-BLA PG01037 (1000 ng), showed no CPA ($p > .05$), demonstrating that intra-BLA D3R transmission is required for the acquisition of aversive opiate withdrawal memories.

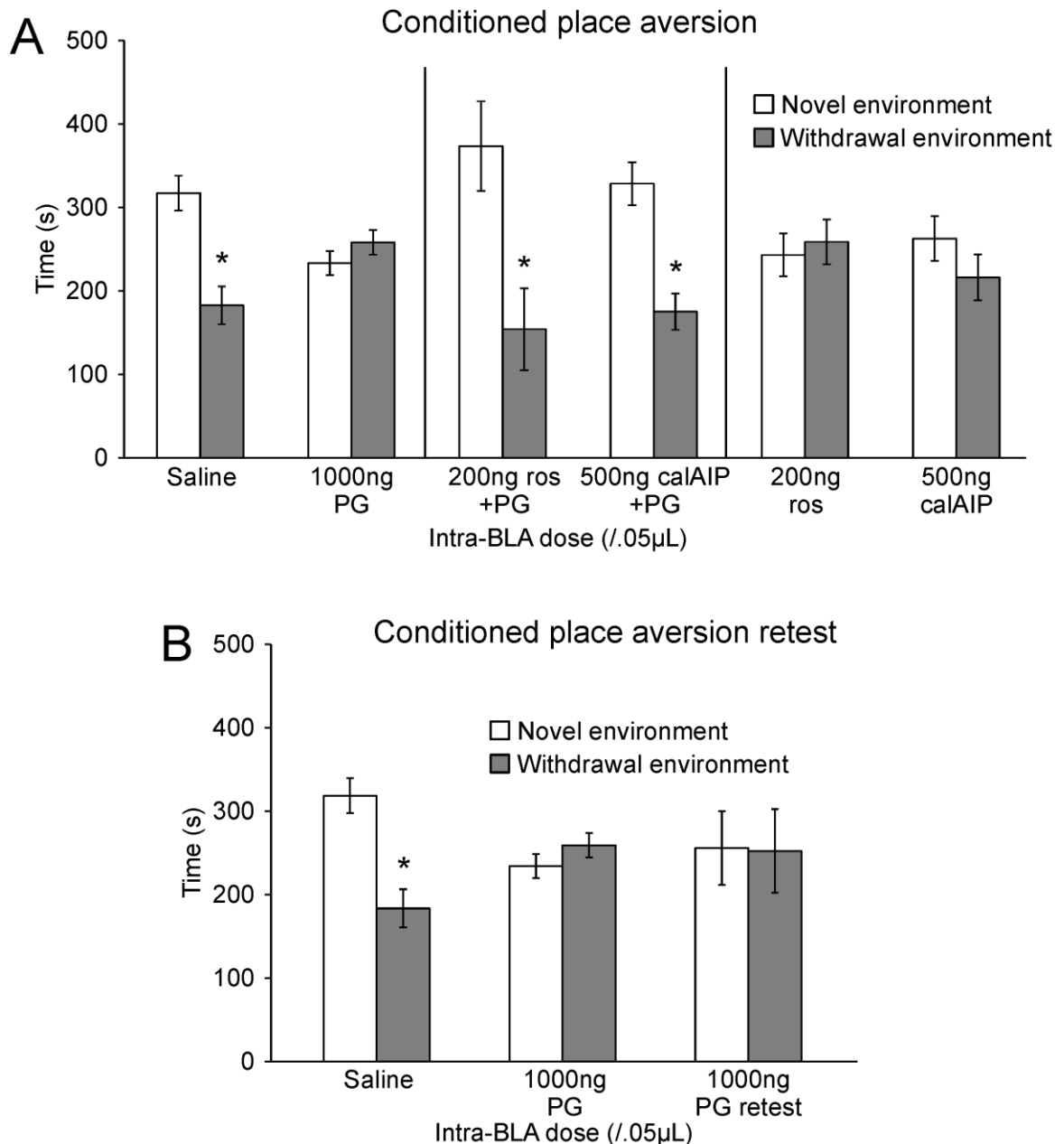


Figure 3.6 Intra-BLA D3-Cdk5/calcineurin signaling is required for CPA acquisition

Aversive withdrawal memories are sensitive to pharmacological manipulations of D3R and its downstream signaling targets. (A) Microinfusion of 1000 ng PG01037 (PG) blocks the acquisition of a CPA. Co-infusion of either roscovitine (ros) or calcineurin AIP (calAIP) with 1000 ng PG01037 recovers the PG-induced blockade of CPA acquisition. Group *n*'s: saline *n* = 7, 1000 ng PG01037 *n* = 7, 200 ng ros + PG01037 *n* = 5, 500 ng calAIP +PG01037 *n* = 7, 200 ng ros *n* = 8, 500 ng calAIP *n* = 6. (B) The block of CPA by PG01037 is not restored by infusing 1000 ng PG01037 immediately prior to testing. Groups receiving intra-BLA saline during conditioning, as well as those receiving

1000 ng intra-BLA PG01037 during conditioning but not testing are presented again from panel (A) for ease of comparison. Group n 's: saline $n = 7$, 1000 ng PG01037 $n = 7$, 1000 ng PG01037 retest $n = 7$. Time (s) represents time spent in withdrawal or novel environment on testing day. Asterisks represent $p < .05$.

Given our previous findings demonstrating that reversing chronic-opiate induced up-regulation of Cdk5 and calcineurin in the BLA reversed the effects of D3R blockade on opiate reward memory formation, we next tested if similar intra-BLA manipulations of Cdk5 or calcineurin may reverse D3R effects on opiate withdrawal memory formation.

Using the highest effective doses from our previous dose-response curves, we found that groups receiving 1000 μ g PG01037 co-infused with either 200 ng roscovitine ($n = 5$) or 500 ng calAIP ($n = 7$) demonstrated significant CPAs for the withdrawal environment (p 's $< .01$). Thus, similar to the effects of D3R blockade observed on opiate reward memory formation, pharmacological reversal of calcineurin or Cdk5 upregulation in the BLA fully reversed the functional effects of D3R blockade on opiate withdrawal memory formation.

Finally, we investigated whether intra-BLA administration of Cdk5 or calcineurin inhibitors alone would be sufficient to block the aversive effects of opiate withdrawal and inhibit the formation of a CPA to the withdrawal environment. Indeed, administration of intra-BLA 200 ng roscovitine ($n = 8$) or 500 ng calAIP ($n = 6$) alone resulted in the block of an acquired CPA, as demonstrated by a lack of significant difference between the time spent in the novel and withdrawal environments (p 's $> .05$). Again, to rule out the possibility of state-dependent effects of intra-BLA D3 antagonism on withdrawal-aversion memory formation during the initial CPP test, rats were re-tested in the CPP procedure, but in the presence of intra-BLA PG01037. As with CPP experiments, this effect was not altered by the administration of intra-BLA PG01037 during the CPP testing phase (see Fig 3.6 (B)). Animals tested in the CPA paradigm in the presence of intra-BLA PG01037 did not spend significantly different amounts of time in the novel or withdrawal environments ($p > .05$). Thus, this suggests that intra-BLA D3 blockade of the acquisition of CPA memories is not state-dependent.

3.4 DISCUSSION

Given the fundamental importance of neural learning and memory mechanisms during the opiate addiction process, a critical question concerns how different states of opiate exposure and withdrawal may alter molecular memory substrates linked to the positive and aversive motivational properties of opiates. We have previously demonstrated that chronic opiate exposure profoundly alters intra-BLA DAergic receptor expression and molecular memory substrates, including ERK and CaMKII (Lintas et al., 2011; Lyons et al., 2013; Rosen et al., 2015). However, while neuroadaptations in limbic D3R expression have been observed following chronic drug use (Le Foll et al., 2005; Cosgrove, 2010), the functional and mechanistic role of these adaptations have yet to be investigated in the context of BLA-dependent, opiate-related associative memory formation.

Here, we report that chronic opiate exposure results in a selective and profound down-regulation of intra-BLA D3R expression. These receptor level adaptations were associated with a functional switch in the role of intra-BLA D3R transmission during the acquisition of both opiate associative opiate reward memories as well as memories linked to the aversive effects of opiate withdrawal. Thus, while intra-BLA D3R blockade failed to block the formation of opiate reward memories in non-dependent rats, following chronic opiate exposure and withdrawal, we observed a functional switch to a BLA D3R-dependent opiate memory mechanism, and increased sensitivity to intra-BLA D3R blockade during opiate reward and withdrawal aversion memory formation. Interestingly, these D3R receptor expression alterations were concomitant with profound upregulation of both calcineurin and Cdk5, two signaling pathways that are linked to the functional properties of D3R transmission (Chergui et al., 2004; Chen et al., 2009; Liu et al., 2009), required for associative memory formation (Mansuy et al., 1998; Biala et al., 2005; Baumgärtel and Mansuy, 2012; Lyons et al., 2013), and which are altered by chronic drug exposure (Chiocco et al., 2011; Ramos-Miguel and García-Sevilla, 2012). These molecular adaptations in Cdk5/calcineurin were functionally linked to D3R-dependent memory formation, as pharmacologically inhibiting either calcineurin or Cdk5 was sufficient to reverse the effects of intra-BLA D3R blockade on both opiate reward and withdrawal aversion memory formation, in the chronically exposed and withdrawn states.

Interestingly, antagonism of either Cdk5 or calcineurin alone was sufficient to block the acquisition of a CPA to the withdrawal environment. Thus, intra-BLA roscovitine or calAIP alone was able to bypass D3 receptor-dependent modulation of associative withdrawal memory formation, and directly reverse intracellular upregulation of Cdk5 or calcineurin. This may suggest the potential clinical utility for these compounds to act as targeted therapeutics for treating opiate-related withdrawal aversion memories.

Given that the D3R is a member of the D2-type receptor family, the observed switch between the opiate naïve vs. chronically exposed and withdrawn states is consistent with previous findings showing that opiate reward memory formation is dependent on intra-BLA D2R transmission only after chronic exposure and withdrawal from heroin (Lintas et al., 2011). Furthermore, several previous reports have demonstrated a role for intra-BLA D3R activity in distinct phases of drug-related memory processing, including the incubation and/or recall phases of drug seeking behaviors, including cocaine and nicotine (Vorel et al., 2002; Beninger and Banasikowski, 2008; Di Ciano, 2008; Khaled et al., 2014; Xi et al., 2014). Our observed opiate-induced alterations to D3R expression are also consistent with preclinical reports of opiate-induced alterations in BLA D2R expression (Rosen et al., 2015) and aberrant neural D3R expression in substance abusing clinical populations (Le Foll et al., 2005; Cosgrove, 2010; Hou et al., 2012). While little is known regarding how chronic opiate-dependence selectively alters D3R expression in the human brain, previous studies have reported decreases of > 40% in the amygdala of chronic alcoholics (Tupala et al., 2001). Furthermore, homozygosity at the D3R gene has been linked to measures of increased sensation seeking in human heroin addicts (Duaux et al., 1998).

Interestingly, we found that the functional effects of intra-BLA D3R and associated calcineurin and Cdk5 signaling were similar during the formation of both rewarding and withdrawal-related aversive opiate-related memory formation, consistent with the involvement of the BLA in processing both reward and aversion-related associative memories (Lintas et al., 2011; Tan et al., 2011). What functional mechanism(s) may account for the presently observed relationship between BLA D3R transmission and calcineurin/Cdk5 signaling? One potential explanation may be related to the role of the

D3R as a presynaptic autoreceptor with a high affinity for DA (Missale et al., 1998; Boileau et al., 2015). For example, the D3R is particularly sensitive to increases in DA activity relative to the D1R and D2R. However, D3R activation results in inhibition of further DA release (Tang et al., 1994; Sokoloff et al., 2006; Chen et al., 2009). This process involves D3R suppression of presynaptic GABAergic inputs (Diaz et al., 2011; Avalos-Fuentes et al., 2013), and activation of both calcineurin and downstream Cdk5 signaling (Hernandez-Lopez et al., 2000; Chen et al., 2009). Consequently, one possibility is that at low tonic DA activity states, the D3R remains active, suppressing DA release (Avalos-Fuentes et al., 2013). However, in response to high levels of DA release (e.g. following mesolimbic DA activation in states of opiate withdrawal and increased BLA DA release (Wise, 2004; Luscher, 2013), the D3R may detect resulting elevations in intracellular Ca^{2+} and become inactivated, thus causing augmented disinhibition of BLA DA release and enhanced sensitivity to BLA DA blockade during opiate reward learning (Liu et al., 2009).

Given that intra-BLA DA release is necessary for the formation of affective associative memories (Rosenkranz and Grace, 1999; Grace and Rosenkranz, 2002), it is conceivable that DA release via activity-dependent deactivation of the D3R is involved in the formation of context-dependent drug memories. Indeed, previous reports have demonstrated that D3R modulation can influence the formation of place preference memories for drugs of abuse (Le Foll et al., 2002; Sokoloff et al., 2006). Furthermore, functional interactions between the D3R and CaMKII α signaling are responsible for activity-dependent reduction in D3R efficacy following high, but not low, frequency rates of action potential firing (Liu et al., 2009; Avalos-Fuentes et al., 2013), consistent with earlier reports that the D3R controls synaptic transmitter release only above a set spike frequency or burst duration threshold (Kuzhikandathil and Oxford, 1999). Liu et al. (2009) have further demonstrated that cocaine strengthens the CaMKII α -D3R association, and that disruption of that link subsequently restores the ability of the D3R to attenuate responses to subsequent cocaine administration. Pairing our current finding that the D3R is selectively downregulated following chronic opiate exposure with previous reports of opiate-induced reductions in intra-BLA CaMKII α expression (Lyons et al.,

2013), one possibility is that the CaMKII α -D3R complex is highly sensitive to antagonism of either D3R or CaMKII α during states of chronic opiate exposure and withdrawal. This hypothesis is supported both by the D3R-antagonist-dependent block of CPP and CPA formation reported here, and previous reports that CaMKII α blockade inhibits CPP acquisition selectively in states of chronic opiate exposure and withdrawal (Lyons et al., 2013).

D3R activation is linked to an increase in both calcineurin and Cdk5 activation (Chen et al., 2009). In the present study, we report that both calcineurin and Cdk5 activity are upregulated following chronic opiate exposure, while D3R expression is selectively downregulated. It is possible that while calcineurin and Cdk5 activity may remain augmented following chronic opiate exposure, the D3R is downregulated as a compensatory mechanism in response to over-stimulation by repeated opiate administration. Over-expression of Cdk5 has been reported to increase D3R-mediated suppression of DA release, likely via activation of calcineurin (Chen et al., 2009). Furthermore, Cdk5 inhibitors have previously been shown to increase DA release (Chergui et al., 2004). It is therefore possible that by blocking the chronic opiate-induced increases to calcineurin and Cdk5 activity, any remaining D3R-mediated suppression of DA release is removed, allowing for postsynaptic DA transmission, and the formation of associative memory formation. Given that Cdk5 is reported to be a downstream target of calcineurin in a D3R signaling cascade (Chen et al., 2009), it is likely that the observed restoration of reward and withdrawal aversion associative memory by co-administration of a calcineurin inhibitor may be a result of indirectly blocking downstream Cdk5, although this question requires further investigation.

3.4.1 Conclusions

The present findings demonstrate that chronic opiate exposure and withdrawal alters both the expression of the D3R in the BLA, and the function of the D3R in the context of opiate associative reward and withdrawal aversion memories. Furthermore, chronic opiate exposure and withdrawal induced functional adaptations in both Cdk5 and

calcineurin, two signaling pathways closely linked to the functional properties of the D3R. These findings add to a growing body of evidence that chronic opiate exposure results in profound alterations to the expression and function of DA-D3R transmission and associated downstream signaling pathways, and identifies a novel plasticity mechanism within the BLA linked to both reward and withdrawal-aversion related associative memory formation. The demonstration that the effects of opiate exposure and withdrawal on intra-BLA molecular memory mechanisms are reversible with targeted pharmacological manipulations of the Cdk5/calcineurin signaling pathways adds to growing evidence demonstrating the plastic nature of opiate-induced neuroadaptations and the potential clinical significance of targeting opiate-related associative memory formation as a potential treatment for persistent opiate-related addiction memories.

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Chapter 4

4 A TEMPORAL ANALYSIS OF OPIATE-INDUCED ALTERATIONS TO INTRA-BLA SIGNALING MOLECULES IMPLICATED IN THE FORMATION OF ASSOCIATIVE DRUG MEMORIES

4.1 INTRODUCTION

In the mammalian brain, regions associated with reward learning and memory are profoundly altered by chronic exposure to drugs of abuse. The basolateral amygdala (BLA) is the primary center for the processing of first-order associations between affective drug states and contextual cues via dopaminergic signaling. Interestingly, dopamine (DA) signaling is functionally altered by chronic opiate exposure such that the processing of opiate-related memories transitions from the use of a D1-dependent to a D2/D3-dependent mechanism between an opiate-naïve and an opiate-dependent and withdrawal state (Lintas et al., 2011; Lyons et al., 2013; Rosen et al., 2017).

Recent evidence further suggests that downstream signaling targets related to the D1R and D2/3R, also undergo changes in function and expression within the BLA following chronic heroin exposure (Lyons et al., 2013; Rosen et al., 2015, 2017). Specifically, extracellular signal-related kinase 1/2 (ERK1/2), Ca²⁺/calmodulin-dependent protein kinase type II α isoform (CaMKII α), calcineurin A (calcineurin), and cyclin-dependent kinase 5 (Cdk5) have each been identified as molecular targets that are subject to opiate-induced neuroplastic alterations, particularly in the context of synaptic plasticity events and drug-related associative memory formation (Mansuy et al., 1998; Ferrer-Alcón et al., 2003; Biala et al., 2005; Gerdjikov and Beninger, 2005; Narita et al., 2005; Xie et al., 2009; Baumgärtel and Mansuy, 2012; Lyons et al., 2013; Rosen et al., 2015).

Although the evidence of changes to DA signaling in mesocorticolimbic regions as a result of opiate exposure is growing, there is little understanding of the temporal dynamics of the molecular switching that occurs as a consequence of opiate addiction. Understanding the time course of cellular and molecular alterations resulting from opiate addiction is profoundly important from a clinical perspective. Opiate addiction is highly resistant to extinction, and memories linked to the addiction process can remain latent for years after abstinence, only to be unexpectedly triggered by exposure to associative cues that reinstate opiate seeking compulsive behaviours. This has raised the question of whether addiction is a permanent disorder.

Thus, it is critical to assess the permanence of both molecular and behavioural changes within mesocorticolimbic regions following chronic heroin exposure. Gao and colleagues (2013) have found that DA transmission (via D1 and D2Rs) within the nucleus accumbens shell region and caudate putamen is involved in opiate-seeking behaviour, even after prolonged abstinence. However, there is little work that directly compares intra-BLA DA signaling between opiate-dependent brains in acute withdrawal versus those at various stages of opiate recovery.

Here, we address the temporal dynamics and permanence of alterations to intra-BLA DA receptor signaling targets following the cessation of chronic heroin exposure. Using a western blotting procedure, we examined the expression of intra-BLA ERK1/2, CaMKII α , calcineurin and Cdk5 at multiple time points, ranging from before the onset of heroin withdrawal to 30 days following the cessation of heroin treatment.

We report that changes to signaling molecules within the BLA do not share a uniform time course relative to latency from spontaneous heroin withdrawal. Interestingly, particular signaling targets in heroin-treated animals appear to demonstrate peak change relative to opiate-naïve animals at distinct time points. This suggests that protracted opiate dependence may be a dynamic process with multiple phases, rather than a single neurobiological switch. Furthermore, we report that specific signaling pathways may represent the most promising targets for the treatment of long-term opiate addiction.

4.2 MATERIALS AND METHODS

4.2.1 Animals

All procedures were performed in accordance with the Canadian Council on Animal Care and approved by the Western University Council on Animal Care. Male Sprague-Dawley rats (350-400 g; Charles River) were used for all experiments.

4.2.2 Drug treatments and Comparison of Opiate Exposure States

Experimental groups consisted of heroin- or vehicle-treated animals. For chronic opiate exposure groups, rats were pre-treated with once-daily injections of 0.5 mg/kg heroin (s.c.) (diacetyl-morphine, MacFarlane Smith; dissolved in physiological vehicle; pH adjusted to 7.4) in their home cage for a total of 15 days. Non-dependent rats received vehicle (saline) injections over the same time course, in place of heroin. This regimen produces aversive motivational effects (conditioned place aversion) similar to those produced by a 3-weeks of morphine administration (Bechara et al., 1995; Laviolette and van der Kooy, 2004), thus indicating a state of potent opiate dependence. The 15-day regimen also allows for results that are easily compared to previous work (Lyons et al., 2013; Rosen et al., 2015)

Four time points were chosen for analysis, each labeled for the latency of analysis from the final treatment injection and are summarised in Table 4.1. Although much research has been conducted on the effects of chronic heroin on reward memory acquisition during opiate dependence and withdrawal (Lintas et al., 2011; Lyons et al., 2013; Rosen et al., 2015, 2017), it is unclear whether some observed effects are related to opiate dependence or withdrawal specifically. Thus, we assessed tissue from animals in a state of opiate dependence, but not withdrawal, 3 hr following the final heroin injection.

Previous work has demonstrated that a functional recovery of D1 signaling within the BLA occurs following chronic heroin exposure after as little as 7 days following cessation of heroin treatment (Lintas et al., 2011). We therefore included a 7d recovery group in our analyses. The 14d and 30d recovery groups were included to assess two long-term points of assessment. Thus, protein extractions occurred at four separate time points, measured by their latency from the 15th daily injection: 3hr, 7d, 14d, 30d, with labels outlined in Table 4.1.

Time from final injection	Heroin-treated	Vehicle-treated
3 hours	H3hr	V3hr
7 days	H7d	V7d
14 days	H14d	V14d
30 days	H30d	H30d

Table 4.1 Summary of experimental group names

4.2.3 Western blot procedure

At each time point, $n = 8$ non-dependent and $n = 8$ chronically exposed animals were sacrificed for tissue extraction. Upon sacrificing, animals' brains were rapidly removed and sectioned in order to obtain a micro-punch of the amygdala. Tissue samples were manually homogenized with a Dounce homogenizer and protein was isolated using a lysis buffer with a protease inhibitor (HaltTM protease and phosphatase inhibitor cocktail; ThermoFisher). Following homogenization, samples were mixed with 2X Laemmli loading buffer and heated to 95°C for 5 minutes prior to storage in a -80°C freezer.

For blotting, 12.5µg of protein from time yoked non-dependent and chronically exposed animals were loaded in an alternating pattern onto 10 or 12% denaturing SDS-Page gels. A BLUeye prestained protein ladder (FroggaBio) was loaded onto each gel in order to confirm the molecular weight of proteins of interest. Gel electrophoresis was run for 1 h at 125V in a Mini-PROTEAN 3 Western blot apparatus (Bio-Rad). Protein was then transferred to a nitrocellulose membrane (Bio-Rad) via a Mini Trans-Blot Cell (Bio-Rad). Membranes were stained with Ponceau S to ensure approximately equal protein volume across lanes and to verify the integrity of the protein before probing.

Blots were then blocked with 2.5% non-fat dried milk (NFDM; Carnation) in TBS-T for 1 h at room temperature on a rocker. Blots were then incubated overnight with rocking at 4°C in a TBS-T solution containing the primary antibody of interest and either 2.5% Bovine Serum Albumin Fraction V (Calbiochem) or 2.5% NFDM as recommended by the manufacturer.

Primary antibody dilutions were as follows: phosphorylated Cdk5 [Tyr 15] (pCdk5; 1:100; Santa Cruz Biotechnology), Cdk5 (1:200; Santa Cruz Biotechnology), Calcineurin A (CnA; 1:100,000; Sigma-Aldrich) and α -tubulin (1:120,000; Sigma-Aldrich). All secondary antibodies (Thermo Scientific) were used at a 1:10,000 dilution. Following incubation with the primary antibody, blots were washed with TBST (3 \times each for 5 min). Primary antibody dilutions are summarized in Table 4.2.

Blots were then incubated for 1h at room temperature with secondary antibodies (IRDye® goat anti-rabbit, goat anti-mouse; each at 1:10,000; LI-COR Biosciences; Table 4.3). Membranes were then washed with TBST (3 \times each for 5 min) followed by TBS (5 min).

Target	Species	Dilution
Calcineurin A	Mouse	1:100,000
pCaMKII α	Rabbit	1:1,000
CaMKII α	Rabbit	1:2,000
pERK1/2	Rabbit	1:1,000
ERK1/2	Rabbit	1:2,000
pCdk5	Goat	1:200
Cdk5	Rabbit	1:200
α -tubulin	Mouse	1:1,000,000
α -tubulin	Rabbit	1:5,000

Table 4.2 Summary of primary antibodies used

Antibody	Colour	Catalog #	Dilution
Donkey anti-rabbit	Green	926-32213	1:10,000
Donkey anti-goat	Green	926-32214	1:10,000
Donkey anti-mouse	Red	926-68072	1:10,000
Donkey anti-rabbit	Red	926-68073	1:10,000

Table 4.3 Summary of secondary antibodies used

4.2.4 Data Visualization and Analysis

Visualisation of protein levels was performed with the Odyssey Imaging System (LI-COR Biosciences). Densitometry values for western blots were obtained with Kodak digital analysis software. Outliers were identified using Chauvinet's criteria, and excluded from analysis. Main effects were analyzed with two-way analysis of variance (ANOVA), and post hoc analyses were performed with Fisher's least significant difference (LSD) test where appropriate.

4.3 RESULTS

4.3.1 Chronic heroin exposure alters calcineurin expression during acute dependence

Representative blots of calcineurin expression across all timepoints is presented in Fig. 4.1A. A comparison between heroin-treated and vehicle-treated animals revealed no significant effect of treatment or time from final injection on the expression of calcineurin ($F_{(1,51)} = 2.094, p = .154, F_{(3, 58)} = 1.894, p = .142$, respectively; see Fig. 4.1B). A trend towards significance was found for a time by group interaction $F_{(3, 58)} = 2.164, p = .104$. Post-hoc analyses revealed that heroin-treated animals showed an increase in calcineurin expression as compared to vehicle-treated animals 3 hours following final treatment injection ($p < .01$). Animals in the H3hr group also displayed significantly more calcineurin than animals in H7d, H14d and H30d (p 's $< .05$).

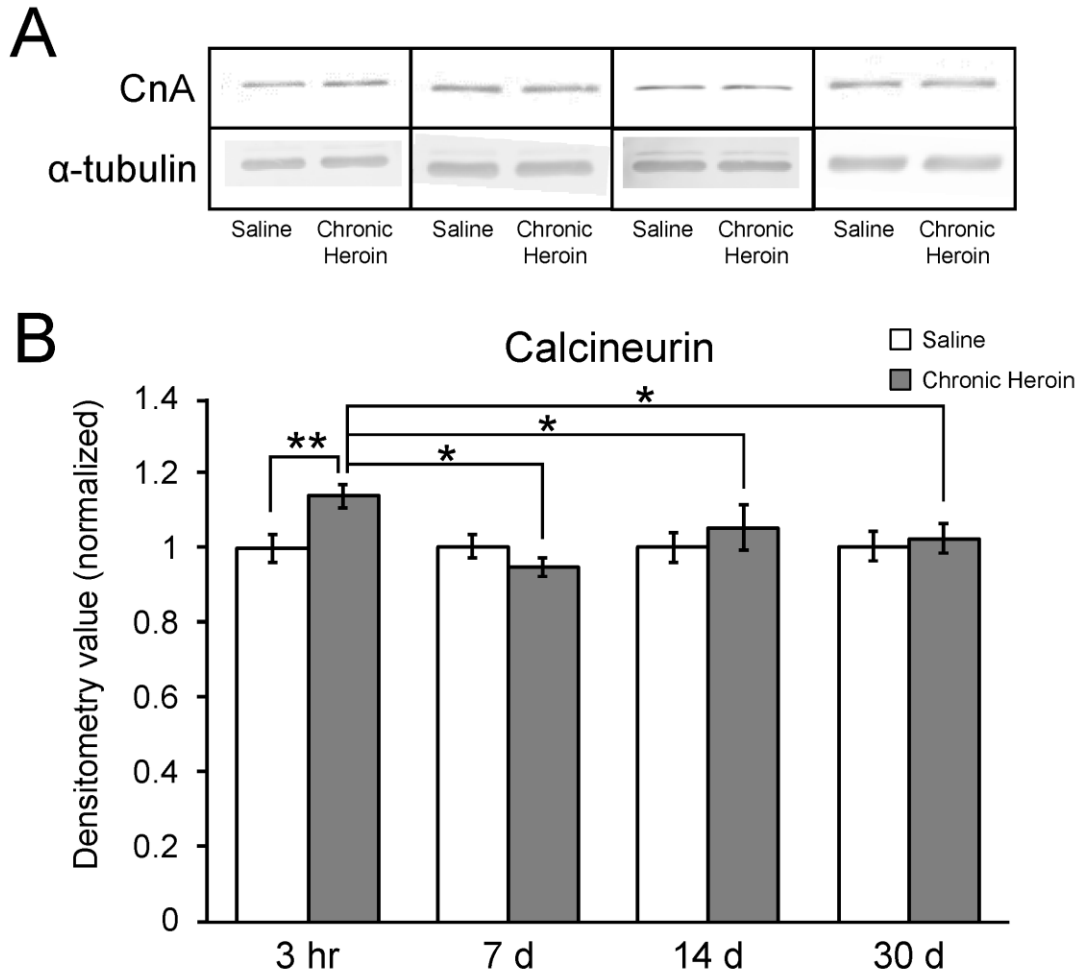


Figure 4.1 Intra-BLA calcineurin expression following the cessation of chronic heroin treatment

Chronic heroin exposure differentially affects the expression of calcineurin in the BLA in a time-dependent manner. (A) Representative western blots for calcineurin expression over time. (B) Densitometry analyses revealed time-dependent alterations to calcineurin expression. ** $p < .01$, * $p < .05$.

4.3.2 Chronic heroin exposure does not alter CaMKII α expression in animals outside the withdrawal state

Previous reports of CaMKII α expression in heroin-exposed animals showed a dramatic downregulation in both pCaMKII α and CaMKII α 21 hours following cessation of heroin treatment when animals are in a state of acute withdrawal (Lyons et al., 2013).

Interestingly, we did not observe any effects of heroin exposure when animals are not in a

state of withdrawal (representative blots shown in Fig. 4.2A, C). Heroin-treated and vehicle-treated animals showed no significant effect of heroin treatment or time from final injection on the expression of pCaMKII α ($F_{(1,54)} = 0.313$, $p = .578$, $F_{(3, 61)} = 0.795$, $p = .502$, respectively). Additionally, no significant interaction between treatment and time was observed $F_{(3, 61)} = 0.482$, $p = .696$ (Fig. 4.2B).

Furthermore, a comparison between heroin-treated and vehicle-treated animals revealed no significant effect of heroin treatment or time from final injection on the expression of CaMKII α ($F_{(1,53)} = 1.777$, $p = .188$, $F_{(3, 60)} = 0.093$, $p = .963$ respectively). Additionally, no significant interaction between treatment and time was observed $F_{(3, 60)} = 0.258$, $p = .855$ (Fig. 4.2D).

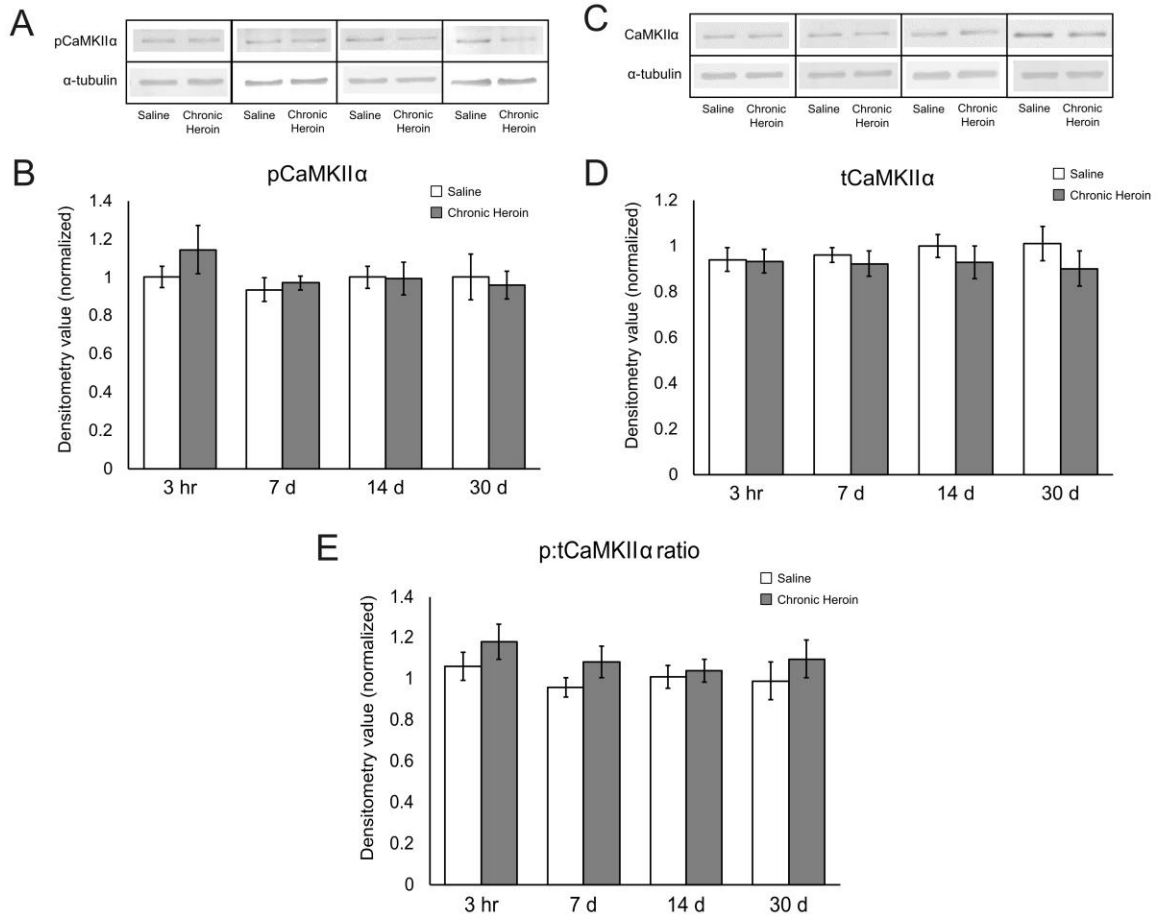


Figure 4.2 Intra-BLA CaMKII α expression following the cessation of chronic heroin treatment

Chronic heroin exposure does not alter the expression of CaMKII α . (A) Representative western blots for pCaMKII α over time. (B) Densitometry analyses found no effect of treatment or time on the expression of pCaMKII α . (C) Representative western blots for CaMKII α over time. (D) Densitometry analyses found no effect of treatment or time on the expression of CaMKII α . (E) Densitometry analyses found no effect of treatment or time on the ratio of phosphorylated to total CaMKII α expression.

Finally, analysis revealed no significant effect of heroin treatment or time from final injection on the ratio of phosphorylated to total CaMKII α ($F_{(1,51)} = 2.99, p = .090, F_{(3, 58)} = 0.722, p = .544$, respectively). No significant interaction between treatment and time was observed $F_{(3, 58)} = 0.164, p = .920$ (Fig 4.2E).

4.3.3 ERK1 expression is altered by chronic heroin exposure

Representative pERK1 blots are presented in Fig. 4.3A. Western blot analysis revealed no significant effect of treatment ($F_{(1,54)} = 0.591, p = .445$), time ($F_{(3, 61)} = .690, p = .562$) or a group by time interaction ($F_{(3, 61)} = 1.574, p = .206$) on the expression of pERK1. Post-hoc analysis, however, revealed a significant decrease in pERK1 between H3hr and H14d ($p < .05$; Fig 4.2B).

Furthermore, no significant effect of treatment or time on the expression of ERK1 (Fig. 4.3D) was found ($F_{(1,51)} = 2.078, p = .156, F_{(3, 58)} = 0.174, p = .913$, respectively). Additionally, no significant treatment by time interaction was observed ($F_{(3, 58)} = 0.829, p = .484$; Fig 4.3E).

Analysis revealed no significant effect of time and no significant time by treatment interaction on the ratio of phosphorylated to total ERK1 ($F_{(3, 56)} = 0.539, p = .658, F_{(3, 56)} = 0.942, p = .428$, respectively; Fig. 4.3G). A significant treatment effect was observed ($F_{(1,49)} = 4.561, p = .038$). Post-hoc analysis revealed that animals in the H14d group showed a significantly lower p:tERK1 ratio than V14d ($p < .05$). Animals in the H14d group also showed a significantly lower p:tERK1 ratio than H3hr ($p < .05$).

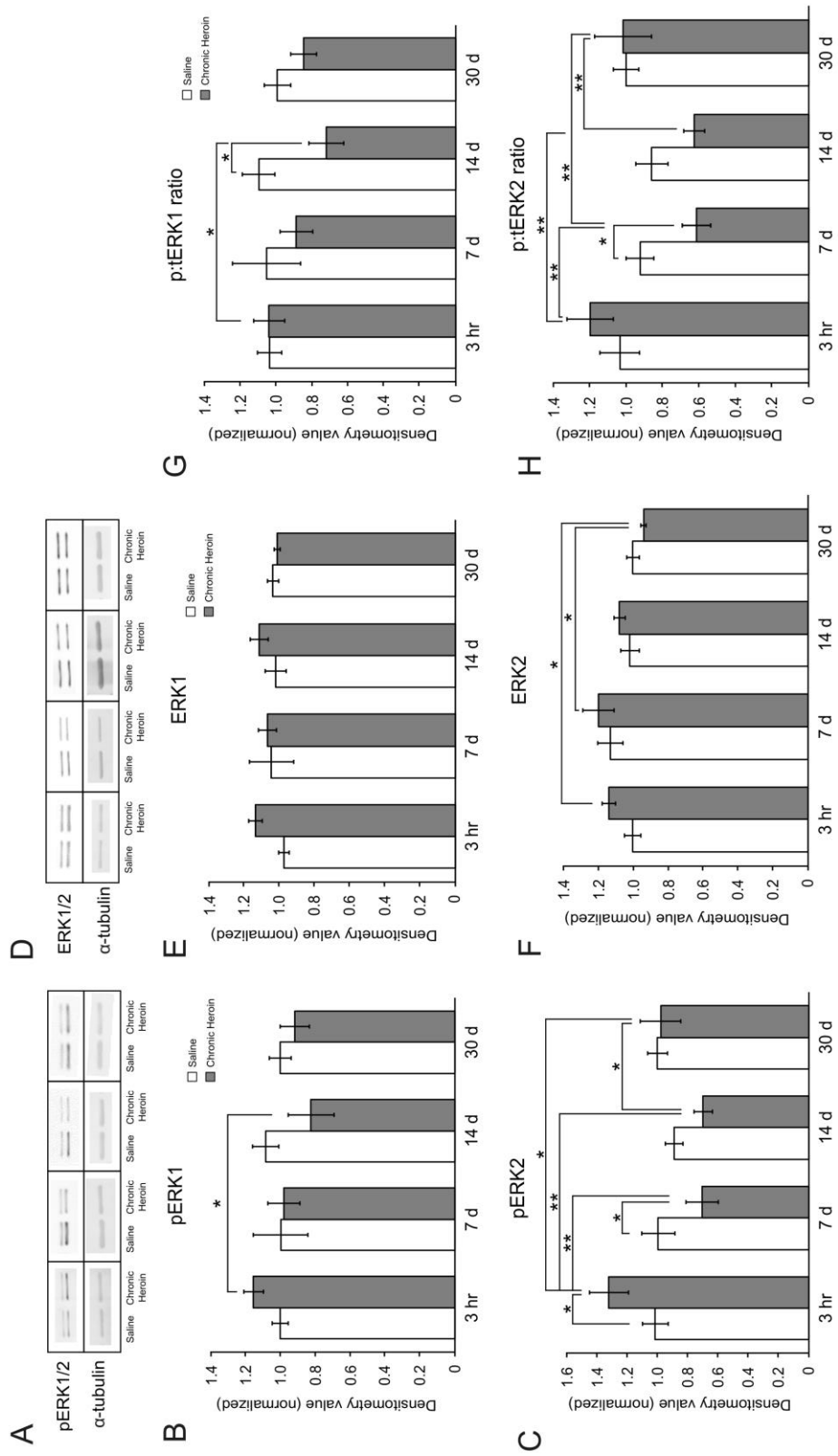


Figure 4.3 Intra-BLA ERK1/2 expression following cessation of chronic heroin treatment

Chronic heroin exposure differentially affects the expression of ERK1/2 in the BLA in a time-dependent manner. (A) Representative western blots for pERK1/2 over time. (B) Densitometry analyses revealed a decrease in pERK1 expression in heroin-treated animals between the 3 hr and 14 d time points. (C) Densitometry analyses revealed time-dependent alterations to pERK2 expression. (D) Representative western blots for pERK1/2 over time. (E) Densitometry analyses found no effect of treatment or time on the expression of ERK1. (F) Densitometry analyses found a decrease in ERK2 expression in heroin-treated animals from the 3 hr and 7 d time points to the 30 d time point. (G) Densitometry analysis revealed a decrease in the p:tERK1 ratio between heroin-treated and saline-treated animals at the 14 d time point. Heroin-treated animals also had a significantly lower p:tERK1 ratio relative to heroin-treated animals at the 3 hr time point. (H) Densitometry analysis revealed both time- and treatment-dependent effects on the p:tERK2 ratio. ** $p < .01$, * $p < .05$.

4.3.4 ERK2 expression is altered by chronic heroin exposure

Representative pERK2 blots are presented in Fig. 4.3A. A significant effect of time and a significant time by treatment interaction were found on the expression of pERK2 ($F_{(3, 60)} = 5.851$, $p = .002$, $F_{(3, 60)} = 3.617$, $p = .019$, respectively; Fig. 4.3C). Post-hoc analysis revealed that at 3 hours after final treatment injection, H3hr animals expressed significantly more pERK2 than V3hr animals ($p < .05$). At 7d, H7d animals expressed significantly less pERK2 than V7d animals ($p < .05$). H3hr animals expressed significantly more pERK2 than H7d ($p < .01$), H14d ($p < .01$) and H30d ($p < .05$). H30d expressed significantly more pERK2 than H14d ($p < .05$).

Analysis showed no significant effect of treatment, or a significant treatment by time interaction ($F_{(1, 52)} = 1.748$, $p = .192$, $F_{(3, 59)} = 1.135$, $p = .484$, respectively) on the expression of ERK2 (representative blots shown in Fig 4.2D). A significant effect of time was observed ($F_{(3, 59)} = 4.360$, $p = .008$; Fig. 4.3F). Animals in H30d expressed significantly less ERK2 than animals in H3hr ($p < .05$) and H7d ($p < .01$).

There was no observed effect of treatment on the phosphorylated to total ERK2 expression ($F_{(1, 49)} = 1.730$, $p = .195$; Fig 4.3H). A significant effect of time ($F_{(3, 56)} = 6.956$, $p = .001$) and a trending significant time by treatment interaction ($F_{(3, 56)} = 2.488$, $p = .071$) were observed. Post-hoc analyses revealed that H7d expressed a significantly

smaller p:tERK2 ratio than V7d ($p < .05$). H3hr had a significantly bigger p:tERK2 ratio than H7d ($p < .01$) and H14d ($p < .01$). Furthermore, H30d had a significantly greater p:tERK2 ratio than H7d ($p < .01$) and H14d ($p < .01$).

4.3.5 Cdk5 is altered by chronic heroin exposure following prolonged abstinence

Representative pCdk5 blots are shown in Fig 4.4A. Analysis revealed no significant main effect of treatment on the expression of pCdk5 ($F_{(1,54)} = 0.099$, $p = .754$; Fig 4.4B). A trend toward a significant effect of time on the expression of pCdk5 was observed ($F_{(3, 61)} = 2.272$, $p = .091$). A significant treatment by time interaction was observed ($F_{(3, 61)} = 4.367$, $p = .008$). H30d animals expressed significantly greater levels of pCdk5 than V30d animals ($p < .01$). Animals at H30d also expressed significantly higher levels of pCdk5 than H3hr ($p < .01$), H7d ($p < .05$) and H14d ($p < .01$).

Representative Cdk5 blots are shown in Fig 4.4C. A trending effect of both treatment ($F_{(1,55)} = 3.684$, $p = .060$) and time following final treatment injection ($F_{(3, 62)} = 2.660$, $p = .057$) was observed. No significant treatment by time interaction was observed ($F_{(3, 62)} = 2.038$, $p = .119$). Animals in the H7d group expressed significantly less Cdk5 than V7d animals ($p < .05$). Animals in the H3hr group expressed significantly more Cdk5 than the H7d group ($p < .05$), the H14d group ($p < .01$) and the H30d group ($p < .05$).

Heroin treatment had a significant effect on the ratio of phosphorylated to total Cdk5 ($F_{(1,53)} = 7.494$, $p = .008$; Fig 4.4E). Time from final treatment injection, however, had no effect on the phosphorylated:total Cdk5 ratio ($F_{(3, 60)} = 1.663$, $p = .186$; Fig 4.4D). The treatment by time interaction was trending towards significant ($F_{(3, 60)} = 2.511$, $p = .069$). Post-hoc analysis revealed that animals in H14d had a significantly higher p:tCdk5 ratio than V14d ($p < .01$), and that H30d showed a significantly greater p:tCdk5 ratio than V30d ($p < .05$). H3hr had a significantly lower p:tCdk5 ratio than both H14d and H30d (p 's $< .01$).

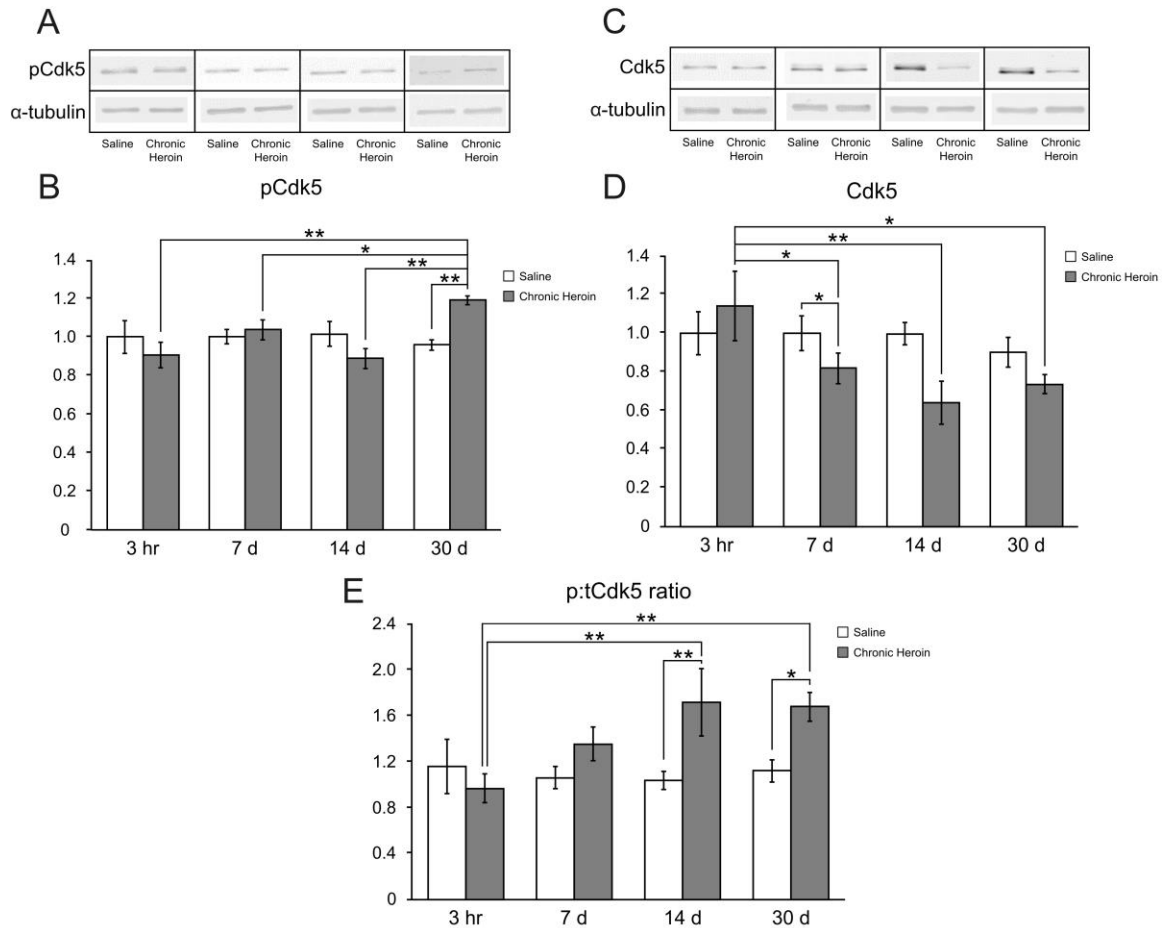


Figure 4.4 Intra-BLA Cdk5 expression following cessation of chronic heroin treatment

Chronic heroin exposure differentially affects the expression of Cdk5. (A) Representative western blots for pCdk5 over time. (B) Densitometry analysis revealed both time- and treatment-dependent effects on pCdk5. (C) Representative western blots for Cdk5 over time. (D) Densitometry analysis revealed both time- and treatment-dependent effects on Cdk5. (E) Densitometry analysis revealed both time- and treatment-dependent effects on the p:tCdk5 ratio. ** $p < .01$, * $p < .05$.

4.3.6 Summary of Results

The above results demonstrate a complex pattern of heroin-induced alterations to the expression of our proteins of interest based on time elapsed from the final heroin injection and signaling molecule. By taking into account heroin-induced changes to signaling molecules that have been previously observed 21 hrs following the cessation of

heroin treatment (i.e. during a state of opiate dependence and withdrawal; Lyons et al., 2013; Rosen et al., 2015; Rosen, Rushlow, & Laviolette, 2017), it appears that the individual signaling molecules demonstrate peak alterations at different time points. In particular, calcineurin shows the most profound changes at early time points, followed by CaMKII α during opiate dependence and withdrawal. At later time points (i.e. 7 d after the cessation of heroin treatment and beyond), the most notable heroin-induced alterations to the expression of signaling molecules are seen in ERK1/2 and Cdk5. A summary of this pattern of results is outlined in Table 4.4

	3hr	21hr	7d	14d	30d
Calcineurin	↑↑	↑↑	-	-	-
pCaMKII α	-	↓↓	-	-	-
tCaMKII α	-	↓↓	-	-	-
CaMKII ratio	-	-	-	-	-
pERK1	-	↓↓	-	-	-
tERK1	-	-	-	-	-
ERK1 ratio	-	↓↓	-	↓↓	-
pERK2	↑↑	↓↓	↓↓	↓	-
tERK2	-	-	-	-	-
ERK2 ratio	-	↓↓	↓↓	↓	-
pCdk5	-	↑↑	-	-	↑↑
tCdk5	-	-	-	↓↓	↓
Cdk5 ratio	-	↑	-	↑↑	↑↑

Table 4.4 Summary of results

Summary of heroin-induced changes to molecular signaling molecules as a function of time elapsed from the cessation of heroin treatment. Double arrows represent statistical significance ($p < .05$), and single arrows indicate a statistical trend ($p < .1$). Results for the 21 hr time point were published previously (Lyons et al., 2013; Rosen et al., 2015, 2017) and included here for ease of comparison.

4.4 DISCUSSION

The BLA is the primary brain region responsible for processing first-order associative drug memories via DAergic signaling (Guo et al., 2008; Lintas et al., 2011; Luo et al., 2013; Lyons et al., 2013). Anatomically, the BLA serves as a critical nexus point in the opiate addiction process as it receives inputs from other addiction-related brain regions, such as the VTA DAergic efferents, inputs from the PFC, and inputs from the hippocampus signaling contextually relevant information (Pitkänen et al., 2000; Ford et al., 2006). Given the functional importance of VTA DAergic inputs to the BLA, it is not surprising that chronic exposure to opiates has been shown to alter the expression of DA receptors and downstream signaling molecules that are responsible for the formation of these memories. Furthermore, these alterations have been demonstrated to affect opiate-related reward and aversion memories (Lyons et al., 2013; Rosen et al., 2015, 2017). The time course of these changes, however, remains poorly understood. Whether chronic opiate exposure results in a single major neuronal event or a longer-term series of changes has not been previously investigated. Here, we report that chronic heroin exposure results in differential, time-dependent alterations to signaling targets downstream of DA receptors. Interestingly, the profile of changes to these signaling targets is not uniform over time. Rather, it appears that specific signaling molecules demonstrate their most profound changes in expression over unique timelines.

4.4.1 Calcineurin

The expression of calcineurin is increased in heroin-treated animals only 3 hr following the cessation of heroin treatment, and returns to control levels by the 7 d timepoint. Previous reports have found that chronic heroin exposure results in a dramatic increase in intra-BLA calcineurin expression (Rosen et al., 2017). The effects found here are relatively modest when compared to our previous study of calcineurin in the BLA 21 hours following heroin exposure (Rosen et al., 2017). It is therefore possible that chronic heroin results only in shorter-term changes to calcineurin. This may reflect involvement of calcineurin in early acquisition of associative opiate memories, and is consistent with

reports that calcineurin is critical for transitioning memory traces from short- to long-term storage (Mansuy et al., 1998).

4.4.2 CaMKII α

In this study, heroin-treated animals demonstrated no change in the expression of either phosphorylated or total CaMKII α expression relative to control animals. These results are in stark contrast to previous reports that chronic heroin exposure leads to a drastic downregulation of CaMKII α (Lyons et al., 2013). This suggests that the previously observed opiate-induced changes to CaMKII α may be more attributable to opiate withdrawal, rather than dependence. Previous studies have found that the formation of opiate reward memories is blocked by both intra-BLA and intra-PFC CaMKII α antagonism when the CaMKII α antagonist AIP is applied when animals are in a state of opiate withdrawal (Lyons et al., 2013; Rosen et al., 2015). Whether the restoration of CaMKII α expression seen here may render opiate associative memory formation insensitive to AIP administration when applied at later time points (i.e. 7 d and beyond) is an interesting question for future study. For instance, previous work has shown that the switch between intra-BLA D1 and D2 receptor function that is observed following chronic opiate exposure is reversed following a 7-day recovery period (Lintas et al., 2011). It is also possible that opiate-induced behavioural changes to CaMKII α during the withdrawal process may gate further changes related to opiate associative memory formation at later time points.

4.4.3 ERK1/2

Our investigation into heroin-induced changes to ERK expression revealed no difference in the expression of intra-BLA pERK1 or ERK1 between heroin- and saline- treated animals. This is in contrast to previous reports that ERK1 function and expression are altered by chronic heroin treatment when animals are observed 21 hr following the cessation of heroin treatment (Lyons et al., 2013; Rosen et al., 2015). However, we did observe that reductions in ERK1 activation in heroin animals between the 3 hr and 14 d timepoints results in an alteration in the ratio of phosphorylated to total ERK1 between

heroin- and saline-treated animals. It is unknown whether the decrease in the p:tERK1 ratio at the 14 d timepoint would result in any functional changes to opiate associative memory formation, but it is a valuable question for future study.

In contrast to ERK1 expression, heroin-induced changes to ERK2 expression are more profound and complex. The expression of pERK2 in heroin-treated animals is significantly higher than saline-treated controls at the 3 hr timepoint, but drops relative to saline controls by the 7 d timepoint before recovering to control levels. The differences in p:tERK2 ratio between heroin- and saline-treated animals follows a similar pattern, showing a decrease at longer-term time points. This is consistent with previous reports of a lowered ratio of phosphorylated to total ERK1/2 in morphine-dependent rats (Ramos-Miguel et al., 2011).

The greater changes in ERK2 expression as a result of heroin exposure are inconsistent with reports that ERK1 and ERK2 are similarly affected by long-term heroin treatment at the 21 hr timepoint (Lyons et al., 2013). However, there is evidence that chronic opiate exposure has differential effects on the expression of ERK1 and ERK2 within the mPFC (Rosen et al., 2015). The behavioural implications for distinct patterns of these results in these two molecules is unclear, as the functional difference between ERK1 and ERK2 is not well understood (Kyosseva, 2004). There is evidence that ERK1 knockout mice are viable, but mice lacking ERK2 are not (Pouysségur et al., 2002), suggesting that implications of large changes to ERK2, but not necessarily ERK1, may warrant further investigation.

4.4.4 Cdk5

Our Cdk5 analyses showed that an increase in pCdk5 relative to saline control animals is only observed at the 30 d time period, indicating that long-term changes to Cdk5 may encode previous history of heroin exposure. This is accompanied by decreases in total Cdk5, and an increase in the p:tCdk5 ratio at later (14 d, 30 d) time points. This suggests that Cdk5 may be involved in the longer-term neuroadaptations that result as a consequence of heroin exposure, and could even be a candidate target for understanding

why previously opiate-exposed animals and humans alike remain prone to relapse long after drug dependence has passed. (Li et al., 2008; Pickens et al., 2011). This finding is in accordance with other studies that have suggested that sustained morphine exposure results in Cdk5-dependent long-term neuroplastic effects in mesolimbic regions (Ramos-Miguel and García-Sevilla, 2012). Interestingly, Chen et al. (2016) have suggested that alterations to Cdk5 signaling may reflect a compensatory mechanism that limits further heroin-seeking. Combined with the results presented here, Cdk5 appears to be a promising target in understanding the long-term neuroplastic adaptations that occur as a result of heroin addiction.

4.4.5 Summary and Future Directions

All together, the pattern of results in this study suggests that addictive states are more likely to result in a cascade of neurobiological adaptations over time, rather than one, discrete event. However, one major outstanding question related to the results outlined here is the effect of these molecular changes on drug-related memories and behaviours. Previous investigations into the effects of heroin on intra-BLA DA receptors and their downstream targets have found not only changes in expression, but alterations to their role in opiate reward and aversion memory acquisition (Lintas et al., 2011; Gholizadeh et al., 2013; Lyons et al., 2013; Rosen et al., 2017).

Furthermore, previous reports have investigated the role of interconnected mesocorticolimbic regions in the acquisition, maintenance and retrieval of opiate-related memories. The PFC, VTA and nucleus accumbens have all demonstrated functional alterations to DAergic signaling following chronic heroin exposure (Laviolette et al., 2002; Lane et al., 2008; Tan et al., 2014; Rosen et al., 2015). Understanding the shifting patterns of expression and function in memory-related molecules as a function of opiate exposure would thus be of value in these regions, as well

Understanding the time-dependence of these changes is also critical for future investigation. For instance, the PFC has previously shown a complementary pattern of molecular changes following chronic opiate exposure relative to the BLA (Rosen et al.,

2015). It would be interesting to assess whether the pattern of these changes relative to the BLA are expressed in the opposite direction at time points other than during acute opiate withdrawal.

To gain a better understanding of the cascading alterations to reward memory molecules as a result of chronic heroin exposure, there are a number of potentially interesting molecular targets to investigate. For instance, dopamine- and cAMP-regulated phosphoprotein, Mr 32 kDa (DARPP-32; also known as PPP1R1B) mediates neuronal activity of many drugs of abuse, including opioids (reviewed in Svenningsson et al., 2005). DARPP-32 can be found colocalized with DA receptors in the rat cortex and striatum (Rajput et al., 2009), and its activity is affected by opioid receptor activation via DAergic mechanisms (Lindskog et al., 1999). Furthermore, DARPP-32 is directly regulated by molecules involved in memory formation, including Cdk5 (Greengard et al., 1999) and calcineurin (Chiocco et al., 2010).

Given its role as a Cdk5 activator and mediator of MEK/ERK, PP1 and calcineurin signaling with respect to long-term plasticity events, p25 is also a promising target of future study into the effects of chronic opiate exposure (Sharma et al., 2002; Takahashi et al., 2005; Seo et al., 2014). Indeed, some studies have already found p25 expression to be altered by chronic morphine administration, and may be involved in long-term neuroplastic alterations resulting from opiate exposure (Ramos-Miguel and García-Sevilla, 2012).

Perhaps one of the most exciting implications of this study is that opiate addiction may not be, as often believed, a disease that results in permanent, untreatable neurobiological changes (see Self, 2004; Satel and Lilienfeld, 2014). Furthermore, this may address critical issues surrounding traditional treatments for opiate addiction (i.e. naloxone, methadone maintenance) that are aimed at preventing the aversive effects of withdrawal to reduce compulsive drug seeking, but have limited long-term efficacy (Petitjean et al., 2001; De Maeyer et al., 2011). Perhaps the results outlined here may guide more targeted treatment for the recovery from opiate addiction at various stages of the disease.

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Chapter 5

5 GENERAL DISCUSSION

5.1 SUMMARY OF RESULTS AND CONCLUSIONS

5.1.1 Chronic opiate exposure results in a functional switch of D2-CaMKII α signaling within the BLA-mPFC circuit

Following the discovery of an opiate exposure-dependent molecular switch between the role of D1 vs D2 signaling in the BLA in the formation of opiate reward memories (Lintas et al., 2011), we aimed to extend this work into other regions within the mesocorticolimbic circuitry. Given its bidirectional connections with the BLA, the mPFC was a prime target for expanding our investigation.

We found that within the mPFC, opiate exposure state predicts a similar molecular switch between D1 and D2 function in the acquisition of opiate reward memories. Animals in an opiate naïve state required D2 activity to form a conditioned place preference to a morphine environment, but D2 antagonism in opiate dependent and withdrawn animals did not affect the formation of an opiate reward memory. Like previous reports of intracellular DA target signaling in a similar paradigm (Lyons et al., 2013), the role of CaMKII α is similarly reversed between opiate exposure states, with antagonism of intra-PFC CaMKII α signaling resulting in the block of opiate memory formation in the opiate naïve state, but not the chronically exposed/withdrawn state. Interestingly, the intra-PFC switch we observed in Chapter 2 is functionally opposite to the one found previously in the BLA (Lintas et al., 2011; Lyons et al., 2013).

Chapter 2 further explored the role of an integrated BLA-mPFC circuit in the acquisition of opiate reward memory. We found that functional dissociation of the BLA and mPFC resulted in a block of opiate reward memory formation.

Together, these results not only extend evidence for a functional molecular switch in the context of reward memory formation following the transition to opiate dependence, but also further underscore the importance of the BLA-mPFC circuit in these processes. Furthermore, we found that the magnitude of observed changes to the PFC following what was previously reported in the BLA as a result of chronic opiate exposure (Lyons et al., 2013), was more modest. As a result, we identified the BLA as a more promising

target region for further understanding opiate-exposure-induced changes to the molecular substrates underlying first-order associative opiate memory formation (Chapter 3).

5.1.2 Chronic opiate exposure alters intra-BLA D3-Cdk5/calceineurin signaling in both reward and aversion memory formation

Given the magnitude of both molecular and behavioural changes seen in BLA vs. mPFC in Chapter 3, we shifted focus towards better understanding the extent of the molecular memory switch that occurs within the BLA as a result of opiate exposure. Previous studies investigating the switch in D1/D2 function and expression as a consequence of opiate exposure did not explore the role of distinct D2 subtypes (Lintas et al., 2011; Lyons et al., 2013; Rosen et al., 2015). The D3 receptor has become a promising target in understanding addiction-induced neuroplastic changes, thus we decided to explore how D3 signaling in the BLA may contribute to the acquisition of both opiate reward and withdrawal aversion memory formation following chronic opiate exposure.

We found that opiate reward memory formation is insensitive to D3 blockade in previously opiate naïve animals. In opiate-dependent animals, however, the formation of both opiate reward and withdrawal aversion memories were dependent upon intra-BLA D3 receptor signaling. This functional switch was accompanied by increases in Cdk5 and calcineurin expression, and a decrease in D3 expression. By inhibiting the increases in Cdk5 and calcineurin, we were able to return intra-BLA reward and aversion memory formation back to a D3-independent state in opiate dependent animals.

These findings add to the growing body of evidence that chronic heroin exposure results in alterations to the function and expression of intra-BLA DA transmission in the context of drug-related memory formation. The demonstration that the observed effects could be functionally reversed by restoring intracellular signaling targets back to an opiate naïve phenotype highlights the plastic nature of opiate-induced effects on the mesocorticolimbic system. Thus, these findings demonstrate the potential clinical

significance of targeting associative opiate memory formation for treating pervasive opiate addiction.

5.1.3 Chronic opiate exposure alters the expression of intracellular targets underlying drug memory formation in a time-dependent manner

Following the completion of Chapter 3, we began to uncover the extent of the DA receptors and targets that are affected by chronic opiate exposure. Until this point, however, all of our analyses had been performed while animals were in a state of opiate dependence and withdrawal (Rosen et al., 2015, 2017). This left a number of questions unanswered in regards to the time course of opiate-induced neuroplastic changes to the BLA. Specifically, 1) which of the changes observed to date have been a function of opiate dependence and which are a result of the acute withdrawal state and 2) what is the permanence of these changes?

Thus, we examined the expression of DA receptor targets that we had previously found to be altered by chronic heroin exposure. We probed for changes 3 hours following the cessation of chronic heroin administration (i.e. when animals were in a state of opiate dependence, but not withdrawal), as well as 7 days, 14 days and 30 days following the cessation of chronic heroin treatment.

We identified time-dependent changes in the expression of intra-BLA calcineurin, ERK1/2 and Cdk5. Specifically, the expression of calcineurin appeared to be altered only in a state of acute heroin dependence, and returned to baseline levels beyond 7 days following the cessation of heroin treatment. The expression of ERK1/2 appeared to be affected at both the short and longer-term time points following chronic heroin cessation, but also returned to normal near 30 days after chronic heroin treatment. Interestingly, the maximal changes to Cdk5 expression were observed 14 days and 30 days following the cessation of chronic heroin exposure, suggesting that Cdk5 could be involved in the persistence of opiate addiction long after opiate use has stopped.

We did not identify any changes to CaMKII α expression at the time points examined in this chapter, suggesting that the changes observed previously (Lyons et al., 2013; Rosen et al., 2015) were indeed a result of acute withdrawal. Given that CaMKII α antagonism has still been shown to result in the block of associative opiate memory formation in the drug-free state, this suggests that a lack of alteration to CaMKII α at the later time points may not in fact be indicative of a lack of behavioural effect, but this hypothesis will require further study.

5.2 LIMITATIONS

As outlined in Chapter 1, the mesocorticolimbic system is a complex, interconnected circuit of multiple brain regions. When studying such complex systems, one must consider the relationship of reciprocal connections in addition to synergistic effects of multiple neurotransmitters. For instance, the role of glutamatergic signaling within the mesocorticolimbic system is well documented (Fields and Margolis, 2015). Not only has glutamate been implicated in the development of opiate dependence (Siggins et al., 2003; Martin et al., 2004), alterations to D1 and D2 receptors have been found to influence the function of glutamatergic receptors, particularly in the context of synaptic strengthening (Lane et al., 2008; Madhavan et al., 2013). Ultimately, a more complete view of the mechanisms underlying drug memory processing will require a thorough investigation into dynamics between neurotransmitter systems.

Similarly, the work outlined in this thesis has provided only a rudimentary analysis of the intracellular mechanisms involved in opiate memory formation. Future work will be required to uncover the complex interplay of intracellular signaling substrates in the context of opiate associative memory processing. For instance, the ERK1/2 signaling pathway is linked to more than just downstream D1 activity. Girault and colleagues (2007) have proposed that the role of ERK1/2 may be to act as an integration hub of DA and glutamate signals within the striatum, and that its role in processing drug-related memories could indeed be widespread throughout the limbic system, and recruit multiple neurotransmitter systems (Valjent and Pascoli, 2005; Girault et al., 2007; Philibin et al.,

2011; Zamora-Martinez and Edwards, 2014). For instance, in the context of memory formation, there is evidence that both ERK1/2 and CaMKII α may be regulated by serotonin signaling (Cammarota et al., 2008). DARPP-32 has also been proposed as an integrator of signals converging at DAergic neurons via a variety of neurotransmitters and neuromodulators, including glutamate (Svenningsson et al., 2004; Fernandez et al., 2006), thus representing another viable target for studying the convergence of multiple transmitter systems.

Furthermore, DA receptors regulate multiple signaling pathways not explored in this thesis that have been studied in relation to addiction processes and could also be critical for gaining a complete understanding of drug memory processing. Some of these targets include the Akt-GSK3 β -mTORC1 pathway and β -arrestin signaling (Kim et al., 2005; Rozenfeld and Devi, 2007; Beaulieu and Gainetdinov, 2011; Salles et al., 2013; Miller et al., 2014).

Broadly speaking, there are some inherent limitations to the above studies that need to be considered when extending this work to a clinical research. By not using a self-administration model of opiate addiction, we cannot relate the current findings to drug memory formation in users that actively seek out drugs, nor can we shed light on how opiate associative memories relate directly to the persistence of drug use despite adverse consequences. For instance, chronic stress has been identified as a characteristic shared by many addicts (Kreek and Levran, 2012). Stress in itself has been found to have effects on habit-forming and decision making (Dias-Ferreira et al., 2009).

Evidently, opiate addiction is a complex disorder that does not exist in isolation of psychosocial factors, and future work that can more closely tie basic research of reward circuitry to the understanding of whole individuals living with a disorder would be of immense value to developing an effective treatment for opiate addiction.

5.3 FUTURE DIRECTIONS

As outlined in Chapter 1, the BLA-mPFC link is just one of many within the highly interconnected mesocorticolimbic system. Further extending the work outlined in this thesis to the greater mesocorticolimbic circuitry could help us to better understand the behavioural significance of opiate associative memory processing. For example, examinations of opiate-induced changes to NAc processing could help us to evaluate the neurobiological underpinnings for how individuals act on cravings. As a primary brain structure underlying memory formation, future studies of hippocampal activity in drug-related memory processing would also be of value.

As discussed in Chapter 4, many questions remain about the behavioural significance of long-term, opiate-induced alterations to intracellular DA signaling targets. In particular, it would be interesting to evaluate the involvement of these long-term changes in the incubation of drug craving. Both humans and rodents demonstrate a time-dependent increase in cue-induced drug seeking following withdrawal from opiates, cocaine and alcohol (Pickens et al., 2011). Whether some of the changes observed in Chapter 4 underlie this phenomenon is a potentially interesting area for future investigation.

The investigations of opiate-induced alterations to associative memory formation in this thesis were focused exclusively on changes to the neurobiological substrates underlying memory acquisition. Indeed, memory processing is complex and multi-faceted. A more complete picture of how opiate exposure results in alterations to memory processing will require examination of the consolidation, recall and extinction of associative memories, as well. It would be particularly interesting to directly study the resistance of opiate-related memories to extinction in the context of guiding the development of addiction treatments (Everitt, 2014). Along these lines, studies of synaptic-level learning in opiate related memory processing would also be necessary. Long-term potentiation and long-term depression are commonly studied mechanisms of learning and memory, and have been identified as candidate mechanisms for understanding opiate-induced disruptions in neural circuitry (Hyman and Malenka, 2001).

Certainly, there are many compelling research questions to pursue in both basic and clinical research on opiate addiction. Ultimately, it will be the convergence of many techniques and approaches that will best guide the development of more effective treatments and/or pharmacotherapies for addictive disorders. In this thesis, my integrative research projects have revealed a series of novel molecular adaptations within the BLA-mPFC circuitry related not only to how the brain physically adapts during the process of opiate dependence, addiction and withdrawal, but how these neuroadaptations are functionally linked to learning and memory processing and their associated behavioural outcomes. The mechanistic role of these neuroadaptations in the context of processing opiate-related addiction memories underscores not only the complexity of neurophysiological and psychological underpinnings associated with opiate addiction, but also the importance of taking an integrative approach to developing more effective approaches to addiction treatment.

For example, most current pharmacotherapies for opiate dependence (e.g. methadone therapy, naloxone and buprenorphine) primarily serve to target the underlying pharmacological substrates required for maintaining opiate-related euphoria and/or preventing the negative physiological and psychological effects of opiate withdrawal. Nevertheless, evidence from my research highlights the importance of targeting specific receptor systems and their downstream molecular signaling targets in order to block and/or prevent the formation of opiate-related reward or withdrawal-related associative memories, and how these interventions can lead to improved behavioural outcomes. Thus, combining and integrating research and treatment modalities that target both opiate-induced molecular adaptations in brain pathways such as the BLA-mPFC circuit as well as the psychological underpinnings of opiate-related memory formation (e.g. cognitive behavioural therapies), might ultimately serve as the most effective strategy for combatting the devastation of the opioid epidemic.

5.4 REFERENCES

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Appendices

Appendix A: Animal Protocol Approval



2010-247::6:

AUP Number: 2010-247

AUP Title: Characterization of an Opiate and Nicotine Addiction Switching Mechanism in the Mammalian Brain: Behavioural and Electrophysiological Investigations

Yearly Renewal Date: 11/01/2016

The YEARLY RENEWAL to Animal Use Protocol (AUP) 2010-247 has been approved, and will be approved for one year following the above review date.

1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

REQUIREMENTS/COMMENTS

Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Schoeller, Marianne
on behalf of the Animal Use Subcommittee

The University of Western Ontario
Animal Use Subcommittee / University Council on Animal Care
Health Sciences Centre • London, Ontario • CANADA - N6A 5C1
PH: 519-661-2111 ext. 86768 • FL: 519-661-2028
Email: aupsc@uwo.ca • <http://www.uwo.ca/animal/website/>

Curriculum Vitae

Laura Gillian Rosen

Education

2017	Doctor of Philosophy in Neuroscience The University of Western Ontario
2012	Master of Science in Neuroscience Queen's University at Kingston
2010	Honours Bachelor of Science in Psychology Queen's University at Kingston

Scholarships and Awards

Sept 2016 - Dec 2016	Western Graduate Research Scholarship	\$2,334
Sept 2016	Doctoral Excellence Research Award	\$1,666
Sept 2015 - Aug 2016	Western Graduate Research Scholarship	\$7,000
July 2015	Mediterranean Neuroscience Society Travel Award	€500
May 2015 - Apr 2017	Natural Science and Engineering Research Council of Canada (NSERC) Canada Graduate Scholarship	\$70,000
Sept 2014 - Aug 2015	Western Graduate Research Scholarship	\$7,000
Sept 2014 - Apr 2015	Ontario Graduate Scholarship	\$10,000
July 2014	International Narcotics Research Conference Travel Award	\$800
Sept 2013 - Aug 2014	Ontario Graduate Scholarship	\$15,000
Sept 2013 - Aug 2014	Western Graduate Research Scholarship	\$7,000
Jan 2013 - Aug 2013	Western Graduate Research Scholarship	\$9,216
Sept 2011 - Aug 2012	Queen's Graduate Award	\$6,000
Sept 2010 - Aug 2011	Queen's Graduate Award	\$6,000

Professional Service

Spring 2017: Appointed member of the University Secretary Search Committee, The University of Western Ontario

Fall 2016: Appointed member of working group to review the University Research Board terms of reference

Fall 2016: Reviewer for Psychology and Neuroscience

September 2016-2017: Graduate Student Representative, University Research Board, The University of Western Ontario

June 2016-2017: Student Representative, University Discipline Appeal Committee, The University of Western Ontario

January 2016-2017: Student Representative, Senate Nominating Committee, The University of Western Ontario

June 2015-2017: Student Representative, Senate Review Board Academic, The University of Western Ontario

June 2015-2017: Graduate Student Senator, The University of Western Ontario

Teaching

Fall 2016: Lecture “Neuroplasticity of reward memory in opiate addiction” for:

- Integrative Neuroscience, ACB 5551F
- Integrative Neuroscience, ACB 4451F
- Neuroscience 9551

Fall 2011: Teaching Assistant: Introduction to Comparative Cognition, Psyc 205, Queen’s University

Media and Outreach

April 2014: Finalist in the University of Western Ontario 3 Minute Thesis. “Forget Me Not: The Story of Heroin's Grasp on the Brain”

Sept 12, 2013: Broadcast interview on XFM News, 106.9 CIXX FM discussing the findings and significance of Lyons (2013) paper in the *Journal of Neuroscience*.

Sept 2013: “Discovery could help treat opiate addiction”, The Gazette (Western University campus newspaper) <http://www.westerngazette.ca/2013/09/discovery-could-help-treat-opiate-addiction/>

Peer-Reviewed Publications

- Rosen, L.G.**, Rushlow, W.J. & Laviolette, S.R. (2017). "Opiate exposure controls dopamine D3 receptor and cdk5/calceinurin signaling in the basolateral amygdala during reward and withdrawal aversion memory formation." *Progress in Neuropsychopharmacology and Biological Psychiatry*, 79: 59-66.
- Renard, J., **Rosen, L.**, Rushlow, W.J. & Laviolette, S.R. (2016). "Role of the Prefrontal Cortex in Addictive Disorders." *The Cerebral Cortex in Neurodegenerative and Neuropsychiatric Disorders: Experimental Approaches to Clinical Issues*. Eds. David Cechetto and Nina Weishaupt. Elsevier. 289-309. Print and eBook.
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Habib D., Tsui C.K., **Rosen L.G.**, Dringenberg H.C. (2013). Occlusion of Low-Frequency-Induced, Heterosynaptic Long-Term Potentiation in the Rat Hippocampus *In Vivo* Following Spatial Training. *Cerebral cortex*. 24(11): 3090-6.

Hogsden J.L., **Rosen LG.**, Dringenberg H.C. (2011). Pharmacological and deprivation-induced reinstatement of juvenile-like long-term potentiation in the primary auditory cortex of adult rats. *Neuroscience*. 186: 208-19.

Presentations (* presenting author)

Christina E.L. Jobson*, Justine Renard, **Laura G. Rosen**, Walter J. Rushlow, Steven R. Laviolette. (May 2017) Adolescent nicotine exposure induces long-term anxiety and depression-like symptoms through molecular and neuronal dysregulation of mesocorticolimbic dopamine D1 and ERK1/2 signaling pathways. Southern Ontario Neuroscience Association Annual Meeting, St. Catharine's, Canada

Laura G. Rosen*, Walter J. Rushlow, Steven R. Laviolette. (May 2017). Temporal analysis of intra-BLA molecular changes following chronic opiate exposure. Southern Ontario Neuroscience Association Annual Meeting, St. Catharine's, Canada

Justine Renard*, Cecilia Kramar, Hanna Szkudlarek, **Laura G. Rosen**, Walter J. Rushlow, Steven R. Laviolette. (June 2016). Involvement of prefrontal GABAergic transmission in schizophrenia-like behaviour induced by chronic adolescent THC exposure. Department of Psychiatry Academic Research Day, London, Canada

Christina E.L. Jobson*, Justine Renard, **Laura G. Rosen**, Walter J. Rushlow, Steven R. Laviolette. (June 2016). Long-term effects of chronic adolescent nicotine exposure on anxiety and social interaction: Implications for psychiatric disorders. Department of Psychiatry Academic Research Day, London, Canada

Justine Renard, Cecilia Kramar, Hanna Szkudlarek, **Laura G. Rosen***, Walter J. Rushlow, Steven R. Laviolette. (June 2016). Involvement of prefrontal GABAergic transmission in schizophrenia-like behaviour induced by chronic adolescent THC exposure. Canadian Student Health Research Forum CIHR poster competition, Winnipeg, Canada

Laura G. Rosen*, Walter J. Rushlow, Steven R. Laviolette. (June 2016). Dopamine D3 receptor activity and its downstream signaling targets are altered within the basolateral amygdala following chronic opiate exposure. Canadian Association for Neuroscience Annual Meeting, Toronto, Canada

Laura G. Rosen*, Walter J. Rushlow, Steven R. Laviolette. (May 2016). Dopamine D3 receptor activity and its downstream signaling targets are altered within the basolateral amygdala following chronic opiate exposure. Western Neuroscience 25th Anniversary Research Day, London, Canada

Laura G. Rosen*, Walter J. Rushlow, Steven R. Laviolette. (March 2016). Dopamine D3 receptor activity and its downstream signaling targets are altered within the basolateral amygdala following chronic opiate exposure. London Health Research Day, London, Canada

Christina E.L. Jobson*, Walter J. Rushlow, **Laura G. Rosen** & Steven R. Laviolette. (March 2016). Long-term effects of chronic adolescent nicotine exposure: Implications for psychiatric disorders. London Health Research Day, London, Canada

Justine Renard*, Cecilia Kramar, Hanna Szkudlarek, **Laura G. Rosen**, Walter J. Rushlow, Steven R. Laviolette. (March 2016). Involvement of prefrontal GABAergic transmission in schizophrenia-like behaviour induced by chronic adolescent THC exposure. London Health Research Day, London, Canada

Laura G. Rosen*, Walter J. Rushlow, Steven R. Laviolette. (October 2015). Dopamine D3 receptor activity and downstream calcium/calmodulin signaling targets are altered within the basolateral amygdala as a function of opiate exposure state. Society for Neuroscience Annual Meeting, Chicago, United States

Justine Renard*, Michael Loureiro, **Laura G. Rosen**, Walter J. Rushlow, Steven R. Laviolette. (October 2015). Adolescent cannabinoid exposure leads to molecular and neuronal alterations in the mesocorticolimbic system and behavioural impairments resembling schizophrenia symptomology. Society for Neuroscience Annual Meeting, Chicago, United States

Michael Loureiro*, Justine Renard, **Laura G. Rosen**, Steven R. Laviolette. (October 2015). Cannabinoid transmission in the ventral hippocampus modulates excitatory neuronal activity in the nucleus accumbens and induces schizophrenia-like disturbances in emotional processing and social cognition behaviors. Society for Neuroscience Annual Meeting, Chicago, United States

Justine Renard*, Michael Loureiro, **Laura G. Rosen**, Walter J. Rushlow, Steven R. Laviolette. (July 2015) Cannabidiol's Antipsychotic Effects in the Mesolimbic System: Role for mTOR/p70S6 Kinase Signaling. 25th International Cannabinoid Research Society, Wolfville, Nova Scotia, Canada

Justine Renard*, Michael Loureiro, **Laura G. Rosen**, Walter J. Rushlow, Steven R. Laviolette. (September 2015) Identification of a Novel Molecular Pathway for Cannabidiol's Antipsychotic Properties in the Mesolimbic Pathway: Role of mTOR/p70S6 Kinase Signaling. The 5th European Conference on Schizophrenia research, Berlin, Germany

Laura G. Rosen*, Walter J. Rushlow, Steven R. Laviolette. (June 2015). Chronic opiate exposure alters dopamine D3 receptor signaling within the basolateral amygdala. Department of Psychiatry Academic Research Day, London, Canada

Laura G. Rosen* (June 2015) Dopamine D3 receptor signaling and its downstream signaling targets are altered as a function of opiate exposure state in the basolateral amygdala. Mediterranean Neuroscience Society Meeting, Santa Margherita di Pula, Italy (oral presentation)

Laura G. Rosen*, Walter J. Rushlow, Steven R. Laviolette. (April 2015). Opiate Exposure State alters Dopamine D3 receptor signaling within the Basolateral Amygdala. London Health Research Day, London, Canada

Laura G. Rosen (July 2014). Exploring the molecular switching of opiate addiction memories in the amygdala-prefrontal cortical pathway. International Narcotics Research Conference, Montreal, Canada (invited talk)

Laura G. Rosen*, Danika Lyons, Walter J. Rushlow, Steven R. Laviolette. (July 2014). Opiate exposure state controls a molecular switch in opiate reward memory formation in the basolateral amygdala-prefrontal cortical pathway. International Narcotics Research Conference, Montreal, Canada

Laura G. Rosen*, Danika Lyons, Walter J. Rushlow, Steven R. Laviolette. (June 2014). Opiate exposure state controls a molecular switch in opiate reward memory formation in the basolateral amygdala-prefrontal cortical pathway. Department of Psychiatry Academic Research Day, London, Canada

Laura G. Rosen*, Danika Lyons, Walter J. Rushlow, Steven R. Laviolette. (May 2014). Opiate exposure state controls a molecular switch in opiate reward memory formation in the basolateral amygdala-prefrontal cortical pathway. Southern Ontario Neuroscience Association Annual Meeting, London, Canada

Laura G. Rosen*, Danika Lyons, Walter J. Rushlow, Steven R. Laviolette. (April 2014). Opiate exposure state controls a novel, CaMKII α / ERK1/2-dependent molecular opiate reward memory switch in the basolateral amygdala-prefrontal cortical pathway. London Health Research Day, London, Canada

Laura G. Rosen*, Danika Lyons, Walter J. Rushlow & Steven R. Laviolette. (2013). Opiate exposure state controls a novel, CaMKII α /ERK1/2-dependent molecular opiate

reward memory switch in the basolateral amygdala-prefrontal cortical pathway. Society for Neuroscience Annual Meeting, San Diego, United States

Laura G. Rosen*, Chloe N. Soutar, Hans C. Dringenberg. (2013). Sensory deprivation alters properties of short- and long-term plasticity in the rat auditory cortex. Canadian Association for Neuroscience Annual Meeting, Toronto, Canada

Laura G. Rosen*, Hans C Dringenberg. (2012). Cortical zinc application restores developmentally-dependent declines in plasticity of the rat primary auditory cortex. Canadian Society for Brain, Behaviour and Cognitive Science Annual Meeting, Kingston, Canada

Laura G. Rosen*, Hans C. Dringenberg. (2012). Cortical zinc application restores developmentally-dependent declines in plasticity of the rat primary auditory cortex. Queen's Neuroscience Research Day, Kingston, Canada

Laura G. Rosen (2012). Alteration of thalamocortical plasticity in the rat auditory cortex by exogenous zinc. Brain, Behaviour and Cognitive Science lunch series, Kingston, Canada (invited talk)

Laura G. Rosen*, Jennifer L. Hogsden, Hans C. Dringenberg. (2011). Pharmacological and deprivation-induced reinstatement of juvenile-like long-term potentiation in the primary auditory cortex of adult rats. Canadian Association for Neuroscience Annual Meeting, Quebec, Canada