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Identification of a Molecular Opiate-Addiction Memory Switch in the Basolateral Amygdala

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A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

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by

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Abstract

The molecular mechanisms involved in acquiring opiate-related associative memories are largely unknown. One neural region implicated in the formation of opiate-related memories is the basolateral nucleus of the amygdala (BLA). Transmission through dopamine (DA) receptors within the BLA controls the formation of opiate-related reward memories (Lintas et al., 2011; Lintas et al., 2012). Specifically, transmission through DA D1 receptors controls opiate reward memory formation in animals that are previously naïve to opiate exposure. However, once opiate dependence and withdrawal are present, intra-BLA DA-mediated control of opiate reward memory processing switches to a DA D2 receptor substrate. These findings demonstrate a DA receptor D1→D2 functional switching mechanism controlling the acquisition of opiate-related memories. However, it is unclear what are the underlying molecular substrates controlling the transition from a D1 to a D2 receptor-dependent memory mechanism are, and how chronic opiate exposure controls the process. Using a Pavlovian Place Preference (CPP) paradigm combined with molecular protein analyses, we tested the hypothesis that D1 and D2-like receptors in the BLA control the acquisition of opiate reward memory through modulation of intracellular phosphorylated MEK and phosphorylated CaMKII levels, respectively. We report that intra-BLA blockade of MEK inhibits opiate reward memory acquisition in previously opiate-naïve but not opiate dependent/withdrawn animals. Conversely, inhibition of CaMKII phosphorylation blocks the acquisition of opiate-related reward memory only in opiate dependent/withdrawn animals. Furthermore, chronic opiate exposure down-regulates the phosphorylation of MEK, and dramatically down-regulates expression levels of CaMKII. Our findings demonstrate a novel opiate exposure-state dependent molecular switch within the BLA controlling the formation of opiate-related reward memories.

Keywords: Opiate, Addiction, MEK, CaMKII, Dopamine, Basolateral Amygdala, Ventral Tegmental Area, Conditioned Place Preference

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α	alpha
μ	mu
BLA	basolateral nucleus of the amygdala
Ca^{2+}	calcium
CaMK	calcium/calmodulin kinase
CaMKII	calcium/calmodulin-dependent kinase II
CPP	conditioned place preference
DA	dopamine
D1R	dopamine D1-like receptor
D2R	dopamine D2-like receptor
DWD	opiate-dependent/withdrawn
ERK	extracellular regulated kinase
GABA	gamma-aminobutyric acid
GLU	glutamate
IP	intraperitoneal
LTP	long term potentiation
MAPK	mitogen activated protein kinase
MCLS	mesocorticolimbic system
MEK	mitogen-activated protein kinase kinase
MMT	methadone maintenance treatment
mPFC	medial pre-frontal cortex
NAc	nucleus accumbens
NAcC	nucleus accumbens core
NAcS	nucleus accumbens shell
pCaMKII	phosphorylated CaMKII
PFC	pre-frontal cortex
pMEK	phosphorylated MEK
PN	previously opiate-naive
Ser	serine
SQ	sub-cutaneous
Thr	threonine
Tyr	tyrosine
VTA	ventral tegmental area

1. Introduction

1.1 Opiate Addiction in Canada

Opiate addiction is a complex pathological disorder of learning and memory that represents a pervasive healthcare issue across Canada and most of North America (Fischer, Patra, Cruz, Gittins, & Rehm, 2008; Nosyk et al., 2012). Addiction is characterized by a transition from voluntary to persistent, compulsive drug use even in the face of adverse physical, psychological, and social consequences associated with continued drug use. It is also characterized by a high likelihood for relapse even after extended periods of successful abstinence (Everitt et al., 2008; Trigo, Martin-García, Berrendero, Robledo, & Maldonado, 2010). The extremely potent compulsive thoughts focused on obtaining drugs, no matter the cost, and the high rate of relapse following recovery, not only adversely affect the individual suffering from the disorder but also society as a whole. The societal costs associated with opiate abuse and dependence includes health-related expenses, law enforcement costs, and loss of productivity costs. In actual dollar amounts, the cost to society for each addict over a lifetime can range, on average, anywhere from $736,800 to over $1,000,000 (Nosyk et al., 2012). As such, opiate addiction represents a costly, chronic, intermittently recurring disorder treated primarily with a therapeutic procedure that is only minimally effective.

Methadone maintenance treatment (MMT) is currently the most common form of treatment offered to people suffering from opiate addiction. However, research has shown repeatedly that MMT is not especially effective (Nosyk et al., 2012; Ward, Hall, & Mattick, 1999). Although MMT reduces the frequency of heroin use and risk of contracting diseases

transferrable by needle sharing, such as HIV and Hepatitis C, it has a high rate of attrition and non-compliance. Furthermore, the MMT treatment itself can be dangerous, and the facilities and supplies required to sustain the treatment are quite expensive (S. Johnson, Macdonald, Cheverie, Myrick, & Fischer, 2012; Nosyk et al., 2012; Ward et al., 1999). A number of alternative treatment strategies for opiate addiction have been introduced over the years some of which have yielded moderate success in reducing societal costs related to addiction but none of which relieve the core, underlying neurobiological mechanisms sustaining the opiate addiction cycle. Decades of neuroscience research in both human and animal models of opiate addiction suggest that the primary feature of the opiate addiction process involves the triggering of intrusive and compulsive associative memories linked to environmental cues associated with the rewarding effects of opiates. The activation of these associative memories in turn, drives drug seeking behavior in both humans and other animals. These memories, triggered by drug-related cues in the environment, seem to take precedence over all other cogent thought and decision-making and are extremely resistant to extinction. However, it is still unclear how drug-related sensory experiences gain their tremendous incentive salience, how they maintain their saliency status, or what the most effective therapeutic procedure would be to extinguish these memories. The high prevalence of opiate addiction across North America, the high financial burden associated with opiate addiction, and the low to moderate success rate of current treatments, speak to the urgency of basic neurobiological opiate addiction research and the development of more effective pharmacotherapeutic treatments.

1.2 Reward Learning and the Mesocorticolimbic System

Recent theories of drug addiction underscore the importance of neural plasticity, learning, and memory formation, recall, and extinction as critical processes underlying the persistence of chronic opiate abuse. Opiates are highly euphorigenic and produce potent associative memories linked to the drug taking experience. In fact, the ability of opiate-related memory cues to trigger opiate craving is a primary factor underlying persistent opiate relapse, even after years of successful abstinence. It is unclear how, at the level of the individual neuron, these drug-related changes take place. Although, it is well documented that chronic drug use causes long-lasting changes in neuronal structure and function within the neural circuitry involved in encoding reward-related and/or emotionally salient memories (Robinson & Berridge, 1993). That circuitry, referred to as the mesocorticolimbic system (MCLS), consists of a series of interconnected structures including the ventral tegmental area (VTA), the basolateral nucleus of the amygdala (BLA), the pre-frontal cortex (PFC), and the nucleus accumbens (NAc) (see Figure 1). Each of these structures, individually and in concert with one another, contributes to the ascription of incentive salience to sensory stimuli and to the formation of associative memories that drive drug-seeking behaviours.

Figure Caption 1:

Proposed model for the formation of associative memories within the MCLS.

Proposed model for the formation of opiate-related reward memory. Opiates bind to μ-opiate receptors on GABAergic interneurons in the VTA removing GABAergic inhibition over local DA neurons thereby facilitating DAergic release to the BLA and NAc. The increased DA concentration causes a release of GABAergic inhibition over local pyramidal neurons while simultaneously stimulating integration of associative signaling from the sensory cortex. Associative memories, between the motivational effects of opiates (VTA DA signal) and sensory information from convergent cortical sensory inputs, form in BLA neuronal populations. GLUergic release from the BLA towards the mPFC and NAc become active and NAc GLUergic projections to motor cortex facilitated.

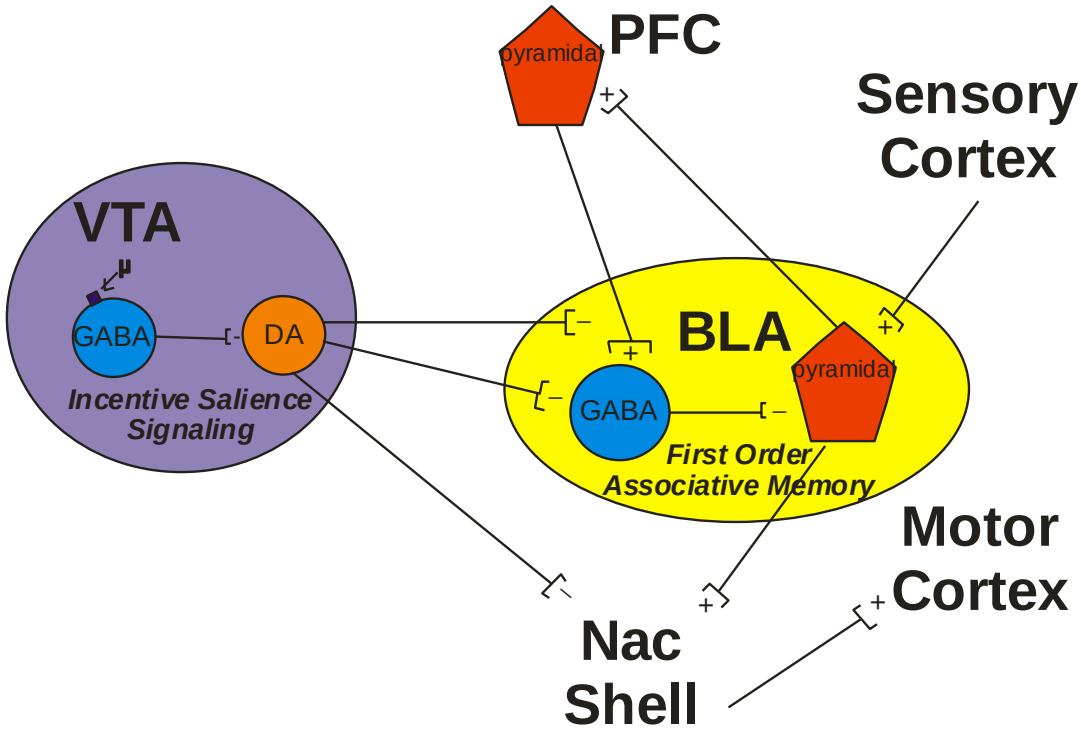

PFC
pyramidal
Sensory
Cortex
VTA
μ
GABA
DA
Incentive Salience
Signaling
BLA
pyramidal
GABA
First Order
Associative Memory
Motor
Cortex
Nac
Shell

Figure 1

1.2.a The Ventral Tegmental Area

DAergic projections within the MCLS originate from DAergic neurons in the VTA and terminate throughout the MCLS. DAergic projections are involved in encoding the primary rewarding effects of opioids but not the subjective experience of drug craving following abstinence (Breiter et al., 1997). Put another way, the VTA is the primary area in which the rewarding "high" of drugs of abuse are processed. As an example, Olmstead and Franklin (1997) tested the rewarding effects of opiates when administered into various regions of the central nervous system. They reported that direct, intra-VTA administration of morphine elicits observable drug seeking behaviors while direct administration of morphine to other areas of the MCLS failed to produce comparable motivational effects. The VTA is an area rich in μ-opiate receptors making it a primary target for the binding of opioid molecules. Consequently, opiates achieve their euphoric effects by binding to μ-opiate receptors in the VTA, which are associated with 'non-DA', GABAergic interneurons. Since μ-opiate receptors are inhibitory in nature, their activation in turn inhibits the inhibitory GABAergic inputs to the VTA DA neurons, leading to the disinhibition, and indirect activation of the VTA DA neurons. Indeed, studies show that pharmacological blockade of μ-opiate receptors within the VTA impairs the encoding of the rewarding effects of opiates (Olmstead & Franklin, 1997).

Binding of opioids to μ-opiate receptors facilitates DAergic output from the VTA to all of the other areas of the MCLS and facilitates an increase in the overall concentration of DA throughout the MCLS (Adinoff, 2004; Ford, Mark, & Williams, 2006). These DAergic projections from the VTA excite neighboring structures (see Figure 1). Specifically, DA neurons located in the VTA are dynamically regulated by local GABAergic inter-neurons (S. W. Johnson

& North, 1992; Klitenick, DeWitte, & Kalivas, 1992; Steffensen et al., 2006). When exogenous opioids are introduced into the system, opioid molecules bind to opiate receptors located on GABAergic cells, releasing the GABAergic inhibition over DA cells (S. W. Johnson & North, 1992; Klitenick et al., 1992), and indirectly facilitating DAergic release to the other areas of the MCLS (De Vries & Shippenberg, 2002). This DAergic neurotransmission plays an important role in modulating neuronal plasticity within the system and smoothes the process of experiential learning.

1.2.b The Basolateral Nucleus of the Amygdala

The BLA receives DAergic projections from the VTA. These DAergic projections indirectly modulate neuronal plasticity within the BLA while the BLA simultaneously receives neural signaling from areas of the sensory cortex (area Te3) (Bissière, Humeau, & Lüthi, 2003; Kröner, Rosenkranz, Grace, & Barrionuevo, 2005; Rosenkranz & Grace, 2003). The increase in DA concentration in the BLA facilitates GLUergic output from the BLA to areas such as the NAc and PFC (Kröner et al., 2005; Rosenkranz & Grace, 2003). Thus, the BLA is an ideal area for the integration of opiate-mediated salience signaling from the VTA with concurrent sensory experiences leading to the formation of experience-related associative memories. In keeping with this idea, studies have demonstrated a critical role for the BLA in the formation of associative memories that carry emotional significance. For instance, although opiates hold no rewarding effect when delivered directly into the BLA (Olmstead & Franklin, 1997) inactivation of the BLA causes a deficit in the formation of emotionally-relevant opiate-related conditioned associations (Fuchs & See, 2002). In other words, when the BLA is inactive the animal is unable to learn a simple classically conditioned association between a rewarding or aversive stimuli and

an external sensory experience temporally paired with it. Similarly, activation of the BLA promotes the formation of associative memories (Adinoff, 2004; Grace & Rosenkranz, 2002; Lintas et al., 2012). Presumably then, the BLA is associated with the acute formation of associative memories within the MCLS. However, the molecular mechanism(s) by which the BLA is able to integrate opiate-related euphoria encoded by the VTA and sensory signaling from sensory cortices, occurring in tandem, to form potent associative drug-related memories is unclear.

1.2.c The Pre-Frontal Cortex

The pre-frontal cortex (PFC) is an area important for higher order cognition, decision-making, and for regulating subcortical structures. Similarly, within the context of experience-based learning and memory, the PFC plays an important role in regulating the activity of its neighboring structures within the MCLS such as the BLA. However, the PFC is a very large region with many different subdivisions all structurally and functionally distinct from one another. The medial pre-frontal cortex (mPFC) is the portion of the PFC implicated for its' role in regulating the formation, maintenance, and extinction of associative memories. The mPFC includes the pre-limbic cortex (PLC) and the infra-limbic cortex (ILC) with the PLC located superior to the ILC (Paxinos & Watson, 2005).

As mentioned previously, the BLA is important for acute formation of associative memories. The formation of emotionally salient associative memories within the BLA depends upon maintenance of a delicate balance of reciprocal interconnections between the BLA and the mPFC. The mPFC receives DAergic neurotransmission from the VTA and GLUergic

transmission from the BLA. In turn the mPFC sends inhibitory GLUergic projections back to the BLA (Grace & Rosenkranz, 2002; Peters, Kalivas, & Quirk, 2009; Rosenkranz & Grace, 2001). It is through these neural signaling pathways that the mPFC and BLA dynamically modulate each other's activity to control the formation, recall, and extinction of associative memories.

The projections from the mPFC to the BLA are inhibitory in nature, thereby influencing the likelihood that an associative memory will be formed (Peters et al., 2009; Rosenkranz & Grace, 2001, 2003). For example, inhibition of the PLC, within the mPFC, dramatically potentiates the saliency of normally non-salient stimuli and facilitates the formation of opiate-related associative memories (Bishop, Lauzon, Bechard, Gholizadeh, & Laviolette, 2011; Lauzon, Ahmad, & Laviolette, 2011). Once a memory has been encoded, neuronal activation within the mPFC influences recall and extinction of the memory (Grace & Rosenkranz, 2002; Peters et al., 2009; Sun et al., 2011; Sun & Laviolette, 2012). However, chronic drug use modifies the DAergic transmission throughout the MCLS, including transmission between the mPFC and BLA thus altering the balance of interplay between the mPFC and BLA as well as the formation of associative memories dependent on the mPFC and BLA.

1.2.d. The Nucleus Accumbens

The nucleus accumbens (NAc) is responsible for translating neural signaling from other areas of the MCLS into appropriate behavioral responses, and as such, is often referred to as a "limbic-motor interface" (Ambroggi, Ishikawa, Fields, & Nicola, 2008). It has two important subdivisions, the NAc shell, and core (NAcS, NAcC). The main function of the NAcS is to strengthen stimulus-drug associations while the NAcC is responsible for the expression of drug-

seeking behaviour associated with the stimulus-drug association (Di Chiara, 2002; Di Chiara et al., 2004). For instance, DA concentration within the NAcS increase upon introduction of salient sensory stimuli and this increase is associated with a decrease in latency to learn a reinforcement contingency such as a lever press for reward (Beyene, Carelli, & Wightman, 2010). Hence, exposure to novel stimuli increases DA concentration within the NAcS and consequently facilitates encoding of action-consequence relationships. In contrast, inactivation or ablation of the NAcC results in a significant decrease in the number of lever presses an animal will make for a given reward (Ambroggi et al., 2008). Thus, the NAcC is important for the expression of reward-driven behaviors rather than the ability to learn a reinforcement contingency. Put another way, the animal is able to learn the reward contingency but appears to be less motivated to acquire the reward. For example, where a healthy animal may push a lever to get a food reward ten times in one minute, an animal with a NAcC deficit may only push the lever four times.

Similar to the VTA, the NAc is rich in μ-opiate receptors and direct administration of opiates to the NAcS elicits drug-seeking behavior (Olmstead & Franklin, 1997). Opiates achieve their motivational effect by increasing the concentration of DA in the NAc through DAergic input from the VTA (McDonald, 1991a, 1991b; Stuber et al., 2011). Blockade of DA transmission within the NAc impairs expression of reward seeking behaviors (Ambroggi et al., 2008; Nicola, Taha, Kim, & Fields, 2005). In addition, the NAc receives GLUergic inputs from the BLA (McDonald, 1991a, 1991b; Stuber et al., 2011) which work in concert with DAergic input from the VTA to facilitate the expression of drug seeking behaviors. Functionally, DA transmission within the BLA strongly modulates neuronal activity specifically within the NAcS. Thus, blockade or activation of DA D1 or D2 receptors directly within the BLA can either

increase or decrease NAcS neuronal sensitivity to opiate administration (Lintas et al., 2011; Lintas et al., 2012). Again, this BLA DA-mediated modulation of NAcS neuronal responsiveness is dependent upon the opiate exposure history of the animal, with D1 receptors important only when in an opiate-naïve state, and D2 receptors only important when in an opiate dependent and withdrawn state (Lintas et al., 2011; Lintas et al., 2012). Blockade of DAergic or GLUeric receptors within the NAcS attenuates drug-related neuronal activation and subsequent drug-seeking behaviors (Ambroggi et al., 2008; Bassareo, Musio, & Di Chiara, 2011; Lecca, Valentini, Cacciapaglia, Acquas, & Di Chiara, 2007; Lintas et al., 2011; Lintas et al., 2012). Thus, the shell and core subdivisions of the NAc play an important role in translating cell signaling within the MCLS into behavioral output. Once again, the introduction of exogenous opiates into the system modifies the functionality of the NAc and thereby modifies the behavioral output generated by cellular signaling within the MCLS.

All components of the MCLS, the VTA, BLA, PFC, and NAc, play important but discrete roles in the formation and expression of drug-related behaviors. However, the ability of each component to perform its role effectively often depends upon their interconnections between one another. The introduction and chronic use of exogenous opiates modifies the structure and function of individual neurons and their synaptic connections between one another within the structures which make up the components of the MCLS. These neuronal and synaptic modifications eventually translate into behavioral modifications, ultimately resulting in the maladaptive compulsion to use drugs even in the face of adverse consequences. However, it is unclear exactly how these modifications take place within and between neurons and it is unclear how the changes are maintained on a long term basis. Thus, the opiate-related modifications in

DA neurotransmission in the MCLS following chronic opiate use are a key target of research studies attempting to answer those questions.

1.3 Opiate Reward Learning and Dopamine

Acute and chronic opiate use differentially modulate DAergic neurotransmission within the MCLS (Berridge & Robinson, 1998; Laviolette, Nader, & van der Kooy, 2002; Self & Nestler, 1998) making DA a key target of investigation in the opiate addiction literature. Dopamine, often thought of as the "reward" neurotransmitter, conveys information about rewarding stimuli, or events that may signal impending reward. However, there is extensive evidence which would suggest that DA is important for conveying the "incentive salience" of stimuli rather than their rewarding properties per se (Berridge & Robinson, 1998; De Vries & Shippenberg, 2002; Robinson & Berridge, 1993). For instance, in the context of ordinary, experience-based learning, DA concentration throughout the MCLS increases in response to novel stimuli regardless of the rewarding or aversive valence of the stimulus (Adinoff, 2004; Berridge & Robinson, 1998; Lamb et al., 1991). In addition, novel stimuli-related increase in DA neurotransmission is attenuated following repeated exposure to the stimulus but increased once again if the reward contingency associated with the stimulus is changed (Horvitz, 2000). These studies indicate that regardless of the emotional valence of the stimuli (rewarding or aversive) DA transmission is facilitated whenever a novel stimuli or event is encountered and decreases as the stimuli becomes more familiar with repeated exposures.

Abnormalities in DA transmission are implicated in a number of neuropsychological disorders including opiate addiction. Long-term opiate use alters the usual fluctuations in DA

transmission initiated by novel experiences (Adinoff, 2004; Di Chiara, 2002; Volkow, Fowler, Wang, Baler, & Telang, 2009). For example, whereas repeated exposure to previously novel stimuli would normally decrease DA release, in the context of chronic opiate exposure there is no decrease in DA transmission following repeated exposures. Abstinence from drug use following extended periods of chronic opiate use results in a dramatic drop in DA concentration throughout the MCLS and aversive somatic withdrawal symptoms (Di Chiara, 1999; Volkow, Fowler, Wang, & Goldstein, 2002). This suggests that opiates alter DA transmission within the MCLS to facilitate the allocation of attention and ascription of incentive salience to stimuli that would otherwise hold little or no incentive salience. This dramatic boost in the motivational salience of objects or events also results in a higher likelihood of the formation of associative memories between the experience of drug use and the surrounding environment at the time of use. The associative memories later serve as potent cues for reinstatement and/or relapse of drug seeking behaviour following withdrawal and/or long term abstinence.

1.3.a DA D1-like versus D2-like Receptors

There are two broad families of DA receptors. These receptor subtypes are classified into the ‘D1-like’ and ‘D2-like’ families of receptors. The D1 family (herein referred to as D1R) includes the D1- and D5-type receptors and the D2 family (herein referred to as D2R) includes the D2-, D3-, and D4-type receptors (Missale, Nash, Robinson, Jaber, & Caron, 1998; Vallone, Picetti, & Borrelli, 2000). The two families of receptors are both G-protein coupled metabotropic receptors with seven transmembrane domains. However, they are distinct from one another in terms of their locations on neurons, which G protein subunit they couple to, and their influence on two distinct intracellular downstream signaling cascades. D1R's tend to be located post-

synaptically; they couple to the $G_{\alpha s}$ subunit, and activate the intracellular PKA/cAMP pathway. D2R's, on the other hand, tend to be located pre-synaptically, as autoreceptors, and post-synaptically. D2R's couple to the $G_{\alpha i/o}$ subunit, and inhibit the PKA/cAMP pathway (Berke & Hyman, 2000; Meador-Woodruff, Damask, & Watson, 1994; Vallone et al., 2000). However, chronic stimulation of DA receptors through continuous drug use modifies the expression, function, and binding affinity of D1R and D2R's on individual neurons and does so differentially for each family of receptors (Bhargava & Gulati, 1990; Collins et al., 2011; Novak & Seeman, 2010; Volkow et al., 1993).

Numerous studies have reported dissociable roles for D1R and D2R's in relation to the formation and expression of drug seeking behaviors depending upon the state of previous drug exposure one is in at the time of encoding (Grieder et al., 2012; Lauzon, Bishop, & Laviolette, 2009; Laviolette, Lauzon, Bishop, Sun, & Tan, 2008; Lintas et al., 2011; Lintas et al., 2012). Specifically, for animals in a previously opiate-naive state (PN), the ability to encode opiate-related associative memories depends upon activation of D1R's in the BLA. Conversely, for animals in an opiate-dependent/withdrawn state (DWD), activation of D2R's in the BLA is required for the formation of opiate-related associative memories (Lintas et al., 2011; Lintas et al., 2012). The reason behind the functional dissociation between D1R and D2R's is not clear although there is some indication that the distinct intracellular signaling pathways initiated by the activation of D1R and D2R's may provide some explanation. For instance, Lintas and colleagues (2011, 2012) demonstrated that in PN animals the D1R antagonist-mediated blockade of associative opiate-reward memory can be rescued by co-administration of a cyclic-AMP/PKA activator. Hence, by restoring the activation of cyclic-AMP/PKA normally initiated by D1R

activation, the formation of the associative memory was able to occur as it normally would. Further, in DWD animals a D2 antagonist-mediated blockade of associative memory can be rescued by co-administration of a cyclic-AMP/PKA antagonist (Lintas et al., 2011). Taken together this research suggests that the intracellular signaling pathways altered by the activation of D1 and D2 receptors activation may reflect their functional differences under acute versus chronic morphine exposure states. Even more importantly, considerable evidence points to functional linkages between the D1-like receptors with downstream signaling of mitogen-activated protein kinase (MAPK) and between D2-like receptors with downstream signaling of the calcium/calmodulin-dependent kinase (CaMK) signaling pathways.

1.4 Intracellular Signaling Cascade Kinases Linked to D1 or D2 Receptor Transmission

Intracellular signaling cascades are important for transducing extracellular signaling to the relevant intracellular effecter sites regulating cellular processes including gene synthesis and transcription, neurotransmitter synthesis, packaging, and release, as well as growth and pruning of synaptic connections, and cell death. There are many extracellular as well as intracellular variables which work together to determine which signaling cascades will be activated and what the consequences of their activation will be for cell structure and function. Two signaling kinases critical for learning and the formation of synaptic LTP are the mitogen-activated protein kinase pathway (MAPK) and the calcium/calmodulin-dependent protein kinase pathway (CaMK). MAPK and CaMK are functionally interconnected with D1R and D2R's, respectively (Acquas et al., 2010; Fricks-Gleason & Marshall, 2011; Gangarossa et al., 2011; Gu & Yan, 2004; Ibba et al., 2009; Liu et al., 2009; Novak & Seeman, 2010; O'Sullivan et al., 2008).

The D1R-related MAPK pathway is a well characterized intracellular signaling pathway which includes the protein kinases MEK(1/2) and ERK(1/2) in its chain of signaling proteins (Seger & Krebs, 1995). The MEK kinase is located in the cytoplasm and is phosphorylated by tyrosine (Tyr) and serine/threonine (Ser/Thr). When Tyr and Ser/Thr phosphorylate at the cell membrane they in turn phosphorylate MEK farther down the signaling chain in the cytoplasm of the neuron (Anderson, Maller, Tonks, & Sturgill, 1990). Once phosphorylated, MEK can autophosphorylate until its eventual deactivation via Tyr phosphatase (CD45) or protein-Ser/Thr phosphatase 2A (PP2A) (Anderson et al., 1990). When MEK phosphorylates and is in its active state it is able to phosphorylate downstream targets in the signaling cascade.

An important consequence of MEK phosphorylation is facilitation of the encoding of new memories. As an example, an up-regulation in pMEK levels during the encoding phase of learning and a drastic impairment in the ability to encode new memories when MEK phosphorylation has been artificially inhibited has been reported (Alvarez-Jaimes, Feliciano-Rivera, Centeno-González, & Maldonado-Vlaar, 2005; Fricks-Gleason & Marshall, 2011; Kelly, Laroche, & Davis, 2003; Lin, Wang, Ji, & Yu, 2010). Interestingly, activation of D1R's at the cell membrane is associated with increased levels of phosphorylated MEK (pMEK1/2, pERK1/2) during the encoding phase of associative learning. Conversely, blockade of D1R's or blockade of MEK phosphorylation impairs encoding of associative memories (Alvarez-Jaimes et al., 2005; Fricks-Gleason & Marshall, 2011; Kelly et al., 2003; T. Li et al., 2010; Lintas et al., 2011; Pan, Zhong, Sun, & Liu, 2011). Therefore, an important link exists between the activation of D1R's and the phosphorylation of MEK during the formation of associative memories.

Acute as well as continuous exposure to a wide variety of drugs of abuse, including opiates, varies pMEK levels and D1R's expression within the MCLS (Bhargava & Gulati, 1990; Bilecki et al., 2005; Lin et al., 2010; Ma, Zheng, Powell, Jhamandas, & Quirion, 2001). Expressly, acute exposure to caffeine, ethanol, cocaine, and morphine all substantially increase pMEK levels throughout many regions of the brain (Acquas et al., 2010; Bilecki et al., 2005; Ferrer-Alcón, García-Fuster, La Harpe, & García-Sevilla, 2004; Fricks-Gleason & Marshall, 2011; Ibba et al., 2009; Ma et al., 2001; Seo et al., 2008). In addition, exposure to drug-paired cues, rather than the drug itself, also increases pMEK levels. Also of interest is the fact that pharmacological blockade of D1R's can prevent drug exposure-induced increases in pMEK levels and suppression of MEK phosphorylation impairs the formation of drug-related associative memories (Acquas et al., 2010; Fricks-Gleason & Marshall, 2011; Ibba et al., 2009; Z. Li et al., 2011). Therefore, not only are pMEK levels increased during the formation of new memories and in synchrony with D1R's, but pMEK levels are also modified by drug exposure and exposure to drug-paired cues. The implications of these drug-related modifications in pMEK levels for the acquisition of drug-related associative memories within the BLA are unclear at this point. However, research to date suggests that pMEK does play an important role in the formation of drug-related memories, and that role may differ depending upon the drug-exposure state one is in at the time of memory encoding.

The D2R-related calcium/calmodulin-dependent protein kinase (CaMKII) pathway is also important for the formation of new memories (Fink & Meyer, 2002; Frankland, O'Brien, Ohno, Kirkwood, & Silva, 2001; Lisman, Schulman, & Cline, 2002). An increase in intracellular concentration of calcium (Ca^{2+}) promotes binding of calmodulin to the kinase on its catalytic

domain (Thr286) and transforms CaMKII into its active phosphorylated state. Once in this state, CaMKII can autophosphorylate independent of Ca^{2+}/calmodulin until its eventual dephosphorylation by phosphatase (Fink & Meyer, 2002).

The active form of CaMKII, phosphorylated CaMKII (pCaMKII), phosphorylates its neighboring proteins in the CaMK pathway leading to modifications in neuronal structure and function (Frankland et al., 2001; Lisman et al., 2002; Shen & Meyer, 1999). Through this chain of phosphorylation, pCaMKII is able to influence the formation of new memories in a similar fashion as pMEK. For instance, pCaMKII levels, similar to pMEK levels, are increased during the formation of new memories while pharmacological blockade of pCaMKII impairs the formation of new memories (Fan, Wang, Qiu, Ma, & Pei, 1999; Lauzon et al., 2011; Narita et al., 2004). However, in contrast to pMEK, pCaMKII level regulation is closely associated with the activation of D2R's such that D2R activation leads to increased pCaMKII levels (Gu & Yan, 2004; Liu et al., 2009; Novak & Seeman, 2010).

Acute and chronic drug use differentially modifies the expression of D2R's and in a comparable fashion; acute and chronic drug use dynamically modulates pCaMKII levels (Christian, Alexander, Diaz, Robinson, & McCool, 2012; Frankland et al., 2001; Lisman et al., 2002; Lou, Zhou, Wang, & Pei, 1999; Lu, Zeng, Liu, & Ceng, 2000; Wang & Wang, 2006; Wanjerkhede & Bapi, 2008). Where acute opiate use frees Ca^{2+} from intracellular cystolic stores and increases pCaMKII levels for a short period of time, chronic opioid use results in a much longer lasting up-regulation of pCaMKII levels (Christian et al., 2012; Fan et al., 1999; Greenstein, Novak, & Seeman, 2007; Lou et al., 1999; Seo et al., 2008; Wang & Wang, 2006).

Evidence suggests that this long lasting up-regulation of pCaMKII levels contributes to the expression of drug tolerance whereby individuals require increased doses of the drug in order to achieve the subjective experience of drug-related euphoria (Fan et al., 1999; Wang & Wang, 2006). In fact, amphetamine sensitized rats, a model often used to mimic human behaviors such as hyperactivity and impulsivity characteristic of people with drug addictions, display an up-regulation in pCaMKII levels (Greenstein et al., 2007).

Therefore, pCaMKII not only assists in the development of new memories, and does so in synchrony with D2R activation, but it also plays a role in the development and persistence of drug seeking behaviors. Much of the research to date concerning drug-related modulation of pCaMKII levels has focused on regions of the brain other than the amygdala. As a result, the role of pCaMKII in modulating neuronal plasticity within the BLA is largely unclear. However, the fact that CaMKII phosphorylation is important for learning and memory and the evidence suggesting that pCaMKII levels are affected by chronic drug use provide a compelling platform for speculating about its role in drug-related learning and memory. For instance, pCaMKII may modulate the formation of associative memories in the BLA and may do so differentially depending on the drug-exposure state one is in at the time of encoding of the associative memory.

1.5 Research Purpose and Hypotheses

We have previously reported identification of a novel opiate-exposure-state dependent functional switch between DA D1R and D2R's in the acquisition of opiate-related reward memories in the BLA (Lintas et al., 2011). However, we were not able to characterize how this

switch was accomplished or what intracellular mechanisms might have been contributing to the change. Research suggests that phosphorylation of MEK and CaMKII, following DA D1R and D2R activation, respectively, may mediate the functional switch occurring at the level of the DA receptors. **The overarching aim of the experiments conducted in this research project were to:**

1. **Determine the extent to which pMEK and pCaMKII are important for the formation of associative opiate-related memories in the BLA**
2. **Determine the extent to which pMEK and pCaMKII's functional roles vary based on opiate-exposure state**
3. **Determine the extent to which their roles depend upon activation of D1R and D2Rs**

It was predicted, that the formation of associative memories depends upon increased intra-BLA pMEK levels in opiate-naive animals and increased intra-BLA pCaMKII levels in opiate-dependent/withdrawn animals. Further, we hypothesized that increased expression of pMEK and pCaMKII would reflect D1R and D2R activation, respectively.

2. Materials and Methods

This project included 23 independent experimental groups. Each group consisted of N=6-8 adult male Sprague-Dawley (Charles River Canada) rats. In the following section, the materials and methods used in this project including surgical and histological procedures, drug treatments, behavioral paradigms, and statistical analysis are discussed.

2.1 Surgical Procedures

Male Sprague-Dawley rats (Charles River, 350-400g at the start of the experiments) were anaesthetized with a ketamine (80 mg/ml) xylazine (6 mg/ml) mixture and placed into a stereotaxic device. Stainless steel guide cannulae (22 gauge; Plastics One) were bilaterally implanted into the brain regions of interest using the following stereotaxic coordinates (in mm). For the BLA (0˚ angle): from bregma AP -2.6, L ±5.0; from the dural surface, V -7.2. For the VTA (10˚ angle): from bregma, AP -6.0, L ±2.6; from the dural surface, V -8.0. The cannulae were secured using stainless steel screws and dental acrylic. Following the completion of experiments, animals were anesthetized and intracardially perfused first with isotonic saline and then with 10% formalin. Brains were then removed from the skull and placed in 10% formalin in the fridge over night and transferred to a formalin-sucrose solution for long-term storage in the fridge for at least 48 hours before sectioning. The brains were sectioned using a cryostat, at -30˚ Celsius, into 40μm slices and mounted onto microslides. The sections were stained with Cresyl violet and the BLA and VTA cannulae placements were verified using light microscopy according to the anatomical boundaries defined by Paxinos and Watson (2005). Any animals with cannulae placements outside of the BLA and/or VTA were excluded from statistical analysis.

2.2 Drug Treatments

The drugs used in this project included a MEK inhibitor (UO-126, Tocris), a CaMKII inhibitor (KN-62, Tocris), a DA D1 receptor agonist (SKF-81297, Tocris), a DA D2 receptor agonist (Quinpirole, Sigma), morphine (morphine hydrochloride, MacFarlane Smith), and heroin (diacetylmorphine, MacFarlane Smith). All drugs were dissolved in filtered isotonic saline. Bilateral intra-BLA or intra-VTA microinjections of 0.5μl per side took place over a 60 second

time interval by means of plastic tubing connected to a 1μl Hamilton micro-syringe. Following which, an additional 60 second interval was allowed to ensure ample diffusion of the drugs from the injector tip. All intra-BLA microinfusions (UO-126, KN-62, UO-126+SKF-81297, KN-62+Quinpirole, saline vehicle) were performed immediately prior to systemic or intra-VTA injections of morphine or saline. Following morphine or saline injection, animals were then placed into one of the two randomly assigned place conditioning environments. For intra-BLA DA receptor activation coupled with signaling kinase inhibition, the DA D1 agonist (SKF-81297) and MEK inhibitor (UO-126) as well as the DA D2 agonist (Quinpirole) and CaMKII inhibitor (KN-62) were co-administered. Both "supra-threshold" (5 mg/kg; i.p.) as well as "sub-threshold" (0.05 mg/kg; i.p.) doses of morphine were employed throughout this project. All sub-threshold doses were administered systemically while supra-threshold doses were administered both systemically and intra-cranially. For systemic morphine administration experiments, a sub-threshold dose does not normally produce a significant preference for a morphine-paired environment, whereas a supra-threshold dose does produce a significant preference for a morphine-paired environment (Bishop et al., 2011; Lintas et al., 2011; Lintas et al., 2012). For experiments using intra-VTA morphine administration, a supra-threshold dose of intra-VTA morphine (0.5 μg/0.5 μl) will produce a significant preference for the morphine-paired environment (Laviolette, Gallegos, Henriksen, & van der Kooy, 2004; Laviolette & van der Kooy, 2004; Nader & van der Kooy, 1997).

2.3 Conditioned Place Preference Paradigm

An motivationally neutral, and highly robust Pavlovian conditioned place preference procedure (CPP) was used to train animals to associate the rewarding effects of morphine with

one of two environments which differed in terms of colour, texture, and smell as described in Laviolette and van der Kooy (2003), and Laviolette, Nader, and van der Kooy (2002). Following recovery from surgery, animals were randomly assigned to an experimental group. All animals were exposed to a pre-conditioning phase where they were placed into a motivationally neutral grey box for 20 minutes. The following day, the 8 day conditioning phase commenced. The conditioning phase took place in one of two 15 X 15 inch Plexiglas conditioning environments. One environment was white with a wire-mesh floor covered in woodchips different from those in the animals' home cage. The other was black with a smooth Plexiglas floor wiped down with 2% acetic acid immediately before the animal was placed into it. Thus, the conditioning environments in the procedure differed in terms of visual, tactile, and olfactory cues. The type of treatment received (saline or morphine) in each of the two environments were counterbalanced such that each animal was randomly assigned to receive morphine in either the white or the black environment and vice versa when receiving saline. Animals display no baseline preference for either of these environments (Laviolette & van der Kooy, 2003).

During CPP conditioning experiments, animals received an equal number of morphine-environment versus saline- environment pairings. Therefore, over the eight-day procedure animals received four 30 minute morphine-environment pairings and four 30 minute saline-environment pairings. Animals received three to five (opiate-naïve groups) or five to seven (opiate-dependent groups) days to recover following the conditioning phase to ensure they were in a "drug-free" state when tested. During testing, animals were placed on a narrow grey zone that separates the white and black environments and the times spent in each of the black and white environments were digitally recorded, using two stopwatches, and scored separately for

each animal over a 10 minute test session. Animals must have had their front two paws and head located in the environment in order for them to be considered within that environment (see Figure 2A).

Figure Caption 2

Conditioned Place Preference Paradigm Diagram

(A) Standard CPP procedure: On day 1 all animals are placed into a neutral grey box for 20 minutes. On days 2 through 9 animals receive an injection (i.p.) of either saline (S) or morphine (M) and are placed into either a white box or a black box for 30 minutes. Animals receive a total of 4 saline and 4 morphine pairings fully counterbalanced over the 8 day conditioning phase. On days 10 through 12 animals are left in their home cages to recover and to return to a "drug-free" state. On day 13 animals are placed into the test box on the neutral grey strip for 10 minutes. Animals may move freely between either side of the test box and the times they spend on either side are recorded for later statistical analysis.

(B) Procedure used to create a state of opiate dependence and the standard CPP procedure when animals are in an opiate-dependent/withdrawn state: On day 1 all animals are placed into a neutral grey box for 20 minutes. On days 2 through 8 animals receive an injection (subcutaneous) of heroin in their home cage (yellow box). On days 9 through 16 animals receive an injection (i.p.) of either saline (S) or morphine (M) and are placed into either a white box or a black box for 30 minutes. Animals receive a total of 4 saline and 4 morphine pairings fully counterbalanced over the 8 day conditioning phase. On days 17 through 20 to 23 animals are left in their home cages to recover and to return to a drug free state. On day 24 animals are placed into the test box on the neutral grey strip for 10 minutes. Animals may move freely between either side of the test box and the times they spend on either side are recorded for later statistical analysis.

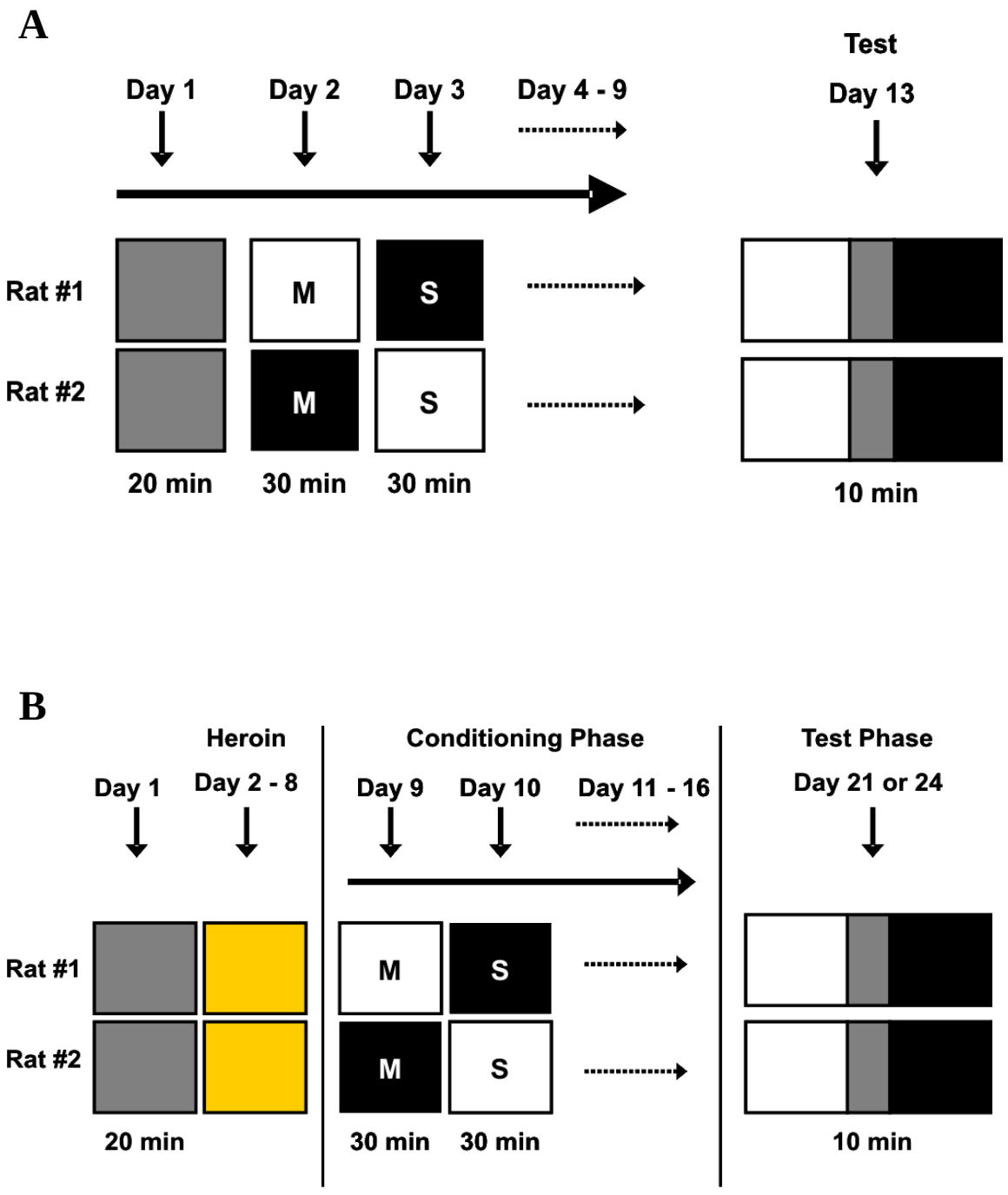

A
Day 1
Day 2
Day 3
Day 4 - 9
Test
Day 13
Rat #1
Rat #2
M
S
M
S
20 min
30 min
30 min
10 min
B
Heroin
Day 1
Day 2 - 8
Conditioning Phase
Day 9
Day 10
Day 11 - 16
Test Phase
Day 21 or 24
Rat #1
Rat #2
M
S
M
S
20 min
30 min
30 min
10 min

Figure 2

2.3.a Induction of Opiate Dependence and Withdrawal

For experiments exploring behaviour in an opiate-dependent state, the animals were conditioned/trained in a state of opiate dependency and withdrawal (DWD) as described in (Bechara & van der Kooy, 1992; Laviolette et al., 2002; Laviolette & van der Kooy, 2004) (see Figure 2B). Following recovery from surgery, animals were pre-conditioned for 20 minutes in a neutral grey box. The following day the animals were given their first of seven daily 0.5 mg/kg subcutaneous (s.c.) injections of heroin in order to create a state of heroin dependency. Animals were then conditioned 21 hours after their last heroin injection. Behavioural signs of withdrawal are evident in rodents following at least 16 hours of drug-abstinence (Bechara & van der Kooy, 1992). During the conditioning phase, heroin injections were administered as a maintenance dose 2.5 hours after the termination of each conditioning session. This 2.5 hour gap ensured animals did not develop any associative memory between the conditioning environments and their daily heroin injections. Over the course of the experiments, animals thus received a total of 15 heroin injections (7 before conditioning, 8 during conditioning). The formation of opiate dependence and subsequent signs of opiate-withdrawal observed following this regimen of heroin administration is qualitatively similar to signs of dependence and withdrawal following a 3-week regimen of morphine administration (Bechara & van der Kooy, 1992; Laviolette et al., 2002; Laviolette & van der Kooy, 2004). However, by employing a shorter 7-day heroin regimen the likelihood for formation of infections and blockages in in-dwelling cannulae was decreased compared to a 3-week morphine regimen.

In experiments examining recovery from chronic opiate exposure animals were subjected to a 7-day clearing period (drug free) after a 7-day heroin treatment as described above.

Behavioural signs of withdrawal in rodents are no longer evident following a 7-day clearing period (Bechara & van der Kooy, 1992). The clearing period was used to assure animals were conditioned in a drug-free state. Following the induction of heroin dependence and the clearing period, animals underwent the standard 8-day conditioning procedure, 3 to 5 day recovery period, and testing phase.

2.4 Western Blot Procedure

Two groups of 8 animals were given 15 days of injections in order to mimic the number of injections received during the formation of opiate dependence and withdrawal followed by morphine place preference conditioning. The first group of 8 received daily saline injections and the other 8 received daily heroin (0.5 mg/kg; s.c.) injections. Twenty-one hours following their last injection, animals were sacrificed, their brains rapidly removed, and a micro-punch of the amygdala isolated. The amygdalar tissue samples were then homogenized using a Dounce homogenizer and protein isolated using RIPA lysis buffer containing a protease inhibitor tablet (mini complete tablets; ROCHE) and phosphatase inhibitors (Calbiochem phosphatase inhibitor cocktail set 4, Sigma phosphatase inhibitor cocktail 2). Following homogenization, the samples were mixed with 2X Laemmli loading buffer and heated to 95 degrees Celsius for 5 minutes prior to storage in a -80 degrees Celsius freezer until needed for Western blotting.

Twenty-five μg of protein from the control (PN) and treatment (DWD) samples were loaded onto 12% denaturing SDS-Page gels. The samples were then subjected to electrophoresis using a Bio-Rad Mini Protein 3 Western blotting apparatus under 125V of electricity for 1.5 hours to separate the proteins according to size. Bio-Rad Kaleidoscope molecular weight (MW)

standards were also included on the gels to confirm the molecular weight of the proteins of interest. Following electrophoresis, protein was transferred from the gels onto a nitrocellulose membrane (Bio-Rad) using a mini trans-blot apparatus (Bio-Rad) in a Tris-Glycine (Bio-Rad Cube Solutions) containing 20% methanol under 75V of electricity for 1 hour. To ensure proper saturation/transfer and that relatively equal volumes of protein were loaded on the gels, the membranes were stained using Ponceau S.

To identify changes in the level of phosphorylation state of the proteins of interest, the blots were first blocked with 5% BSA in TBS-T for 1 hour at room temperature with rocking. Following blocking the membranes were incubated in a solution containing 5% BSA in TBS-T along with an antibody directed against the protein of interest for one hour at room temperature (ERK1/2, CaMKII) or overnight at 4 degrees Celcius (pERK1/2, pCaMKII). Following incubation in the primary antibody solution, the blots were washed in TBS-T three times for 15 minutes each and once in TBS for 5 minutes. Lastly, a solution containing 5% dried non-fat milk in TBS-T and a secondary antibody (HRP-conjugated goat-anti-rabbit antibody; see table) was applied to the blots and left to incubate for 1 hour at room temperature. The proteins of interest (ERK1/2, pERK1/2, CaMKIIα, pCaMKIIα; see Table 1) along with the loading control (alpha-tubulin) were detected using chemiluminescence (Super-Signal; THERMO Scientific) and Kodak X-OMAT LS film. Densitometry measurements were obtained using Kodak digital analysis software and the data analyzed using Microsoft Excel.

Table 1

Name	Source	Species	Dilution	Catalogue #
alpha-tubulin	Sigma	rabbit	1/50,000	SAB3500023
ERK1/2	Cell Signaling	rabbit	1/10,000	9102
pERK1/2	Cell Signaling	rabbit	1/2000	4370
CaMKIIα	Cell Signaling	rabbit	1/2000	3357
pCaMKIIα	Cell Signaling	rabbit	1/5000	3361
HRP-conjugated goat	Thermo	rabbit	1/20,000	31210

2.5 Statistical Analysis

All behavioral data were tested individually for statistical normality and analyzed using standard two-way repeated-measures ANOVA. Fisher's LSD post-hoc analyses were then performed to examine group differences for statistical significance within or across levels of analysis. All protein density data were analyzed by standard T-test. In graphical representations of the data the vertical bars on the graphs represent group means with error bars representing the standard error of the mean (SEM). One asterisk (*) indicates a difference between two groups that is statistically significant at $p < 0.05$. Two asterisks (**) indicate a level of significance of $p < 0.01$.

3. Results

3.1 Effects of inhibition of intracellular signaling kinases within the BLA on the acquisition of associative opiate reward learning in previously opiate-naïve animals

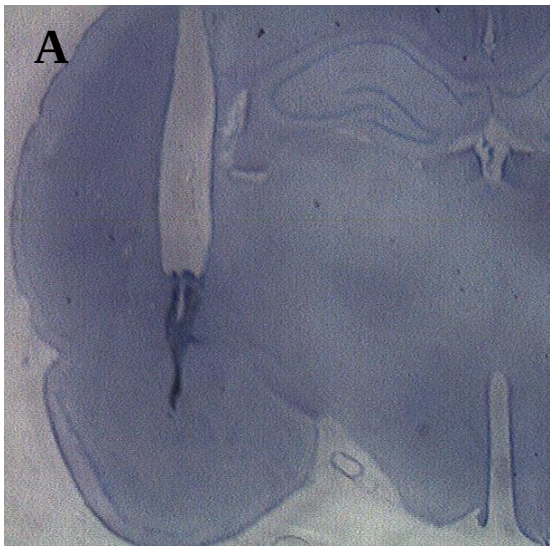
To explore the role of the intracellular signaling kinases, MEK and CaMKII, in the encoding of associative opiate-related reward learning, previously opiate-naïve animals received bilateral intra-BLA micro-infusions of the potent non-competitive MEK inhibitor UO-126 (0.1-1.0 μg/0.5 μl) or CaMKII inhibitor KN-62 (.05-1.0 μg/0.5 μl) followed by a systemic injection of a previously established supra-threshold dose of morphine (5 mg/kg; i.p.) (Bishop et al., 2011; Lintas et al., 2011; Lintas et al., 2012) or a systemic injection of physiological saline prior to placement in one of two conditioning environments. A representative microphotograph of bilateral intra-BLA micro-injector tip placements is shown in Figure 3A. Figure 3B and C show

a schematic representation of bilateral intra-BLA injector tip placements for four representative experimental groups.

Figure Caption 3

Depiction of intra-BLA Guide Cannulae Placement

(A) Micro-photograph of a representative bilateral intra-BLA guide cannulae and injector tip placement. (B) Schematic representation of bilateral intra-BLA injector tip placements. ■ = previously opiate-naïve animals receiving intra-BLA UO-126 (1.0 μg/0.5 μl). □ = previously opiate-naïve animals receiving intra-BLA KN-62 (1.0 μg/0.5 μl). (C) Schematic representation of bilateral intra-BLA injector tip placements. ● = opiate-dependent/withdrawn animals receiving intra-BLA KN-62 (1.0 μg/0.5 μl). ○ = opiate-dependent/withdrawn animals receiving intra-BLA UO-126 (1.0 μg/0.5 μl).



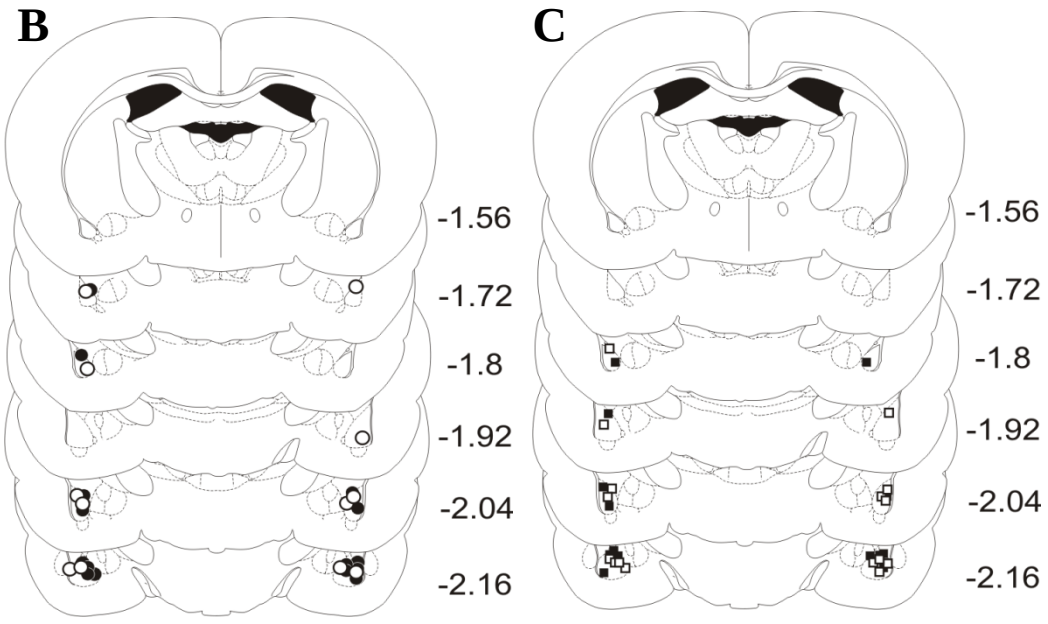


Figure 3

The results of the place conditioning experiments revealed that bilateral micro-infusions of the MEK inhibitor (UO-126), but not the CaMKII inhibitor (KN-62), dose-dependently (1.0 μg/0.5 μl) blocked the encoding of opiate-reward learning in previously opiate-naïve animals. At the high dose of the MEK inhibitor (1.0 μg/0.5 μl) previously opiate-naïve animals failed to display a significant morphine-related CPP while control animals receiving intra-BLA saline, low dose UO-126 (0.1 μg/0.5 μl), and all doses of KN-62 (.05-1.0 μg/0.5 μl) displayed a significant morphine CPP.

The omnibus two-way repeated-measures ANOVA revealed a significant main effect of group (intra-BLA saline, 0.1 μg, 1.0 μg UO126) ($F_{(2, 45)} = 5.6$, $p<.01$), a main effect of treatment (saline vs. morphine) ($F_{(1, 45)} = 188.8$, $p<.0001$), and a significant group (intra-BLA saline, 0.1 μg, 1.0 μg UO-126) X treatment (saline vs. morphine) interaction on times spent in the saline versus morphine-paired environments ($F_{(2, 45)} =29.8$, $p<0.0001$) for animals receiving intra-BLA UO-126. Fisher's LSD post hoc analyses were conducted to examine the interaction further. The post hoc analyses revealed that animals treated with intra-BLA UO-126 at the high concentration (1.0 μg) spent an equal amount of time in both the morphine and saline-paired environments ($p>.05$) whereas intra-BLA UO-126 at the low concentration (0.1 μg) and control animals treated with intra-BLA saline spent significantly more time in the morphine than saline-paired environment (p's<.01) (Figure 4A). Comparing only times spent in the morphine-paired environments, a significant difference between groups on the time spent in the morphine-paired side was revealed. Animals in the intra-BLA saline group and the low dose UO-126 group spent significantly more time in the morphine-paired side than those in the high dose UO-126 group (p's<.01).

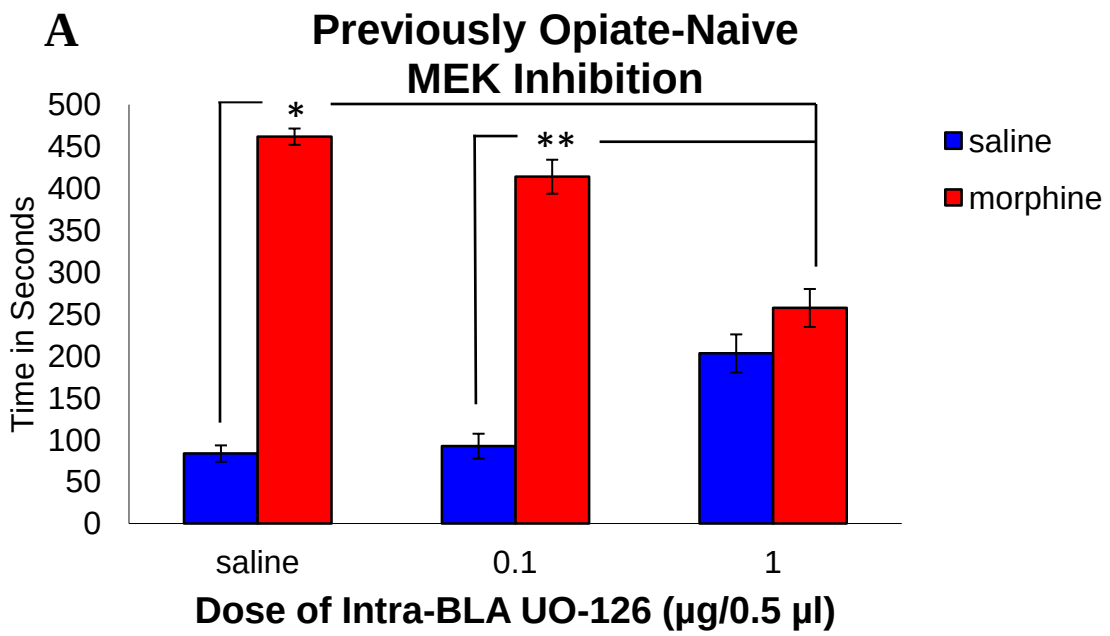
Figure Caption 4

Effects of intra-BLA kinase inhibition on opiate reward conditioning in previously opiate-naïve animals.

(A) In previously opiate-naïve animals, a well established supra-threshold dose of morphine (5 mg/kg; i.p.) produces a robust morphine CPP in control animals receiving intra-BLA saline as well as in those receiving the low dose of UO-126 (0.1 μg/0.5 μl). However, animals receiving the high dose of UO-126 (1.0 μg/0.5 μl) fail to display a significant difference in time spent in either the morphine or saline-paired environment.

(B) In previously opiate-naive animals a supra-threshold dose of morphine (5 mg/kg; i.p.) produces a robust morphine CPP in animals receiving all doses (.05 μg, 0.5 μg, 1.0 μg/0.5 μl) of intra-BLA KN-62. Therefore, in previously opiate-naïve animals the high dose of KN-62 fails to block the acquisition of morphine CPP.

Note: error bars represent standard error (SE); ** denotes significance of $p<.01$; * denotes significance of $p<.05$


A
Previously Opiate-Naive
MEK Inhibition
Time in Seconds
500
450
400
350
300
250
200
150
100
50
0
*
**
saline
morphine
saline
0.1
1
Dose of Intra-BLA UO-126 (µg/0.5 µl)

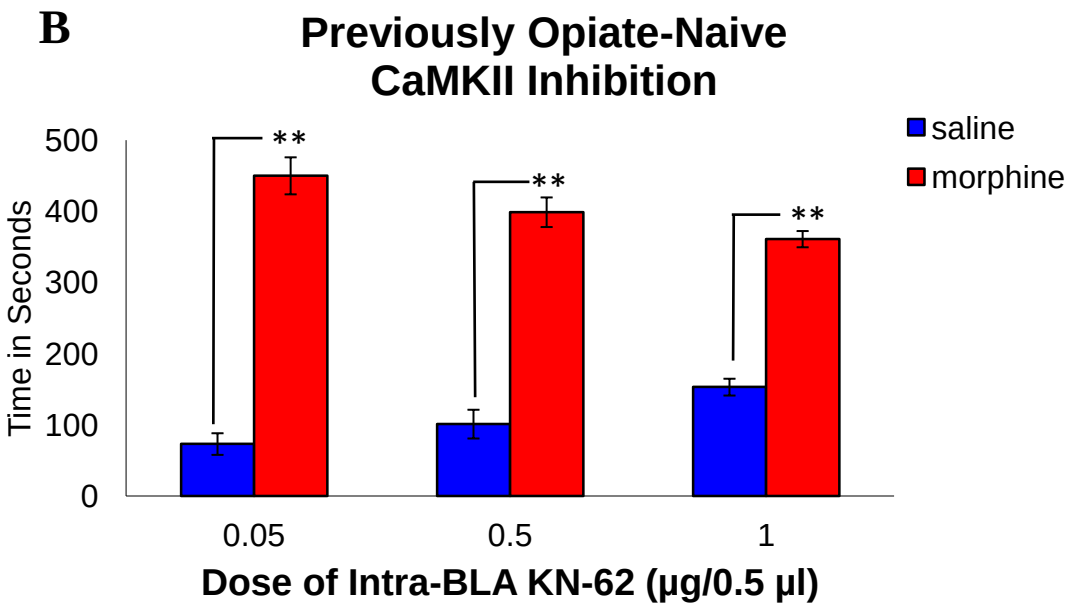

B
Previously Opiate-Naive
CaMKII Inhibition
Time in Seconds
500
400
300
200
100
0
**
**
**
saline
morphine
0.05
0.5
1
Dose of Intra-BLA KN-62 (µg/0.5 µl)

Figure 4

To explore the effect of CaMKII inhibition on the encoding of associative opiate-related memory, bilateral intra-BLA micro-infusions of KN-62 (.05-1.0 μg/0.5 μl) were administered in animals in a previously opiate-naive state. At all dose levels (.05-1.0 μg/0.5 μl) intra-BLA injections of the CaMKII inhibitor, KN-62, failed to block the acquisition of morphine CPP in previously opiate-naive animals.

The two-way repeated-measures ANOVA revealed a significant main effect of treatment (saline vs. morphine) ($F_{(1,43)} = 216.3$, $p<.0001$) and a group (intra-BLA .05 μg, 0.5 μg, 1.0 μg KN62) X treatment (saline vs. morphine) interaction on times spent in the morphine versus saline-paired environments ($F_{(2,43)} = 6.0$, $p=.009$) in animals receiving intra-BLA KN-62. Post hoc analyses revealed that animals treated with intra-BLA KN-62 spent significantly more time in the morphine than saline-paired environment at all doses of KN-62 (.05-1.0 μg/0.5 μl) (p's <.01) (Figure 4B). These experiments indicate that pharmacological inhibition of MEK phosphorylation, but not CaMKII phosphorylation, within the BLA dose-dependently blocks the acquisition of associative opiate reward memory in animals in a previously opiate-naïve state.

3.2 Effect of Inhibition of intracellular signaling kinases within the BLA on the acquisition of associative opiate-related reward learning in opiate-dependent/withdrawn animals

To explore the role of the intracellular signaling kinases, CaMKII and MEK, in the encoding of associative opiate-reward learning, opiate-dependent/withdrawn animals received bilateral intra-BLA micro-infusions of the CaMKII inhibitor KN-62 (0.5-1.0 μg/0.5 μl) or the MEK inhibitor UO-126 (1.0 μg/0.5 μl) immediately prior to conditioning. The results of the

place conditioning experiments revealed that bilateral intra-BLA micro-infusions of the CaMKII inhibitor, but not the MEK inhibitor, dose-dependently (1.0 μg/0.5 μl) block the encoding of opiate-reward learning in opiate-dependent/withdrawn animals. At the high dose of the CaMKII inhibitor (1.0 μg/0.5 μl) animals did not display a significant morphine-related CPP. Control groups receiving intra-BLA saline, low dose KN62 (0.5 μg/0.5 μl), and high dose UO-126 (1.0 μg/0.5 μl) did display a significant morphine CPP.

The two-way repeated-measures ANOVA revealed a significant main effect of treatment (saline vs. morphine) ($F_{(1,39)} = 108.0$, $p<.0001$) and a significant group (intra-BLA saline, 0.5 μg, 1.0 μg KN-62) X treatment (saline vs. morphine) interaction on times spent in the saline versus morphine-paired environments ($F_{(2, 39)} =40.4$, $p<.0001$) in animals receiving intra-BLA KN-62. Post hoc analyses revealed that animals treated with KN-62 at the high dose (1.0 μg) spent equal amounts of time in morphine and saline-paired environments ($p>.05$) whereas control animals treated with intra-BLA saline and intra-BLA KN-62 at the low dose (0.5 μg) spent significantly more time in the morphine than saline-paired environment (p's<.01) (Figure 5A). Comparing only times spent in the morphine-paired environments, a significant difference between groups on the time spent in the morphine-paired side was uncovered. Animals in the intra-BLA saline group and the low dose KN-62 group spent significantly more time in the morphine-paired side than those in the high dose KN-62 group (p's<.01).

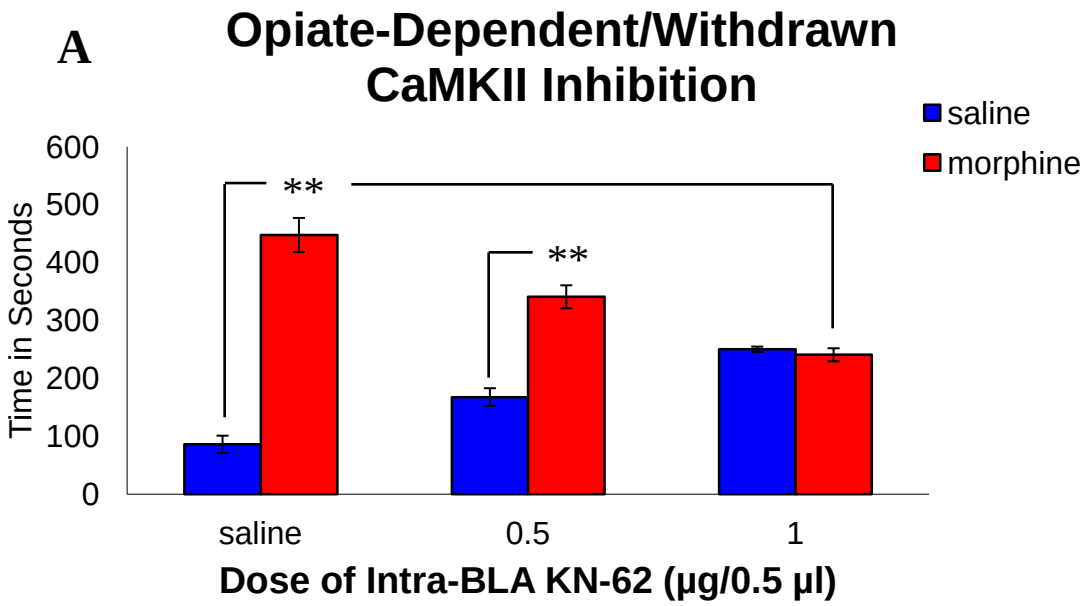
Figure Caption 5

Effect of intra-BLA kinase inhibition on opiate reward conditioning in opiate-dependent/withdrawn animals.

(A) In opiate-dependent/withdrawn animals a well established supra-threshold dose of morphine (5 mg/kg; i.p.) produces a robust morphine CPP in control animals receiving intra-BLA saline as well as in those receiving the low dose of KN-62 (0.5 μg/0.5 μl). However, animals receiving the high dose of KN-62 (1.0 μg/0.5 μl) fail to display a significant difference in time spent in either the morphine or saline-paired environment.

(B) In opiate-dependent/withdrawn animals a supra-threshold dose of morphine (5 mg/kg; i.p.) produces a robust morphine CPP in control animals receiving intra-BLA saline as well as in those receiving the effective high dose of UO-126 (1.0 μg/0.5 μl) in previously opiate-naïve animals. Therefore, in opiate-dependent/withdrawn animals the high dose of UO-126 fails to block the acquisition of morphine CPP.

Note: error bars represent standard error (SE); ** denotes significance of $p<.01$; * denotes significance of $p<.05$


A
Opiate-Dependent/Withdrawn
CaMKII Inhibition
saline
morphine
Time in Seconds
600
500
400
300
200
100
0
**
**
saline
0.5
1
Dose of Intra-BLA KN-62 (µg/0.5 µl)

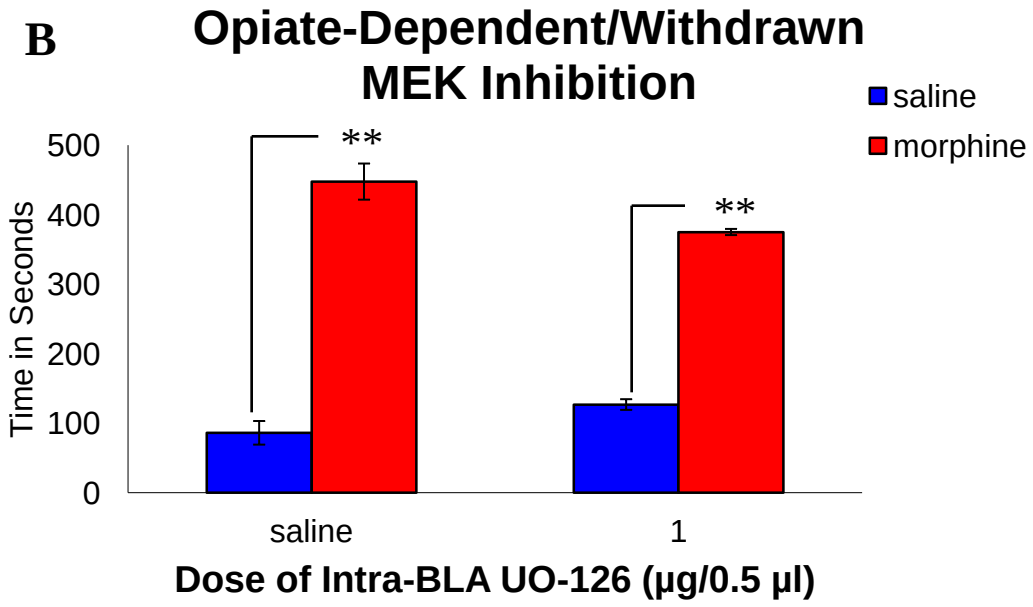

B
Opiate-Dependent/Withdrawn
MEK Inhibition
saline
morphine
Time in Seconds
500
400
300
200
100
0
**
**
saline
1
Dose of Intra-BLA UO-126 (µg/0.5 µl)

Figure 5

To explore the effect of MEK inhibition on the encoding of associative opiate-related memory bilateral intra-BLA micro-infusions of UO-126 (1.0 μg/0.5 μl) were administered in animals in an opiate-dependent/withdrawn state. The results revealed no effect of MEK inhibition on the acquisition of opiate-related memory. The previously established effective dose of UO-126 (1.0 μg/0.5 μl) in previously opiate-naïve animals failed to block the acquisition of morphine CPP in opiate-dependent/withdrawn animals (Figure 5B).

The ANOVA revealed a significant main effect of treatment (saline vs. morphine) ($F_{(1,23)}$ = 191.3, p<.0001) and a group (intra-BLA saline, 1.0 μg UO-126) X treatment (saline vs. morphine) interaction on times spent in the morphine versus saline-paired environments ($F_{(1,23)}$ = 6.5, p=.028) in animals receiving intra-BLA UO-126. Post hoc analyses revealed that animals treated with intra-BLA saline and high dose UO-126 (1.0 μg/0.5 μl) spent significantly more time in the morphine than saline-paired environment (p's <.01) (Figure 5B). Taken together these experiments indicate an opiate exposure state-dependent effect of pharmacological inhibition of CaMKII activation within the BLA. Where inhibition of CaMKII does-dependently blocks the acquisition of associative opiate reward memory in opiate-dependent and withdrawn animals, inhibition of MEK fails to block the acquisition of opiate-related learning and memory in opiate-dependent/withdrawn animals.

3.3 Effects of Intra-BLA intracellular kinase inhibition on intra-VTA morphine reward acquisition

We administered a dose of morphine previously shown to produce a robust morphine CPP when micro-infused directly into the VTA (Laviolette & van der Kooy, 2004; Lintas et al.,

2012; Nader & van der Kooy, 1997) to investigate the effects of kinase inhibition on the acquisition of associative reward learning via morphine reward cues directly from the VTA. Figure 6A shows a representative microphotograph of bilateral intra-VTA micro-injector tips. Figure 6B shows a schematic representation of the bilateral intra-VTA micro-injector tip placements of two experimental groups described in this section.

Figure Caption 6

(A) Microphotograph of a representative bilateral intra-VTA guide cannula and injector tip placement. (B) Schematic representation of bilateral intra-VTA injector tip placements. ■ = previously opiate-naïve animals receiving intra-VTA morphine (0.5 μg/0.5 μl). □ = opiate-dependent/withdrawn animals receiving intra-VTA morphine (0.5 μg/0.5 μl).

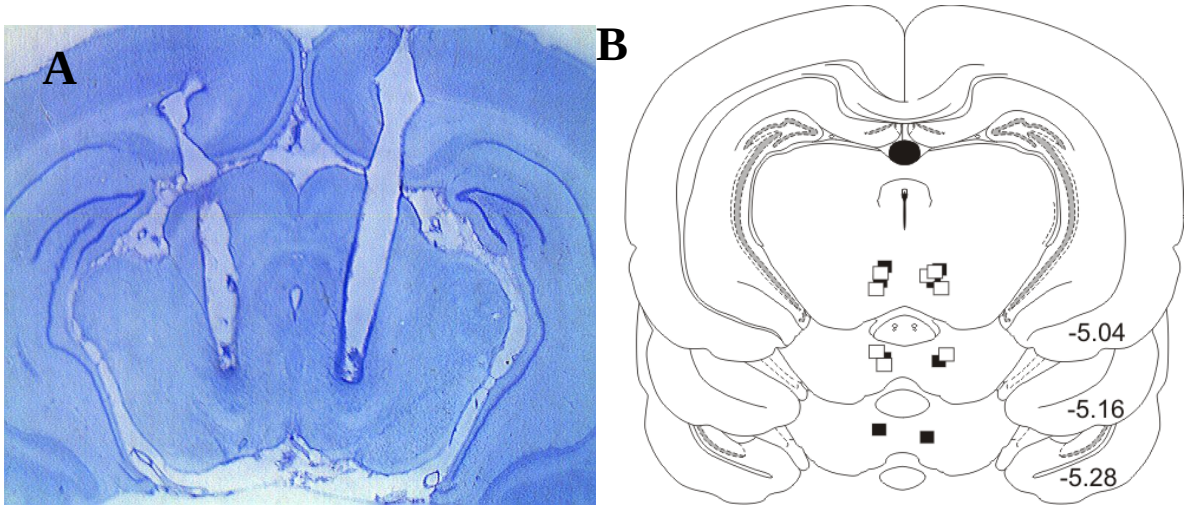


Figure 6

The same dissociation between MEK inhibition and CaMKII inhibition in previously-opiate-naïve versus opiate-dependent/withdrawn animals observed during systemic administration of morphine was also observed during intra-VTA morphine administration. At the established effective dose of UO-126 (1.0 μg/0.5 μl) in previously opiate-naïve animals, the supra-threshold dose of intra-VTA morphine (0.5 μg/0.5 μl) failed to produce a significant morphine CPP. However, this same dose of UO-126 when administered in opiate-dependent/withdrawn animals did result in a significant morphine CPP. Further, in opiate-dependent/withdrawn animals the effective dose of KN-62 (1.0 μg/0.5 μl) for the blockade of morphine CPP in opiate-dependent/withdrawn via systemic morphine injection also blocked morphine CPP via intra-VTA morphine micro-injection. Conversely, this dose of KN-62 was unable to block morphine CPP in previously opiate-naïve animals.

An ANOVA was conducted to examine the effect of intra-BLA MEK inhibition on the acquisition of morphine-related reward memory via direct intra-VTA micro-infusion of a supra-threshold dose of morphine (0.5 μg/0.5 μl). This analysis revealed a significant main effect of treatment (saline vs. morphine) ($F_{(1,27)} = 12.6$, $p<.01$) and a significant group (previously-opiate naïve vs. opiate-dependent/withdrawn) X treatment (saline vs. morphine) interaction on time spent in the morphine versus the saline-paired environment ($F_{(1,27)} = 21.4$, $p<.01$). Further post hoc exploration of the interaction revealed no significant difference between the times spent in the morphine versus saline-paired environment in previously opiate-naïve animals ($p>.05$). However, animals in an opiate-dependent/withdrawn state spent significantly more time in the morphine-piared than in the saline-paired environment ($p<.01$) (Figure 7A). When comparing the times spent in the morphine-paired environment between groups, post hoc analysis revealed a

significant difference between groups on the time spent in the morphine-paired environment; the opiate-dependent/withdrawn animals spent more time in the morphine-paired environment than the previously-opiate naïve animals ($p<.05$).

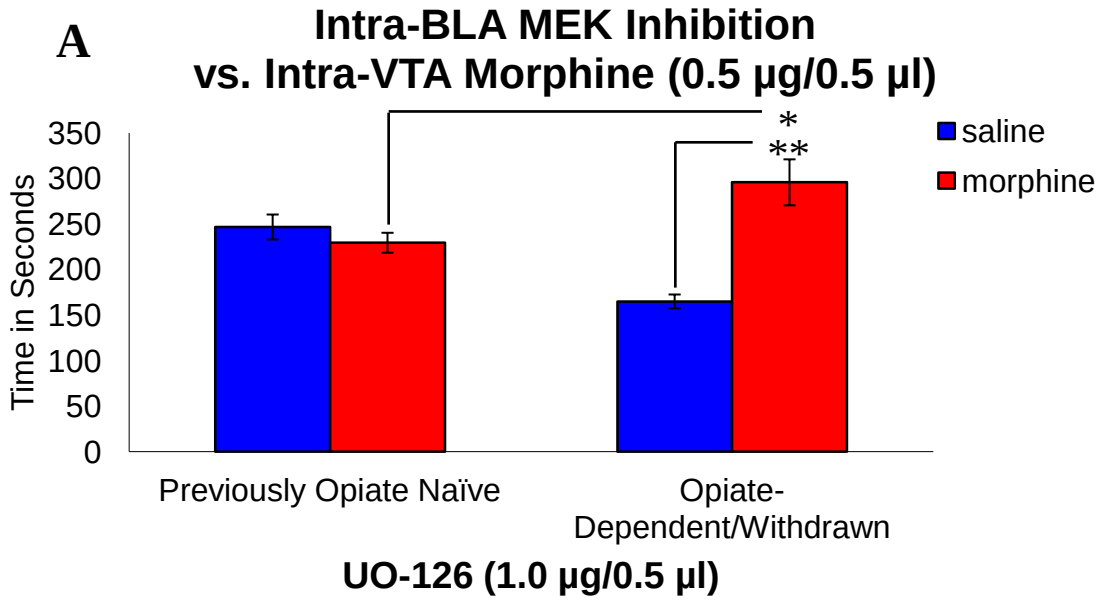
Figure Caption 7

Effect of intra-BLA kinase inhibition on opiate reward conditioning via direct intra-VTA morphine administration.

(A) In previously opiate-naive animals a well established supra-threshold dose of intra-VTA morphine (0.5 μg/0.5 μl) fails to produce a morphine CPP in animals receiving the effective high dose (1.0 μg/0.5 μl) of intra-BLA UO-126. However, opiate-dependent/withdrawn animals receiving the high dose of UO-126 (1.0 μg/0.5 μl) do spend significantly more time in the morphine than the saline-paired environment.

(B) In previously opiate-naive animals a well established supra-threshold dose of intra-VTA morphine (0.5 μg/0.5 μl) produces a robust morphine CPP in animals receiving the effective high dose of intra-BLA KN-62 (1.0 μg/0.5 μl). However, opiate-dependent/withdrawn animals receiving the high dose of KN-62 (1.0 μg/0.5 μl) fail to produce significant morphine CPP such that they spend an equal amount of time in both the morphine and saline-paired environments.

Note: error bars represent standard error (SE); ** denotes significance of $p<.01$; * denotes significance of $p<.05$


A
Intra-BLA MEK Inhibition
vs. Intra-VTA Morphine (0.5 µg/0.5 µl)
Time in Seconds
350
300
250
200
150
100
50
0
*
**
saline
morphine
Previously Opiate Naïve
Opiate-
Dependent/Withdrawn
UO-126 (1.0 µg/0.5 µl)

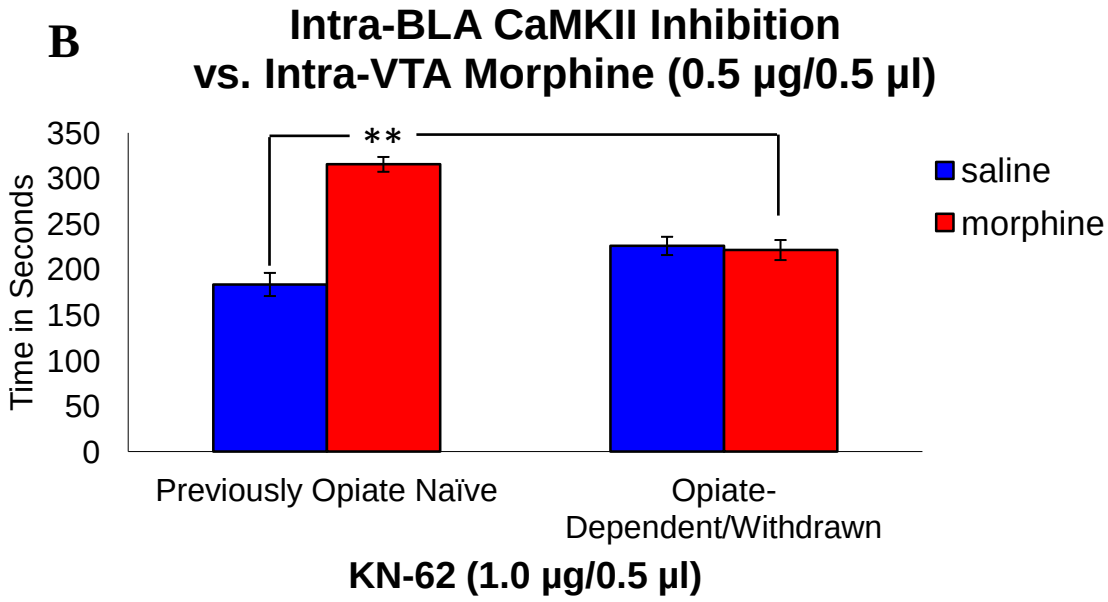

B
Intra-BLA CaMKII Inhibition
vs. Intra-VTA Morphine (0.5 µg/0.5 µl)
Time in Seconds
350
300
250
200
150
100
50
0
**
saline
morphine
Previously Opiate Naïve
Opiate-
Dependent/Withdrawn
KN-62 (1.0 µg/0.5 µl)

Figure 7

The analysis conducted to examine the effect of CaMKII inhibition on the encoding of opiate-reward via intra-VTA morphine revealed a significant main effect of group (previously opiate-naïve vs. opiate-dependent/withdrawn) ($F_{(1,27)} = 10.9$, $p<.01$), a significant main effect of treatment (saline vs. morphine) ($F_{(1,27)} = 32.6$, $p<.01$), and a significant group (previously opiate-naïve vs. opiate-dependent/withdrawn) X treatment (saline vs. morphine) interaction ($F_{(1,27)} = 24.9$, $p<.01$) on times in the morphine or saline-paired environment. Post hoc analysis showed that previously opiate-naïve animals spent significantly more time in the morphine rather than the saline-paired environment ($p<.01$) while opiate-dependent/withdrawn animals did not differ in their time spent in either environment ($p>.05$) (Figure 7B). In comparing the two groups total time spent in the morphine-paired environment, post hoc analysis revealed a significant difference between groups in their times spent in the morphine-paired environment such that previously opiate-naïve animals spent significantly more time in the morphine-paired environment than the opiate-dependent animals ($p<.01$).

These results closely mirror the opiate exposure state-dependent dissociation between MEK and CaMKII inhibition on the acquisition of morphine-reward memory when morphine was delivered systemically. The results suggest a state-dependent switch within the mesocorticolimbic system from an opiate-naïve MEK dependent pathway to an opiate-dependent/withdrawn CaMKII dependent pathway in the formation of opiate-related associative memories.

3.4 Examining the permanency of the functional switch from previously opiate-naive MEK to opiate-dependent/withdrawn CaMKII pathways in the encoding of associative opiate-related memory.

Next, the longevity of the functional switch from a MEK-dependent pathway to a CaMKII-dependent pathway in the acquisition of opiate-reward related associative memories was examined. The animals were exposed to the standard seven day schedule of heroin injections followed by a seven day recovery period to ensure animals were in a drug free state on the first day of CPP conditioning. The results of these experiments indicated that the functional switch from pMEK-dependent encoding of associative memory to pCaMKII-dependent encoding of associative memory is long lasting. The group of opiate-recovered animals receiving intra-BLA MEK inhibitor (1.0 µg/0.5 µl) displayed a significant morphine-CPP while animals receiving intra-BLA CaMKII inhibitor (1.0 µg/0.5 µl) failed to display a significant morphine-CPP.

The statistical analysis conducted to examine the effect of intra-BLA MEK inhibition revealed a significant main effect of treatment (saline vs. morphine) ($F_{(1,43)} = 114.1$, $p<.0001$) and a significant group (previously opiate-naive, opiate-dependent/withdrawn, opiate-recovered) X treatment (saline vs. morphine) interaction for times spent in the morphine versus saline-paired environments ($F_{(2,43)} = 13.8$, $p<.001$). Post hoc analyses revealed no significant difference between times spent in the morphine or saline paired side for animals in a previously opiate-naive state ($p>.05$). However, animals in an opiate-dependent/withdrawn and opiate-recovered state spent significantly more time in the morphine- than the saline-paired environment ($p's<.01$). Furthermore, when comparing the total times spent in the morphine-paired environments between groups the opiate-dependent/withdrawn and opiate-recovered groups spent significantly

more time in the morphine-paired environment than the previously opiate-naive group (p's<.01) (Figure 8A).

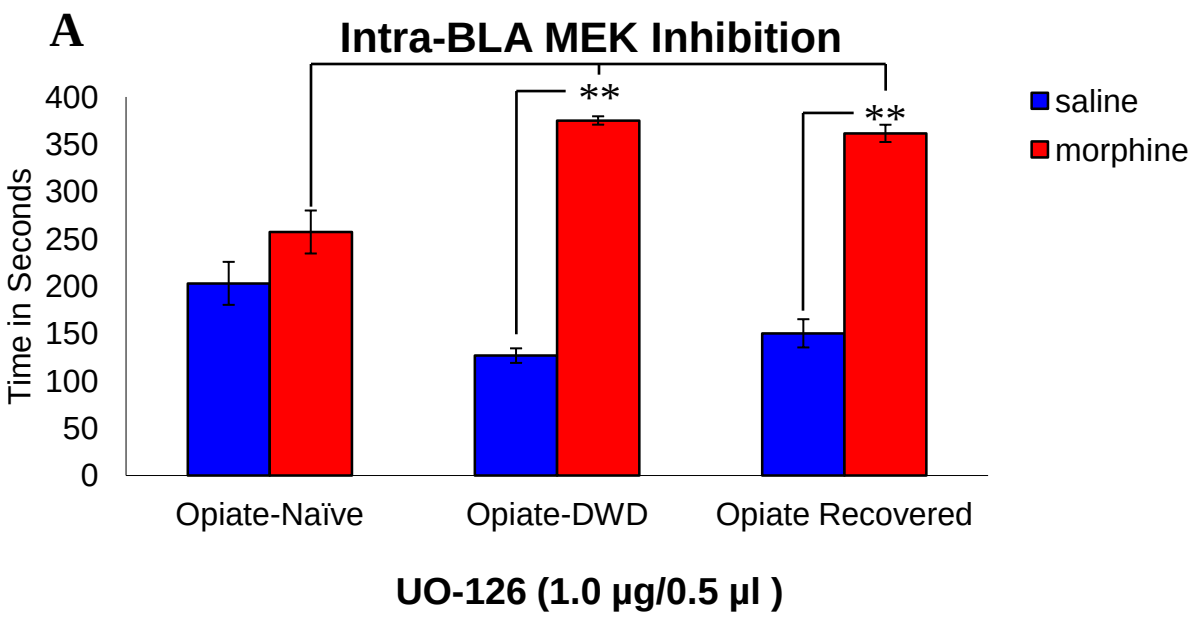
Figure Caption 8

Plasticity of intra-BLA kinase inhibition on opiate reward conditioning.

(A) In previously opiate-naive animals receiving the effective high dose (1.0 μg/0.5 μl) of intra-BLA UO-126, a supra-threshold dose of morphine (5 mg/kg; i.p.) fails to produce morphine CPP. However, opiate-dependent/withdrawn and opiate-recovered animals receiving the high dose of UO-126 (1.0 μg/0.5 μl) versus the supra-threshold dose of morphine (5 mg/kg; i.p.) did spend significantly more time in the morphine than the saline-paired environment.

(B) In previously opiate-naive animals receiving the effective dose (1.0 μg/0.5 μl) of intra-BLA KN-62 a supra-threshold dose of morphine (5 mg/kg; i.p.) produced a robust morphine CPP. However, opiate-dependent/withdrawn and opiate-recovered animals receiving the high dose of KN-62 (1.0 μg/0.5 μl) failed to display a significant morphine CPP such that they spend an equal amount of time in both the morphine and saline-paired environments.

Note: error bars represent standard error (SE); ** denotes significance of $p<.01$; * denotes significance of $p<.05$


A
Intra-BLA MEK Inhibition
Time in Seconds
400
350
300
250
200
150
100
50
0
**
**
saline
morphine
Opiate-Naïve
Opiate-DWD
Opiate Recovered
UO-126 (1.0 µg/0.5 µl)

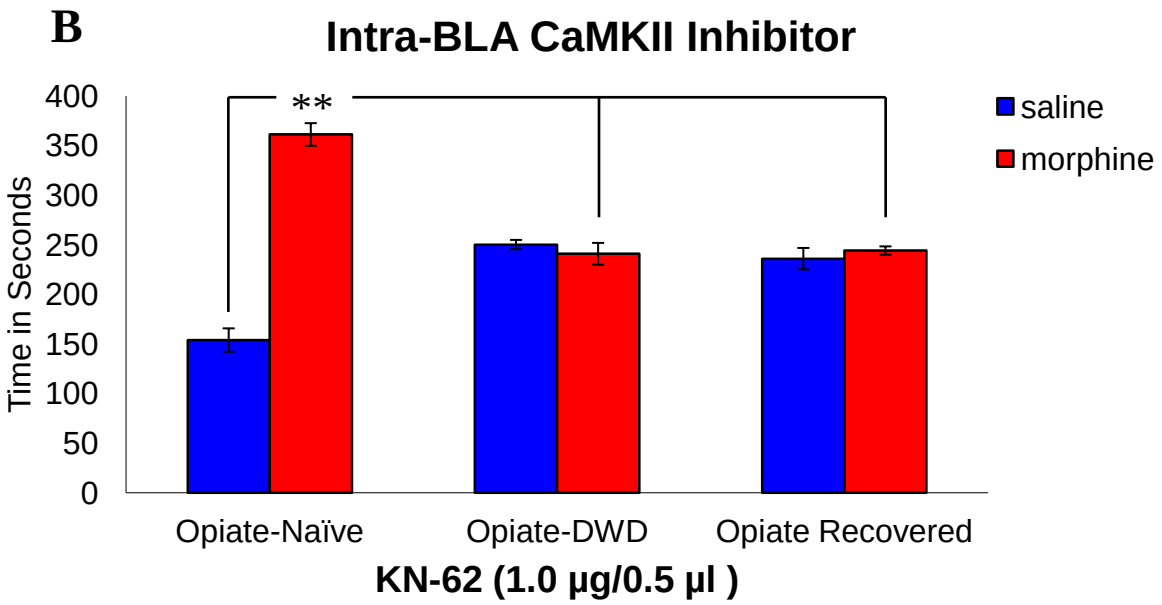

B
Intra-BLA CaMKII Inhibitor
Time in Seconds
400
350
300
250
200
150
100
50
0
**
saline
morphine
Opiate-Naïve
Opiate-DWD
Opiate Recovered
KN-62 (1.0 µg/0.5 µl)

Figure 8

The ANOVA conducted to examine the effect of intra-BLA CaMKII inhibition revealed a main effect of treatment (saline vs. morphine) ($F_{(1,41)} = 47.6$, $p<.0001$) and a significant group (previously opiate-naive, opiate-dependent/withdrawn, opiate-recovered) X treatment (saline vs. morphine) interaction on times spent in the morphine- versus saline-paired environments ($F_{(2,41)} = 48.5$, $p<.001$). Post hoc analyses revealed that animals in a previously opiate-naive state spent significantly more time in the morphine- than the saline-paired environment ($p<.01$). Those in the opiate-dependent/withdrawn and opiate-recovered groups however spent an equal amount of time in both the morphine- and the saline-paired environments (p's>.05). Comparing the times spent between groups in the morphine-paired environment the previously opiate-naive group spent significantly more time in the morphine-paired environment than both the opiate-dependent/withdrawn and opiate-recovered groups (p's<.01) (Figure 8B).

These results clearly indicate that the functional switch from a MEK dependent pathway in previously opiate-naive animals to a CaMKII dependent pathway in opiate-dependent/withdrawn animals is a long lasting one. Opiate-recovered animals receiving intra-BLA MEK inhibitor displayed a strong morphine CPP while those receiving intra-BLA CaMKII inhibitor failed to display morphine CPP.

3.5 Pharmacological inhibition of intracellular signaling kinases blocks the DA receptor mediated potentiation of morphine CPP to a sub-threshold dose of morphine

Previous research has demonstrated that intra-BLA agonism of DA D1-like and D2-like receptors in previously opiate-naive and opiate-dependent/withdrawn animals, respectively, can potentiate morphine CPP to a sub-threshold dose of morphine (Lintas et al., 2011; Lintas et al.,

2012). Normally a sub-threshold dose (.05 mg/kg; i.p.) of morphine fails to produce a significant morphine CPP (Bishop et al., 2011; Lintas et al., 2011; Lintas et al., 2012) however, in previously opiate-naive animals receiving an intra-BLA DA D1-like receptor agonist the sub-threshold dose of morphine successfully produces a morphine CPP. Similarly, in opiate-dependent/withdrawn animals receiving an intra-BLA DA D2-like receptor agonist this same sub-threshold dose of morphine successfully produces morphine CPP. Given the functional link between DA D1-like receptors and MEK activation (Fricks-Gleason & Marshall, 2011; O'Sullivan et al., 2008; Pan et al., 2011) we hypothesized that pharmacological agonism of intra-BLA D1-like receptors coupled with MEK inhibition would result in a blockade of the previously established potentiation of a sub-threshold dose of morphine in previously opiate-naive animals. Further, based on previous research suggesting a link between DA D2-like receptors and CaMKII activation (Gu, Jiang, Yuen, & Yan, 2006; Gu & Yan, 2004; Liu et al., 2009), it was hypothesized that pharmacological agonism of intra-BLA D2-like receptors coupled with CaMKII inhibition would block the potentiation of a sub-threshold dose of morphine in opiate-dependent/withdrawn animals. To explore this idea we first replicated the D1R and D2R mediated potentiation of morphine CPP in previously opiate-naive and opiate-dependent/withdrawn animals, respectively. Subsequently, we co-administered a D1R agonist (SKF-81297) with the MEK inhibitor (UO-126) into the BLA of animals in a previously opiate-naive state and co-administered a D2R agonist (Quinpirole) with the CaMKII inhibitor (KN-62) into the BLA of opiate-dependent/withdrawn animals during the conditioning phase against a sub-threshold dose of morphine (.05 mg/kg; i.p.).

The results of these experiments revealed a significant morphine CPP in previously opiate-naive animals receiving sub-threshold morphine and intra-BLA saline or SKF-81297 (1.0 µg/0.5 µl) and a blockade of morphine CPP in animals receiving sub-threshold morphine with intra-BLA co-administration of SKF-81297 and UO-126 (1.0 µg/0.5 µl). Further, opiate-dependent/withdrawn animals receiving sub-threshold morphine and intra-BLA saline or Quinpirole (1.0 µg/0.5 µl) displayed a significant morphine CPP while those receiving intra-BLA co-administration of Quinpirole and KN-62 (1.0 µg/0.5 µl) did not show a significant morphine CPP.

The results from the analysis conducted to examine the effect of intra-BLA D1R agonism as well as D1R agonism coupled with MEK inhibition against a sub-threshold dose of morphine (.05 mg/kg; i.p.) revealed a significant group (Saline, SKF-81297, SKF-81297+UO-126) X treatment (saline vs. morphine) interaction on times spent in the morphine versus the saline paired environments ($F_{(2,45)} = 19.3$, $p<.0001$). Post hoc analyses revealed that those in the SKF-81297 group spent significantly more time in the morphine- than the saline-paired environment ($p<.01$). However, the saline and the SKF-81297+UO-126 groups spent an equal amount of time in both the morphine- and saline-paired environments ($p>.05$). In addition, when comparing times spent in the morphine-paired environment between groups, the SKF-81297 group spent significantly more time in the morphine-paired environment than the saline and SKF-81297+UO-126 groups ($p<.01$) (Figure 9A).

Figure Caption 9

Blockade of DA receptor mediated potentiation of sub-threshold morphine CPP by intracellular signaling kinase inhibition.

(A) In previously opiate-naive animals receiving intra-BLA SKF-81297 (1.0 μg/0.5 μl), a sub-threshold dose of morphine (.05 mg/kg; i.p.) produces a significant morphine CPP. However, in animals receiving intra-BLA saline or SKF-81297+UO-126 (1.0 μg/0.5 μl), a sub-threshold dose of morphine (.05 mg/kg; i.p) failed to produce a significant morphine CPP.

Note: Intra-BLA saline data courtesy of Bishop et. al., (2011)

(B) In opiate-dependent/withdrawn animals receiving intra-BLA Quinpirole (1.0 μg/0.5 μl), a sub-threshold dose of morphine (.05 mg/kg; i.p.) produced a significant morphine CPP. However, in animals receiving intra-BLA saline or Quinpirole+KN-62 (1.0 μg/0.5 μl), a sub-threshold dose of morphine (.05 mg/kg; i.p.) failed to produce a significant morphine CPP.

Note: Intra-BLA saline data courtesy of Lintas et. al., (2012); ** denotes significance of $p<.01$; * denotes significance of $p<.05$

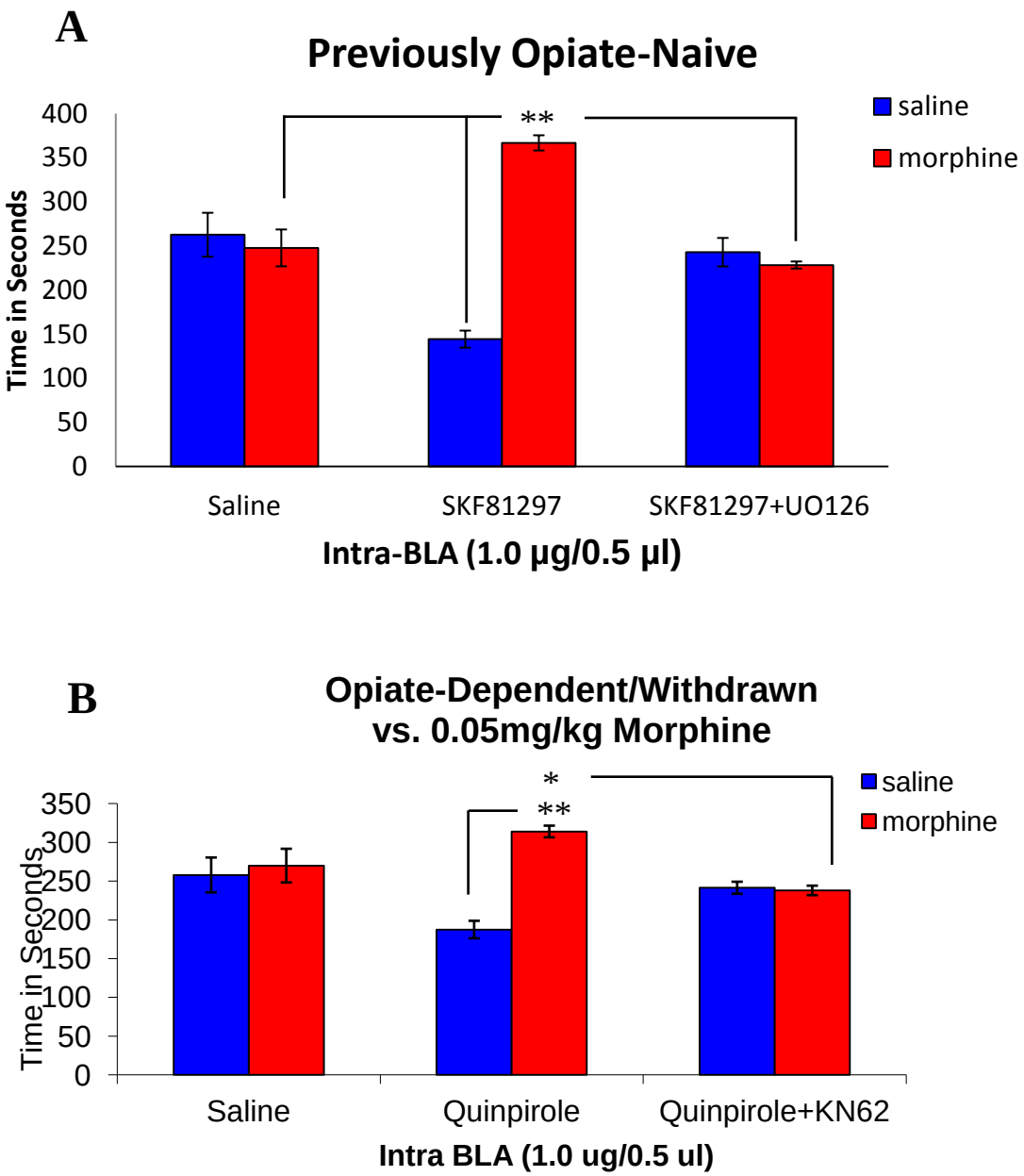

A
Previously Opiate-Naive
saline
morphine
**
Time in Seconds
400
350
300
250
200
150
100
50
0
Saline
SKF81297
SKF81297+UO126
Intra-BLA (1.0 µg/0.5 µl)
B
Opiate-Dependent/Withdrawn
vs. 0.05mg/kg Morphine
saline
morphine
*
**
Time in Seconds
350
300
250
200
150
100
50
0
Saline
Quinpirole
Quinpirole+KN62
Intra BLA (1.0 ug/0.5 ul)

Figure 9

The two-way ANOVA conducted to examine the effect of intra-BLA D2R agonism as well as D2R agonism coupled with CaMKII inhibition against a sub-threshold dose of morphine (.05 mg/kg; i.p.) revealed a significant group (Saline, Quinpirole, Quinpirole+KN-62) X treatment (saline vs. morphine) interaction on times spent in the morphine versus the saline paired environments ($F_{(2,45)} = 5.92$, $p<.01$). Post hoc analyses revealed that those in the Quinpirole group spent significantly more time in the morphine- than the saline-paired environment ($p<.01$). However, the saline and Quinpirole+KN-62 groups spent an equal amount of time in both the morphine- and saline-paired environments ($p>.05$). In addition, when comparing times spent in the morphine-paired environment between groups, the Quinpirole group spent significantly more time in the morphine-paired environment than the Quinpirole+KN62 group ($p<.05$) (Figure 9B).

3.6 Opiate-exposure state modulates the density of signaling kinase protein expression

There is an extensive literature which suggests that acute and chronic exposure to drugs as well as abstinence or withdrawal from drugs cause significant changes in the levels of pMEK(ERK1/2) and pCaMKII expressed within the MCLS. To explore this phenomenon in the BLA, we exposed one group of animals to 15 days of saline injections (s.c.) and one group to 15 days of heroin injections (0.5 mg/kg; s.c.). The heroin and saline injections were carried out for 15 days in order to mimic the number of injections our previous DWD groups received during the formation of dependence and subsequent conditioning phase. Twenty-one hours following their last injection the animals were sacrificed and a section of their BLA removed for analysis.

Western Blot analyses of the density of protein expression of total ERK1 (ERK1) and pERK1 in PN animals versus DWD animals revealed no change in ERK expression between groups (PN, DWD) ($p>.05$) but a significant difference in the expression of pERK1 in DWD animals compared to the PN animals. The difference was such that DWD group displayed a significant decrease in pERK1 expression compared to the PN group ($t_{(12)} = 3.75$, $p<.01$). Similarly, the analysis of ERK2 and pERK2 revealed no change in ERK2 expression between groups (PN, DWD) ($p>.05$) but a significant difference in pERK2 expression between PN and DWD groups. Again, there was a significant decrease in the expression of pERK2 in DWD animals compared to the PN animals ($t_{(12)} = 3.11$, $p<.05$). (Figure 10).

Figure Caption 10

Withdrawal from chronic opiate-exposure modulates the expression of phosphorylated ERK1/2 within the BLA.

(A) Graph of the expression of total ERK1 and 2 (ERK1/2) and picture of protein blots in PN and DWD groups of α-tubulin control, ERK1, and ERK2. Within the BLA ERK1 and 2 expression is unchanged between animals in an opiate-naive (PN) and opiate-dependent/withdrawn (DWD) state.

(B) Graph of the expression of phosphorylated ERK1 and 2 (pERK1/2) and picture of protein blots in opiate-naive (PN) and opiate-dependent/withdrawn animals (DWD) groups of α-tubulin control, pERK1, and pERK2. The expression of the phosphorylated form of ERK1 and 2 (pERK1/2) within the BLA is significantly decreased in DWD in comparison to animals in a PN state.

(C) Graph of the ratio of the pERK1/2 to the total ERK1/2 expressed in the opiate-naive (PN) group compared to the opiate-dependent/withdrawn (DWD) group. The ratio of pERK1/2 to ERK1/2 expressed in the DWD group is significantly less than that of the PN group

Note: error bars represent standard error (SE); ** denotes significance of $p<.01$; * denotes significance of $p<.05$

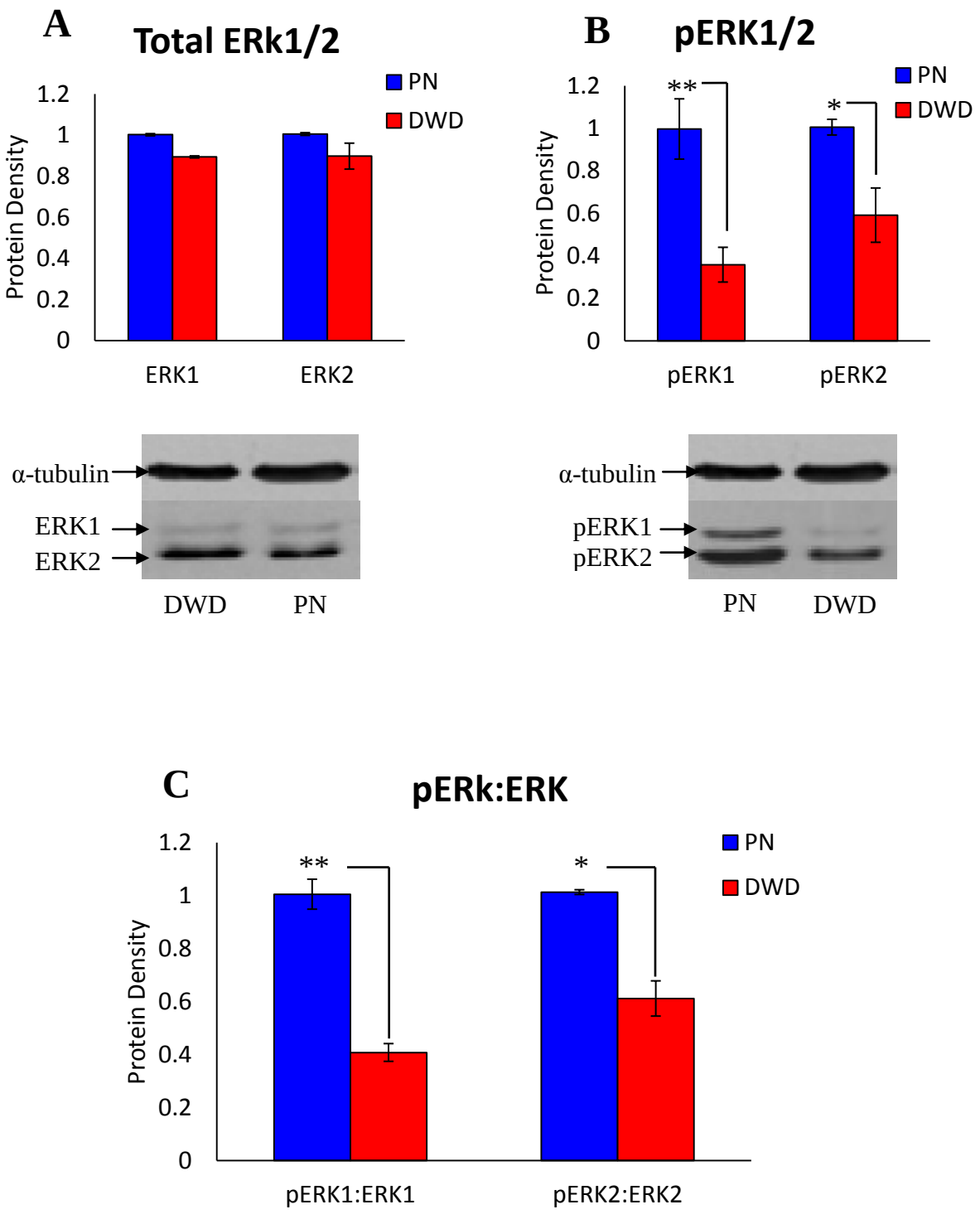

A
Total ERk1/2
PN
DWD
Protein Density
1.2
1
0.8
0.6
0.4
0.2
0
ERK1
ERK2
α-tubulin
ERK1
ERK2
DWD
PN
B
pERK1/2
**
*
PN
DWD
Protein Density
pERK1
pERK2
α-tubulin
pERK1
pERK2
PN
DWD
C
pERk:ERK
**
*
PN
DWD
Protein Density
pERK1:ERK1
pERK2:ERK2

Figure 10

Preliminary analyses of the density of protein expression of total CaMKIIα (CaMKIIα) and phosphorylated CaMKIIα (pCaMKIIα) in PN animals versus DWD animals revealed a large difference in CaMKIIα levels and pCaMKIIα expression between groups (PN, DWD). There was a dramatic decrease in total CaMKIIα levels and pCaMKIIα expressions in the DWD group compared to the PN group (Figure 11). *The data for the quantification of CaMKIIα and pCAMKIIα protein density are still being analyzed. However, preliminary examination of the Western Blots indicates a chronic opiate exposure-mediated decrease in total and phosphorylated CaMKII.

Figure Caption 11

Withdrawal from chronic opiate-exposure modulates total CaMKII and phosphorylated CaMKIIα within the BLA.

(A) Photograph of protein blots in PN and DWD groups of α-tubulin control, CaMKIIα, and pCaMKIIα. Within the BLA CaMKIIα and pCaMKIIα expression is dramatically decreased between animals in an opiate-naive (PN) and opiate-dependent/withdrawn (DWD) state.

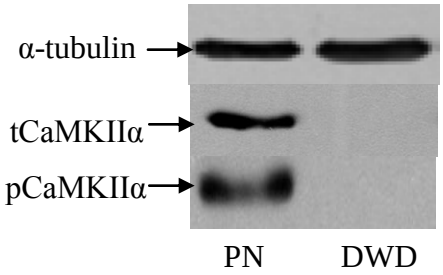


Figure 11

4. Discussion

We previously reported a functional switch within the BLA between DA D1- and D2-like receptor transmission in the formation of opiate-related associative memories as a function of opiate-exposure state (Lintas et al., 2011). However, the underlying intracellular molecular mechanism(s) responsible for this switch was unclear. It was hypothesized that modulation of intracellular MEK phosphorylation, a molecule important in altering plasticity in cellular structure and function, may be partly responsible for the D1-mediated formation of associative memories in previously opiate-naive animals. Further, it was hypothesized that modulation of intracellular CaMKII phosphorylation, also important in controlling neuronal plasticity, might serve as an underlying mechanism for D2R-mediated modulation of opiate-related associative memory formation, in the opiate-dependent/withdrawn state. The present results strongly implicate both MEK and CaMKII as critical molecular substrates sub-serving the functional effects of intra-BLA D1/D2 transmission during the formation of associative opiate reward memory. In addition, the results demonstrate a novel molecular alteration between phosphorylation of MEK and CaMKII levels as a functional consequence of chronic opiate exposure.

4.1 Overview of Behavioral Results

Using an unbiased CPP behavioral paradigm, a novel molecular-memory switch within the BLA was identified. Specifically, the results show that the formation of opiate-related associative memories in the BLA depends upon the phosphorylation of MEK (pMEK) in previously opiate-naive animals but switches to a phosphorylated CaMKII (pCaMKII)-dependent mechanism in opiate-dependent/withdrawn animals. The results demonstrate that in

previously opiate-naive animals, intra-BLA administration of a pharmacological inhibitor of MEK phosphorylation before conditioning impaired the animals' ability to encode a morphine-related conditioned associative memory. However, the same pMEK inhibitor administered to opiate dependent and withdrawn animals had no influence on the encoding of morphine-related associative memory. In opiate dependent and withdrawn animals, intra-BLA administration of a pharmacological inhibitor of CaMKII phosphorylation before conditioning blocked the encoding of a morphine-related conditioned associative memory. However, intra-BLA inhibition of CaMKII signaling was entirely ineffective in modulating opiate reward memory formation in previously opiate-naïve animals.

The VTA is not the only structure within the MCLS that contains μ-opiate receptors. Indeed, opiate-class drugs act on opiate receptor subtypes beyond the μ subtype, that trigger neurotransmission within other neural pathways extrinsic to the MCLS (Ford et al., 2006; Olmstead & Franklin, 1997). To bypass structures other than the VTA, such as the NAc, containing μ-opiate receptors, and focus on the DAergic pathway between the VTA and BLA specifically, morphine was administered directly into the VTA. The results obtained from intra-VTA morphine administration mirrored those found when morphine was administered systemically. Thus, the results demonstrate that the molecular-switch does rely upon the activation of DAergic projections from the VTA specifically. More importantly, these findings implicate the BLA as a critical modulator of opiate-related motivational information arising directly from the VTA.

It is important to identify long lasting changes in neuronal structure and function taking place following chronic drug use. This is especially essential given that there is such a high risk for opiate abuse relapse, even following years of successful abstinence. The molecular switch observed in the current investigation appears to be a long lasting change such that inhibition of pCaMKII, but not pMEK, continued to block the formation of associative memories even when animals were in an opiate-recovered state, following chronic exposure. This evidence suggests a long-lasting molecular change occurring in response to chronic drug exposure. This is a very important observation when considering possible implications for novel treatment models for opiate addiction. A common focus of research in the field of opiate addiction is to identify long lasting neurobiological and/or behavioral changes which occur following chronic drug exposure because they may speak to the long lasting susceptibility for relapse following abstinence in people suffering from drug addictions. While future studies are required to address these issues, our findings identify a novel molecular switch specifically within the BLA, which may ultimately underlie the longer term effects of chronic opiate exposure, dependence, and relapse.

Finally, MEK and CaMKII are ubiquitous signaling molecules, expressed within many neuronal sub-types in multiple neural regions. In addition, both molecules are modulated by many extra- as well as intra-cellular events (Fink & Meyer, 2002; Seger & Krebs, 1995). For this reason, the functional relationship between the activation of D1R and D2R's and the subsequent phosphorylation of MEK and CaMKII, respectively, was examined. Intra-BLA administration of a D1R-agonist prior to morphine conditioning with a sub-threshold conditioning dose of morphine potentiates the reward salience of opiates in previously opiate-naive animals but not opiate-dependent and withdrawn animals (Lintas et al., 2012). However,

administration of a D2R-agonist prior to conditioning potentiates the reward salience of morphine specifically in opiate-dependent and withdrawn animals but not previously opiate-naive animals (Lintas et al., 2012). In the current investigation, by coupling the D1-agonist with the MEK inhibitor in previously opiate-naive animals, we were able to block the D1-agonist mediated potentiation of morphine-conditioned place preference. Similarly, by coupling the D2-agonist with the CaMKII inhibitor in opiate-dependent/withdrawn animals, we were able to block the D2-agonist mediated potentiation of morphine-conditioned place preference. These findings strongly support our suggestion of a functional relationship between the DA D1-like and DA D2-like families of receptors and the MEK and CaMKII intracellular signaling cascades, respectively.

4.2 Overview of Molecular/Protein Expression Experimental Results

A series of Western Blot protein expression analyses were conducted to investigate the changes in protein expression occurring in response to chronic opiate exposure and withdrawal within the BLA. In our original hypothesis, we expected to find equal expression of total protein levels between opiate-naive and opiate-dependent and withdrawn groups and increased levels of pERK1 and 2 in opiate-naive groups. In addition, based on our behavioural observation of increased sensitivity to CaMKII inhibition specifically following chronic opiate exposure, our hypothesis predicted increased levels of pCaMKII in opiate-dependent and withdrawn groups. In agreement with our expectations regarding MEK signaling, we found expression of total ERK1 and 2 to be equal across groups and phosphorylated ERK1 and 2 levels to be higher in the opiate-naive than the opiate-dependent/withdrawn group. However, interestingly, total CaMKII

expression as well as pCaMKII levels were dramatically decreased in the opiate-dependent and withdrawn group compared to the opiate-naive group.

Although unexpected, our findings do follow what similar investigations of CaMKII expression following chronic administration and withdrawal from addictive substances have reported. Specifically, pCaMKII levels increase from baseline during chronic drug exposure and dramatically decrease during abstinence and/or withdrawal from drug exposure (Christian et al., 2012; Greenstein et al., 2007; Lou et al., 1999; Papaleo et al., 2012; Wang & Wang, 2006; Zhong et al., 2006). In the current investigation, animals that received 15 days of heroin injections were in 21 hours of withdrawal when they were sacrificed for protein density analysis. Hence, the dramatic down-regulation of total CaMKII expression uncovered in the analysis coincides with research demonstrating a similar down-regulation of CaMKII in animals following chronic exposure to and withdrawal from addictive substances. However, the current investigation is unique from those previously reported because it examined protein density specifically within the amygdala. Past research concerning CaMKII protein expression and pCaMKII levels following chronic drug exposure and withdrawal examined larger areas such as the PFC or hippocampus and reported a down-regulation in pCaMKII levels but little or no change in total CaMKII levels (Lou et al., 1999; Wang & Wang, 2006). The results reported here show a dramatic decrease in total CaMKII expression within the amygdala of opiate-dependent and withdrawn animals in comparison to opiate-naive animals. The decrease in pCaMKII levels logically follows from the dramatic drop in total CaMKII expression. This evidence is intriguing; it suggests that chronic opiate use and withdrawal does not just affect the phosphorylation state of CaMKII but total protein levels in and of themselves.

4.3 Dopamine D1-like Receptors and pMEK

As mentioned previously, activation of D1 receptors within the MCLS is associated with increased levels of pMEK (Alvarez-Jaimes et al., 2005; Fricks-Gleason & Marshall, 2011; Kelly et al., 2003; T. Li et al., 2010; Pan et al., 2011). As we have previously reported, D1R activation in the BLA is necessary for the formation of associative memories in PN animals (Lintas et al., 2011). Similarly, in the current project intra-BLA pMEK levels were higher in opiate-naive than in opiate dependent and withdrawn animals and MEK phosphorylation was necessary for the formation of opiate-related reward memories in previously opiate-naive but not opiate-dependent and withdrawn animals. The fact that pMEK levels were significantly lower in opiate-dependent and withdrawn than in opiate-naive animals suggests that D1R activity decreased and MEK phosphorylation was no longer necessary for the formation of associative memories in opiate-dependent and withdrawn animals. Furthermore, the fact that we were unable to disrupt the formation of associative memories in opiate-dependent and withdrawn animals via pharmacological blockade of pMEK also supports the notion that MEK phosphorylation is no longer necessary for the formation of associative memories in an opiate-dependent and withdrawn state.

4.4 Dopamine D2-like Receptors and pCaMKII

As discussed previously, evidence suggests that the expression of D2 receptors is decreased (Collins et al., 2011; Volkow et al., 1993) and their binding affinity or activity state increased (Novak & Seeman, 2010) following chronic drug use and withdrawal. Similarly, withdrawal from chronic drug administration is also associated with a decrease in pCaMKII levels (Christian et al., 2012; Frankland et al., 2001; Lisman et al., 2002; Lou et al., 1999; Lu et

al., 2000; Wanjerkhede & Bapi, 2008). Interestingly, research shows that the expression of CaMKII within the cell is modulated differentially by D2R's depending on the activity state D2R's are in at the time. When in a low activity state, D2R activation is associated with an increase in pCaMKII levels through mobilization of intracellular calcium stores but when in a high activity state, D2R activation is associated with a decrease in pCaMKII levels (Gu & Yan, 2004). Thus, there are two potential mechanisms implied by the behavioural and protein density analyses reported here. First, the overall decrease in total CaMKII within the BLA in opiate-dependent and withdrawn animals suggests that the total number of D2R's in the BLA decreased. Second, the decrease in pCaMKII observed in opiate-dependent and withdrawn animals suggests that those remaining D2R's were in a high affinity, high activity state. The fact that intra-BLA pharmacological blockade of D2R's (Lintas et al., 2011) and pCaMKII inhibition impair the formation of associative memories in opiate-dependent and withdrawn but not previously opiate-naive animals, suggests that when in an opiate-dependent/withdrawn state, DA transmission from the VTA preferentially activates D2R's in their high affinity state and facilitates the formation of associative memories through a CaMKII-dependent molecular substrate. In addition, although total intra-BLA CaMKII levels were dramatically reduced in the opiate-dependent withdrawn state, CaMKII signaling is required for the formation of opiate-related associative memory, as blockade of CaMKII signaling specifically in the opiate-dependent and withdrawn state completely prevents the formation of opiate-related associative memories. In direct contrast, inhibition of CaMKII signaling in the previously opiate-naïve state has no influence on opiate-related associative memory acquisition.

4.5 Proposed Model of Opiate Exposure State-Dependent Associative Learning

The MCLS is a highly malleable circuit, constantly modifying itself in order to maintain a level of balance and homeostasis between its component parts. The MCLS is able to adapt to changes on an ongoing basis through the plasticity of individual neurons and through the plasticity of synaptic connections between groups of neurons. In addition to previous research, the results presented in the current investigation demonstrate clear and concise evidence of the ability of the MCLS to adapt to drug-induced changes. For example, in response to chronic opiate use, an up-regulation in DA transmission followed by a decrease in DA concentration during withdrawal occurs. The number of DA receptors available on neurons decreases and their binding affinity modulated such that D2R binding affinity increases. The chronic drug use-related decrease in D1R expression results in decreased pMEK levels as well as decreased D2R expression coupled with increased D2R binding affinity that leads to decreased total and pCaMKII levels. These drug-related modifications translate into an observable switch in the ability of a D1-antagonist or pMEK-inhibitor and D2-antagonist or pCaMKII-inhibitor to effect the acquisition of opiate-related reward memories based on opiate-exposure state at the time of encoding. Put another way, when animals are in a previously opiate-naive state the MCLS is functioning naturally, an increase in DA transmission due to the introduction of morphine into the system preferentially activates D1R's triggering phosphorylation of MEK in the BLA, facilitating the formation of associative memories. Following chronic exposure to opiates and chronic increases in DA concentration within the MCLS, the number of DA receptors decreases. Once opiate concentrations decrease within the MCLS, the concentration of extracellular DA drops exponentially, the binding affinity of D2R's increases, and the expression of phosphorylated CaMKII decreases. Presumably then, once opiates are reintroduced into the system, DA transmission increases once again and preferentially targets D2R's in their high

affinity state possibly increasing Ca^{2+} mobilization and phosphorylation of CaMKII and facilitating associative memory formation (see Figure 12 for depiction of proposed model).

Figure Caption 12

Proposed model of the formation of associative memories in previously opiate-naïve versus opiate-dependent/withdrawn animals.

Scenario A) *Blue DA projection*. Opiates bind to μ-opiate receptors on GABAergic interneurons in the VTA releasing GABAergic inhibition over local DA neurons and facilitating DAergic release to the BLA and NAc. In the opiate-naive state the DAergic projection from the VTA to the BLA (blue DA projection) preferentially activates D1R's on GABAergic neurons and promotes MEK(ERK) phosphorylation. This causes GABAergic inhibition over local pyramidal neurons to be released. Simultaneously, sensory cortex input to the BLA is facilitated. This series of events facilitates GLUergic BLA output towards the mPFC and NAc, and subsequently NAc GLUergic projections to motor cortex.

Scenario B) *Red DA projection*. Through repeated activation of this system via chronic opiate exposure GLUergic projections from the mPFC to the BLA are modulated and the number of D2R's is increased and the expression of pCaMKII is increased. During opiate withdrawal there is a decrease in DAergic transmission coupled by an increase in D2R sensitivity and a decrease in the expression of pCaMKII as well as tCaMKII. Presumably then, the system is primed for direct inhibition of GLUergic projections from the mPFC towards GABAergic neurons in the BLA (red DA projection) and facilitating BLA Gluergic projections towards the NAc upon subsequent administration of opiates.

Note: VTA: ventral tegmental area; BLA: basolateral nucleus of the amygdala; NAc: nucleus accumbens; mPFC: medial pre-frontal cortex; DA: dopaminergic neuron; GABA: GABAergic neuron; pMEK: phosphorylated MEK; pCaMKII: phosphorylated CaMKII.

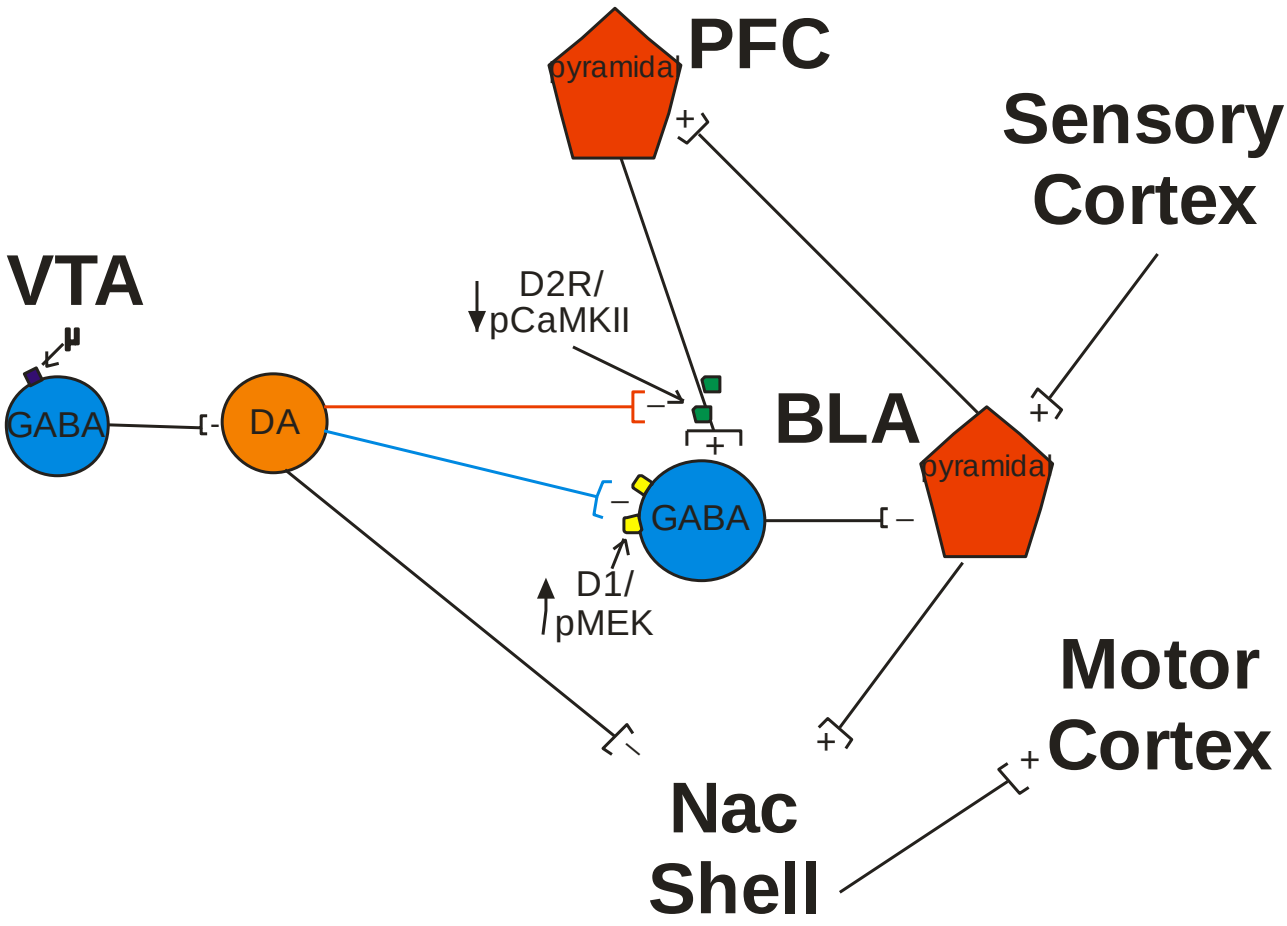

PFC
pyramidal
Sensory
Cortex
VTA
D2R/
pCaMKII
GABA
DA
BLA
pyramidal
GABA
D1/
pMEK
Motor
Cortex
Nac
Shell

Figure 12

5. Future Directions

The protein density analyses presented in the investigation presented here revealed decreased levels of pMEK and decreased total and phosphorylated CaMKII levels suggesting that DA D1R expression had decreased, D2R expression had decreased, and D2R binding affinity had increased in opiate-dependent/withdrawn animals. Further studies are required to confirm the down regulation of DA D1R and D2R expression in the amygdala following chronic opiate administration and withdrawal.

The investigation of intra-BLA MEK and CaMKII phosphorylation inhibition revealed an opiate-exposure state-dependent molecular switch in the acquisition of opiate-related associative memories. However, intra-BLA activation of MEK and CaMKII phosphorylation was not investigated. It is possible to potentiate the formation of an associative opiate-related memory to a sub-threshold conditioning dose of morphine by pharmacologically activating D1R and D2R's in previously opiate-naive and opiate-dependent/withdrawn animals, respectively (Lintas et al., 2012). It follows logically that intra-BLA activation of pMEK in previously opiate-naive and pCaMKII in opiate-dependent/withdrawn animals should potentiate the formation of an opiate-related associative memory to a sub-threshold dose of morphine. If intra-BLA pMEK and pCaMKII activation revealed a similar opiate-exposure state-dependent molecular-switch in potentiation of opiate-related associative memories it would add to evidence suggesting a functional link between D1R's and pMEK, and D2R's and CaMKII. Confirmation of a functional link between the two families of DA receptors and the two intracellular signaling cascades has important implications for the development of new treatments for opiate addiction. For example, pharmacological interventions designed to modulate DA levels to correct neuropsychological

disorders, are associated with a number of adverse long term side effects. A treatment designed to target the intracellular protein kinases associated with DA receptor activation rather than DA levels directly, may be able to bypass the adverse side effects associated with DA agonists or antagonists.

The molecular switch to a pCaMKII-dependent mechanism for the formation of opiate-related associative memories observed in the current investigation remained following recovery from withdrawal. This evidence suggests that opiate exposure-induced changes in CaMKII expression and CaMKII phosphorylation may represent a stable molecular switch taking place within the BLA. Further investigation to test the longevity of the molecular-switch to a pCaMKII-dependent mechanism for reward related memory formation may have important therapeutic implications. In fact, the goal of most addiction-related research is to elucidate any permanent neurobiological changes taking place following chronic drug use because such changes, if reversed, may assist with the treatment of opiate-addiction related craving and relapse phenomena. Thus, further testing to explore the longevity of the molecular switch to a pCaMKII-dependent pathway in opiate-related memory formation is necessary. If the evidence suggested the switch to a pCaMKII-dependent pathway in the formation of opiate-related memories was permanent, an important next step would be to investigate the effect of a treatment designed to restore CaMKII levels to normal levels in preventing cue-induced relapse.

6. Conclusions

Taken together the results strongly indicate a novel molecular-memory switch taking place within the BLA due to chronic opiate exposure and withdrawal. This evidence is an

important step forward in understanding the long-lasting neuromolecular changes caused by chronic opiate exposure. In addition, understanding the underlying molecular substrates responsible for the formation and persistence of opiate-related associative memories will provide a clearer understanding of opiate-related relapse and craving in human addicts. Further research is required to elucidate the long-term behavioral significance of the findings. However, the results have revealed some important molecular targets for future research which may hold significance in the development of pharmaco-therapeutic treatments for people suffering from opiate addiction.

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Appendix

Figure 4

A

Group	saline	0.1µg UO-126	1.0µg UO-126
Size	7	8	8
Mean Saline Side	83.4	92.3	203
SD Saline Side	25.9	42.2	64.2
SEM Saline Side	9.8	14.9	22.7
Mean Morphine Side	461.7	413.9	257.3
SD Morphine Side	25.8	57.5	64.1
SEM Morphine Side	9.7	20.3	22.7
t-Value	-11.4	-10.4	-1.8
p	<.01	<.01	>.05

B

Group	.05µg KN-62	0.5µg KN-62	1.0µg KN-62
Size	7	7	8
Mean Saline Side	73.3	101	153.8
SD Saline Side	40.3	53.4	42.7
SEM Saline Side	15.2	20.2	11.8
Mean Morphine Side	450.7	398.9	361
SD Morphine Side	68.9	55	32.3
SEM Morphine Side	26	20.8	11.4
t-Value	-10.7	-8.4	-6.3
p	<.01	<.01	<.01

Figure 5

A

Group	saline	0.5µg KN-62	5.0µg KN-62
Size	6	7	7
Mean Saline Side	86	167.7	250.1
SD Saline Side	41.7	41	12.5
SEM Saline Side	15	15.3	4.7
Mean Morphine Side	447.5	340.9	240.7
SD Morphine Side	63.5	52.6	29.2
SEM Morphine Side	29.5	19.9	11
t-Value	-11.8	-6.1	0.33
p	<.01	<.01	>.05

B

Group	saline	1.0µg UO-126
Size	6	6
Mean Saline Side	86	126.7
SD Saline Side	41.7	18.8
SEM Saline Side	17	7.7
Mean Morphine Side	447.5	375
SD Morphine Side	63.5	10.8
SEM Morphine Side	26	4.4
t-Value	-11.9	-8
p	<.01	<.01

Figure 7

A

Group	PN	DWD
Size	7	7
Mean Saline Side	246.7	165.1
SD Saline Side	37.1	20.2
SEM Saline Side	13.7	7.6
Mean Morphine Side	229.4	295.7
SD Morphine Side	29	57.7
SEM Morphine Side	10.9	25.3
t-Value	0.76	-5.8
p	>.05	<.01

B

Group	PN	DWD
Size		
Mean Saline Side	183.6	225.9
SD Saline Side	33.6	26.4
SEM Saline Side	12.7	10
Mean Morphine Side	315.4	221.4
SD Morphine Side	21.5	29
SEM Morphine Side	8.1	11
t-Value	-7.6	-0.5
p	<.01	>.05

Figure 8

A

Group	PN	DWD	RCV
Size	8	6	8
Mean Saline Side	203	126.7	150.3
SD Saline Side	64.2	18.8	40.1
SEM Saline Side	22.7	7.7	14.9
Mean Morphine Side	257.3	375	361.4
SD Morphine Side	64.1	10.8	25.8
SEM Morphine Side	22.7	4.4	9.1
t-Value	-2.1	-8.2	-8
p	>.05	<.01	<.01

B

Group	PN	DWD	RCV
Size	8	7	6
Mean Saline Side	153.8	250.1	235.8
SD Saline Side	42.7	12.5	26.2
SEM Saline Side	11.8	4.7	10.8
Mean Morphine Side	361	2400.7	244.3
SD Morphine Side	32.3	29.2	10.3
SEM Morphine Side	11.4	11	4.2
t-Value	-12.9	0.55	-0.46
p	<.01	>.05	>.05

Figure 9

A

Group	Saline	SKF	SKF+UO-126
Size	7	8	8
Mean Saline Side	262.7	144.4	242.9
SD Saline Side	65.8	27.4	45.8
SEM Saline Side	24.9	9.7	16.2
Mean Morphine Side	247.8	366.8	228.4
SD Morphine Side	59.2	24.4	11.3
SEM Morphine Side	20.9	8.6	4
t-Value	0.5	-7.9	-1
p	>.05	<.01	>.05

B

Group	Saline	Quinpirole	Quinpirole +KN-62
Size	8	8	8
Mean Saline Side	257.9	187.4	241.3
SD Saline Side	63.7	31.9	20.6
SEM Saline Side	22.5	11.3	7.8
Mean Morphine Side	269.9	313.9	237.9
SD Morphine Side	61.3	21.3	16.2
SEM Morphine Side	21.7	7.5	6.1
t-Value	-0.42	-4.4	0.11
p	>.05	<.01	>.05

Figure 10

A

Group	tERK1	tERK2
Size	7	7
Mean Saline Side	1	1
SD Saline Side	0.01	0.02
SEM Saline Side	0.005	0.007
Mean Morphine Side	0.89	0.9
SD Morphine Side	0.01	0.17
SEM Morphine Side	0.005	0.063
t-Value	1.73	0.66
p	0.11	0.52

B

Group	*pERK1*	*pERK2*
Size	7	7
Mean Saline Side	0.996	1.01
SD Saline Side	0.14	0.009
SEM Saline Side	0.141762	0.036824
Mean Morphine Side	0.358	0.591
SD Morphine Side	0.062	0.114
SEM Morphine Side	0.081722	0.127694
t-Value	3.75	3.11
p	0.003	0.008

C

Group	pERK1:tERK1	pERK2:tERK2
Size	7	7
Mean Saline Side	1	1.013
SD Saline Side	0.149	0.024
SEM Saline Side	0.056	0.009
Mean Morphine Side	0.408	0.611
SD Morphine Side	0.089	0.176
SEM Morphine Side	0.033	0.066
t-Value	3.238	2.377
p	0.007	0.035

Curriculum Vitae

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Education

- Master of Sciences – Neuroscience 2010-2012

  University of Western Ontario, London, ON

  - Completed MSc Thesis 2012

- Honours Bachelor of Arts – Psychology 2006-2009

  University of Waterloo, Waterloo, ON

  - Completed an honours thesis 2008-09
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Awards and Scholarships

- Ontario Graduate Scholarship 2011-2012
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- Psychology Memorial Award 2008-2009
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Teaching Experience

- Teaching Assistant, Applications of Psychology – PSYCH 2990 (Sept 2010 – Dec 2010)
- Teaching Assistant, Introduction to Psychology – PSYCH 1000 (Jan 2011 – April 2011)

Research Talk and Poster Presentations

- Lyons, D., Laviolette, S.R. (May 2011). *Exploring the molecular signaling involved in the acquisition of opiate-related reward learning and memory*. Talk presented in the Neuroscience Student Seminars, London, ON.

- Lyons. D., Laviolette, S.R. (November 2011). *Exploring the role of signaling kinases, MEK and CaMKII, in the acquisition of opiate-related reward learning and memory in the basolateral amygdala*. Poster presented at the 2010/2011 Society for Neuroscience Conference, Washington, D.C.
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- Lyons, D., Laviolette, S.R. (March 2012). *Opiate exposure state dependent functional switch in the acquisition of opiate-related reward learning and memory*. Poster presented at the 2011/2012 London Health Research Day, London ON