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Prefrontal Cortex Dopamine Transmission Regulates Emotional Memory Processing and Morphine Reward Salience: Implications for Post-Traumatic Stress Disorder and Addiction Comorbidity

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Abstract

Post-Traumatic Stress Disorder (PTSD) and addiction are strongly comorbid. However, the underlying neural mechanisms by which traumatic memory recall may increase addiction liability are poorly understood. The inability to suppress memory recall related to either stressful or rewarding, drug-related experiences may be an underlying neuropsychological feature capable of triggering both PTSD or addiction-related behaviours. Our previous research has shown that transmission through dopamine (DA) D_4 and D_1 receptor subtypes (D_4R, D_1R) within the prefrontal cortex (PFC) strongly modulates emotional memory acquisition and recall (Lauzon et al., 2009). Using olfactory fear conditioning and morphine conditioned place preference (CPP) procedures in rats, combined with molecular protein expression analyses, we examined if ***1)*** associative fear memory recall would increase subjects' sensitivity and vulnerability to morphine reward salience; ***2)*** if blocking fear memory recall with intra-PFC D_1R stimulation may block the potentiation of morphine reward salience; ***3)*** if PFC D_4R stimulation would potentiate morphine reward salience by modulating the emotional salience of fear memories during memory acquisition. Furthermore, we concomitantly examined the underlying PFC molecular signaling pathways associated with these behavioural effects. We report that rats receiving supra-threshold (0.8 mA) fear conditioning showed strong associative fear memories and heightened morphine reward sensitivity (with either systemic or intra-ventral tegmental area [VTA] administered morphine). Inhibition of fear memory recall with intra-PFC D_1R activation reduced the potentiated morphine CPP through cyclic adenosine monophosphate (cAMP) and extracellular-signal-related kinases 1 and 2 (ERK1/2) dependent molecular pathways. In addition, PFC D_1R stimulation selectively increased phosphorylation levels of ERK 1/2. In contrast, PFC D_4R activation bi-directionally controlled fear memory acquisition and morphine CPP behaviours through a calcium/calmodulin dependent kinase II (CaMKII)-dependent mechanism wherein D_4R activation selectively stimulated phosphorylation of PFC CaMKII. Our findings reveal for the first time a novel DA-receptor dependent mechanism in the mammalian PFC capable of controlling both fear-related associative memory formation and the salience of morphine-related reward memories.

Key words: post-traumatic stress disorder, opioid addiction, medial prefrontal cortex, ventral tegmental area, dopamine, cAMP, ERK, CaMKII, morphine conditioned place preference, olfactory fear conditioning, western blot

Co-Authorship Statement

The thesis was written by Jing Jing Li with inputs from Dr. Steven R. Laviolette, experimental procedures and data analysis were performed by Jing Jing Li, Dr. Hanna Szkudlarek, Dr. Justine Renard. Dr. Steven R. Laviolette provided intellectual input and experimental protocol design.

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ADHD	Attention-deficit hyperactivity	GABA	gamma-Aminobutyric acid
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid	i.p	intraperitoneally
ATP	Adenosine triphosphate	NMDA	N-methyl-D-aspartate
ANOVA	Analysis of variance	NAc	nucleus accumbens
BLA	basolateral nucleus of the amygdala	mPFC	medial prefrontal cortex
BOLD	blood oxygen level-dependent	MOP	Mu opioid receptor
Ca^{2+}	calcium ion	pCaMKII	phosphorylated CaMKII
CaMKII	calcium/calmodulin dependent kinase II	pERK1	phosphorylated ERK1
CNS	central nerve system	pERK2	phosphorylated ERK2
CPP	conditioned place preference	PFC	prefrontal cortex
cAMP	cyclic adenosine monophosphate	PKA	protein kinase A
DA	dopamine	PP1	protein phosphatase 1
D_1R	dopamine D_1-like receptor	SN	substantia nigra
D_4R	dopamine D_4-like receptor	TBI	traumatic brain injury
ERK1	extracellular-signal-related kinases 1	VNTR	variable number tandem repeat
ERK2	extracellular-signal-related kinases 2	VTA	ventral tegmental area

1. Introduction

1. 1 Post-traumatic Stress Disorder (PTSD)

PTSD is classified as an axis I anxiety disorder that can develop after individuals experience repeated or singular traumatic events, typically involving the threat of serious injury, physical harm or death (*Diagnostic and Statistical Manual of Mental Disorders*. 5th ed., 2013). PTSD remains one of the most frequent neuropsychiatric disorders, with a lifelong prevalence in the US estimated at 8.3%. Approximately 1 in 12 adults will experience PTSD-related symptoms during their lives (Breslau et al., 1998; Schöner, Heinz, Endres, Gertz & Kronenberg, 2017). Some professions, such as soldiers, first responders (police, firefighters, etc), are at higher risk of trauma exposure and subsequently developing PTSD. The symptoms of this disorder are a complex constellation of symptoms including hyperarousal (sleep disturbances, difficulty concentrating), behavioural avoidance (diminished participation in activities to avoid people and/or places that are associated with the traumatic experience), emotional numbing, and re-experiencing phenomena. The hallmark of PTSD symptoms is characterized by intrusive memory recall of the traumatic event, triggered by exposure to the related cues, typically associated with intense and prolonged anxiety (*DSM* 5th Ed, 2013). For example, a veteran could link the sound of a lawn mower to the sound of a helicopter in the battle field, which in turn triggers the stressful memory recall and feelings of anxiety. This extreme and sustained maladaptive response to the stressful events may persist for years, and is often associated with significant disability and distress (Schöner et al., 2017). The types of stressors can also influence the likelihood of developing PTSD, as well as the severity of the symptoms. Depending on the nature of the traumatic event, longer exposure and greater intensity of the experience can increase the risk for the subsequent development of PTSD. Stressor severity was also the strongest predictor of symptoms at both 3 and 12 months after trauma exposure (Wolfe, Erickson, Sharkansky, King & King, 2000). Experiences related to interpersonal assaults, such as rape or violent combat, have a greater likelihood of causing PTSD compared to events like natural disasters and/or traffic accidents (Breslau et al., 1998). However, not all individuals that experience traumatic events will go on to develop PTSD. Brewin, Andrews and Valentine (2000) summarized the risk factors associated with the later development of PTSD: trauma severity,

previous trauma history (such as childhood abuse), comorbid mental disorders, gender, lower education, introverted personalities, the availability of post-traumatic support services, are all contributing factors to the development and prognosis of the disorder.

The etiology of PTSD is complex and multifactorial; and the symptoms of PTSD have been linked to a variety of underlying neuropathological mechanisms (Brewin et al., 2000; Pizzimenti & Lattal, 2015). Over the past several decades, research has largely shifted attention from the potential role of the endocrine system, to specific neural circuits which are involved in fear memory regulation. Currently, PTSD is generally conceptualized as a neural disorder related to abnormal memory extinction. For example, a significant body of neuroimaging studies have revealed that PTSD patients show hyperactivity in the amygdala, hypoactivity in the medial prefrontal cortex (mPFC), and abnormal hippocampal function, when processing stimuli with negative emotional valence. Due to the inhibitory nature of the PFC projection to the amygdala, it has been proposed that the re-experiencing of traumatic memories could result from a lack of inhibition from the PFC to the amygdala (Shvil, Rusch, Sullivan & Neria, 2013; Tipps, Raybuck & Lattal, 2013). In the review of Shvil et al. (2013), the authors summarized several pieces of evidence regarding the etiology of PTSD. The authors pointed out that PTSD patients show deficiencies in processing danger-safety related contextual information compared to trauma exposed non-PTSD individuals. With the persistent re-experiencing of traumatic memories, PTSD individuals not only reconsolidate the stressful events, but also maintain the physiological hyperarousal state which leads to the dysfunction of other physiological systems. Indeed, Shvil et al. (2013) also pointed out that PTSD patients have distinct abnormal psychophysiological features, such as heightened resting heart rate, persistent elevated heart rate during exposure to traumatic stimuli, which is an indication of weakened regulation of psychophysiological responses. Due to these constant states of anxiety (both psychological and physiological), it is perhaps not surprising that PSTD patients will often self-medicate with drugs of abuse in an attempt to relieve their symptoms (Bradizza, Stasiewicz & Paas, 2006).

1. 2 Opiate Addiction

Across the wide range of drugs of abuse, opiate-class drugs represent the most addictive by producing potent rewarding effects in the early stages of exposure, and the formation of potent associative memories linked to the drug-taking experience (Hyman, Malenka & Nestler,

2006). While the initial/early drug exposure phase is generally controlled and voluntary; compulsive and uncontrolled drug taking is maintained due to the euphoric effects of drugs combined with a need for withdrawal symptoms alleviation (Everitt et al., 2008). This stage is concomitant with neurobiological changes in various brain regions, including the prefrontal cortical areas and subcortical areas, such as the mesolimbic DA system, which in turn can modify drug-seeking behaviours (Everitt & Robbins, 2005). After repeated drug exposure, full-blown addiction and dependence develops, along with a wide variety of associated neurobiological and behavioural changes (Everitt et al., 2008). At this stage, drugs exert controlling power over behaviours, characterized by compulsive drug taking and/or obsessive craving for the drug of abuse (Hyman et al., 2006). Repeated drug use also results in molecular and pharmacological neuroadaptions involving tolerance to the drugs rewarding effects, leading to greater and greater consumption levels in order to achieve the desired hedonic state of reward (Nestler & Aghajanian, 1997).

Current theories point out the importance of learning and memory dysregulation during the addiction process, involving maladaptive forms of Pavlovian and instrumental learning (Hyman et al., 2006). As mentioned previously, drugs of abuse hijack the neural mechanisms involved in emotional memory formation, such as the mPFC, which controls executive functions and emotional memory processing (Hyman et al., 2006). Importantly, addicted individuals usually associate drugs and their euphoric effects with specific, associative environmental cues. Re-encountering these cues can then elicit drug-related memories, which in turn can trigger compulsive drug seeking behaviours and relapse.

Opioids are currently the most effective analgesics for clinical pain management. Nevertheless, there is currently a national and international opioid addiction epidemic due to the wide spread availability of both prescription and illicit forms of opiate-class compounds and a concomitant dramatic rise in opioid-related overdose deaths (Popova, Patra, Mohapatra, Fischer & Rehm, 2009). According to the International Narcotic Control Board (2006), Canada ranked first (in per capita use) for consumption of hydromorphone, second in morphine and oxycodone use and third in hydrocodone use. In 2003, there were an estimated 321,000-914,000 abusers of non-medical prescription opioids. This number has increased 24% from 2002 to 2005 (Popova et al., 2009). Based on the OPICA study conducted in five Canadian cities (Vancouver, Toronto,

Montreal, Edmonton and Quebec City, Fischer et al., 2005), 48.9% of addicts are non-medical prescription opioid users, 27.8% are using illicit heroin, and 23.2% combine opioid drugs with other classes of drugs. However, recreational opioid abuse is most likely underestimated due to the clandestine nature in which these drugs are consumed and reported, both in Canada and globally (Fischer et al., 2005; Popova et al., 2009).

Opioid drugs produce potent euphoric effects and have exceptionally high abuse liability. Long term exposure results in both psychological and physical dependence. Withdrawal leads to emotional and motivational symptoms, such as anhedonia, dysphoria and craving. Unlike other classes of drugs, such as psychostimulants, opioid withdrawal also results in a series of physical symptoms: hypertension, abdominal pain, flu-like symptoms, tremor and possibly death (Hyman et al., 2006).

A tremendous body of research has identified many of the neuronal mechanisms involved in opioid addiction. Endogenous and exogenous opioid molecules bind the mu opioid receptor subtype (MOP), which are widely spread in the brain and highly concentrated in the ventral tegmental area (VTA, Fields & Margolis, 2005), which is the origin of mesocorticolimbic dopamine (DA) pathway (Missale, Nash, Robinson, Jaber & Caron,1998). The MOP is inhibitory in nature and usually located on the terminals of GABA interneurons in the VTA; once activated by opioids, the VTA MOR can inhibits GABA inhibitory effects on DA neurons in the VTA (Fields & Margolis, 2005). In other words, MOP activation disinhibits the inhibitory effects of GABA neurons on VTA DA neurons, thereby indirectly increasing activity of the mesocorticolimbic DA pathway. Since increased VTA DA activity is associated with the processing of reward-related motivational information, one potential mechanism underlying the rewarding and addictive properties of opioids is by increasing DA transmission to other limbic areas during drug use (Everitt et al., 2008; Fields & Margolis, 2005; Hyman et al., 2006). Substantial research has demonstrated the critical role of the VTA in opioid addiction. For example, Olmstead and Franklin (1997) have reported that intra-VTA morphine administration elicited drug seeking behaviours, but this effect was not observed following morphine infusions into other brain regions, such as the amygdala and hippocampus. Blocking the MOP in the VTA has been shown to impair drug seeking behaviours and mice with MOP gene knock-out in the VTA show impaired heroin conditioned place preference (CPP, Zhang et al., 2009).

Although the VTA is believed to mediate the initial rewarding effects of opioids, it is not responsible for the perception of craving during the withdrawal period. Rather, the increased DA activity in the PFC has been proposed to encode drug relate associative memory and other emotional salient information (Lauzon, Bishop & Laviolette, 2009; Laviolette, Lipski & Grace, 2005; Sun et al., 2011)

1.3 PTSD is highly comorbid with addiction

The high rate of comorbidity between PTSD and addiction was first reported in the early 80s with the study of veterans. Kulka et al. (1988) and Keane and Kaloupek (1997) reported that in Vietnam veteran PTSD patients, 64-84% of them met life time criteria for alcohol abuse and 40%-44% were comorbid with other drugs of abuse. In the following decades, similar results were also detected in civilian populations. Jacobsen et al. (2001) reviewed and summarized recent epidemiological studies in the general PTSD population, in which the life time prevalence of addiction at 22%-43%, was far higher than the prevalence of substance abuse disorder alone (8.1%-24.7%). Vice versa, whereas the prevalence of PTSD in the general population is 8.3%, this reaches 36%-50% when patients are sampled from substance abuse treatment centers. Addiction comorbid with PTSD predicted faster relapse rates and worse treatment outcomes of substance abuse, with the severity of re-experiencing symptoms as a significant predictor of relapse (Bradizza et al., 2006). Pre-clinical animal research has found similar effects and shown that various type of stressors, such as food restriction, social stress or foot shock, can increase the consumption of heroin, morphine and alcohol tested in self-administration and CPP paradigms (Edwards et al., 2013; Ribeiro Do Couto et al., 2006; Shalev, 2012).

Thus, both clinical and pre-clinical evidence demonstrates that subjects experiencing PTSD-related symptoms are at higher risk of developing a substance use disorder and more likely to relapse to drug abuse. Drug abusing individuals have a reported elevated risk to develop PTSD when encountering traumatic experiences as compared to non-addicted individuals (Bradizza et al., 2006; Brady et al., 2009; Jacobsen et al 2001; Pizzimenti & Lattal, 2015; Steward, Pihl, Conrod & Dongier, 1998). Stewart et al. (1998) and Brady et al. (2009) summarized the possible reasons for the high comorbidity between PTSD and addiction. First, it is possible that the influence of drugs increases the probability of experiencing trauma (such as, higher traffic accident rates during alcohol intoxication). Second, chronic drug abuse or

withdrawal may lead to elevated anxiety levels, which predisposes individuals for the development of PTSD when exposed to traumatic events. Third, PTSD patients are more likely to abuse drugs in order to relieve the symptoms, such as avoiding the vivid reoccurrences of the traumatic experiences. For example, CNS depressants such as heroin or alcohol acutely relieve the anxiety arousal symptoms. Fourth, drug abuse may exacerbate and prolong the PTSD symptoms by interfering with neuronal circuits regulating associative emotional memory processing. Fifth, some drug withdrawal symptoms may overlap with the PTSD symptoms; for example, withdrawal from alcohol is accompanied with increased anxiety, sleep disturbance and irritability, all of which resemble certain PTSD symptoms. In this case, patients may misinterpret the withdrawal symptoms as a sign of anxiety, thus further increasing the arousal level and attempts to self-mediate with drugs of abuse. Lastly, stress and anxiety may modulate the rewarding salience of drugs, thereby influencing relapse to drug taking in drug-dependent individuals (Liu & Weiss 2002).

Behaviourally, both disorders are characterized by the inability to suppress and extinguish persistent associative memories linked to either catastrophic events in the case of PTSD or drug-related rewarding experiences in addiction. In either case, the spontaneous memory recall related to these experiences maybe an underlying neuropsychological feature triggering anxiety in the case of PTSD, or relapse/drug seeking in addiction (Lauzon et al., 2013). In addition, both traumatic and addiction-related associative memories are extremely resistant to extinction. The recall of the memory is usually associated with specific environmental triggers, suggesting that abnormal encoding and recall of emotional memory is a key component underlying the neurobiological etiology of both PTSD and addiction (Pizzimenti & Lattal, 2015).

As noted above, the high comorbidity and symptom overlap between addictive behaviours and PTSD would suggest a common neurobiological mechanism responsible for the aberrant memory processing in PTSD and addiction. Indeed, as will be discussed next, research targeting emotional associative memory neural circuits has yielded a wide body of compelling evidence, identifying the mammalian PFC as a critical neural region underlying the pathology of both PTSD and addiction (Pizzimenti & Lattal, 2015, Sun et al., 2011, Lauzon et al., 2013).

1.3a Role of the prefrontal cortex (PFC) in emotional memory regulation

Converging clinical and pre-clinical evidence has identified the PFC as a brain region that plays a key role in the acquisition, encoding, recall and extinction of aversive and rewarding emotionally salient associative learning and memory (Laviolette et al., 2005; Lauzon et al., 2009; Morgan, Romanski & LeDoux, 1993; Shin & Liberzon 2010; Sun et al., 2011). For example, stimulation of the DA receptors directly in the mPFC during fear-related memory encoding and recall stages impaired rats' ability to acquire and express associative fear memories (Lauzon et al., 2009). *In vivo* electrophysiological evidence has also indicated that at the single neuron level, neurons in the mPFC processed and encoded associative learning of olfactory cues and aversive stimuli. Laviolette et al. (2005) reported that mPFC neurons show associative responding to fear-related cues, both in terms of increased associative firing and bursting rates following fear-related cue presentations. Earlier studies using mPFC lesions and more recent electrophysiological studies have revealed the vital importance of this area in fear extinction. For example, PFC lesioned rats showed impaired extinction of conditioned fear (Morgan et al., 1993); Milan and Quirk (2002) reported increased PFC neuronal activity during extinction learning to auditory cues previously paired with foot shocks. Inhibition of the PFC attenuated the recall of conditioned fear and subjects with lesion in the same brain area took significantly longer to extinguish fear memories and recovered faster when the cue was paired with the aversive stimuli again (Quirk, Likhtik, Pelletier, & Paré, 2003; Quirk, Russo, Barron & Lebron, 2000). Similar results have been found in human subjects. For example, one fMRI study found activation of ventromedial PFC during the extinction phase of fear conditioning, with this regional activation positively correlated with memory extinction magnitude (Milad et al., 2007).

In the context of addiction, the mPFC is crucially involved in processing drug related rewarding memory and activation of neuronal circuits in this area elicits drug craving and drug seeking in humans and other animals (Daglish et al., 2001; Sun et al., 2011, Gholizadeh et al. 2013; Rosen et al., 2016). *In vivo* electrophysiological evidence from Sun et al. (2011) reported significant increase of associative mPFC neuronal firing rates during the memory acquisition phase of morphine CPP. Luo et al. (2004) found that heroin priming injection induced blood oxygen level-dependent (BOLD) signal changes primarily in the PFC region in rats. Similar evidence has been reported from human research showing that the presence of drug related cues elicited subjective craving in abstinent individuals, with a concomitant strong increase in PFC BOLD signal activity (Daglish et al., 2001; Langleben et al., 2008; Yang et al., 2009). Goldstein

and Volkow (2002) summarized that, in human studies, most results showed the activation of the PFC at the drug intoxication stage, associated with the subjective perception of intoxication. These authors also reported increased levels of brain activation, such as glucose metabolism, in the PFC during drug abstinence, and the changes significantly correlated with exposure to drug cues. Therefore, proper recall and extinction of rewarding and aversive memories requires optimal PFC function and malfunction of the PFC could impair the extinction of drug-related memories, resulting in persistent and inappropriate memory recall.

The experiencing of trauma does not fully explain the occurrence of PTSD, in fact, the exposure and vivid re-experiencing of the catastrophic events may trigger a cascade of neurobiological sequelae that ultimately lead to the development of PTSD (Segman et al., 2002). Increasing evidence from animal and human research has shown that stress exposure leads to malfunction of the PFC with disrupted neuronal functions, accompanied by morphological abnormalities of the PFC in PTSD patients (Knox, Perrine, George, Galloway & Liberzon, 2010; Richert, Carrion, Karchemskiy & Reiss, 2006; Wang et al., 2016). Rauch et al., (2003) reported patients with phobia have increased cortical thickness compared to healthy controls. Furthermore, Richert et al. (2006) revealed significantly increased gray matter volume in middle and ventral PFC, but decreased volume in dorsal PFC, which was correlated with increased functional impairment in PTSD patients. Wang et al. (2016) reported that PTSD patients have hypoactivity in the dorsal mPFC; but hyperactivity in the ventral mPFC, which corresponds to the grey matter volume changes in the previous study. In a review by Shin and Liberzon (2010), the authors pointed out that compared to healthy subjects, PTSD patients showed reduction of PFC activities during emotional word retrieval.

Brady et al. (2009) pointed out that addiction, PTSD and traumatic brain injury (TBI) often occur simultaneously, and it is especially true in veterans, which suggests that neurobiological deficits associated with one disorder may predispose individuals to the other. Another study found that TBI increases the probability of relapse to alcohol abuse and that the neurotoxic effects of alcohol interact with TBI, which in turn predicted worse recovery outcomes (Jorge et al., 2005).

The mammalian PFC regulates higher levels of cognitive function, such as decision making, behavioural flexibility and emotional regulation. PFC damage is associated with poorer

executive performance and less behavioural inhibitory control. Drug abuse is known to impair PFC function (Goldstein & Volkow 2002). For example, Liu, Matochik, Cadet and London (1998) and Franklin et al. (2002) reported decreased PFC volume and reduction of grey matter in cocaine, alcohol and heroin abusers. In an animal study, Robinson et al. (2001) reported that psychostimulant abuse was associated with increased numbers of dendritic branching and dendritic spine density in the PFC. During states of drug withdrawal, lower brain metabolism was detected in the PFC region in cocaine and alcohol abusers compared to healthy controls (Goldstein & Volkow 2002). To summarize, the impairment of PFC function further interferes with the ability to regulate emotional memory, and to assign appropriate emotional salience to incoming environmental stimuli. This cascade of events may lead to a pathological cycle, thereby increasing susceptibility to the development of other psychiatric disorders, such as PTSD or depression.

1.3b Dopamine transmission in PTSD and addiction

The neurotransmitter DA has received considerable research attention due to its vital role in the control of emotion, cognition, locomotion, reinforcement, as well as neuroendocrine secretion. There are two types of DA receptor: the D_1 like receptor, which includes D_1 (D_1R) and D_5 (D_5R) subtypes; the D_2 like receptor type, which includes D_2 (D_2R), D_3 (D_3R) and D_4 (D_4R) subtypes. Each type of receptor is involved in different functions, as well as interacting with other neurotransmitter systems (Messale et al., 1998). D_1R is the most widespread DA receptor in the brain, whereas the D_4R is primarily expressed in the frontal cortex, amygdala and hippocampus. The experiments conducted in this project, the role of DA in emotional memory regulation is studied with a focus on the mesocorticolimbic pathway, comprising DAergic projections from the VTA to the nucleus accumbens and PFC. Pharmacologically, my experiments focus selectively on the D_1R and D_4R since our lab has previously implicated their roles in the modulation of emotional associative learning and memory and opiate addiction (Lauzon et al., 2009; 2013; Lauzon & Laviolette, 2010; Sun et al., 2011).

In the context of addiction, the mesocorticolimbic DA pathway mediates the initial hedonic effects of drugs by increasing DA transmission in the VTA and associated regions like the nucleus accumbens and PFC (Fields & Margolis 2005; Volkow, Fowler, Wang, Baler & Telang, 2009). VTA DA cells are activated in response to emotionally salient stimuli and drugs

of abuse will thereby increase attention and arousal as well as facilitate conditioned learning and motivation associated with the drug taking experience. Chronic drug use results in higher levels of DA transporter activity and increases the thresholds required for dopamine cells to fire, which leads to a reduced rewarding salience of natural reinforcers as well as escalated levels of drug abuse to compensate the anhedonia and dysphoric conditions associated with dependence and withdrawal (Volkow, Fowler, Wang & Goldstein, 2002). This DAergic hypoactivity is believed to contribute to impaired PFC functions, thus leading to less behavioural inhibition, poor decision making and dysregulated emotions. Drug-related cue exposure triggers craving and increases DA transmission in the PFC, which increases the motivation to pursue drugs (Volkow et al., 2009). Pre-clinical research has indicated the importance of DA transmission in addiction; for instance, D_1R gene knock out mice did not reinstate cocaine self-administration after drug priming; similarly, blocking mPFC D_1R impaired recall of morphine rewarding memory in the CPP paradigm. In contrast, mPFC D_2 receptor activation enhanced cocaine self-administration and alterations in the expression and function of D_1R and D_2R were found in the PFC and amygdala of chronic heroin exposed rats (Caine et al., 2007; Lauzon et al., 2013; Rosen et al., 2016; Self, Barnhart, Lehman, & Nestler, 1996). Compared to wild type controls, D_4R gene knock out mice showed locomotor super sensitivity to major psychostimulants and ethanol but decreased reward sensitivity to methamphetamine and amphetamine (Katz et al., 2003; Rubinstein et al., 1997; Thanos et al., 2010).

Current theories have suggested that D_1R transmission is important in memory preservation and recall over temporally delayed periods (Seamans & Yang, 2004). Zahrt, Taylor, Mathew and Arnsten (1997) and Lauzon et al. (2009) showed that pharmacological supra-stimulation of mPFC D_1R blocked the ability of rodents to recall fear memories and impaired spatial working memory without interfering the encoding and acquisition of the memory. Interestingly, blockade of mPFC D_1R, but not D_2R, impaired the ability to use spatial cues in a previously learned spatial memory task (Seamans, Floresco & Phillips, 1998). Therefore, the function of mPFC D_1R produces an inverted U shape, with either hypo or hyper stimulation resulting in malfunction in memory recall and PFC dysfunctions. Thus, optimal levels of mPFC D_1R activity are required to regulate emotional memory and adaptive behavioural responses to environmental stimuli (Seamans & Yang, 2004; Zahrt et al., 1997).

The D_4R is very well characterized from a behavioural and pharmacological perspective and considerable evidence has indicated that the function of D_4R is associated with the acquisition and encoding of emotionally salient memory (Lauzon et al., 2009; Lauzon & Laviolette, 2010). For example, in fear conditioning procedures, local mPFC infusion or systemic injection of a D_4R antagonist produced anxiolytic effects, and prevented the association of cues paired with foot shocks, as well as impaired the acquisition of aversive associative memories (Inoue, Tsuchiya & Koyama, 1994; Shah, Sjovold & Treit, 2004). Recent research has further illustrated that mPFC D_4 transmission bi-directionally controls associative fear memory learning depending on the salience of emotional conditioning stimuli (Lauzon et al., 2009). Specifically, within the hypoactive mPFC (during states of low PFC neuronal activity), D_4R activation potentiated the acquisition of normally non-salient fear conditioning stimuli; while in the hyperactive mPFC (during states of high PFC neuronal activity), D_4R activation impaired the acquisition of normally supra-threshold fear conditioning stimuli. This finding demonstrated that D_4R regulates the learning and encoding of emotionally relevant sensory input from the environment, as well as processing the emotional amplitude of incoming sensory information (Lauzon & Laviolette, 2010). As mentioned above, PTSD and addiction are both characterized by being unable to inhibit emotional memory recall, which might implicate the involvement of cortical DA transmission in both disorders.

In terms of DA abnormalities in PTSD, early studies reported higher levels of urinary and plasma DA in PTSD patients (Hamner & Diamond, 1993; Yehuda, Southwick, Giller, Ma & Mason, 1992). Genetically, Comings, Muhleman and Gysin (1996) found that carrying the D_2 A1 allele significantly increases the susceptibility to the development of PTSD after trauma encounters in veterans, as well as being positively correlated with symptom severity. Interestingly, the high prevalence of the D_4R genotype was also detected in alcohol abusers, and associated with the severity of alcoholism (Coming, Muhleman, Ahn, Gysin & Flanagan, 1994). More recent studies have reported that a variable number tandem repeat (VNTR) polymorphism found in the DA transporter gene is frequently associated with PTSD patients compared to non-PTSD trauma survivors (Segman et al., 2002). Dragan and Oniszczenko (2009) reported that the longer repetition (7-8 repeat) of a VNTR polymorphism located on the exon III of D_4R gene was frequently associated with more intense PTSD symptoms and an over-sensitivity to negative emotional stimuli. Interestingly, this long repetition polymorphism was also detected exclusively

in opiate dependent individuals based on patient samples from China and Israel (Kotler et al., 1997; Li et al., 1997). Shao et al. (2006) further reported that D_4 VNTR long allele carriers displayed higher levels of craving for heroin after exposure to drug-related cues, as well as with severe physical dependence; early onset and higher levels of craving in alcohol abusers (Hill, Zezza, Wipprecht & Neiswanger 1999; Hutchison, McGeary, Smolen, Bryan & Swift, 2002). Together, this evidence may suggest a common genetic abnormality of the DA D_4R system associated with both PTSD and opiate addiction vulnerability.

1.3c Downstream molecular pathways of DA receptor transmission involved in PTSD and addiction

D_1R stimulation links to the activity of an important downstream signaling molecule, cyclic adenosine monophosphate (cAMP), which is critically involved in major intracellular signalling pathways. Ligand binding to D_1R activates the receptor's G-protein, which then converts ATP to cAMP. cAMP next activates the protein kinase A (PKA) phosphorylation process, ultimately leading to the phosphorylation of various intracellular proteins and regulation of downstream neurobiological responses which in turn can modify a number of behaviours (Dwivedi & Pandey 2008).

Interestingly, over the last several decades, several studies have found linkages between cAMP activity states and both PTSD and addiction. For example, one clinical study (Lerer, Ebstein, Shestatsky, Shemesh & Greenberg, 1987) found PTSD patients have lower levels of cAMP, suggesting that abnormal cAMP levels may serve as a bio-marker for the diagnosis. Similarly, in rodents, cAMP inhibition in the amygdala or the mPFC blocked fear memory consolidation and interfered with long term memory formation tested in an auditory fear conditioning paradigm (Schafe & Ledoux 2000). Lauzon et al (2012) reported that the effects of PFC D_1R activation on the attenuation of fear or reward memory recall is cAMP dependent and that inhibition of this pathway reversed the effects of PFC D_1R stimulation on memory recall. Similar effects were also observed in cocaine self-administration and lever pressing for food in rats; in both paradigms, inhibition of cAMP/PKA in the nucleus accumbens (NAc) attenuated lever pressing for rewards; while activation of cAMP facilitated these behaviours (Baldwin, Sadeghian, Holahan & Kelley, 2002; Self et al., 1998). Furthermore, the effects of cAMP on reward memory processing are not limited to the recall phase, but also the acquisition of the

reward memory. Beninger, Nakonechny and Savina (2003) and Sutton, McGibney and Beninger (2000) have reported that cAMP inhibition during the conditioning phase attenuated the learning of amphetamine associated rewarding effects and amphetamine-induced locomotion.

D_1R activation is also closely linked to the extracellular-signal-related kinases 1 and 2 (ERK1/2), which is a neuronal molecular pathway highly involved in several types of synaptic plasticity, learning and memory and the processing of drug-related reward behaviours (Rosen, Sun, Rushlow & Laviolette, 2015; Valjent, Pagès, Hervé, Girault & Caboche, 2004) as well as aversive memory processing (Cestari, Rossi-Arnaud, Saraulli & Costanzi, 2014). Recent PTSD research has revealed several roles for the ERK pathway in emotional memory processing (Cestari et al., 2014). For example, Ardi, Ritov, Lucas and Richter-Levin (2014) and Jeon et al. (2012) reported that rats re-exposed to a stress-related environment showed significantly higher ERK activation levels, and PTSD-like symptoms, suggesting that the ERK pathway is involved in conditioned fear memory recall. Furthermore, the ERK pathway is critical for memory extinction and consistent increases in phosphorylated ERK2 (pERK 2) levels in the PFC have been detected during fear extinction learning in mice (Cestari et al., 2014; Matsuda et al; 2015); similarly, Fischer et al. (2007) have reported that ERK1/2 shows increased phosphorylation levels in fear memory extinction trials. Thus, ERK signaling is required for the acquisition, recall and extinction of cue-related conditioned fear and is similarly involved in processing drug related reward memory (Gholizadeh et al; 2013; Lysons et al., 2013; Rosen et al., 2015 & 2016).

ERK is an important molecular substrate by which drugs may exert their effects by modulating the phosphorylation of ERK through a D_1R transmission-dependent mechanism (Valjent, Corbille, Bertran-Gonzales, Herve & Girault, 2006; Valjent et al., 2004). For example, an *in vitro* study using SH-SY5Y cells revealed that acute treatments with morphine or other opioids stimulated the phosphorylation of ERK1/2. However chronic opiate exposure was shown to reduce these phosphorylation levels (Bilecki et al., 2005). Antagonizing D_1R transmission reverses the effects of several drugs, such as ethanol and cocaine, on ERK phosphorylation in the mPFC and the NAc (Acquas et al., 2010; Fricks-Gleason & Marshall, 2011; Ibba et al., 2009). In addition, D_1R activation increased phosphorylation of ERK1/2 in the mPFC and striatum which demonstrated a close functional connection between D_1R transmission and ERK signalling during substance abuse (Xue, Mao, Jin & Wang, 2015). ERK is also highly associated with the

formation and the consolidation of associative morphine reward memory. For example, mice with ERK gene knock out exhibit deficient reactions to morphine and inhibition of ERK was shown to attenuate previously formed morphine CPP memories (Mazzucchelli et al., 2002; Valjent et al., 2006). Gholizadeh et al. (2013) have demonstrated that ERK inhibition in the basolateral amygdala (BLA) or the mPFC blocked recent and remote morphine reward memory recall, accompanied by a reduction of pERK in the BLA and increased pERK in the PFC following chronic opiate exposure (Lyons et al; 2013; Rosen et al., 2016).

As discussed previously, D_4R- mediated transmission in the PFC regulates emotional memory acquisition, and this process requires the participation of calcium/calmodulin dependent kinase II (CaMKII, Lauzon et al., 2012), a molecule critically involved in synaptic plasticity, as well as learning and memory (Frankland, O'Brien, Ohno, Kirkwood & Silva 2001; Silva, Paylor, Wehner & Tonegawa 1992). *In vitro* studies have shown that activation of PFC D_4R bi-directionally regulates the levels of CaMKII. Specially, during high cortical neuronal activity states (corresponding to states of high emotional arousal/salience), D_4R activation induced dramatic reductions in local CaMKII activity. This mechanism requires the reduction of protein kinase A (PKA), leads to increased protein phosphotase-1 (PP1) levels and ultimately dephosphorylates and reduces the activity of CaMKII (Gu & Yan, 2004). Whereas during low neuronal activity states (corresponding to low emotional arousal/salience), D_4 activation stimulates phospholipase C, which results in an increased intracellular calcium (Ca2+) concentration, subsequently promoting calmodulin to bind the kinase and phosphorylate CaMKII to its activated state, phosphorylated CaMKII (pCaMKII, Gu & Yan, 2004). *In vivo* studies examining the role of CaMKII have reached agreement with the previous *in vitro* research. For example, Lauzon et al. (2012) reported that in subthreshold fear conditioning, which resembles the lower baseline neuronal activities, CaMKII inhibition reverses the effects of D_4R activation on facilitation of fear memory learning. While in supra-threshold fear conditioning, which is the analogy of higher neuronal activity states, blocking CaMKII activity by stimulation of PKA/inhibition of PP1 rescues the effect D_4R stimulation on attenuating conditioned fear acquisition.

CaMKII expression is more concentrated in the forebrain; once activated, it is translocated to post synaptic sites concomitant with increasing AMPA and decreasing NMDA

receptor activity though a D_4-dependent mechanism (Gu, Jiang, Yuan & Yan, 2006; Wang, Zhong & Yan, 2003). Given the well-established role of AMPA and NMDA receptors in learning and memory, as well as their involvement in psychiatric disorders, such as schizophrenia, it is not surprising that aberrations in CaMKII signalling is correlated with various behavioural and neurobiological impairments (Lauzon & Laviolette, 2010). Indeed, *in vivo* research has shown that mice with a CaMKII gene knock out display deficiencies in spatial learning and long term spatial memory retrieval (Silva et al 1992). CaMKII gene deficient mice also showed slower learning of foot shock and lower behavioural responses to fear-related cues (Chen, Rainnie, Greene & Tonegawa, 1994). CaMKII transduction also dynamically affects the ability of subjects to attribute reward learning at different drug exposure stages. In drug-naïve subjects, consolidation of morphine reward memories required BLA CaMKII signaling whereas the recall of long term opiate rewarding memory involves PFC CaMKII signaling (Gholizadeh et al., 2013). Chronic exposure or withdrawal to opiates was shown to cause a dramatic reduction in BLA CaMKII-α levels concomitant with increasing CaMKII-α levels in the mPFC (Lyons et al., 2013; Rosen et al., 2016). Thus, CaMKII signaling linked to the D_4R, is involved importantly not only in fear-related memory processing, but also in the opiate addiction and memory formation process.

1.4 Pre-clinical animal modelling of PTSD

PTSD is a devastating disorder with high prevalence and considerable functional disability. Thus, there is an urgent need for a better understanding of the underlying neurobiological mechanisms of PTSD. Over several decades, a rich variety of pre-clinical rodent models have been developed to help gain further insights into this disorder (Schöner et al, 2017). The establishment of an effective pre-clinical animal model should follow 3 criteria, as proposed by Willner (1984): first, face validity: the probed symptoms should resemble those of the modelled disorder. Second, construct validity: the animal model should mimic similar underlying neurobiological mechanisms as those observed in the human patient. Third, predictive validity: the animal model should enable the researcher to make accurate predictions about the treatment effects.

In terms of pre-clinical animal PTSD models, this paradigm should have a fear stimulus, which generates stressful behaviours similar to the symptoms of PTSD. The stressor induced

PTSD-like symptoms should be acute and intensive instead of mild and chronic (Yehuda & Antelman, 1993). More importantly, the hall mark of PTSD is the re-experience symptom associated with traumatic memories and therefore the stress related cues/environments should be able to elicit anxiety and cause memory recall of the traumatic experience. Today, the most accepted pre-clinical animal model for modelling PTSD-like symptoms is the fear conditioning assay (Ross et al., 2017). In terms of construct validity, the neural circuitries involved in fear and anxiety in rodent and the etiology of PTSD in humans are similar, and are highly conserved throughout evolution, including brain areas like the PFC and amygdala (Ross et al., 2017). However, the neurobiological underpinnings of PTSD are far from being understood, and there are currently no effective pharmacological treatments for PTSD, making it difficult to establish clear construct and predictive validities (Schöner et al, 2017).

Associative fear conditioning using foot shock as a proxy for a traumatic experience, has been wildly employed to study anxiety disorders and PTSD in pre-clinical research, primarily using rodents (Van Dijken, Van der Heyden, Mos & Tilders, 1992). In general, this simple, Pavlovian conditioning paradigm requires confining the animal in an inescapable chamber where foot shock can be delivered, allowing for variation in the duration and the intensity of the stimulus. Foot shock models elicit long lasting behavioural effects and associative memories persisting up to 3 weeks (Van Dijken et al, 1992), and it can induce core symptoms of PTSD, such as anxiety, avoidance and hyperarousal, as foot shocked rodents will demonstrate long lasting increased immobility, decreased rearing and grooming behaviours in various tests, such as open field and the elevated plus maze (Pynoos, Ritzmann, Steinberg, Goenjian & Prisecaru, 1996; Van Dijken et al, 1992). One advantage of foot shock fear conditioning models is that they enable researchers to model the re-experiencing symptoms by associating shocks with associative cues (such as tone, context, lights or odours) during the memory acquisition, while presenting the cues alone (without the shock) later during the test phase to elicit the recall of the associative fear memory and evoke PTSD-like anxiety behaviours (Pynoos et al., 1996; Van Dijken et al, 1992; Lauzon et al., 2009). Thus, consistent with the extant literature, my studies employed a well-established model of olfactory fear conditioning in rats combined with morphine reward conditioned place preference, in order to simultaneously model both PTSD and addiction-like behaviours.

1.5 Research Objectives and Hypotheses

The role of D_1R and D_4R transmission within the mammalian PFC, as well as their associated downstream molecular signaling pathways in fear-related and opiate rewarding associative memory processing have been previously identified (Lauzon et al., 2009, 2012, 2013; Rosen et al., 2016). However, the possible relationship between intra-PFC DAergic modulation of fear-related memory and how this may modulate reward-related memory processing has not previously been investigated. Although previous research has suggested that stress itself can reinstate drug taking behaviours, it does not fully explain the high comorbidity between PTSD and opioid addiction phenomena and does not explain how the recall of a traumatic memory might influence sensitivity to reward-related, drug-induced memory formation. ***Our overarching hypothesis is that DAergic transmission within the PFC, by modulating the salience and recall of traumatic, fear-related associative memories, may similarly modulate the ensuing increased sensitivity to opiate-related reward salience and represent a mechanistic link between PTSD and drug addiction comorbidity.*** More specifically, we hypothesized that PFC D_4R stimulation, by selectively ***increasing the emotional salience*** of normally non-salient fear memories during memory acquisition, may simultaneously increase sensitivity to the rewarding effects of opiates, measured in conditioned place preference procedures. In contrast, PFC D_1R stimulation, by selectively ***blocking the recall*** of previously acquired associative fear memories, might simultaneously block the potentiation of morphine reward salience. **For this thesis, my specific experimental AIMS were the following:**

1) **Determine whether fear memory recall potentiates sensitivity to morphine's rewarding effects; and if so:**
2) **Determine the selective roles of intra-PFC D_1 and D_4 receptor transmission during the acquisition vs. recall phases of associative fear memory formation.**
3) **Determine if intra-PFC D_1 vs D_4 modulation of associative fear memory formation modulates sensitivity to the rewarding effects of morphine.**
4) **Determine the downstream molecular pathways associated with intra-PFC D_1R vs. D_4R modulation of fear memory and morphine reward sensitivity, focusing on the cAMP, ERK 1/2 and CaMKII signaling pathways.**

2. Materials and Methods

This project involved 20 independent experimental groups, each consisting of 6-10 adult male Sprague-Dawley rats (Charles River, Canada). This section describes the materials and methods including surgery and drug treatment, behavioural and western blot protocols, and histology and data analysis.

2.1 Animals and surgery

Male Sprague Dawley rats (weight 300 g to 350 g) were purchased from Charles River Laboratories and housed in temperature-controlled (21 °C) room with free access to food and water through out the duration of the experiments. Animals were pair housed before the surgery; and were individual housed after the surgery and during entire experiments in clear Plexiglas cages covered with rat bedding; cages were changed on weekly basis by animal care staff. All procedures were performed at the University of Western Ontario (London, Ontario, Canada) and adhered to regulations outlined by the *Canadian Council on Animal Care*. The animals were anesthetized using a mixture of ketamine (100 mg/mL) and xylazine (20 mg/mL) administered intraperitoneally (115mg/kg, [i.p.]), and subsequently placed on a stereotaxic device. Stereotaxic coordinates were based on the atlas published by Paxinos and Watson (2005). Two stainless-steel guide cannulae (22 gauge, Plastics One, Roanoke, VA, USA) were implanted bilaterally into the medial prefrontal cortex (mPFC) or the ventral tegmental area (VTA) using the following stereotaxic coordinates: bilateral mPFC (15° angle): anteroposterior (+2.9 mm from bregma), lateral (-1.9 mm from bregma), and ventral (-3.0 mm from dural surface). Bilateral VTA placements (10° angle): anteroposterior (-5.0 mm from bregma), lateral (-2.3 mm from bregma), and ventral (-8.0 mm from dural surface). Jeweler's screws and dental acrylic were used to secure the cannulae. The animals had at least 7 days to recover from the surgery, and wet food was placed to the bottom of housing cages to speed up the weight during the first 3 post surgery days.

2.2 Drug administration

Stainless-steel guide cannulae (22 gauge) were implanted bilaterally into the mPFC or the VTA, and drugs were infused through 28-gauge microinfusion injectors (Plastics One), which were cut 1.0 mm longer than the guide cannulae. The 1.0 µL microsyringes (Hamilton Co., Reno,

NV, USA) were connected to the injector by a polyethylene tube. All microinfusions were performed at a constant rate of 1.0 µL/min (0.5 µL/hemisphere), injectors remained in place for an additional 1 min to allow diffusion. All drugs were dissolved in physiological saline (pH 7.4). The ERK inhibitor U0126 was dissolved in 50% dimethyl sulfoxide. Full D_1R agonist SKF 81297 (10 or 100 ng/0.5uL [Tocris, United Kingdomng]), ERK1/2 inhibitor U0126 (1.0 µg/0.5uL [Tocris]), and filtered saline vehicle were infused immediately before the fear memory test phase. The selective D_4R agonist PD 168077 (5 or 50 ng/0.5µL [Tocris]), CaMKII inhibitor autocamtide-2-related inhibitory peptide (AIP, 500 ng/0.5µL [Tocris]), and filtered saline vehicle were infused immediately before the fear conditioning phase. Morphine (0.05 mg/kg, morphine hydrochloride, [MacFarlane Smith, Edinburgh, Scotland]) and saline vehicle were injected (i.p.) immediately before morphine and saline conditioning, respectively. In the VTA experiments, bilateral intra-VTA microinjections of morphine (250 ng/0.5µL) were infused immediately before morphine conditioning. Filtered saline was infused into the VTA immediately before saline conditioning.

2.3 Olfactory fear conditioning

Olfactory fear conditioning was used to test the ability of the animals to recall a fear memory formed by pairing an olfactory cue with foot shocks. Based on experimental purposes, either subthreshold (0.4 mA) or supra-threshold (0.8 mA) foot shocks were delivered to test whether a certain drug treatment could potentiate or block the associative fear memory. Previous studies have reported that subthreshold (0.4 mA) foot shocks fail to elicit conditioned freezing behaviour, and that supra-threshold foot shocks produce robust fear responses (Lauzon et al., 2009). On day 1 (habituation phase), the animals were transported from the housing room to a sound-attenuated room, and were habituated to the new environment for 30 min. The animals were then removed from their cages and placed into one of two well-ventilated, identical size (30 inch × 30 inch) plexiglass conditioning chambers. Each chamber had a distinct background: chamber A had white background with black stripes; chamber B had a white background with black dots. One of the chambers was equipped with a metallic grid-shock floor that could be removed during the test phase. The other chamber had a smooth plexiglass floor. The rats were habituated to each environment for 30 min and were returned to their home cages for 5 min between switching environments. On day 2 (conditioning phase), conditioning occurred in one of

the two distinct environments, and counter-balanced within groups. The resources of the olfactory cues were the almond or peppermint extract oil, which was contained in two separated sealed vials and each vial was connected by the in-flow and the out-flow polyethylene tubes. The animals were placed in the previously assigned shock environment and allowed to explore for 1 min. The olfactory cue (either peppermint or almond [counter-balanced within groups]) was then administered for 19 s, followed by a foot shock (1 s), either 0.4 mA or 0.8 mA. The procedure was repeated 5 times at 4-min intervals (*animals were remained in the conditioning chamber*). The animals were then removed from the conditioning chamber 100 s after the final foot shock and returned to the home cages. On day 3 (test phase), subjects were tested in the previously assigned test environment (where they did not previously receive foot shock). Before presenting the olfactory cue, animals were allowed to explore the environment for 1 min, and baseline level of activity was observed. Odor cues were then presented for 5 min and the length of freeze time was recorded using ANY-maze software (Stoelting Co, Wood Dale, IL, USA). The data were analyzed as percentage of freeze (time spent freezing [s] divided by 300 s), represented as freezing % in the graphs. Freezing was defined as a complete lack of movement other than respiratory-related movement.

2.4 Conditioned place preference

Pavlovian conditioned place preference (CPP) was used to train the animals to form rewarding memory related to morphine's rewarding effects by pairing morphine injection with a specific environment. All rats were conditioned using an unbiased, fully counter-balanced CPP procedure as described previously (Laviolette, Nader & van der Kooy 2002). Conditioning occurred in two distinct environments (both 15 inch × 15 inch) differing in colour, texture, and scent. One environment was white, with a wire mesh floor and covered with wood chips. The other environment was black with a smooth plexiglass floor, wiped with 2% acetic acid solution before each conditioning session and test. Rats display no baseline preference for either environment (Laviolette and van der Kooy, 2003). Treatments received (saline or morphine) in each of the two environments were counter-balanced within the group. For example, 4 animals received morphine injection paired with the white box environment; the other 4 animals received morphine injection paired with the black box. Before olfactory conditioning habituation, the animals were placed in the gray plexiglass boxes for a 30-min habituation period to minimize

stress during future conditioning. Morphine conditioning took place immediately after the fear recall phase in order to temporally probe how fear memory recall may or may not influence morphine reward sensitivity. Subjects received i.p. injections of morphine (0.05 mg/kg) or bilateral intra-VTA morphine microinfusions of morphine (250ng/0.5µL). Previous reports have shown that these morphine treatments are sub-reward threshold and do not normally produce a rewarding CPP effect (Lintas et al., 2012). Immediately after the injections, the rats were placed in the previously assigned morphine environment for 30 min. Twenty-four hours later, they received saline vehicle i.p. injections or intra-VTA saline vehicle microinfusions, followed by 30 min conditioning in another environment. The animals received one drug-environment and one saline-environment conditioning session. At the drug-free test, the rats were placed in a grey narrow zone that separated the two environments, and time spent in each environment over a 10-min period was recorded using two stopwatches. To be scored, the animals had to have their front paws and heads located in one environment. Increased time spent in the drug-paired environment indicated the expression of drug-related rewarding memory, which served as a measure of morphine's rewarding effects or increased motivational state for the drug. The data were analyzed as time spent (s) in each environment. The behavioural protocol for this project is depicted in **Figure 1**.

Figure 1. *Behavioural procedures flow chart.*

Note: fear memory test and morphine conditioning took place in the same day; after the 5-min fear recall phase, subjects were returned to their home cage for 5 minutes and received morphine in their housing rooms. Whereas saline conditioning took place 24h after morphine conditioning session.

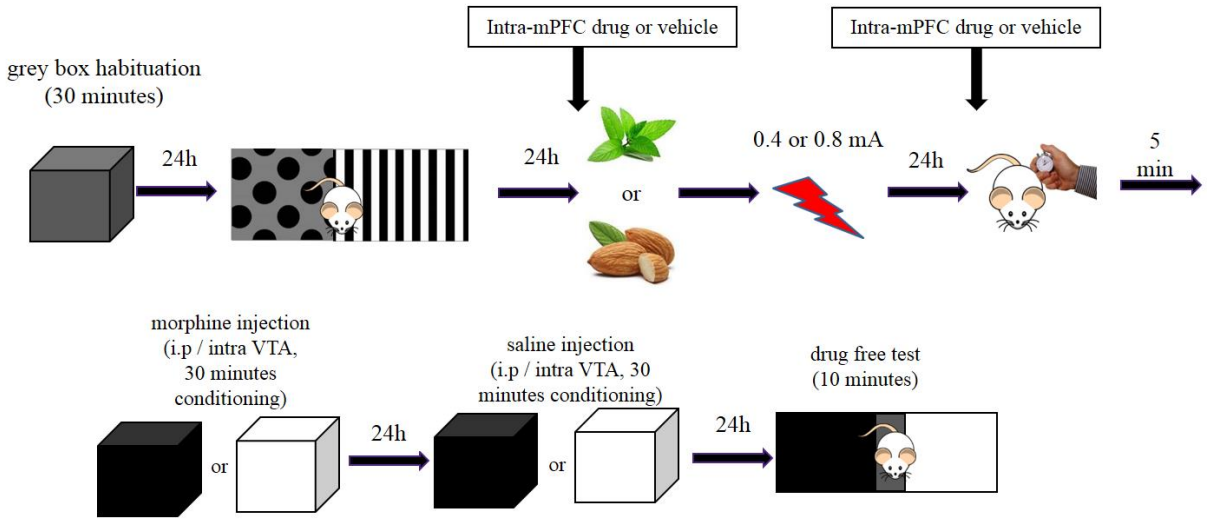

Intra-mPFC drug or vehicle
Intra-mPFC drug or vehicle
grey box habituation
(30 minutes)
24h
24h
or
0.4 or 0.8 mA
24h
5
min
morphine injection
(i.p / intra VTA,
30 minutes
conditioning)
or
24h
saline injection
(i.p / intra VTA, 30
minutes conditioning)
or
24h
drug free test
(10 minutes)

Figure 1

2.5 Western blot protocol

Western blotting was used to detect changes in phosphorylation and total levels of ERK1/2 (pERK 1/2, tERK 1/2) and CaMKII (pCaMKII, tCaMKII). Three groups of animals received intra-mPFC saline vehicle, the selective dopamine D_4 receptor (D_4R) agonist PD 168077 (50 ng/0.5 µL), or full dopamine D_1 receptor (D_1R) agonist SKF 81297 (100 ng/0.5 µL) microinfusions in their housing room. Five minutes after the microinfusions, the animals were euthanized. Brains were removed and stored on dry ice for 5 min, then transferred to the -20°C freezer. The mPFC regions were sectioned into coronal slices (100 µm), and micropunches of the mPFC tissue were obtained. The mPFC tissue samples were then homogenized using a Dounce homogenizer. RIPA lysis buffer (pH 8.0) containing a protease inhibitor tablet (Mini complete tablets, Roche, USA) and phosphatase inhibitors (phosphatase inhibitor cocktail 4 [Calbiochem, San Diego, CA, USA]; phosphatase inhibitor cocktail 2 [Sigma-Aldrich, St Louis, MO, USA]), was used for protein isolation. The sample was then centrifuged for 1.5 h to remove debris, then mixed with an equal volume of 2 × Laemmli loading buffer heated to 95°C for 5 min, then stored in at -20°C for future use.

Protein samples from the saline vehicle or treatment groups (D_1R and D_4R agonists) were loaded onto 10% denaturing sodium-dodecyl sulfate (SDS) polyacrylamide gels and electrophoresed (125 V for 75 min) using a Western blotting apparatus (Mini Protean 3, Bio-Rad, Hercules, CA, USA) filled with Tris/glycine/SDS buffer. After electrophoresis, protein was transferred from the gels to nitrocellulose membranes (Bio-Rad) using a Mini Trans-Blot apparatus (Bio-Rad) filled with a tris/glycine/methanol solution covered with ice at 75 V for 75 min. The nitrocellulose membranes were then blocked in 5% non-fat skim milk (NFSM) tri-buffered saline and Tween 20 (TBS-T) solution for 1 h at room temperature with rocking. The membranes were then incubated in 5% NFSM TBS-T with the appropriate primary antibodies (**Table 1**) directed against the proteins of interest overnight at 4°C on a rocker. The membranes were then washed in TBS-T three times at room temperature (10 min each time on the rocker). Subsequently, the membranes were incubated in the NFSM TBS-T solution containing secondary antibody (alpha tubulin: donkey anti mouse 680RD, 1:1000 [LI-COR, Lincoln, NB, USA]; donkey anti rabbit 800CW, 1:1000 [LI-COR]) for 1 h at room temperature on a rocker. The membranes were then washed with TBS-T two times (10 min each time with rocking) and

once in tri-buffered saline (TBS, 10 min with rocking). The membranes were then scanned (Odyssey Nitrocellulose Membrane Scanner, LI-COR Bioscience). Imagine Studio Lite version 5.2 (LI-COR Bioscience) was used to separate the protein blot and alpha-tubulin images, which were then stored as separate TIF files for densitometry measurements using Molecular Imaging software (Kodak, Rochester, NY, USA). Data were analyzed using Excel (Microsoft Corporation, Redmond, WA, USA).

Table 1

Name	Source	Dilution	Species
alpha tublin	Sigma-Aldrich	1:100000	Mouse
tERK1/2	Cell Signalling Techonology	1:2000	Rabbit
pERK1/2	Cell Signalling Techonology	1:1000	Rabbit
tCaMKII	Cell Signalling Techonology	1:1000	Rabbit
pCaMKII	Cell Signalling Techonology	1:1000	Rabbit

2.6 Histology

After the experiments were completed, the animals were injected (i.p.) with 1.0 mL sodium pentobarbital (240mg/mL, Euthanyl, BI-MEDA Animal Health Inc, Cambridge, ON, Canada) perfused with isotonic saline, then 10% formalin. Brains were removed and stored in formalin (10%) for 24 h, then transferred to a 10% formalin/25% sucrose solution for at least 3 days, and stored at 4°C. The brains were then sectioned into coronal slices (40 μm) using a cryostat, and stained using Cresyl violet, and mounted. The cannulae placements for the mPFC and the VTA were verified under a light microscope. Animals that had cannulation sites outside the anatomical boundaries of the PFC and VTA areas (defined by Paxinos and Watson [2005]) were excluded from data analysis.

2.7 Data analysis

All data were analyzed using one- or two-way ANOVA, or the Student's t test, followed by Tukey post hoc test or Student's t test where appropriate. In the graphical representations of the data, the vertical bars on the group means represent the standard error of the mean, asterisks (*) indicate a level of significance of < 0.05, and double asterisks (**) indicate a level of significance of < 0.01.

3 Results

3.1 Recall of associative fear memory increases sensitivity to morphine's rewarding effects

To investigate the effects of fear memory expression on morphine sensitivity, 3 groups of animals (n=8 in each group) were recruited. Two groups were conditioned to supra-threshold fear conditioning (0.8 mA foot shock); however, 1 of these groups was not presented with cue during the test phase. The third group was included as a control, which did not receive foot shock. Bilateral intra-mPFC microinfusions of saline vehicle for all subjects were administered before the test stage. Systemic injections of the subthreshold dose of morphine (0.05 mg/kg) and saline were administered before the morphine and the saline conditioning phase, respectively. The representative microphotograph of bilateral intra-mPFC microinjector tip placements is illustrated in **Figure 2A**. A schematic representation of bilateral intra-mPFC injector tip placements for the representative groups is presented in **Figure 2B**.

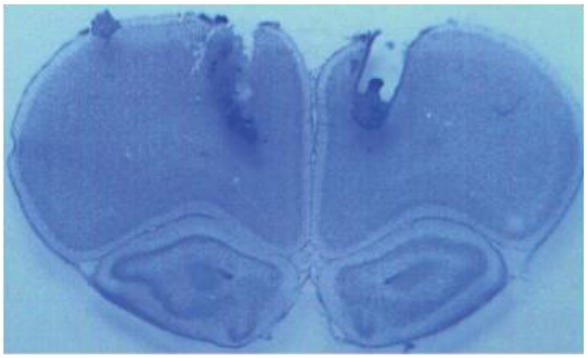
Figure 2

Presentation of intra-mPFC guide cannulae placements

(A) *Microphotograph of the representative bilateral intra- mPFC guide cannulae and injector tip placement.*

(B) *Schematic representation of bilateral intra-mPFC injector tip placements. ○= no fear group, ⌂= fear no recall group, ●= fear recall group, □= SF81297 100ng/0.5ul, filled ⌂= Veh control group (sub-threshold fear conditioning), ■= PD168077 50ng/0.5ul*

A



B

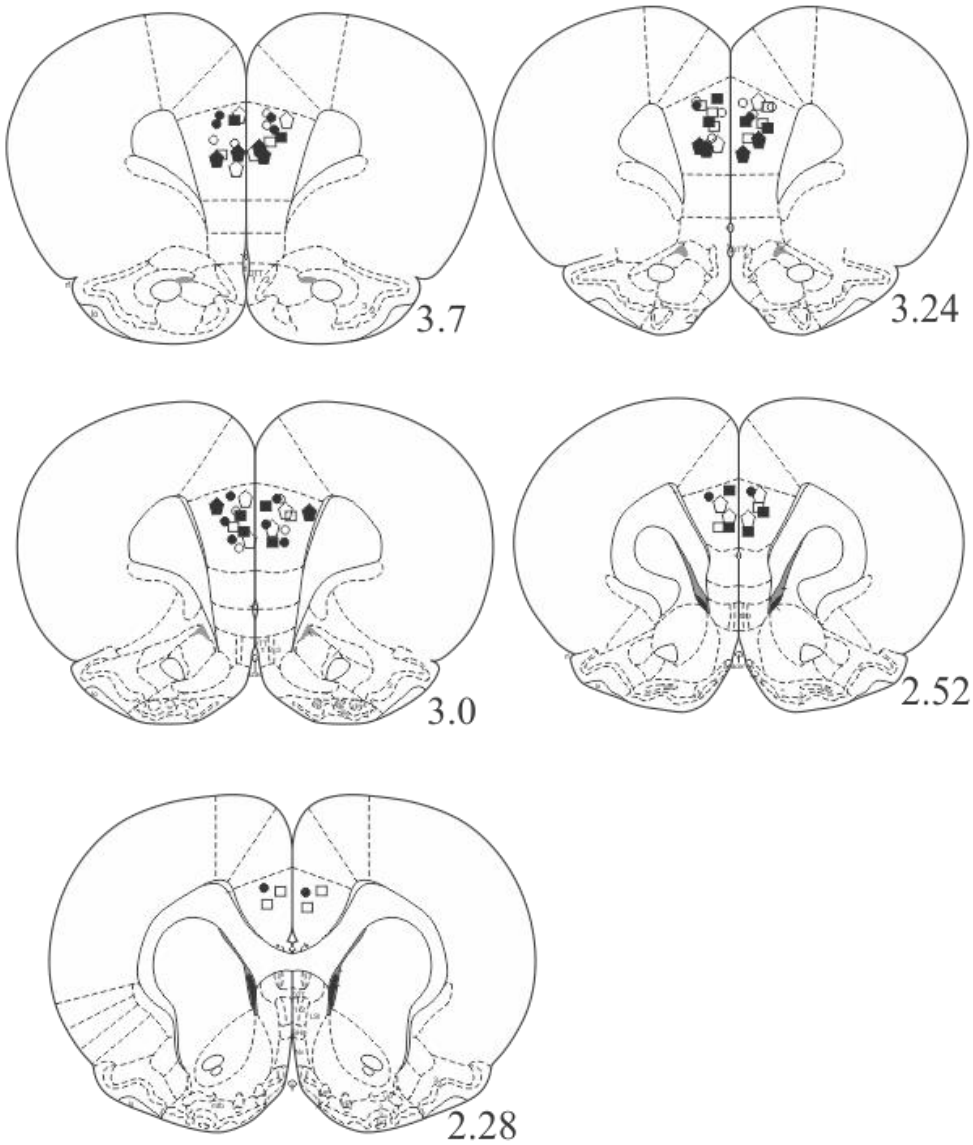


Figure 2

The results of fear conditioning recall testing revealed that groups that were conditioned with supra-threshold fear conditioning showed increased freezing levels relative to the no shock control group. However, relative levels of freezing were much higher in the group receiving the cue presentation during testing (~ 70%), as opposed to the no cue presentation group (~20%; **Figure 3A**). However, in the subsequent morphine CPP test phase, only rats previously receiving the cue presentation during the fear memory recall test showed a significant morphine CPP (**Figure 3B**).

In terms of fear memory recall, one-way ANOVA revealed a significant main effect of group (no shock, fear no cue, fear with cue: $F_{(2, 23)} = 170.399$, $p<0.01$) on freezing behaviours. Post hoc analysis indicated that experiencing foot shock (fear no cue, fear with cue) displayed significantly increased freezing relative to the no fear control group ($p<0.01$). However, rats receiving the cue presentation during the recall testing displayed dramatically increased freezing relative to rats that had experienced foot shock conditioning, but were not presented with the associative cue at testing ($p< 0.01$). Two-way ANOVA analysis of the CPP test revealed a significant main effect of group (no shock, fear no cue, fear with cue: $F_{(2, 21)} = 21.936$; $p<0.01$), environment (morphine, saline: $F_{(1, 21)} = 181.237$; $p<0.01$), and a significant group*environment interaction ($F_{(2, 21)} = 106.803$; $p<0.01$). Post hoc analysis indicated that the fear with cue group exhibited increased morphine preference compared with the corresponding saline environment ($p<0.01$). Comparing only the time spent in the morphine environment, fear groups exhibited significant preference in the morphine environment relative to the no shock and no cue groups ($p<0.01$).

Thus, these findings demonstrate that the selective recall of an associative fear memory, dramatically increases sensitivity to the rewarding effects of a normally non-rewarding conditioning dose of morphine (0.05 mg). Neither rats receiving no shock during conditioning, nor rats that experienced foot shock conditioning (but were not presented with a conditioned cue to trigger the fear memory recall), showed a significant morphine CPP.

Figure 3. *Behavioural effects of supra-threshold foot shock and fear memory expression on conditioned fear response and morphine sensitivity.*
*(**A**) In the no shock group, no demonstrable conditioned fear response was observed. Supra-threshold foot shock (0.8 mA) induced a weak fear response in the fear no cue group and a robust and strong fear memory response in the fear with cue group.*
*(**B**) Only the fear with cue group exhibited significant morphine preference relative to saline-associated context in the conditioned place preference (CPP) test. Comparing only the time spent in the morphine environment, the fear with cue group also spent a significantly longer time in the morphine context compared with the other 2 groups.*

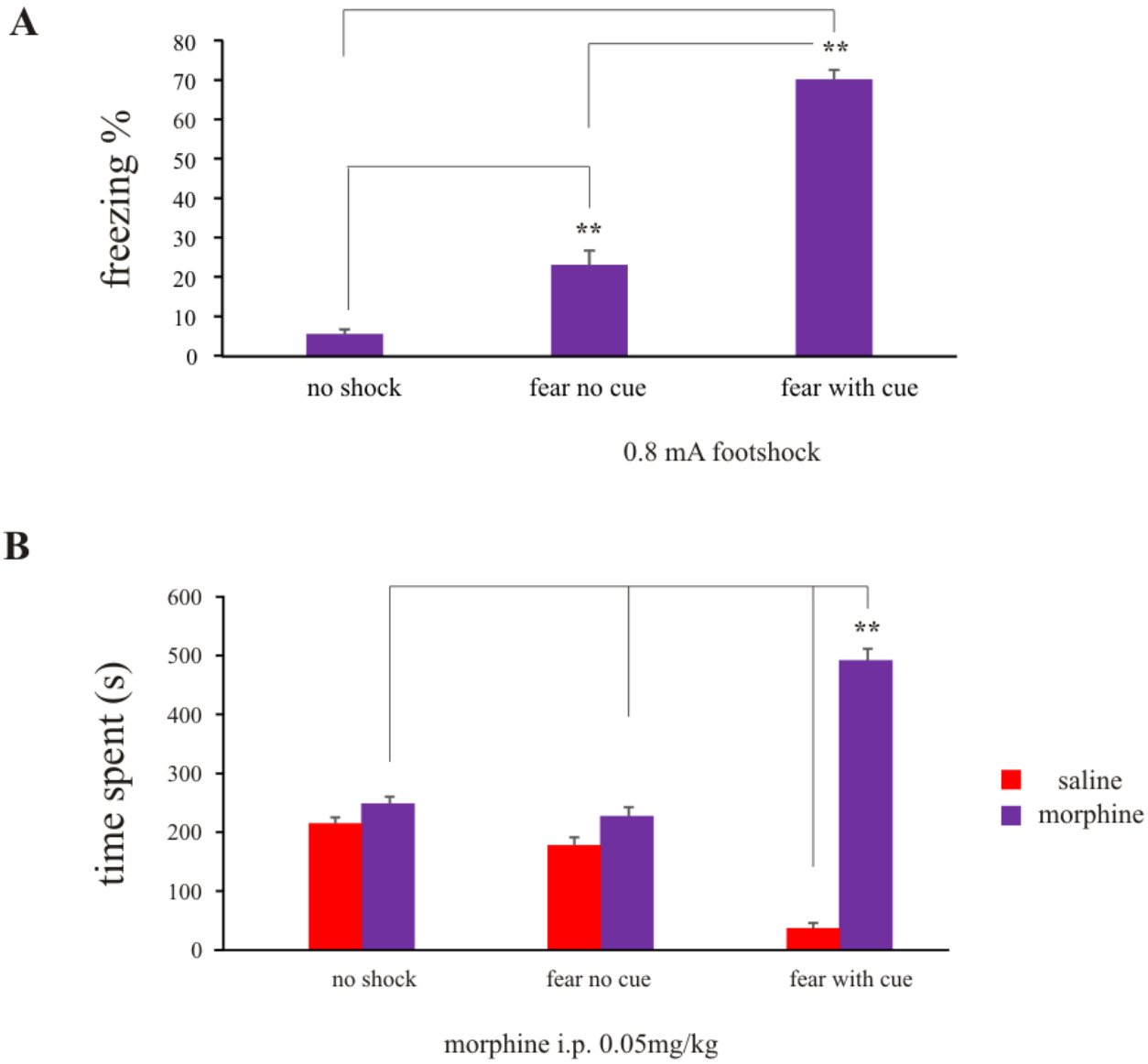

A
freezing %
80
70
60
50
40
30
20
10
0
**
**
no shock
fear no cue
fear with cue
0.8 mA footshock
B
time spent (s)
600
500
400
300
200
100
0
**
no shock
fear no cue
fear with cue
morphine i.p. 0.05mg/kg
saline
morphine

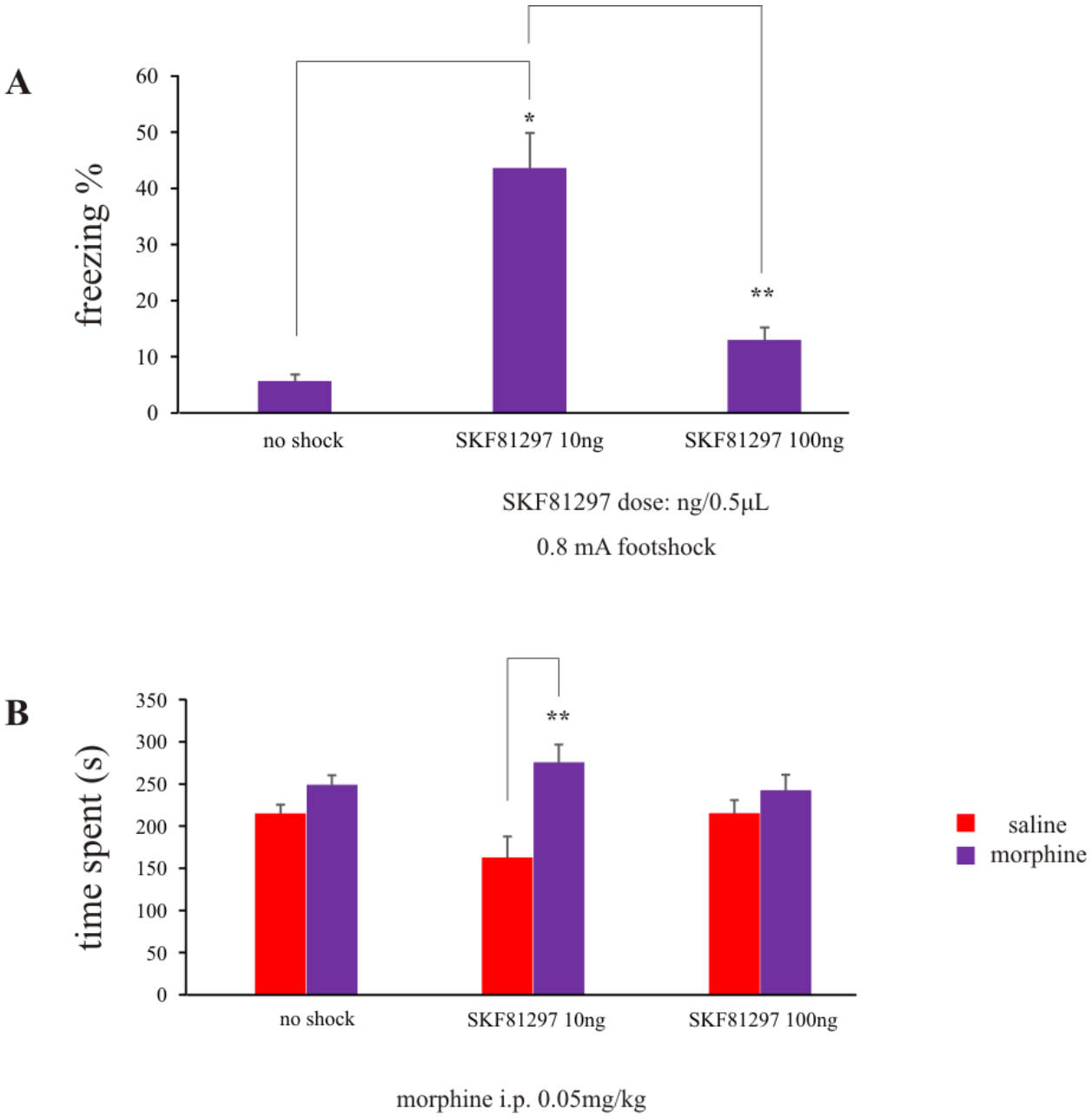
Figure 3

3.2 Activation mPFC D_1R inhibited fear memory expression and blocked potentiated morphine CPP

To further investigate the influence of fear memory expression on morphine sensitivity, it was then decided to test whether inhibition of memory expression could decrease the potentiated morphine sensitivity observed in the previous experiment. Research has shown that mPFC D_1 transmission regulates memory expression (Lauzon et al., 2009); thus, the effects of D_1R super stimulation on both fear and rewarding memory recall were challenged. The experiment included 2 groups, which received different doses of the D_1R agonist SKF81297 (10 ng/0.5 µL [n=8] or 100 ng/0.5 µL [n=8]) immediately before the test stage, followed by i.p. morphine injections administered before morphine conditioning. The no shock group was included in the data analysis and graphs for comparative purposes.

Results showed that bilateral mPFC SKF81297 infusion dose-dependently blocked the expression of fear memory as well as decreased the potentiated morphine preference (**Figure 4 A** and **B**). One-way ANOVA revealed a significant treatment effect (no shock, SKF81297 [10 ng/0.5 µL], SKF81297 [100 ng/0.5 µL]) on freezing behaviour: ($F_{(2, 23)} = 27.143$; $p<0.01$). Post hoc analysis revealed that the higher dose (100 ng/0.5 µL) of SKF81297 fully blocked fear memory recall. The freezing percentage was not different compared with animals in the no shock group ($p>0.05$); whereas the lower dose (10 ng/0.5 µL) failed to attenuate aversive memory expression. The animals exhibited significant higher levels of freezing compared with the higher-dose and no shock groups ($p<0.01$). Two-way ANOVA analysis of the CPP tests revealed a significant main effect of environment (morphine, saline: $F_{(1, 21)} = 9.805$; $p<0.01$). Post hoc tests revealed no morphine preference in the no shock and high dose groups ($p>0.05$), and animals in lower-dose group spent a significantly longer time in the morphine context compared with the saline environment ($p<0.01$).

Figure 4. *Behavioural effects of bilateral medial prefrontal cortex (mPFC) dopamine D_1 receptor (D_1R) activation on fear memory expression and morphine induced place preference. (**A**) Animals in the* no shock *group and those receiving bilateral mPFC infusion of the higher dose of D_1R agonist (SKF81297 [100 ng/0.5 µL]) exhibited no conditioned fear response. Treatment with the lower dose of SKF81297 (10 ng/0.5 µL) failed to block the expression of aversive fear memory because subjects exhibited a significantly higher percentage of freezing. (**B**) Animals in the no shock and higher-dose D_1R agonist group exhibited no significant morphine preference. Animals receiving the lower dose of SKF81297 (10 ng/0.5 µL) spent significantly more time in the morphine-paired environment.*


A
freezing %
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0
*
**
no shock
SKF81297 10ng
SKF81297 100ng
SKF81297 dose: ng/0.5µL
0.8 mA footshock
B
time spent (s)
350
300
250
200
150
100
50
0
**
no shock
SKF81297 10ng
SKF81297 100ng
saline
morphine
morphine i.p. 0.05mg/kg

Figure 4

3.3 Inhibition of cAMP reversed the effects of mPFC D_1R stimulation on aversive memory expression but had no impact on rewarding memory expression

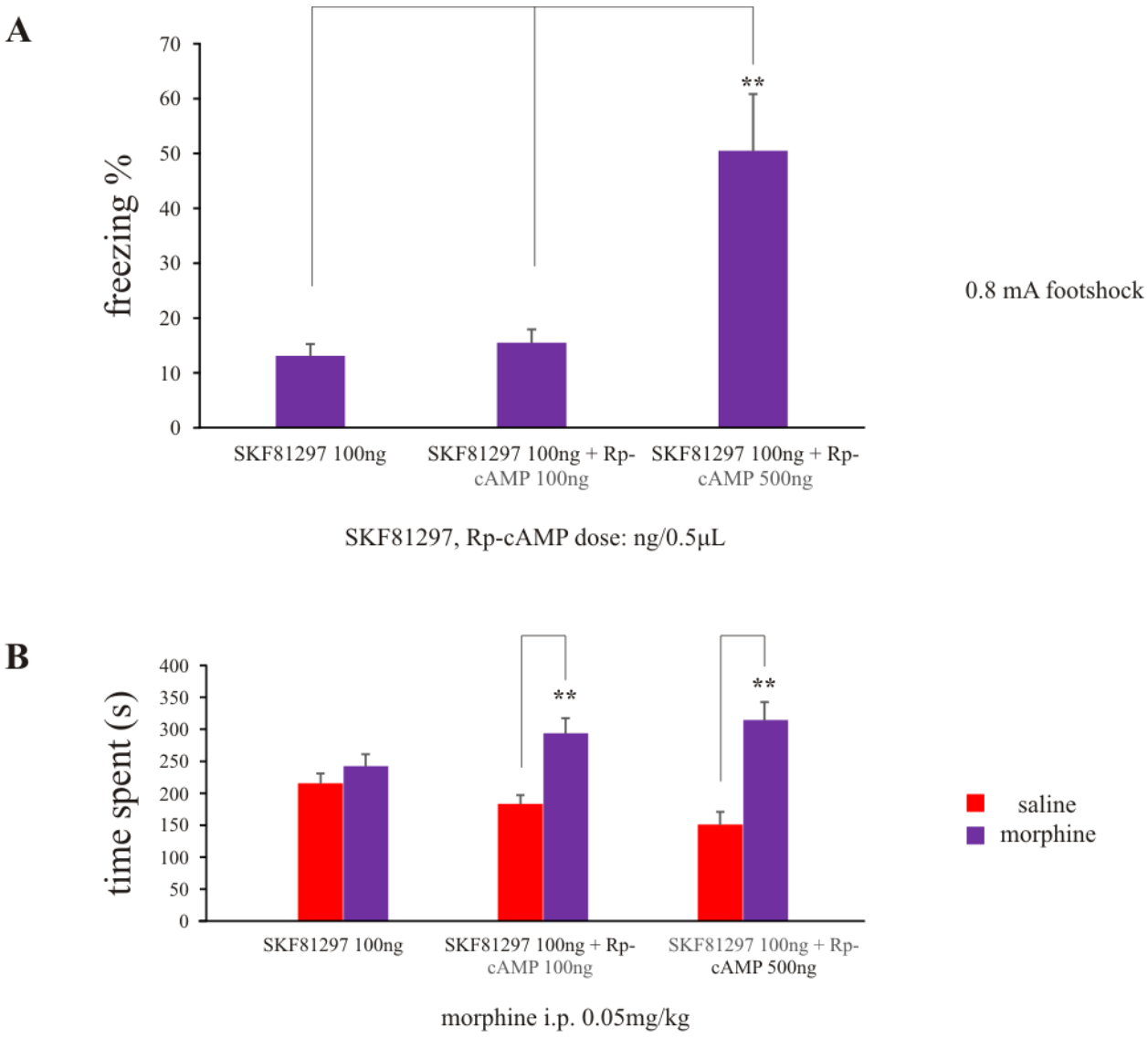
To further examine the role of mPFC D_1 transmission on memory expression and its associated molecular pathways, a reversal experiment was performed with cAMP inhibition, given that previous research has demonstrated mPFC D_1 transmission is associated with cAMP activity (Lauzon et al., 2013). Animals in two groups received bilateral mPFC co-administration of the effective dose of SKF81297 (100 ng/0.5 µL) and different doses of the cAMP inhibitor Rp-cAMP (100 ng/0.5 µL; 500 ng/0.5 µL [n=8 in each group]), before the fear test stage. The following protocols were identical to those previously described. The SKF81297 (100 ng/0.5 µL) group from the previous experiment was included in the data analysis and figures for comparison.

Results indicated that bilateral mPFC Rp-cAMP infusion dose dependently reversed the effect on D_1R activation on fear memory expression, while cAMP inhibition had no effect on regulation of morphine sensitivity and rewarding memory expression (**Figure 5 A** and **B**). One-way ANOVA revealed a significant main effect of treatment (SKF81927 [100 ng/0.5µL] alone, co-administration with Rp-cAMP [100 ng/0.5 µL and 500 ng/0.5 µL] on freezing response ($F_{(2, 23)} = 11.199$; $p<0.01$). Post hoc analysis indicated that, compared with SKF81927 infusion alone, the lower dose of Rp-cAMP (100 ng) failed to reverse its effect on fear memory blocking ($p>0.05$); animals in this group spent the same amount of time freezing. However, the higher dose of Rp-cAMP (500 ng) fully reversed the effect of D_1R activation, and the freezing percentage was significantly higher compared with agonist alone and co-administration with lower-dose Rp-cAMP groups. Two-way ANOVA analysis of the CPP tests revealed a significant main effect of environment (morphine, saline: $F_{(1, 21)} =23.929$; $p<0.01$) and treatment*environment interaction ($F_{(2, 21)} = 3.757$; $p<0.05$). Post hoc analysis indicated that both co-administration groups exhibited significant increased morphine preference compared with their corresponding saline environments ($p<0.01$), which reflects that, although cAMP mediated fear memory expression, it is not the lynchpin mechanism for morphine sensitivity and rewarding memory expression.

Figure 5. *Behavioural effects of bilateral medial prefrontal cortex (mPFC) cAMP inhibition on dopamine D_1 receptor (D_1R) stimulation in the regulation fear memory expression and morphine sensitivity.*

*(**A**) Rp-cAMP dose- dependently reversed the effect of D_1R activation on fear memory expression. Animals receiving SKF81297 alone, and those receiving co-administration of the lower dose of Rp-cAMP (100 ng/*0.5µL*) did not exhibit significant fear response. The group receiving co-administration of the higher dose of Rp-cAMP (500 ng/*0.5µL*) exhibited significant increases in freezing percentage.*

*(**B**) cAMP inhibition failed to block potentiated morphine conditioned place preference (CPP). Both groups received co-administration of SKF81297 and Rp-cAMP spent significantly more time in the morphine environment compared with the saline-paired context. Only SKF81297 infusion alone blocked morphine CPP.*


A
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40
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0
**
SKF81297 100ng
SKF81297 100ng + Rp-cAMP 100ng
SKF81297 100ng + Rp-cAMP 500ng
SKF81297, Rp-cAMP dose: ng/0.5µL
0.8 mA footshock
B
time spent (s)
400
350
300
250
200
150
100
50
0
**
**
SKF81297 100ng
SKF81297 100ng + Rp-cAMP 100ng
SKF81297 100ng + Rp-cAMP 500ng
morphine i.p. 0.05mg/kg
saline
morphine

Figure 5

3.4 mPFC D_4 transmission bi-directionally regulated the acquisition of fear memory and sensitivity to morphine's rewarding effects

Previous research has reported that D_4 transmission mediates the acquisition of memory, and its effects are bidirectional and depend on baseline neuronal activities (Gu & Yan, 2004; Lauzon et al., 2013). To further investigate the effect of aversive memory recall on morphine sensitivity regulation, the effects of mPFC D_4 transmission on the modulation of associative fear and rewarding memory were then examined. First, the effect D_4 transmission in lower baseline neuronal activity was examined; thus, subthreshold fear conditioning was used. Lauzon et al (2009) demonstrated that receiving subthreshold foot shocks (0.4 mA) did not induce significant fear response in rodents. In this experiment, the effect of the D_4R agonist PD168077 on aversive and the rewarding memory acquisition was tested. Three groups were used: saline (Veh [n=9]); PD169077 (5 ng/0.5 µL [n=6]); and PD169077 (50 ng/0.5 µL [n=9]). Intra-mPFC infusions were administered immediately before the fear conditioning stage. Twenty-four hours later, they were tested for fear memory recall and followed by morphine conditioning. Saline conditioning occurred 24 h after morphine conditioning, as previously described.

Results indicated that subthreshold foot shock failed to elicit a strong fear response in both the Veh and lower-dose agonist (5 ng/0.5 µL) groups; however, treating with higher doses (50 ng/0.5 µL) before the acquisition stage potentiated the salience of a subthreshold fear stimulus, animals in this group demonstrated an increased level of fear behaviours during the test (**Figure 6 A)**. This group also exhibited potentiated morphine preference in the CPP; however, the Veh and lower-dose groups did not demonstrate increased morphine sensitivity (**Figure 6 B**).

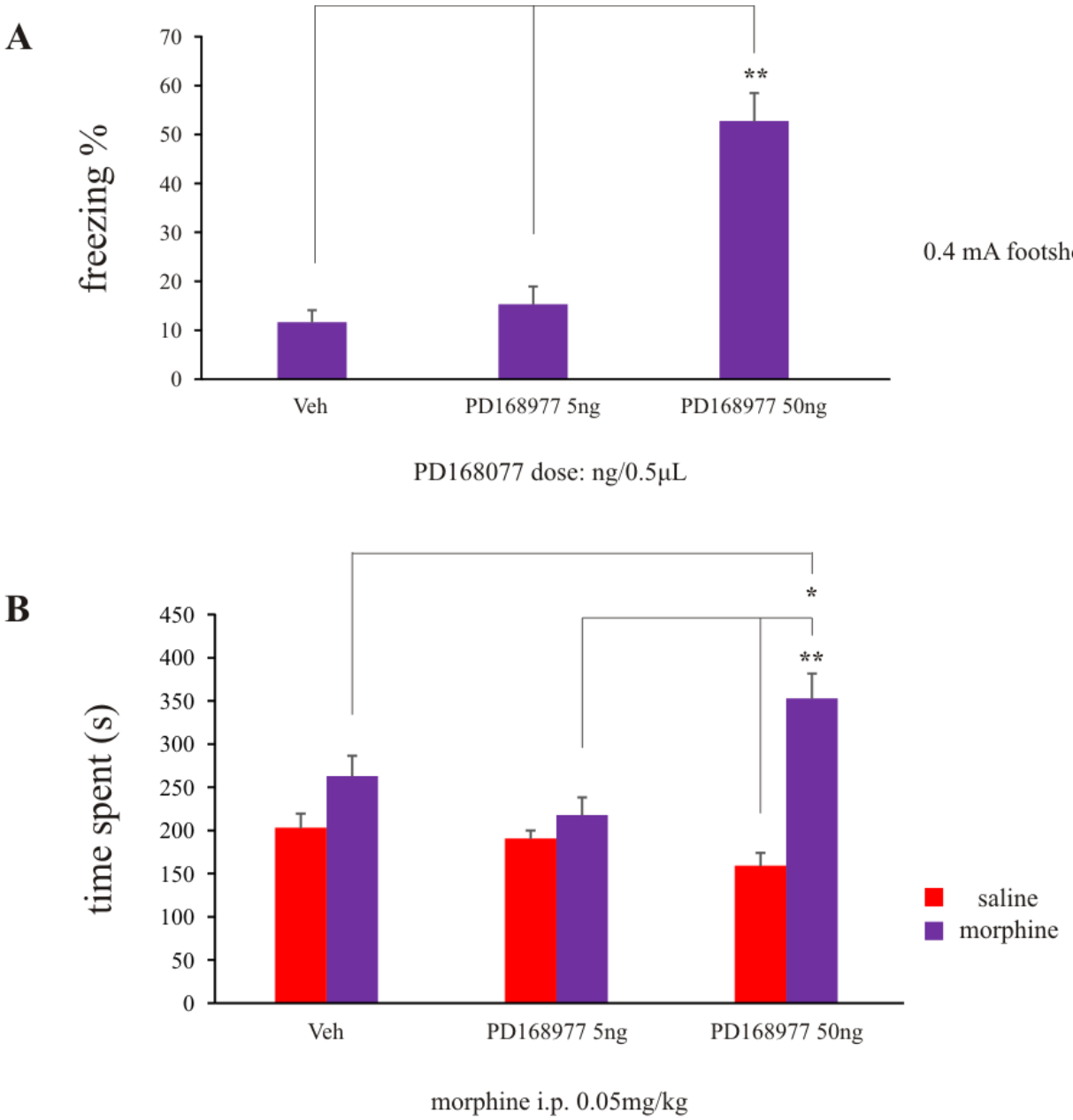
One-way ANOVA test for fear expression revealed a significant main effect of treatment (Veh, PD169077 [5 ng/0.5 µL], and PD169077 [50 ng/0.5 µL] on freezing percentage ($F_{(2, 23)} = 29.817$; $p<0.01$). Post hoc analysis indicated that the group receiving 50 ng/0.5 µL of the D_4R agonist exhibited significantly increased freezing percentage compared with the Veh and lower-dose groups ($p<0.01$). The freezing percentage was not different between the Veh and lower-dose group ($p>0.05$). Two-way ANOVA analysis of the CPP test indicated significant main effects of treatment (Veh, PD169077 [5 ng/0.5 µL], and PD169077 [50 ng/0.5 µL]: $F_{(2, 21)} = 5.764$; $p<0.05$), environment (morphine, saline: $F_{(1, 21)} = 18.799$; $p<0.01$), and treatment*environment interaction ($F_{(2, 21)} = 5.788$; $p<0.05$). Post hoc tests revealed that time

spent in the morphine environment in the PD168077 (50 ng/0.5 μL) group was significantly longer compared with saline ($p<0.01$), and compared with the time spent by the Veh group in the morphine environment ($p<0.05$) and the lower-dose groups ($p<0.01$). The other two groups failed to exhibit potentiated morphine sensitivity in the CPP test ($p>0.05$).

Figure 6. *Behavioural effects of bilateral medial prefrontal cortex dopamine D_4 receptor (mPFC D_4R) activation on fear response and morphine conditioned place preference (CPP) in subthreshold fear conditionings.*

*(**A**) In subthreshold fear conditioning, both the vehicle (Veh) and lower-dose PD168077 (5 ng/0.5 µL) treatments failed to induce strong associative fear memory. The higher dose of PD168077 (50 ng/0.5 µL) potentiated the salience of subthreshold fear stimuli; hence, animals in this group exhibited significant increased freezing percentage.*

*(**B**) Bilateral mPFC D_4R activation in subthreshold fear conditioning subsequently increased morphine preference in the higher-dose agonist group.*


A
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PD168977 5ng
PD168977 50ng
PD168077 dose: ng/0.5µL
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time spent (s)
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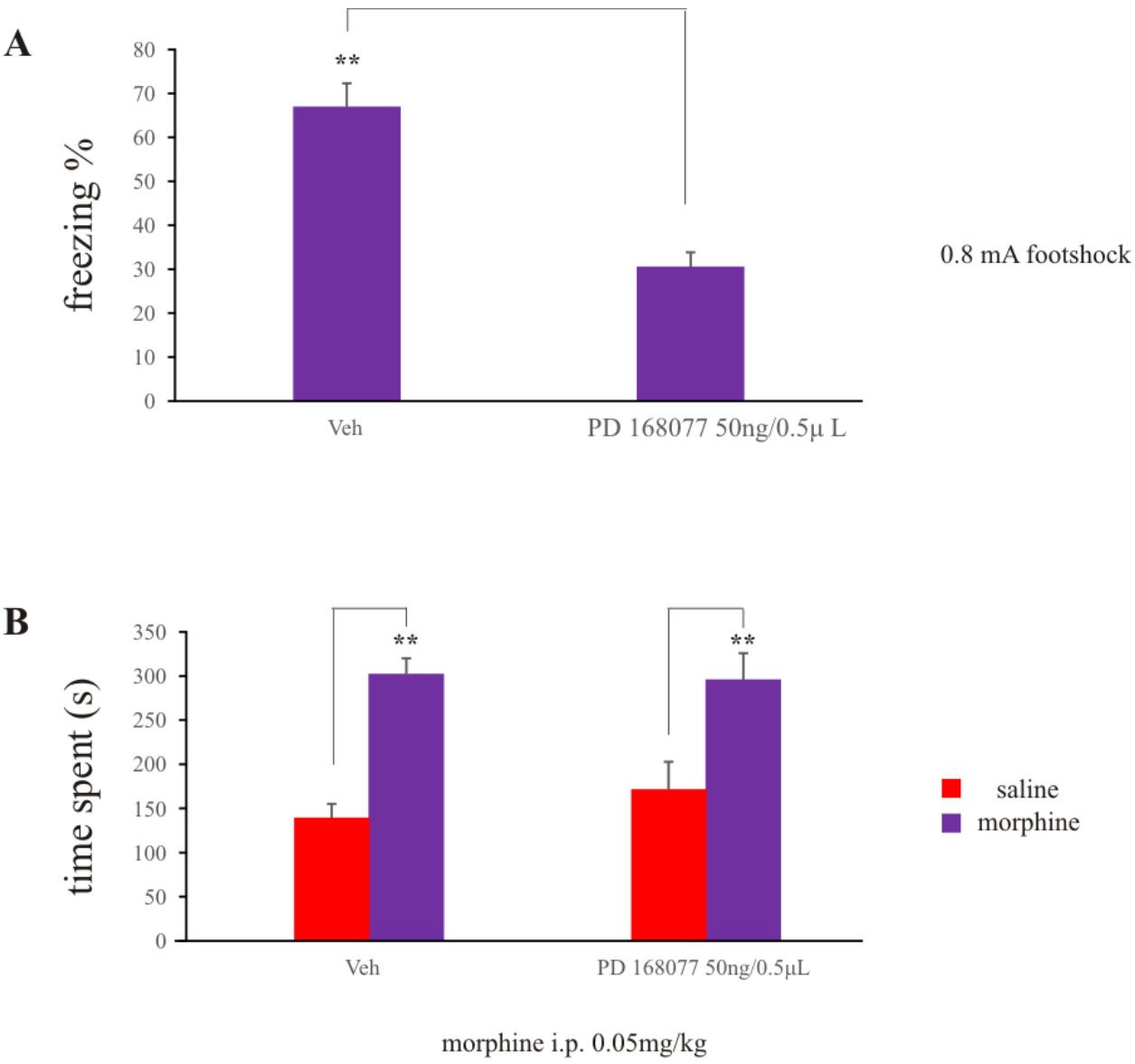
Veh
PD168977 5ng
PD168977 50ng
morphine i.p. 0.05mg/kg
saline
morphine

Figure 6

Next, we examined the effects mPFC D_4R activation on memory acquisition of in the supra-threshold fear conditioning, which assembles the higher baseline neuronal activity. In this experiment, the Veh and the treatment groups (n=8 in each group) received saline vehicle and the effective dose of PD168077 (50ng/0.5 µL) before receiving supra-threshold foot shocks (0.8 mA). The following fear memory test and CPP conditioning tests procedures were identical as previously described.

In supra-threshold fear conditioning, bilateral mPFC D_4R activation attenuated the acquisition of salient fear stimuli because the treatment group did not exhibit elevated freezing percentage compared with the Veh. In the CPP test, both groups exhibited significant morphine preference (**Figure 7 A** and **B**). Student's t test for fear conditioning indicated that the Veh group exhibited significantly higher freezing percentage compared with the treatment group ($p<0.01$). Two-way ANOVA of CPP test results revealed a significant main effect of environment (morphine, saline: $F_{(1, 14)} = 21.385$; $p<0.05$). Post hoc indicated both the Veh and treatment groups spent a significantly longer time in the morphine-paired context.

Figure 7. *Behavioural effects of bilateral medial prefrontal cortex dopamine D_4 receptor (mPFC D_4R) activation on the acquisition of supra-threshold fear stimulus and morphine sensitivity.*
*(**A**) In supra-threshold fear conditioning, bilateral mPFC D_4R activation attenuated the learning of an emotional salient fear stimulus; animals receiving PD168077 (50 ng/0.5 µL) demonstrated a significantly lower freezing percentage compared with the vehicle (Veh) group.*
*(**B**) Morphine conditioned place preference (CPP) tests revealed that both the Veh and treatment groups spent significantly more time in the morphine-paired environment.*


A
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Veh
PD 168077 50ng/0.5µ L
0.8 mA footshock
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350
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Veh
PD 168077 50ng/0.5µL
saline
morphine
morphine i.p. 0.05mg/kg

Figure 7

3.5 Bilateral mPFC D_4R and D_1R co-activation control the acquisition and expression of aversive and rewarding memory

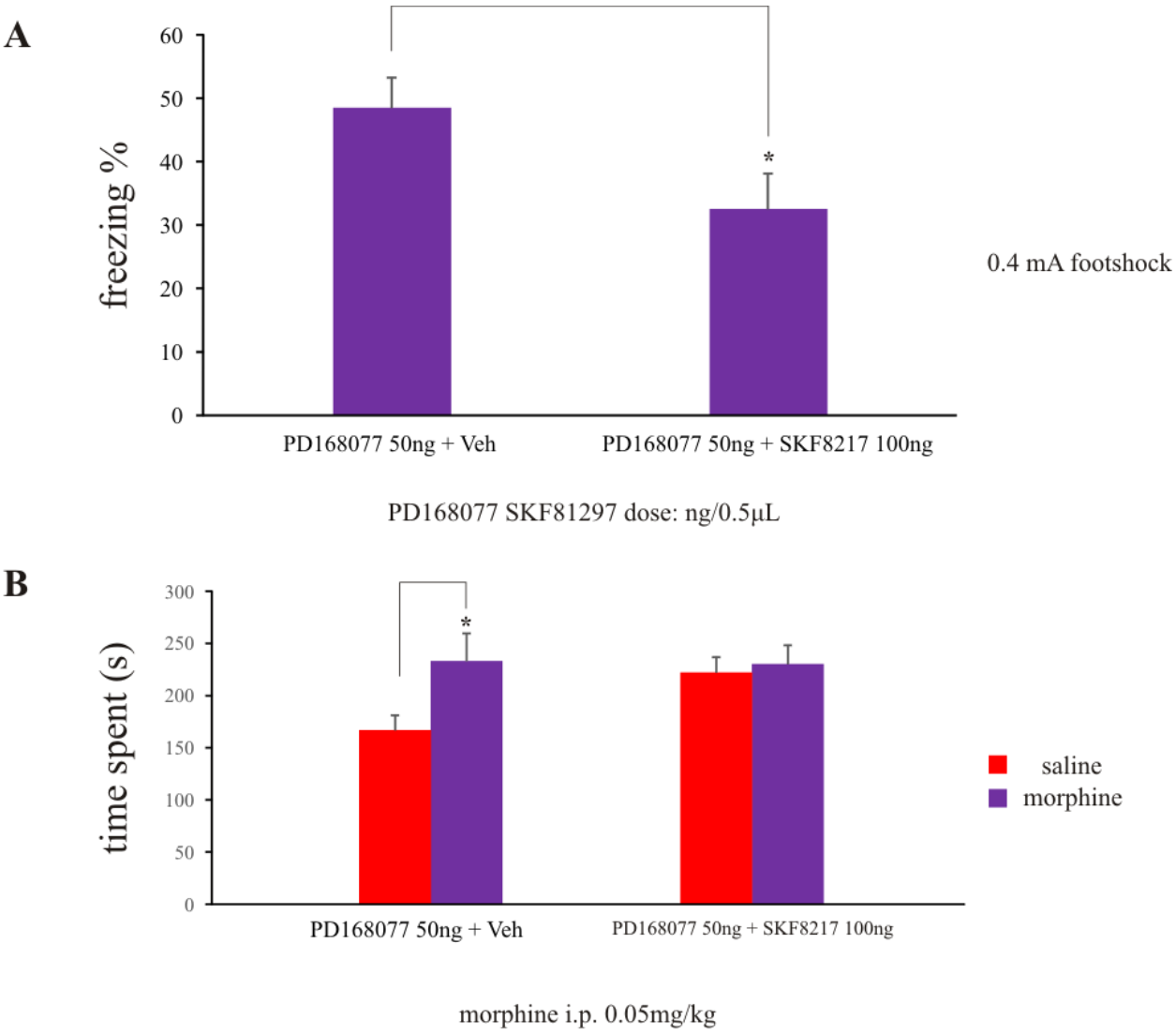
After investigating the role of D_4 and D_1 transmission, an attempt was made to link their functions. Using subthreshold fear conditioning, it was tested whether D_1R activation at the recall stage could block the expression of strong fear memory potentiated by D_4R stimulation during the conditioning stage, and if co-activation of both receptor systems could affect the animals' sensitivity to morphine. Two groups of animals both received the effective dose of D_4R agonist (PD168077 [100 ng/0.5 µL,]) before conditioning. The Veh group (n=8) received saline, and the treatment group (n=7) received the effective dose of D_1R agonist SKF81297 (100 ng/0.5 µL) before the fear test stage. CPP procedures were identical to those described previously.

Results revealed that bilateral mPFC D_4R activation at the conditioning phase potentiated the salience of subthreshold fear stimuli in the Veh group; mPFC D_1R activation at the recall phase was able to block the expression of potentiated fear memory. The CPP tests indicated that D_1R agonist treatment also blocked the expression of morphine preference (**Figure 8 A** and **B**). Student's t test revealed a significant decrease in freezing percentage in the treatment group compared with the Veh group ($p<0.05$). Two-way ANOVA of the CPP test revealed no significant effect. The Student's t test was used to analyze differences in the time spent in the morphine- and the saline-paired environments. In the Veh group, the animals spent significantly more time in the morphine-associated environment.

Figure 8. *Behavioural effects of bilateral medial prefrontal cortex dopamine D_4 receptor (mPFC D_4R) and dopamine D_1 receptor (D_1R) co-activation in subthreshold fear conditioning and morphine preference test.*

*(**A**) Activation of mPFC D_4R facilitated the acquisition of emotionally non-salient fear stimuli because the vehicle (Veh) group exhibited higher freezing percentages. Animals receiving the D_1R agonist SKF81297 (100 ng/0.5 µL) before the test phase blocked the expression of potentiated fear memory.*

*(**B**) The SKF81297 (100 ng/0.5 µL) group spent an equal length of time in the morphine- and saline-paired environments; the Veh group exhibited a strong morphine preference.*


A
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PD168077 50ng + Veh
PD168077 50ng + SKF8217 100ng
PD168077 SKF81297 dose: ng/0.5µL
0.4 mA footshock
B
time spent (s)
300
250
200
150
100
50
0
*
PD168077 50ng + Veh
PD168077 50ng + SKF8217 100ng
morphine i.p. 0.05mg/kg
saline
morphine

Figure 8

3.6 mPFC D_4R and D_1R stimulation increased phosphorylation of CaMKII and ERK1/2

There is substantial literature suggesting that D_4 and D_1 transmissions are closely linked to the activities of CaMKII and ERK1/2. To further investigate the molecular pathways associated with D_4 with D_1 transmission, western blot analysis were performed to examine phosphorylation levels of CaMKII-α and ERK1/2 following D_4R and D_1R stimulation. Three groups of animals received bilateral intra-mPFC infusion of saline (Veh [n=6]), SKF81297 (100 ng/0.5 µL [D_1R activation] [n=6]), and PD168077 (50 ng/0.5 µL [D_4R activation] [n=5]), followed by brain extraction as described in the previous section.

Results indicated that D_1R stimulation significantly increased phosphorylation levels of both ERK 1 and 2. Total ERK1 level was not affected by D_1R stimulation; however, total ERK 2 (tERK 2) was increased by SKF81297 infusion. Both pERK1/ tERK1 and pER2/ tERK2 were increased following D_1R stimulation (**Figure 9, Figure 10**).

One-way ANOVA revealed a significant main effect of treatment (saline Veh; SKF81297 [100 ng/0.5 µL]; PD168077 [50 ng/0.5 µL]) on pERK1 levels: ($F_{(2, 17)} = 4.10136$; $p<0.05$) and pERK2: ($F_{(2, 17)} = 5.44336$; $p<0.05$). Post hoc tests indicated that, compared with the Veh group and D_4R activation, D_1R stimulation led to a significant increase in pERK1 levels ($p<0.05$); the same trend was also observed in pERK2 post hoc analysis ($p<0.01$ vs Veh; $p<0.05$ vs PD168077 [50 ng/0.5 µL]).

One-way ANOVA revealed no significant change in tERK1 levels; however, a significant main effect of treatment on tERK2 level (saline Veh; SKF81297 [100 ng/0.5 µL]; PD168077 [50 ng/0.5 µL]: $F_{(2, 17)} = 6.00827$; $p<0.05$). Post hoc tests indicated that, compared with the Veh group, both D_1R and D_4R activation led to a significant reduction in tERK 2 levels ($p<0.05$ vs SKF81297; $p<0.01$ vs PD168077).

The ratios of pERK1 to tERK1, and pERK2 to tERK2, were also analyzed. One-way ANOVA revealed a significant main effect of treatment (saline Veh; SKF81297 [100 ng/0.5 µL]; PD168077 [50 ng/0.5 µL]) on pERK1/tERK1: ($F_{(2, 17)} = 3.645$; $p<0.05$) and pER2/tERK2 ($F_{(2, 17)} = 5.271$; $p<0.05$). Post hoc analysis indicated that both pERK1/tERK1 and pERK2/tERK2 ratios were significantly elevated in the SKF81297 (100 ng/0.5 µL) group compared with the Veh group.

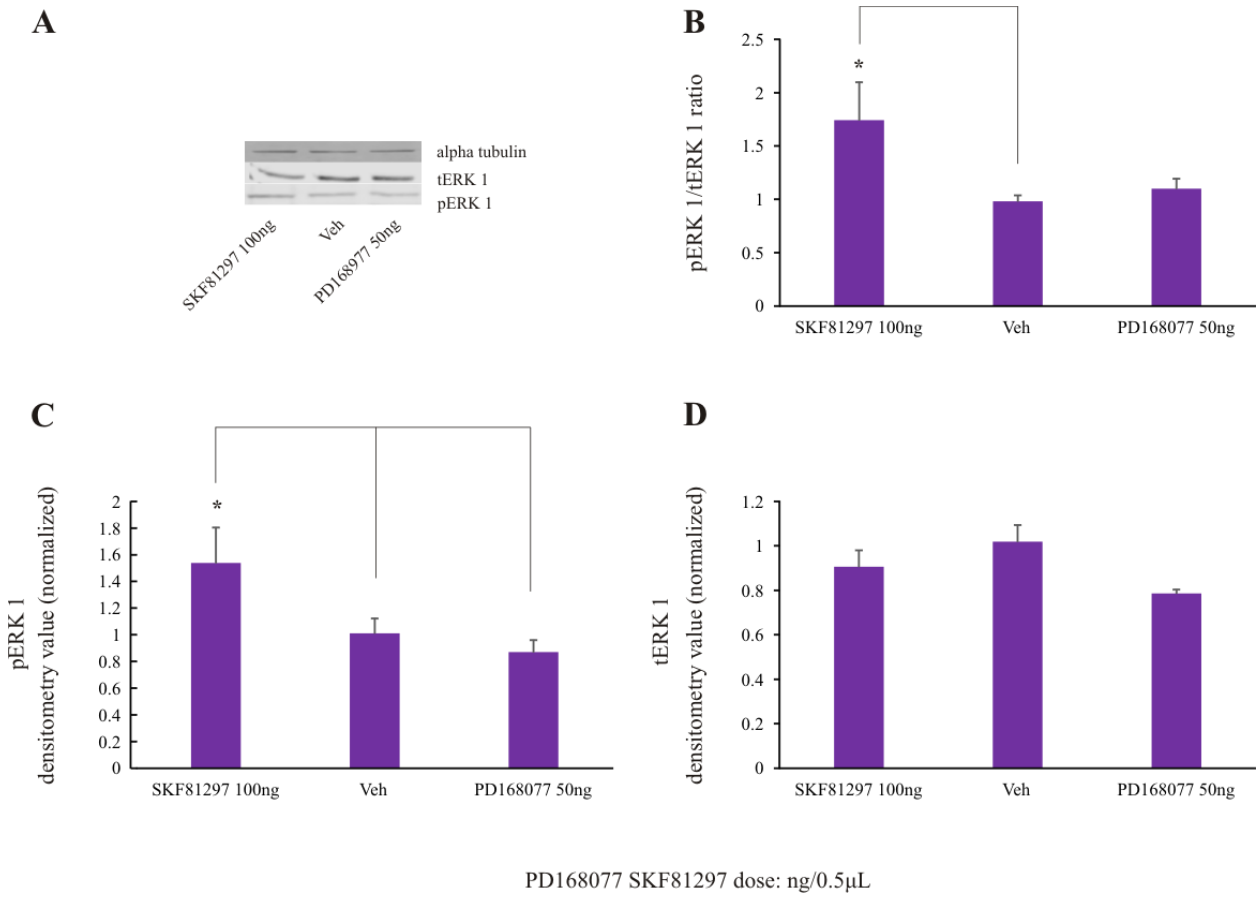
Figure 9. *Western blot analysis of ERK1 following bilateral microinfusions of saline, dopamine D_4 receptor (D_4R) agonist PD168077 (50 ng/0.5 µL)) and D_1R agonist SKF81297 (100 ng/0.5 µL) in to the medial prefrontal cortex (mPFC).*

(**A**) *Representative western blot for tERK1and pERK1 expression.*

(**B**) *The ratio of pERK1/tERK1 was increased in the group receiving SFK81297.*

(**C**) *Densitometry analysis revealed significant increase of pERK1 expression following D_1R activation.*

(**D**) *Densitometry analysis revealed no significant change in tERK1expression following D_1R activation.*



PD168077 SKF81297 dose: ng/0.5µL

Figure 9

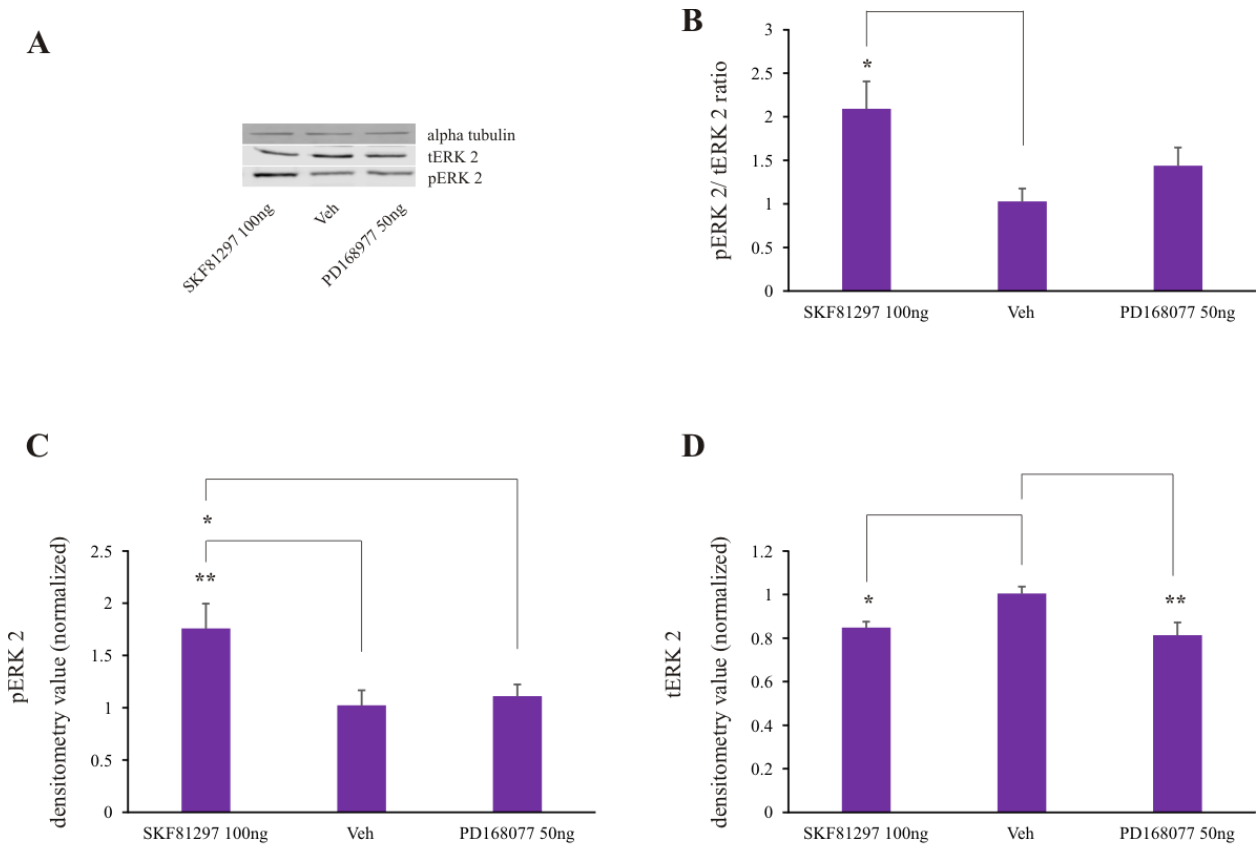
Figure 10 *Western blot analysis of ERK2 following bilateral microinfusions of saline, dopamine D_4 receptor (D_4R) agonist PD168077 (50 ng/0.5 µL)) and D_1R agonist SKF81297 (100 ng/0.5 µL) in to the medial prefrontal cortex (mPFC).*

*(**A**) Representative western blot for tERK2 and pERK2 expressions.*

*(**B**) The ratio of pERK2/tERK2 was increased in group received SFK81297.*

*(**C**) Densitometry analysis revealed significant increase of pERK2 expression following D_1R activation.*

*(**D**) Densitometry analysis revealed a significant reduction in tERK2 expression following D_1R and D_4R activation compared to the Veh group.*



PD168077 SKF81297 dose: ng/0.5µL

Figure 10

Following PFC D_4R stimulation, pCaMKII-α was significantly increased without changing the tCamKII-α expression level (**Figure 11**). One-way ANOVA revealed a significant main effect of treatment (saline vehicle, SKF81297 [100ng/0.5 µL], PD168077 [50ng/0.5 µL]) on pCaMKII-α expression ($F_{(2, 14)} = 6.56259$, $p<0.05$). Post hoc tests indicated that compare to Veh group, D_4R activation led to significant increase of pCaMKII-α level ($p<0.01$). One-way ANOVA revealed no significant change in tCaMKII-α level and pCaMKII-α/ tCaMKII-α ratio.

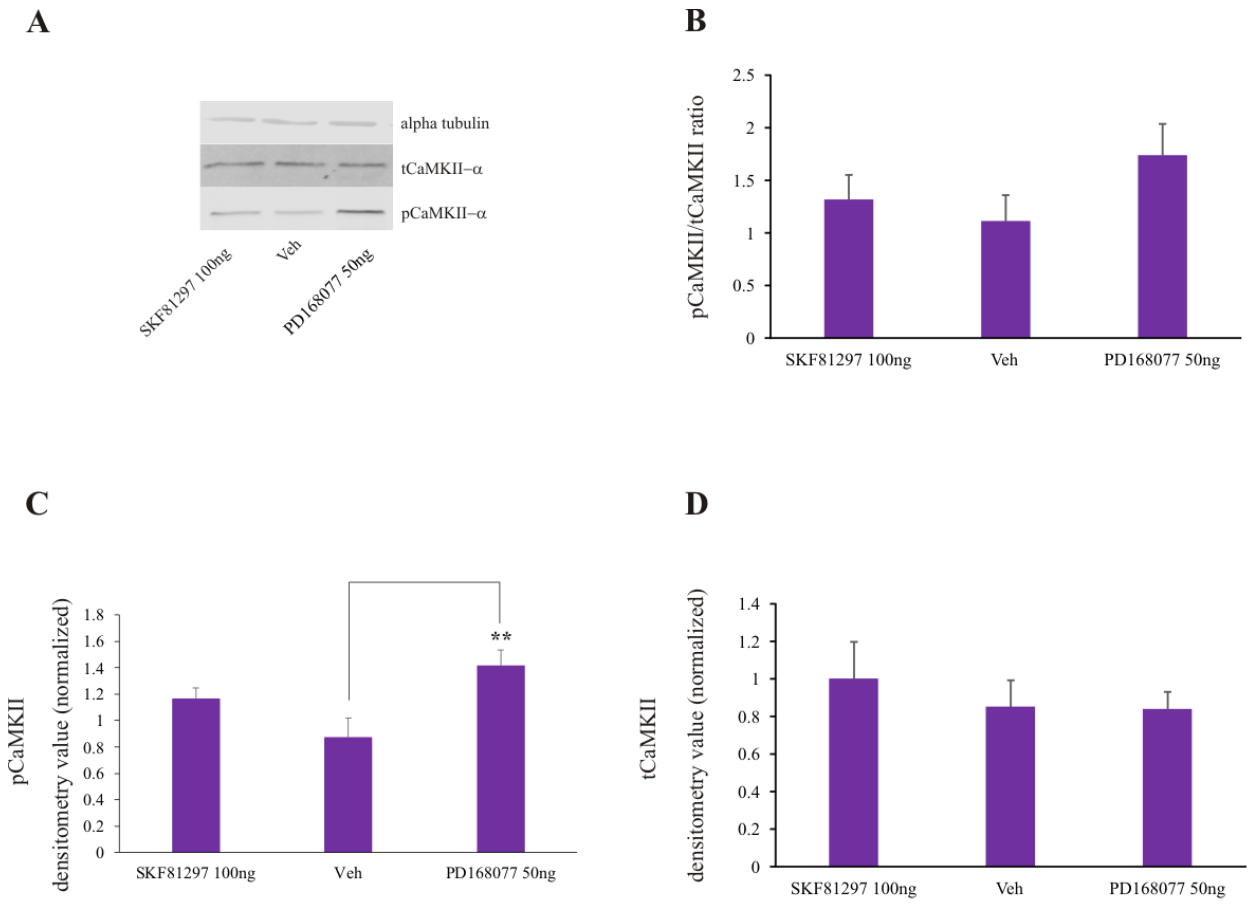
Figure 11 *Western blot analysis of CaMKII-α following bilateral microinfusions of saline, dopamine D_4 receptor (D_4R) agonist PD168077 (50 ng/0.5 µL)) and D_1R agonist SKF81297 (100 ng/0.5 µL) in to the medial prefrontal cortex (mPFC).*

*(**A**) Representative western blot for tCaMKII-α and pCaMKII-α expression.*

*(**B**) The ratio of pCaMKII-α/tCaMKII-α was not changed in all treatment groups.*

*(**C**) Densitometry analysis revealed a significant increase of pCaMKII-α expression following D_4R activation.*

*(**D**) Densitometry analysis revealed no significant change in tCaMKII-α expression following D_4R activation.*



PD168077 SKF81297 dose: ng/0.5μL

Figure 11

3.7 ERK1/2 inhibition reversed the effect of mPFC D_1R stimulation on fear memory expression and potentiation of morphine sensitivity

It was demonstrated that mPFC D_1R activation increased phosphorylation levels of ERK1/2, as well as attenuating both fear and rewarding memory expression at the behavioural level. It was then decided to test whether inhibition of ERK activity could affect the ability of D_1 to regulate fear and rewarding memory. In this experiment, two groups of rats received supra-threshold fear conditioning: one group received the ERK inhibitor U0126 (1000 ng/0.5 µL); the other group received co-administration of U0126 (1000 ng/0.5 µL) and SKF81297 (100 ng/0.5 µL). Bilateral intra-mPFC infusions were performed immediately before the fear recall phase. Two other groups, fear with cue and SKF81297 (100 ng/0.5 µL), were included in data analysis and figures for the purpose of comparison, all groups consisted of 8 animals.

Results demonstrated that bilateral mPFC D_1R activation with SKF81297 alone inhibited the aversive and morphine-related rewarding memory expression compared with the fear with cue group. U0126 alone had no effect on fear response and rewarding memory expression compared with the fear with cue group. Co-administration of ERK inhibitor and D_1R agonist reversed the effect of D_1 activation on aversive and rewarding memory blocking (**Figure 12 A & B**).

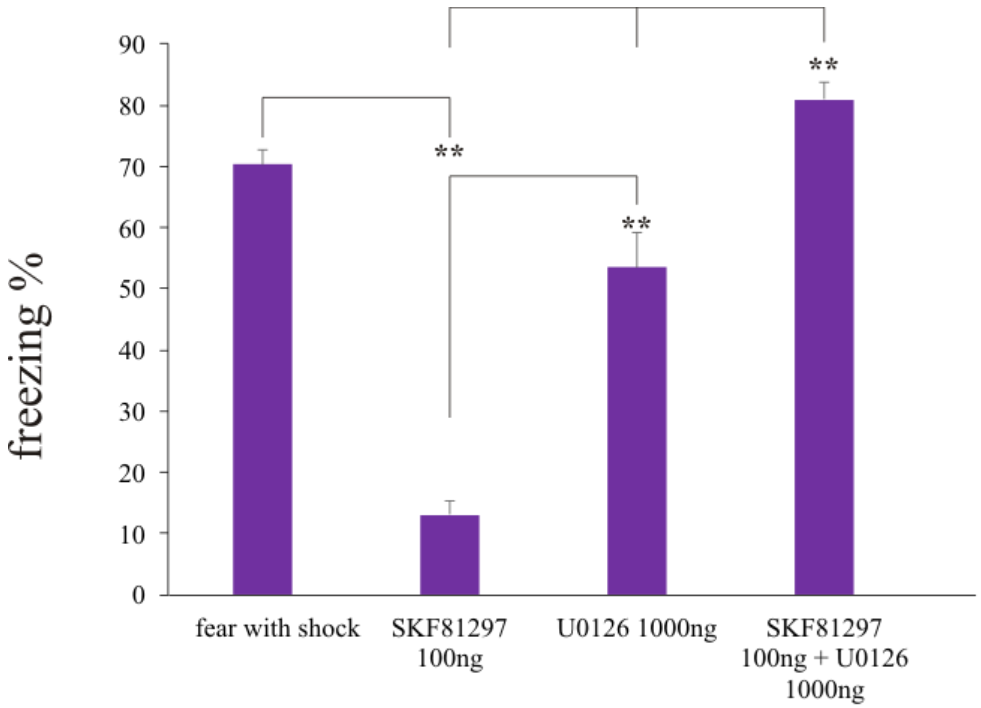
One-way ANOVA analysis of fear tests revealed a significant treatment effect (fear with cue; SKF81297 [100 ng/0.5 µL]; U0126 [1000 ng/0.5 µL]; and U0126 [1000 ng/0.5 µL] + SKF81297 [100 ng/0.5 µL]) on freezing percentage ($F_{(3, 31)} = 55.704$; $p<0.05$). Post hoc tests indicated that U0126 infusion alone had no effect on freezing behaviour compared with the fear with cue group ($p>0.05$). Animals in this group exhibited a significantly strong fear response after receiving 0.8 mA foot shocks compared with the SKF81297 infusion alone ($p<0.01$) and co-administration groups ($p<0.01$). Co-administration with SKF81297 fully reversed the effect of D_1R activation on memory expression: the animals exhibited significantly elevated levels of fear response compared with SKF81297 infusion alone ($p<0.01$), and no change in freezing percentage compared with the fear recall control group ($p>0.05$). Two-way ANOVA analysis of CPP tests revealed significant main effects of treatment (fear with cue, SKF81297 [100 ng/0.5 µL], U0126 [1000 ng/0.5µL], U0126 [1000 ng/0.5 µL] + SKF81297 [100 ng/0.5 µL]: $F_{(3, 30)} = 6.311$; $p<0.01$), environment (morphine, saline: $F_{(1, 30)} = 103.499$; $p<0.01$), and

treatment*interaction ($F_{(3, 30)} = 34.985$; $p<0.01$). Post hoc analysis indicated that U0126 infusion alone had no effect on morphine preference compared with the saline environment ($p>0.05$). Co-administration with SKF81297 reversed the effect of D_1R activation on morphine CPP blockade. Animals in this group spent significantly more time in the morphine-paired context ($p<0.01$). Compared with the morphine environment time-spent alone, the fear with cue group spent significantly more time in the morphine environment compared with the other 3 groups ($p<0.01$).

Figure 12. *Behavioural effects of bilateral medial prefrontal cortex (mPFC) ERK1/2 inhibition on dopamine D_1 receptor (D_1R) activation in supra-threshold fear conditionings and morphine conditioned place preference (CPP).*

(**A**) *In supra-threshold fear conditioning, the fear with cue group, U0126 treatment alone, and co-administration group, exhibited a strong fear response. SKF81297 infusion alone blocked fear memory recall, and the effect was reversed by co-infusion with U0126 (1000 ng/0.5 µL) because the co-administration group demonstrated significant higher freezing percentage compared with SKF81297 infusion alone.*

(**B**) *CPP tests revealed significant morphine preference in fear with cue and co-infusion groups. Animals in the fear with cue group spent more time in the morphine-paired environment compared with the other 3 groups.*



0.8 mA footshock

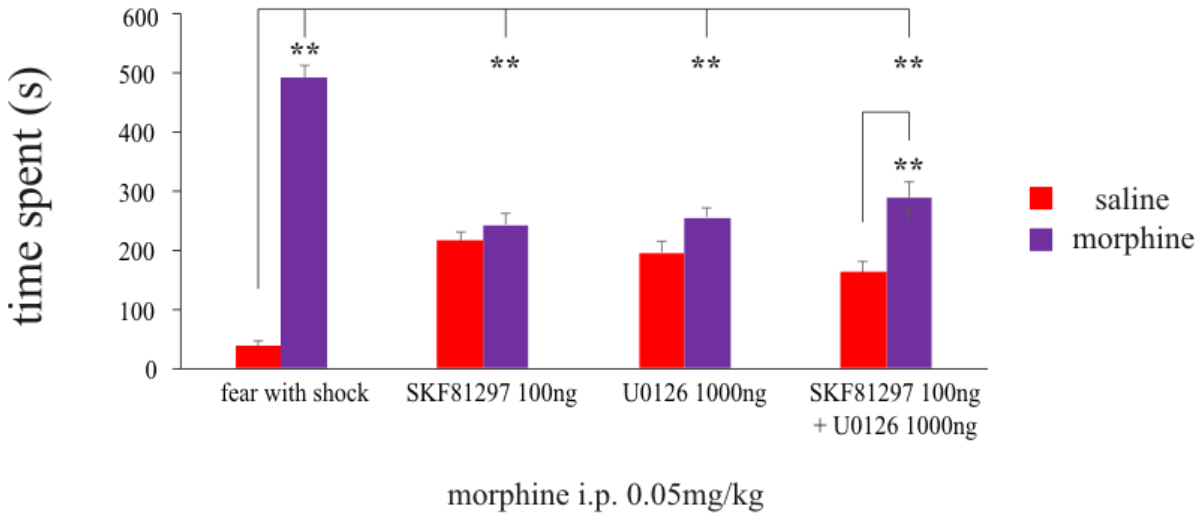


Figure 12

3. 8 CaMKII inhibition reversed the effects of mPFC D_4R activation on fear memory acquisition and decreased potentiated morphine sensitivity

Western blot results indicated increased levels of CaMKII-α phosphorylation following D_4R activation. It was then examined whether inhibition of CaMKII activity in the mPFC could reverse the effect of D_4R activation on fear and rewarding memory regulation. In this experiment, two groups of animals received subthreshold fear conditioning: the first group (n=8) received the CaMKII inhibitor AIP (500 ng/0.5 µL); the other group (n=8) received co-administration of AIP (500 ng/0.5 µL) and PD168077 (50 ng/0.5 µL). Bilateral mPFC infusions were performed immediately before the conditioning phase. The two other groups from the subthreshold fear conditioning experiment (Veh control and PD168077 [50 ng/0.5 µL]) were included in data analysis for comparative purposes.

The fear conditioning results indicated that PD168077 infusion alone potentiated the acquisition of subthreshold fear stimuli and sensitivity to morphine's rewarding effects, AIP infusion alone had no effect on fear acquisition and morphine preference compared with the Veh group. Co-infusion of AIP and PD168077 reversed the effect of mPFC D_4R stimulation on the learning of non-saline fear stimuli because animals in this group failed to exhibit a strong fear response in comparison. This treatment also blocked potentiated morphine CPP (**Figure 13 A** and **B**).

One-way ANOVA test for fear recall indicated a significant main effect of treatment (Veh; PD168077 [50 ng/0.5 µL]; AIP [500 ng/0.5 µL]; and AIP [500 ng/0.5 µL] + PD168077 [50 ng/0.5 µL]) on freezing behaviour ($F_{(3, 33)} = 21.417$; $p<0.01$). Post hoc analysis revealed that, compared with the Veh group, only PD168077 infusion alone elicited a significant increase in freezing percentage ($p<0.01$). Moreover, the amount of elevated fear response was significantly higher compared with the AIP infusion alone and co-infusion groups ($p<0.05$). Two-way ANOVA analysis of CPP results revealed a significant main effect of environment (morphine, saline: $F_{(1, 30)} = 22.425$; $p<0.01$) and significant treatment*environment interaction ($F_{(3, 30)} = 3.607$; $p<0.05$). Post hoc tests revealed that PD168077 infusion alone significantly increased morphine preference compared with the saline environment ($p<0.05$). The time spent in the morphine environment in this group was also significantly longer than the other 3 groups ($p<0.05$).

Figure 13 *Behavioural effects of bilateral medial prefrontal cortex (mPFC) CaMKII inhibition on dopamine D_4 receptor (D_4R) activation in subthreshold fear conditionings and morphine conditioned place preference (CPP).*

*(**A**) In subthreshold fear conditioning, the Veh group, the group received AIP alone and the co-administration group failed to show strong fear response; PD168077 (50ng/0.5 µL) infusion alone potentiated the acquisition of subthreshold fear stimuli.*

*(**B**) CPP tests revealed significant morphine preference only in groups received PD168077 alone, animals in this group spent more time in the morphine-paired environment compared with the other 3 groups.*

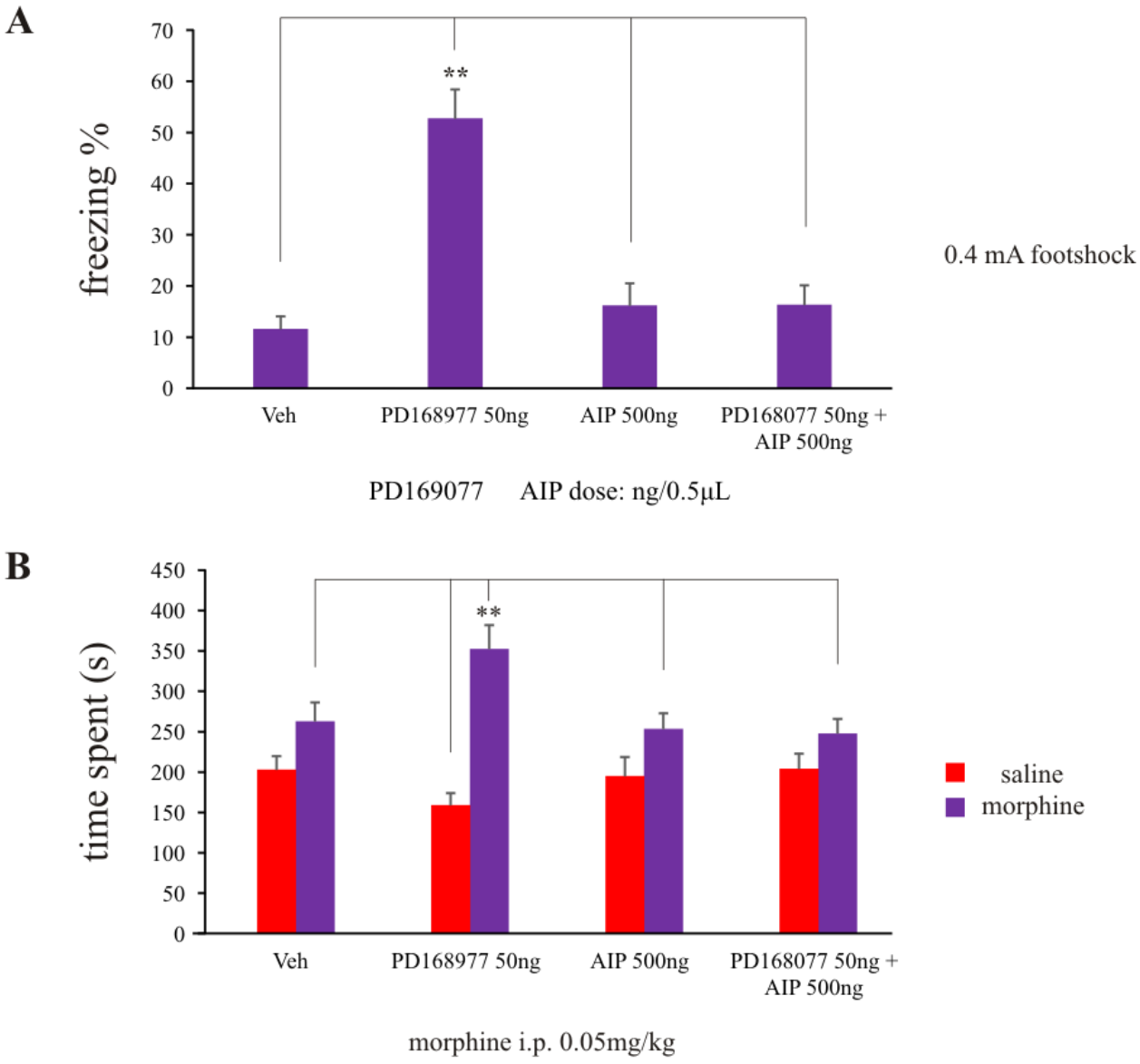

A
freezing %
70
60
50
40
30
20
10
0
**
Veh
PD168977 50ng
AIP 500ng
PD168077 50ng + AIP 500ng
0.4 mA footshock
PD169077 AIP dose: ng/0.5µL
B
time spent (s)
450
400
350
300
250
200
150
100
50
0
**
Veh
PD168977 50ng
AIP 500ng
PD168077 50ng + AIP 500ng
saline
morphine
morphine i.p. 0.05mg/kg

Figure 13

3.9 VTA mediates potentiated morphine sensitivity in a post-traumatic stress disorder rat model

Research in past decades has established the role of the VTA in regulating the rewarding effects of addictive drugs such as morphine and heroin. An attempt was made to investigate whether the VTA is also the brain region that regulates potentiated morphine sensitivity after the animals recall an associative fear memory. Four groups of animals were recruited, all of which underwent quartet cannulations: bilateral in the mPFC; and bilateral in the VTA. In these two experiments, bilateral intra-VTA morphine infusions (250 ng/0.5 µL) were performed before morphine conditioning, instead of i.p. administration of morphine; bilateral intra-VTA saline infusions were given before the saline conditioning sessions.

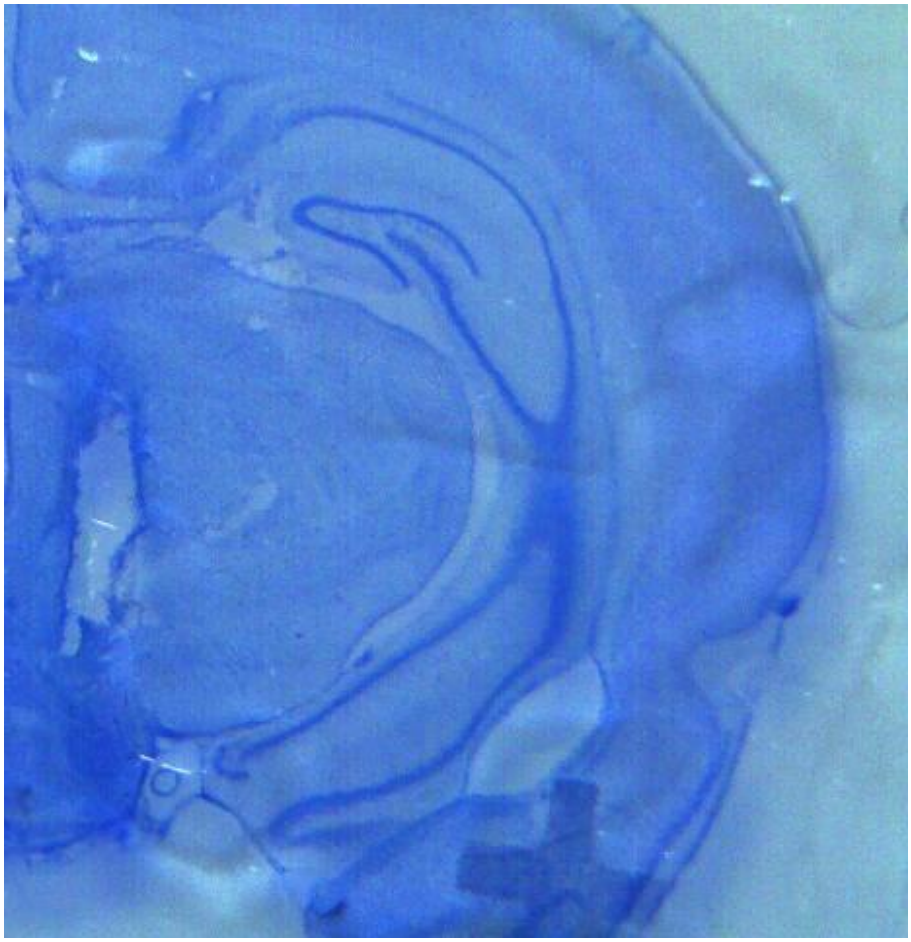
Two groups were included in supra-threshold fear conditioning: the Veh control group (n=7) received saline; and the treatment group (n=6) received the D_1R agonist SKF81297 (100 ng/0.5 µL). Bilateral intra-mPFC infusions were administered before the fear test phase. The remaining two groups were included in subthreshold fear conditioning. Intra-mPFC infusions were administered before the fear conditioning stage, in which the Veh control group (n=7) received saline and the treatment group (n=8) received the D_4R agonist PD 168077 (50 ng/0.5 µL). A representative microphotograph of bilateral intra-VTA microinjector tip placements is presented in **Figure 14 A**. A schematic representation of bilateral intra-VTA injector tip placements is shown in **Figure 14 B**.

Figure 14 *Presentation of intra-VTA guide cannulae placements*

*(**A**) Microphotograph of a representative bilateral intra- VTA guide cannulae and injector tip placement.*

*(**B**) Schematic representation of bilateral intra-VTA injector tip placements.* ○= *Veh control group (sub-threshold fear conditioning),* ●= *PD168077 50ng/0.5ul,* □= *Veh control group (supra-threshold fear conditioning),* ■= *SF81297 100ng/0.5ul*

A



B

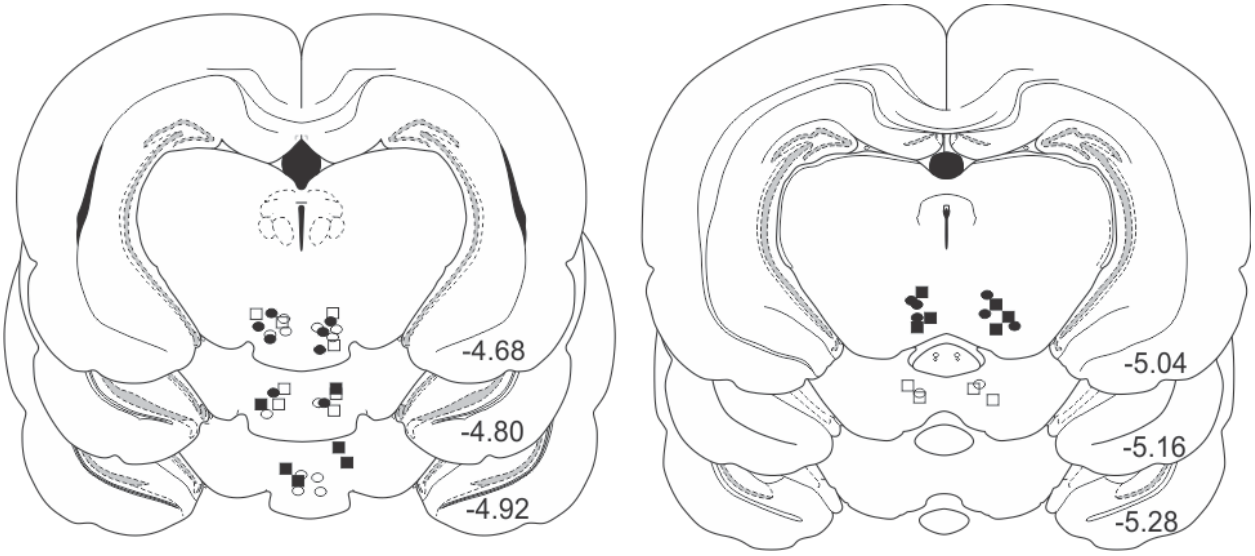


Figure 14

In supra-threshold fear conditioning, bilateral infusions of intra-mPFC SKF81297 decreased expression of a strong associative fear memory compared with the Veh group. Bilateral intra-VTA infusions of morphine following aversive memory recall potentiated morphine preference only in the Veh group but not the treatment group (**Figure 15 A** and **B**). In subthreshold fear conditioning, bilateral infusions of mPFC PD168977 potentiated the salience of subthreshold fear stimuli; the animals exhibited heightened fear response compared with the Veh group. Intra-VTA morphine infusions potentiated the rewarding effects of subthreshold-dose morphine, animals in the treatment group exhibited a strong morphine preference in the CPP test (**Figure 15 C** and **D**).

Student's t test results for supra-threshold fear recall demonstrated a significantly decreased freezing percentage in the treatment group ($p<0.01$). Two-way ANOVA revealed a significant main effect of environment (morphine, saline: $F_{(1, 11)} = 12.157$; $p<0.01$). Post hoc tests indicated that the Veh group spent significantly more time in the morphine-paired environment than the saline environment ($p<0.01$). Student's t test results for subthreshold fear recall revealed significant increases in freezing percentage in the treatment group ($p<0.01$). Two-way ANOVA analysis of the CPP test indicated a significant main effect of environment (morphine, saline: $F_{(1, 13)} = 20.612$; $p<0.01$) and a significant treatment (saline: PD168077 [50 ng/0.5 µL]) * environment interaction ($F_{(1, 13)} = 8.542$; $p<0.05$). Post hoc analysis revealed that in the treatment group, the animals spent significantly more time in the morphine-paired environment compared with the saline environment ($p<0.01$), as well as compared with time spent by the Veh group in the morphine environment ($p<0.01$).

Figure 15 *Behavioural effects of bilateral intra-VTA morphine infusions follow medial prefrontal cortex (mPFC) dopamine D_1 receptor (D_1R) or dopamine D_4 receptor (D_4R) activation in the supra-threshold or the subthreshold fear conditioning.*

*(**A**) Bilateral mPFC infusions of D_1R agonist, SKF81297 (100ng/0.5 µL), fully blocked the fear memory recall; compared to the Veh group, the treatment group displayed significant less freezing percentage.*

*(**B**) Bilateral intra-VTA morphine infusions followed fear memory recall potentiated morphine sensitivity and preference only in the Veh group.*

*(**C**) Bilateral mPFC infusions of D_4R agonist, PD168077 (50ng/0.5 µL), potentiated the acquisition of subthreshold fear stimuli; compared to the Veh group, the treatment group displayed significant higher freezing percentage.*

*(**D**) Bilateral intra-VTA morphine infusions followed fear memory recall potentiated morphine sensitivity and preference in the treatment group.*

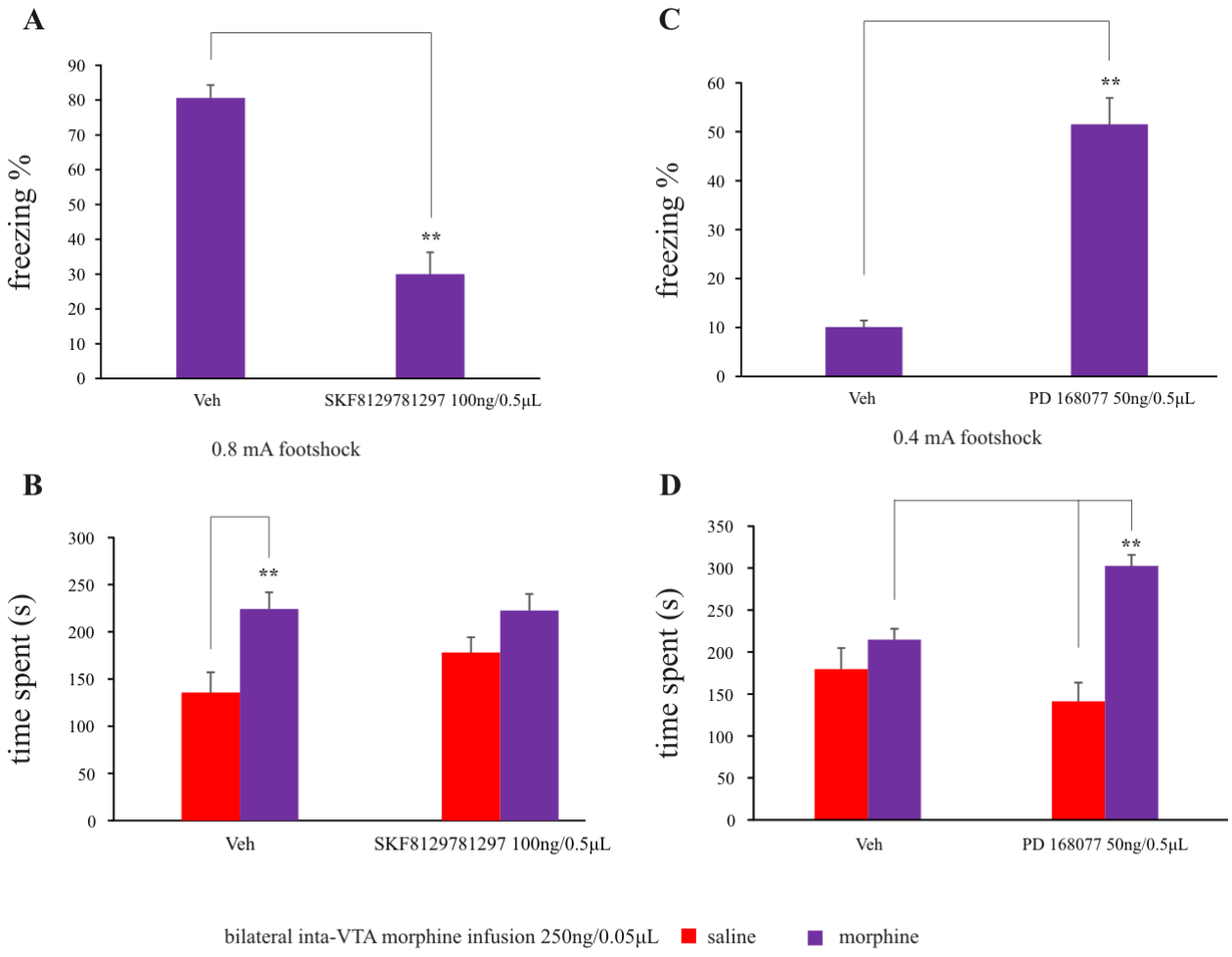

A
freezing %
90
80
70
60
50
40
30
20
10
0
**
Veh
SKF8129781297 100ng/0.5µL
0.8 mA footshock
C
freezing %
60
50
40
30
20
10
0
**
Veh
PD 168077 50ng/0.5µL
0.4 mA footshock
B
time spent (s)
300
250
200
150
100
50
0
**
Veh
SKF8129781297 100ng/0.5µL
D
time spent (s)
350
300
250
200
150
100
50
0
**
Veh
PD 168077 50ng/0.5µL
bilateral inta-VTA morphine infusion 250ng/0.05µL
saline
morphine

Figure 15

4 Discussion

Disturbances in mesocorticolimbic dopamine (DA) transmission are well-established features of comorbid neuropsychiatric disorders involving pathological memory processing, such as post-traumatic stress disorder (PTSD) and opioid addiction. Transmission through PFC DA D_4 receptors (D_4R) has been shown to potentiate the emotional salience of normally non-salient emotional memories whereas transmission through PFC DA D_1 receptors (D_1R) has been demonstrated to selectively block the recall of either reward or aversion-related associative memories. In the present study, using a combination of fear conditioning and opiate reward place conditioning in rats, we examined the role of PFC D_4R/D_1R signaling during the processing of fear-related memory acquisition and recall as well as subsequent sensitivity to opiate reward memory formation. We report that PFC D_4R activation potentiates the salience of normally subthreshold fear conditioning memory cues and simultaneously strongly potentiates the rewarding properties of sub-reward threshold systemic or intra-ventral tegmental area (VTA) morphine conditioning cues. In contrast, selectively blocking the recall of salient fear memories with intra-PFC D_1R activation, blocks the ability of fear memory recall to potentiate systemic or intra-VTA morphine reward salience. These effects are dependent upon dissociable PFC phosphorylation activation states of either calcium-calmodulin-kinase II (CaMKII-α) or extracellular-signal-related-kinase 1/2 (ERK 1/2), following intra-PFC D_4R or D_1R activation, respectively. Together, these findings reveal critical new insights into how aberrant PFC DAergic transmission and downstream molecular signaling pathways may modulate fear-related emotional memory encoding and recall, and how these effects may increase opioid addiction liability during emotional memory recall in traumatic memory disorders such as PTSD.

In summary, the present thesis links the processing of associative fear and rewarding memory to a common, DA D_1 and D_4 transmission directly in the PFC; and demonstrates dynamic interactions between cue-triggered fear memory recall and the potentiation of morphine reward salience. In addition, our results revealed novel roles for D_1R and D_4R-linked signaling through the cAMP, ERK 1/2 and CaMKII pathways, in the context of PFC DAergic regulation of fear and morphine-related reward memory processing.

4.1 Fear memory recall strongly regulates opiate reward salience

Using olfactory fear conditioning combined with morphine CPP, a novel connection between fear memory recall and the perception of morphine rewarding salience was identified. Specifically, while subthreshold conditioning doses of morphine did not induce morphine preference in rats that were not exposed to associative, fear-related recall cues, the cue-induced recall of a previously established associative fear memory induced a robust morphine reward CPP. It is known that various types of stressors, such as social defeat, food restriction, predator threat are capable of reinstating drug taking behaviours in rats (Edward et al., 2013; Do Couto et al., 2006; Shalev 2012). Here, we demonstrated that fear memory recall alone is sufficient to potentiate the reward salience of normally non-rewarding conditioning doses of morphine.

Chronic or acute stress leads to alterations in brain function especially in the mPFC region, including abnormal signal transduction in the mPFC-amygdala pathway (Edwards et al., 2013; Knox et al., 2010; Milad & Quirk 2002). Furthermore, cue-triggered fear memory recall also alters activities in various brain regions, such as the PFC, amygdala and hippocampus (Milad et al., 2007). Nevertheless, the PFC is a vital brain region regulating inhibitory control as well as executive and cognitive functions and is influenced by DA transmission in the mesocorticolimbic DA pathway.

In the present thesis, I found that increasing mPFC D_1 transmission inhibited fear memory recall as well as blocked the potentiation of morphine CPP reward memories. Functionally, mPFC D_1R- mediated transmission increases the excitability of inhibitory GABA interneurons, which in turn may decrease the excitatory tone of PFC pyramidal neurons, increasing feedforward inhibition, which blunts the recall of the associative emotional memories (Lauzon et al., 2013; Seamans, Gorelova, Durstewitz & Yang, 2001). Similar results have been reported previously wherein stimulation of mPFC D_1R impairs the recall of aversive fear memories (Lauzon et al., 2009). Thus, mPFC D_1 receptors play a vital role in emotional memory recall. While there is currently direct clinical evidence for PFC D_1R abnormalities in PTSD clinical populations, one possibility is that chronic PTSD leads to long-term abnormalities in cortical D_1R activation states (or downstream signaling pathways such as cAMP or ERK), leading to abnormal recall of either trauma or addiction-related associative memories.

D_1R- mediated transmission is functionally linked to downstream cAMP activity (Lauzon et al., 2013). In the present thesis, I found that cAMP inhibition fully reversed the effects of mPFC D_1R on aversive memory blockade, but interestingly, had no effect on morphine reward memory recall, suggesting a dissociation in the molecular substrates controlling aversion memory recall vs. reward-related memory recall, directly in the PFC. Previous evidence has demonstrated that the ability of mPFC D_1 transmission block memory recall is due to downstream adenosine cyclase activity, increasing cAMP and protein kinase A (PKA) levels, which in turn increase cortical GABA interneuron activity levels (Lauzon et al., 2013; Seamans et al., 2001). Given the fact that PFC neurons store associative memories linked to emotionally salient events, increasing cAMP/PKA levels through D_1R activation might increase levels of feedforward inhibition on PFC pyramidal neurons, thereby attenuating the ability to recall an associative fear memory. Interestingly, in contrast to the present findings, Lauzon et al. (2013) reported that PFC cAMP inhibition blocked the recall of morphine CPP following a standard 8-day morphine CPP conditioning experiment. However, these differences may be due to experimental paradigm differences. First, Lauzon et al. (2013) used a longer term (8 day) morphine CPP paradigm with a supra-threshold conditioning dose of morphine (5 mg/kg) and rats did not go through a fear conditioning procedure prior to CPP training. In addition, as we have demonstrated in our first experiment, recalling fear memory potentiated morphine reward salience, which in turn may make morphine reward CPP memory more resistant to recall inhibition. In addition, the novel behavioural paradigm we used in this project examined the direct connection between a fear memory and morphine sensitivity, it is possible that the input of other molecular activities is required in this behavioural model. Interestingly, Lauzon et al. (2013) also reported that cAMP inhibition failed to block a morphine priming induced CPP memory recall (only spontaneous memory recall), which is indeed, consistent with the present findings showing that cAMP signaling may be only partially responsible for morphine-related memory processing.

DA D_1 and D_4 transmission in the PFC is known to produce biphasic effects. Thus, whereas D_1 transmission increases intrinsic inhibitory tone, D_4 transmission increases the excitability of PFC neurons and feedforward output (Seamans et al., 2001). In our experiments, we observed similar results, demonstrating that in the subthreshold fear conditioning condition, increased PFC D_4 transmission potentiated the salience of normally non-salient fear stimuli, as

well as potentiated the rewarding salience of the sub-threshold conditioning dose of morphine. Given the fact that D_4Rs are found on both interneurons and the pyramidal neurons, with a preferential location on the GABA interneurons in the mPFC, it is not surprising these receptors are functionally positioned to modulate neuronal activity by mediating local interneuron feedforward levels (Seamans et al., 2001; Wang et al., 2003). Specifically, D_4R activation has been shown to reduce GABA interneurons activity, causing a net decrease in the inhibitory interneuron output as well as potentiation of PFC pyramidal neurons activity levels concomitant with increasing AMPA receptor expression levels (Yuen & Yan 2009). Given the well-established role of AMPA receptor-mediated transmission in learning and memory as well as synaptic plasticity, it is perhaps the ability of PFC D_4R activation to potentiate AMPA PFC transmission and/or modulate the magnitude of excitatory inputs from other brain areas, such as the VTA and BLA is responsible for the potentiation of associative emotional stimuli salience. Thus, increasing PFC D_4R activities might facilitate the acquisition of both aversive and rewarding associative memories, as suggested by the present findings. Using in vitro and in vivo techniques Onne, Wang, Lin and Grace (2005) and Laviolette et al. (2005) both reported that blocking D_4R results in alterations in PFC pyramidal neuron activity levels through GABA transmission, as well as blocking the formation of associative memory related to cue-paired foot shock.

In contrast with our findings with subthreshold fear learning, we found that PFC D_4R stimulation resulted in blunting the learning of a salient fear stimuli during supra-threshold fear conditioning but did not interfere with the potentiation of morphine CPP. Some possible reasons for this might due to the ability of D_4 transmission to decreased CaMKII phosphorylation levels in higher neuronal activity states, which in turn might block formation of normally salient associative fear memories as previously reported (Lauzon et al., 2009). Interestingly, we found that D_4R activation induced blocking of supra-threshold fear memory did not block the potentiation of morphine reward salience. In this experiment, we observed potentiated morphine rewarding salience even though the formation of the fear memory had been blocked by D_4R activation. The reasons for this dichotomy are not clear, however one possibility is that given that supra-threshold fear conditioning would expect to induce a state of high PFC neuronal activity and inhibition of CaMKII levels, the presence of exogenous opioid molecules may have increased mesocorticolimbic DA transmission, thus overriding the effects of decreased CaMKII

levels in the PFC. Alternatively, morphine-induced increased DA transmission to other brain areas (e.g. the BLA or nucleus accumbens) may be able to compensate for the effects of PFC CaMKII reduction associated with the blockade of fear memory formation, allowing for the formation of associative opiate reward memories, independently of fear memory processing. Regardless, this data, similar to the findings observed with concomitant cAMP inhibition and D_1R activation, suggests a functional dissociation between PFC D_4R activation and the potentiation of morphine reward salience, in the context of supra-threshold fear conditioning. Future studies are required to more precisely delineate the underlying mechanisms responsible for these dissociable effects.

4.2 mPFC D_1R and D_4R transmission: functional links to ERK1/2 and CaMKII activity states

A series of western blot analyses were conducted to explore the possible effects of D_1R or D_4R activation on total and/or phosphorylation state changes in the ERK1/2 or CaMKII-α signaling pathways directly in the PFC. First, we found that intra-PFC D_1R activation significantly increased phosphorylation levels of ERK1/2, as well as the pERK/tERK ratio. These findings are consistent with previous reports showing that D_1R agonist activation with SKF81297 can increase ERK1/2 phosphorylation in the striatum and the mPFC, whereas D_2R activation has no effect (Xue et al., 2015). Research has also shown that psychostimulants which act as indirect DA agonists, such as cocaine or amphetamine, potently increase ERK1/2 phosphorylation levels in the mPFC as well as other brain areas, such as the striatum. (Fumagalli et al., 2009; Mao et al., 2013; Valjent et al., 2004). Thus, ERK1/2 phosphorylation states are known to be modulated through a D_1R-dependent pathway. We therefore examined the possible mechanistic role of ERK signaling in our observed behavioural effects of D_1R activation to determine if inhibiting ERK1/2 signaling may reverse the effects of D_1R activation on aversive memory recall and morphine reward sensitivity. Interestingly we found that pharmacological ERK1/2 inhibition with U0126, fully reversed the effects of mPFC D_1R stimulation on both rewarding and aversive memory recall inhibition, such that co-administration with the ERK inhibitor blocked D_1R mediated suppression of fear memory recall and the subsequent formation of a morphine reward CPP, demonstrating a functional role between intra-PFC D_1R activation and control of both aversive or rewarding memory recall effects. We also observed that ERK1/2 inhibition alone was able to partially restore the recall of fear-related memory, but had no effect

on the processing of morphine-related reward memory. These findings are consistent with previous behavioural pharmacological studies. For example, Valjent et al. (2006) reported that administration of the ERK1/2 inhibitor U0126 blocked the recall of previously robust cocaine reward CPP. Gholizadeh et al. (2013) further illustrated that intra- BLA and intra- mPFC local inhibition of ERK1/2 blocked the recall of recent and remote morphine CPP, respectively. In addition, research has reported that ERK1/2 regulates fear memory processing, as stress-related cues induce hyper-phosphorylation of ERK1/2 whereas blocking ERK1/2 was shown to inhibit the behavioural manifestation of conditioned fear responses (Cestari et al., 2013; Yang, Huang & Hsu, 2004). Thus, given the fact that the ERK1/2 pathway is involved in both reward and fear memory processing, it is not surprising that blocking this pathway impaired subject's ability to retrieve emotional memory as observed in both fear and reward conditioning processes. In contrast to our observed effects on ERK 1/2 phosphorylation states, both PFC D_1R and D_4R activation produced slight, but significant decreases in total levels PFC ERK 2, while D_4R activation caused a slight but significant decrease in PFC ERK 1 expression levels. Nevertheless, changes in total ERK 1/2 expression levels mediated by PFC D_4R signaling do not appear to modulate ERK 1/2 phosphorylation states as we observed no concomitant changes in PFC D_4R-mediated ERK 1/2 phosphorylation levels. While beyond the scope of the present thesis, future studies are required to more fully explore the possible functional effects of D_4R activation on total ERK 1/2 expression levels within the PFC.

In addition to our findings with the ERK 1/2 pathway, we observed significant increases in CaMKII-α signaling selectively following mPFC D_4R stimulation. Specifically, we observed a significant and selective increase in PFC expression levels of pCaMKII-α with no corresponding changes in total CaMKII-α expression level. Pharmacological inhibition of the phosphorylation process by treatment with a selective CaMKII phosphorylation inhibitor, AIP, reversed the effects of D_4R activation in the subthreshold fear conditioning and morphine CPP paradigms. Based upon the ability of D_4R activation to increase pCaMKII-α during subthreshold fear conditioning, the present results suggest that CaMKII is a plausible molecular mechanism underlying D_4R- mediated potentiation of non-salient fear and rewarding stimuli. Notably, treatment with the CaMKII inhibitor alone failed to potentiate fear and rewarding memory acquisition, which suggests that pCaMKII itself is not sufficient to facilitate the acquisition of

emotional memory; rather, consistent with *in vitro* work from Gu & Yan (2004), intra-mPFC CaMKII signaling is believed to depend upon the functional input of D_4R substrates for the regulation of perceived emotional memory salience.

4.3 Role of the VTA in PFC-mediated modulation of fear and reward memory processing

Given the importance of connectivity between the PFC and VTA in the mediation of both fear and reward-related associative memory (Lauzon et al., 2009; Bishop, Lauzon, Bechard, Gholizadeh & Laviolette, 2011), we examined if our observed modulation of PFC D_1R and D_4R transmission on opiate reward sensitivity may be mediated directly in the VTA. Decades of research have established the important role of the mesocorticolimbic DA system in processing the initial rewarding effects of opioid drugs (Fields & Margolis, 2015). Similar to the effects of systemically administered morphine, we found that infusions of a subthreshold conditioning dose of morphine directly in the VTA, was similarly modulated by intra-PFC D_1R and D_4R transmission. Thus, activation of PFC D_1R transmission blocked the recall of an aversive fear memory and prevented the potentiation of sub-reward threshold morphine directly in the VTA. In contrast, potentiating the emotional salience of non-salient fear conditioning stimuli with PFC D_4R activation potentiated the normally non-reward effects of intra-VTA morphine, demonstrating the importance of the PFC-VTA pathway in DAergic modulation of opiate-related reward salience. Previous work in our lab has demonstrated the important functional connectivity between the PFC and VTA in modulating opiate-related reward salience. For example, Bishop et al., (2011) found that pharmacological blockade of NMDA receptor signaling in the rat PFC was able to strongly potentiate sub-reward threshold conditioning doses of intra-VTA morphine. In addition, as previously noted, neuronal sub-populations within the PFC are capable of encoding the associative morphine rewarding memory during CPP conditioning (Sun et al., 2011). Nevertheless, future experiments are required to more precisely characterize how DAergic transmission modulation of fear-related associative memories in the PFC can directly modulate sub-cortical DAergic reward sensitivity in the VTA. For example, how does PFC D_1R or D_4R activation modulate either VTA inputs to the PFC or by contrast, the control of descending PFC to VTA projections? Alternatively, future studies are required to explore how intra-PFC D_1R or D_4R modulation regulate functional outputs from the VTA to other reward-related brain processing regions, such as the BLA or nucleus accumbens.

4.4 Implications for understanding PTSD and addiction comorbidity

The present series of experiments reveals several novel mechanisms by which intra-PFC transmission through the D_4R or D_1R can simultaneously modulate the recall and salience of emotionally traumatic associative fear memories, and concomitantly regulate sensitivity to the rewarding salience of opiates. Overall, an important implication for these findings is that the PFC requires an optimal level of DA signal transduction in order to appropriately regulate both the salience and recall of emotionally salient memories, regardless of reward or aversion valence. Aberrant mPFC D_1 transmission may result in disruptive and spontaneous memory recall associated with catastrophic events or euphoric drug experiences, serving as a possible common mechanism underlying the pathology of PTSD and addiction. Interestingly, PTSD has been extensively studied with clinical populations and several reports have indicated abnormally high level of urinary and plasma DA metabolites as well as abnormal levels of cAMP, suggesting that abnormalities in DAergic transmission may be a common clinical feature in PTSD (Hamner & Diamond, 1993; Lerer et al., 1987; Yehuda et al., 1992). PFC D_1 transmission is highly implicated in rewarding memory and addiction behaviours and behavioural neuroscience research has demonstrated a unique role for mPFC D_1 signaling in drug-related learning and memory (Self et al., 1996). Notably, Lauzon et al. (2013) indicated that in the context of both aversive and rewarding associative memory, the ability of mPFC D_1 transmission is limited to the recall stage of memory formation without impacting the stability of the original memory trace. Thus, abnormal memory expression linked to either traumatic experiences or addiction-related reward experiences may share a common link to aberrant cortical D_1R transmission and downstream signaling through the ERK 1/2 molecular cascade.

In contrast to the effects of D_1R transmission in the recall phase of memory processing, the present study confirmed the functional role of PFC D_4R transmission during the acquisition phase of emotional memory formation. Considerable research has highlighted the role mPFC D_4 transduction and its downstream signalling pathway, CaMKII, in various neuropsychiatric disorders, such as PTSD, addiction, schizophrenia and ADHD (Lauzon & Laviolette, 2010). The role of intra-PFC D_4R transmission in these neuropsychiatric disorders is believed to be due to the ability of cortical D_4R transmission to potently modulate the emotional salience of incoming sensory information and associative memory formation. Abnormal signalling through PFC D_4R

substrates has been shown to lead to the misinterpretation of emotional meaning and salience of sensory information resulting in the amplification of normally non-salient emotional stimuli and improper assignment of motivational salience to associative memory cues. In other cases, hyperactive mPFC D_4 transduction leads to an inability to form associative memory which are vital for survival and/or proper cognitive functions. More importantly, mPFC D_4 and CaMKII mediate the appropriate categorization of sensory information and cues based upon their emotional salience, enabling the individual to accurately recognise the meaning of associative cues and to respond appropriately to the environment. In the case of PTSD and addiction, the above processes become aberrant, with patients inappropriately assigning emotional salience to normally neutral/unimportant cues causing misguided attention, pathological ideation and abnormal behavioural output. Thus, consistent with previous findings, the present data suggests that cortical D_4R transmission during the acquisition/encoding phase of either aversion or reward-related associative memory, may be a functional mechanism underlying the emotional memory pathology present in both PTSD and addiction.

4.5 Future Directions

Although the present series of studies revealed critical new roles for cortical PFC D_1R and D_4R transmission in the acquisition and recall of both fear and reward-related memory formation, there are several important questions that remain to be answered.

First, we found dissociations both between the effects of cAMP signaling and D_1R transmission in terms of modulation of fear memory recall and the subsequent regulation of morphine reward sensitivity. Importantly, future experiments should address how and why cAMP appears to selectively modulate the recall of fear (trauma)-related memory, but is independent of the modulation of reward-related memory. Interestingly, we found that ERK 1/2 phosphorylation was capable of regulating the recall of both fear and morphine-reward related memory formation, suggesting that D_1R transmission via the ERK 1/2 signaling pathway may transcend and control the processing of both aversion and reward-related emotional memory formation. Future studies may focus specifically on examining the potential mechanistic links between D_1R, cAMP and ERK 1/2 signaling in the context of both fear and reward-related memory processing.

The current study demonstrated that fear memory recall leads to heightened sensitivity to morphine's rewarding effects, revealed in a robust CPP to a dose of morphine that normally produces no rewarding effects. Since the CPP tests took place 24h after the last conditioning phase, which examined only temporally recent morphine reward memory formation, it is not clear how long lasting and/or persistent these potentiated morphine-related associative memories may be. Thus, it would be interesting in future studies to perform extinction memory tests to explore the memory decay curves of morphine-related associative reward memories, in order to gain deeper insight regarding the comorbidity between PTSD and addiction and how traumatic memories may modulate addiction related memory processing and resistance to abstinence over the longer term. In addition, possible dynamic interactions between fear and rewarding memory extinction curves would be of interest for future studies to gain better insight into how traumatic and/or reward-related associative memories may interact with one another in a temporal manner following initial memory formation.

In terms of opiate exposure states and their relevance to addiction-related memory formation, a limitation of the present study is that experiments used only drug naïve subjects to test their sensitivity to subthreshold morphine reward salience. Thus, it would be very informative to investigate if and how opioid dependent rats react to sub-reward conditioning doses of morphine differently than opiate-naïve subjects. Since opioid dependent states produce different impacts on neural substrates, including D_1, D_2 and ERK 1/2 and CaMKII signaling pathways (Lyons et al., 2013; Rosen et al., 2016), further research should be done in opiate-dependent subjects during long-term withdrawal/abstinence periods.

5. Conclusions

The findings in this thesis reveal several important new insights regarding how the recall of emotionally traumatic memories may directly impact upon vulnerability to the rewarding effects of drugs of abuse. Specifically, the data in this thesis provides novel information regarding how alterations in cortical DAergic transmission through D_1R-ERK 1/2 or D_4R-CaMKII signaling mechanisms may not only strongly modulate the acquisition and recall of associative fear-related memory, but how these mechanisms may simultaneously regulate sensitivity to opiate-related drug reward salience. Given the high comorbidity observed between PTSD and drug abuse liability, particularly, opioid abuse, these findings reveal unique and

promising new directions for the future development of pharmacological and molecular interventions that might be able to target the neurobiological links between PTSD emotional memory pathology and addiction liability.

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Research Talk and Poster Presentation

Poster Presentations

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San Diego, USA

Modulation of traumatic memory acquisition and recall via prefrontal cortical dopamine D4 and D1 receptor transmission differentially controls opiate reward sensitivity: implications for addiction co-morbidity in post-traumatic stress disorder

Jingjing Li, Hanna Szkudlarek, Justine Renard & Steven R Laviolette

Anatomy and Cell Biology Research Day October 2016

London, ON

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London Health Research Day March 2016

London, ON

Role of Prefrontal Cortical Dopamine Transmission in Post-Traumatic Stress Disorder and Opiate Addiction Vulnerability

Jingjing Li, Hanna Szkudlarek, Justine Renard & Steven R Laviolette

Oral Presentation

Psychiatry Research Day June 2016

London, ON

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